

**ECOTOXICOLOGICAL EFFECTS OF PETROCHEMICAL
PRODUCTS ON NATURAL POPULATIONS OF
MYTILUS GALLOPROVINCIALIS INHABITING ROCKY
SHORES ALONG THE NW COAST OF PORTUGAL**



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Author's declaration

The author states that she afforded a major contribution to the conceptual design and technical execution of the work, interpretation of the results and manuscript preparation of the published or under publication articles included in this dissertation.

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ACRONYMS & ABBREVIATIONS

AChE – acetylcholinesterase
AH – aliphatic hydrocarbons
AhR – aryl hydrocarbon receptor
ANOVA – analysis of variance
ANOSIM – analysis of similarities
ATCh – acetylthiocholine
ATP – adenosine triphosphate
BIOENV – biota and/or environment matching
BLAST – basic local alignment search tool
bp – base pairs
CAT – catalase
CARIPOL – Marine Pollution Monitoring Program in the Caribbean
cDNA – complementary deoxyribonucleic acid
CDNB – 1 chloro-2,4-dinitrobenzene
CYP1A – cytochrome P450 1A
DNA – deoxyribonucleic acid
DTNB – 5,5'-dithiobis (2-nitrobenzoic acid)
DTT – dithiothreitol
dw – dry weight
EPA – United States Environmental Protection Agency
GC-MS – gas chromatography-mass spectrometry
GPx – selenium-dependent glutathione peroxidase
GR – glutathione reductase
GSH – reduced glutathione
GSSG – oxidised glutathione
GST – glutathione S-transferases
GDP – guanosine diphosphate
GTP – guanosine 5'-triphosphate
GSx – glutathione equivalents
IDH – NADP⁺-dependent isocitrate dehydrogenase
IOC – Intergovernmental Oceanographic Commission
IOCARIBE – IOC Sub-commission for Caribbean and Adjacent Regions
H₂O₂ – hydrogen peroxide
HSD – honestly significant difference
LB – liquid broth
LPO – lipid peroxides
MDA – malondialdehyde
MDS – multidimensional scaling analysis

N – North
Na₂-EDTA – ethylenediaminetetraacetic acid disodium salt dihydrate
NAD – nicotinamide adenine dinucleotide
NADH – β-nicotinamide adenine dinucleotide
NADP – β-nicotinamide adenine dinucleotide phosphate
NADPH – β-nicotinamide adenine dinucleotide 2'-phosphate reduced
NH₄ – ammonia
NO₂ – nitrite
NO₃ – nitrate
NW – North-west
ODH – octopine dehydrogenase
PAHs – polycyclic aromatic hydrocarbons
PCA – principal component analysis
PCBs – polychlorinated biphenyls
PCR – polymerase chain reaction
PO₄ – phosphates
RACE – rapid amplification of cDNA ends
RDA – redundancy analysis
RNA – ribonucleic acid
rRNA – ribosomal ribonucleic acid
ROS – reactive oxygen species
RT-PCR – reverse transcriptase polymerase chain reaction
S – salinity
SD – standard deviation
SIMPER – similarity percentage test
SOD – superoxide dismutase
T – temperature
TBARS – thiobarbituric acid reactive substances
TBE - Tris/Borate/EDTA
tGSx – total glutathione content
TNB – 5-thio-2-nitrobenzoic acid
Tris – tris(hydroxymethyl)-aminomethane
U – unit
UCM – unresolved complex mixture
UNEP – United Nations Environment Programme
UNESCO – United Nations Educational, Scientific and Cultural Organization
UV – ultraviolet
W – West
WAF – water-accommodated fraction
XO – xanthine oxidase

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ABSTRACT

Global development has increased the demands for fossil fuels over the past decades. As the major centres of population are located near coastal environments, ecological disturbance caused by the chronic release of petrochemical contaminants is an issue of concern due to the ecological and economic value of these ecosystems. The NW coast of Portugal is particularly exposed to petrochemical contamination due to the presence of maritime harbours and an oil refining industry. However, despite the work that has been done in the last years, a scarceness of data regarding the effects of petrochemical contamination in this area of the Iberian Peninsula still exists. Therefore, the present dissertation aimed to assess the effects of petrochemical products on natural populations of the marine mussel *Mytilus galloprovincialis* inhabiting the rocky shores along the NW coast of Portugal. To accomplish the central aim of the current dissertation, a long-term monitoring program was developed. Moreover, considering the limitations that the cytochrome P450 mixed function oxidase system of molluscs presents as an environmental biomarker, and regarding other toxicity mechanisms induced by petroleum hydrocarbons in aquatic organisms (e.g. oxidative stress and carcinogenesis), an attempt was made to develop new tools to assess the effects of petrochemical contamination in mussels, particularly at the transcriptional level.

The long-term monitoring program herein presented was established for more than one year to assess the spatial and temporal trends of petrochemical contamination along the NW coast of Portugal. During this period mussels were collected from five sampling sites for analysis of petroleum hydrocarbon levels. Viana do Castelo harbour, Leixões harbour and Cabo do Mundo, which is located in the vicinity of an oil refinery, were selected due to the presence of putative sources of petrochemical contamination, while Carreço and Vila Chã were selected due to apparent low anthropogenic pressure. Additionally, biochemical parameters involved in key physiological processes (antioxidant defences, detoxification, energetic metabolism and neurotransmission) of mussels were applied as biomarkers to assess the possible consequences that the encountered concentrations of petroleum hydrocarbons may have in the fitness of wild populations of *M. galloprovincialis*. Finally, abiotic parameters quantified in water samples collected from each site aimed to investigate the possible effects of extrinsic factors on the biomarker response. It is fundamental to separate effects due to chemical contamination from those related to the natural fluctuations of water physicochemical parameters and mussels' annual physiological cycle. An initial survey was performed prior to the implementation of the long-term monitoring program to evaluate the suitability of the selected monitoring

strategy in assessing the effects of petrochemical contamination. These initial results showed good correlations between biomarkers responses and the petroleum hydrocarbon levels quantified in mussels' tissues, which allowed the discrimination of the sampling sites into three distinct groups according to the level of petrochemical contamination. The results of the long-term monitoring program corroborated these initial findings, and showed that biomarkers quantified in mussels sampled from less contaminated sites exhibited significant differences in their response throughout the year, while those quantified in mussels sampled from more contaminated sites did not exhibit seasonal fluctuations. This suggests that the effects of high levels of petrochemical contamination may overlap those of abiotic factors.

In addition to the long-term monitoring program, mussels were chronically exposed to petrochemical products under laboratory conditions to determine the specific response of the selected biomarkers to such products. Results showed that the antioxidant enzymes superoxide dismutase (SOD) and catalase (CAT) were the most responsive biomarkers, underlining their role as major defences against oxidative stress induced by contaminants. In light of these results, the putative genes of Cu/Zn-SOD and CAT of *M. galloprovincialis* were isolated and their expression analysed. Results showed that gene expression of CAT, but not Cu/Zn-SOD, corresponded well with its enzymatic activity in mussels chronically exposed to petrochemical products. Finally, considering that some components of petrochemical products are genotoxic and carcinogenic, the status of the *ras* proto-oncogene in *M. galloprovincialis* was also investigated. Results showed that a single *ras* gene mutation at codon 35, though no induction in gene expression levels, occurred in one mussel exposed to petrochemical products under laboratory conditions. This is the first report of a *ras* gene mutation in any invertebrate species. Moreover, a high incidence of polymorphic variation in the *ras* gene of *M. galloprovincialis* may indicate the presence of a second *ras* gene in these species.

In conclusion we suggest that the monitoring strategy implemented to assess the spatial and temporal trends of petrochemical contamination along the NW coast of Portugal was appropriate since it was possible to discriminate the levels of petroleum hydrocarbon contamination present in each sampling site according to biomarker responses quantified in *M. galloprovincialis*. This strategy is therefore recommended for future work. Moreover, regarding the development of new tools to assess the effects of petrochemical contamination at the transcriptional levels in *M. galloprovincialis*, results showed that an increase in the gene expression of CAT, as well as the development of mutational damage in the *ras* gene of mussels chronically exposed to petrochemical products have the potential to be used as biomarkers.

RESUMO

O desenvolvimento global que se tem verificado nas últimas décadas aumentou a procura de combustíveis fósseis. Uma vez que uma parte considerável dos grandes centros populacionais está localizada perto da zona costeira, a libertação crónica de contaminantes petroquímicos para os ecossistemas marinhos tem-se tornado uma questão cada vez mais preocupante devido ao elevado valor ecológico e económico destas áreas. A costa Noroeste de Portugal está particularmente exposta à contaminação por produtos petroquímicos devido à presença de dois grandes portos marítimos e de uma refinaria de petróleo. No entanto, apesar dos estudos que têm sido efectuados nas últimas décadas, ainda existem lacunas de informação sobre os efeitos da contaminação por produtos petroquímicos nesta área da Península Ibérica. Na tentativa de colmatar estas lacunas, a presente dissertação teve como objectivo central avaliar os efeitos dos produtos petroquímicos em populações naturais do mexilhão *Mytilus galloprovincialis* presente nas praias rochosas ao longo da costa Noroeste de Portugal, utilizando um programa de monitorização. Considerando as limitações que o sistema do citocromo P450 de moluscos apresenta enquanto biomarcador ambiental, e tendo em consideração outros mecanismos de toxicidade induzidos por hidrocarbonetos petrolíferos em organismos aquáticos (por exemplo, stress oxidativo e carcinogénese), pretendeu-se desenvolver novas metodologias para avaliar os efeitos da contaminação por produtos petroquímicos em mexilhões, especialmente ao nível de transcrição.

O programa de monitorização aqui apresentado desenvolveu-se por mais de um ano para avaliar a distribuição espacial e temporal dos níveis de contaminação por produtos petroquímicos ao longo da costa Noroeste de Portugal. Durante este período foram recolhidos mexilhões em cinco pontos de amostragem para análise dos níveis de hidrocarbonetos petrolíferos. Enquanto que o porto de Viana do Castelo, o porto de Leixões, e a praia do Cabo do Mundo localizada nas proximidades de uma refinaria de petróleo, foram seleccionados devido à presença de possíveis fontes de contaminação por produtos petroquímicos, as praias de Carreço e Vila Chã foram seleccionadas devido à aparente reduzida pressão antropogénica. Além de análises químicas, parâmetros bioquímicos envolvidos nos principais processos fisiológicos do mexilhão (defesas antioxidantes, desintoxicação, metabolismo energético e neurotransmissão) foram utilizados como biomarcadores para avaliar as possíveis consequências que as concentrações encontradas de hidrocarbonetos petrolíferos podem ter na saúde das populações selvagens de *M. galloprovincialis*. Finalmente, parâmetros abióticos quantificados em amostras de água recolhidas em cada local de amostragem foram

analisados com o objectivo de investigar os possíveis efeitos de factores extrínsecos sobre a resposta dos biomarcadores. É fundamental separar a resposta de biomarcadores devido à contaminação química, da variabilidade relacionada com flutuações naturais dos parâmetros físico-químicos da água, assim como do ciclo fisiológico anual do mexilhão. Antes da execução do programa de monitorização foi efectuado um estudo provisório para aferir a aplicabilidade da estratégia seleccionada para avaliar os efeitos da contaminação por produtos petroquímicos. Estes resultados iniciais mostraram boas correlações entre as respostas de biomarcadores e os níveis de hidrocarbonetos petrolíferos quantificados em tecidos de mexilhões, o que permitiu classificar os pontos de amostragem em três grupos distintos de acordo com o seu nível de contaminação por produtos petroquímicos. Por sua vez, os resultados do programa de monitorização corroboraram estes achados iniciais, e demonstraram que a resposta dos biomarcadores quantificados em mexilhões recolhidos em locais de amostragem menos contaminados apresentaram diferenças significativas ao longo do ano, enquanto que a resposta dos biomarcadores quantificados em mexilhões recolhidos em locais mais contaminados não apresentaram flutuações sazonais significativas. Isto sugere que os efeitos de níveis elevados de contaminação por produtos petroquímicos podem sobrepor-se aos dos factores abióticos.

Além deste programa de monitorização, mexilhões foram expostos a produtos petroquímicos em condições laboratoriais para determinar a resposta específica dos biomarcadores seleccionados, a tais produtos. Os resultados mostraram que as enzimas antioxidantes superóxido dismutase (SOD) e catalase (CAT) foram os biomarcadores mais sensíveis, sublinhando o seu importante papel como defesa contra o stress oxidativo induzido por contaminantes petrolíferos. À luz destes resultados, os genes de Cu/Zn-SOD e CAT de *M. galloprovincialis* foram isolados e a sua expressão analisada. Os resultados mostraram que apenas a expressão do gene da CAT correspondeu com os seus níveis de actividade enzimática, determinada em mexilhões expostos a produtos petroquímicos em condições laboratoriais. Finalmente, considerando que alguns componentes de produtos petroquímicos são genotóxicos e cancerígenos, o proto-oncogene *ras* no mexilhão *M. galloprovincialis* foi também estudado. Foi detectada uma mutação no codão 35 do gene num dos mexilhões expostos a produtos petroquímicos em condições laboratoriais, o que constitui o primeiro relatório de uma mutação do gene *ras* em espécies de invertebrados. No entanto, não se verificou indução dos seus níveis de expressão genética. Verificou-se ainda uma elevada incidência de variação polimórfica no gene *ras* de *M. galloprovincialis* o que sugere a presença de um segundo gene *ras* nesta espécie.

Em conclusão, sugerimos que a estratégia de monitorização implementada para avaliar a distribuição espacial e temporal da contaminação por produtos petroquímicos ao longo da costa noroeste de Portugal se revelou adequada, uma vez que foi possível classificar os locais de amostragem de acordo com os níveis de contaminação por hidrocarbonetos petrolíferos, assim como pela resposta dos biomarcadores quantificados em *M. galloprovincialis*. Esta estratégia é portanto recomendada para trabalhos futuros. Em relação ao desenvolvimento de novas metodologias para avaliar os efeitos da contaminação por produtos petroquímicos ao nível de transcrição em *M. galloprovincialis*, os resultados mostraram que um aumento da expressão do gene da CAT, bem como o desenvolvimento de uma mutação no gene *ras* de mexilhões expostos a produtos petroquímicos, têm potencial para serem utilizados como biomarcadores.

RESUMÉ

Ces dernières décennies le développement global a engendré une augmentation de la demande en énergie fossiles. La majorité des populations étant localisée près des environnements côtiers, les rejets chroniques de contaminants pétrochimiques engendrés entraînent de nombreux troubles écologiques et économiques. La côte Nord-ouest du Portugal est particulièrement exposée aux contaminants pétrochimiques en raison de la présence de ports maritime et d'une raffinerie de pétrole. Cependant, malgré le travail effectué au cours des années passées, un manque de données concernant les effets des contaminants pétrochimiques dans cette région de la Péninsule Ibérique persiste. Ainsi, cette dissertation a pour but d'estimer les effets de substances pétrochimiques sur une population de moule *Mytilus galloprovincialis* vivant sur le rivage rocheux de la côte Nord-ouest du Portugal. Au court de cette étude, un programme de contrôle sur un long terme a été développé. De plus, étant donné les limitations des fonctions du cytochrome P450 des mollusques qui représente un marqueur biologique, ainsi que d'autres mécanismes de toxicité induis par les hydrocarbures chez les organismes aquatiques (expl. stress oxydant et cancerogenese), de nouveaux outils pour évaluer les effets de ces contaminants pétrochimiques chez les moules ont été développés, et plus particulièrement au niveau de l'expression de marqueurs biologiques.

Le programme de contrôle sur long-terme présenté ci-dessous a été établi sur plus d'une année afin d'adresser l'évolution spatiale et temporelle des contaminants pétrochimiques sur la côte nord-ouest du Portugal. Pendant cette période, les prélèvements de moules ont été effectués sur cinq sites indépendants pour analyser le niveau d'hydrocarbure. Le port de Viana do Castelo, le port de Leixões, et Cabo do Mundo, localisés à proximité d'une raffinerie de pétrole, ont été sélectionnés en raison de la présence d'une source possible de contamination pétrochimique, tandis que Carreço et Vila Chã ont été sélectionnés en raison d'une faible pression antropogénétique. De plus, des paramètres biochimiques impliqués dans les processus physiologiques des moules (antioxydation, défense, détoxification, métabolisme énergétique et neurotransmission) ont été utilisé comme marqueurs biologiques afin de déterminer les conséquences de la concentration d'hydrocarbure sur le développement des populations de *M. galloprovincialis*. Pour finir, des paramètres abiotiques ont été quantifiés à partir d'échantillons d'eau collectés à chaque site dans le but d'investir les effets possibles de facteurs extrinsèque sur la réponse des marqueurs biologiques. Il est fondamental de séparer les effets correspondant aux contaminations chimiques de ceux liés aux fluctuations naturelles des paramètres physicochimiques des eaux et du cycle

physiologique annuel des moules. Une enquête préliminaire a été effectuée avant le début du programme de contrôle long-terme afin de juger la pertinence de cette stratégie. Les résultats initiaux ont montrés de bonnes corrélations entre les réponses des marqueurs biologiques et les niveaux d'hydrocarbure quantifiés dans les tissus des moules, ce qui permet la discrimination des échantillons prélevés en trois groupes selon le niveau de contamination pétrochimique. Les résultats du programme de contrôle long-terme ont corroborés ces résultats préliminaires, et ont montrés que les marqueurs biologiques quantifiés dans les échantillons de moules provenant de sites moins contaminés manifestent des différences significatives dans leur réponse sur la période étudiée, tandis que ceux quantifiés dans des moules provenant de sites contaminés ne manifestent pas de fluctuation saisonnière. Cela suggère que les effets de contamination pétrochimique élevée peuvent chevaucher ceux des facteurs abiotiques.

En plus du programme de contrôle long-terme, les moules ont été exposées de façon chronique aux produits pétrochimiques sous condition de laboratoire afin de déterminer la réponse spécifique des marqueurs biologiques sélectionnés à de tels produits. Les résultats démontrent que les enzymes antioxydantes superoxyde dismutase (SOD) et catalase (CAT) sont les marqueurs biologiques les plus réceptifs, soulignant leur rôle en tant que défenseur majeur contre le stress oxydatif induit par les contaminants. À la vue de ces résultats, les gènes de CAT et Cu/Zn-SOD de *M. galloprovincialis* ont été isolés et leur expression analysées. Les résultats démontrent que seule l'expression de CAT correspond à l'activité enzymatique des moules chroniquement exposées aux produits pétrochimiques. Pour finir, à cause de l'influence cancérigène et génotoxique de certains produits pétrochimiques, le statut du proto-oncogène *ras* de *M. galloprovincialis* a également été investigué. Les résultats démontrent qu'une mutation dans le gène *ras* au niveau du codon 35 apparaît dans une moule exposée aux produits pétrochimiques sous conditions de laboratoire. Aucune induction du niveau de l'expression de *ras* n'a été constatée. Ceci représente le premier rapport d'une mutation du gène *ras* dans une espèce d'invertébré. De plus, une fréquence élevée de polymorphisme dans le gène *ras* de *M. galloprovincialis* peut suggérer la présence d'un second gène *ras* dans ces espèces.

Pour conclure, la stratégie de contrôle développée pour juger l'évolution spatiale et temporelle de la contamination pétrochimique sur la côte Nord-ouest du Portugal semble être appropriée en raison de la discrimination possible des niveaux d'hydrocarbure présent dans chaque échantillon en fonction de la réponse des marqueurs biologiques relevés chez *M. galloprovincialis*. Cette stratégie est donc recommandée pour de futurs travaux. De plus, concernant le développement de nouveaux outils afin d'évaluer les

effets de contamination pétrochimique au niveau transcriptionnelle chez *M. galloprovincialis*, les résultats démontrent qu'une augmentation de l'expression du gène de CAT, ainsi que le développement de mutation du gène *ras* chez la moule chroniquement exposée aux produits pétrochimiques peuvent être utilisés en tant que marqueurs biologiques.

PART I

GENERAL INTRODUCTION

GENERAL INTRODUCTION

MYTILUS SPP. AS A BIOINDICATOR IN ECOTOXICOLOGY: GENERAL OVERVIEW AND UNANSWERED QUESTIONS

Over the past decades the degradation of marine and estuarine ecosystems has been increasing worldwide. In particular, the chronic release of contaminants following global industrialisation has become an issue of major concern among environmental legislators and regulators since the high ecological and economic value of these ecosystems may be compromised. Therefore, there has been a growing awareness of the need to develop effective and internationally accepted, long-term monitoring programs to assess the impact of stressors upon marine and estuarine ecosystems ^[1]. Such programs will permit the implementation of effective management strategies, either as precautionary measures to minimise chronic inputs of contaminants into the environment, or as restoration procedures that need to be implemented following accidental releases of contaminants such as oil spills ^[2].

Bivalve molluscs, particularly marine mussels of the genus *Mytilus* (Linné, 1758), have been used as indicator organisms in environmental monitoring programmes since the “Mussel Watch” program established in the mid 1970s ^[3]. These organisms have a wide geographic distribution, being found in boreal and temperate waters of the northern and southern hemispheres ^[4]. In the coast of Portugal we have the Mediterranean mussel *Mytilus galloprovincialis* (Lamarck, 1819), which can also be found in northern areas of the Iberian Peninsula ^[4]. Mussels are considered to be suitable indicators in environmental monitoring programs mainly because of their sedentary lifestyle, and because they are filter-feeders with very low metabolism, which results in the bioaccumulation of many chemicals in their tissues ^[5]. Given that some organic contaminants, such as polycyclic aromatic hydrocarbons (PHAs) or polychlorinated biphenyls (PCBs), are highly biodegradable they do not tend to accumulate in fish tissues in concentrations that reflect long-term exposure, therefore, mussels appear to be more suitable organisms to evaluate the effects of chronic releases of certain organic contaminants into the environment because they have been found to accumulate these products ^[6].

The first studies that used marine mussels as bioindicators only accounted for the accumulation and distribution of organic and inorganic contaminants within mussel

tissues^[7, 8]. However, international organisations and environmental agencies soon recognised that environmental monitoring programs could not be based solely on chemical analyses performed in mussels' tissues because chemical data *per se* does not provide any indication of the deleterious effects that contaminants may have on the ecosystems^[9, 10, 11]. As such, the quantification of biological effects induced by contaminants has been having an increasing importance in the assessment of environmental quality^[9, 10, 11]. Generally, molecular, biochemical and physiological biomarkers have been used in ecotoxicology as early warning indicators of contamination. Since the deleterious effects of some chemicals are usually first displayed at low levels of biological organisation, it is possible to predict effects that may occur later at population, community and ecosystem levels, allowing greater time for the development of preventive measures^[12].

The NW coast of Portugal is particularly exposed to petrochemical contamination due to the presence of maritime harbours and an oil refining industry. However, despite the works that have been done in the last decades, lack of information still exists regarding the effects of petrochemical contamination in this area of the Iberian Peninsula. To address this problem a long-term monitoring program was established, and a battery of biomarkers involved in key physiological processes (antioxidant defences, detoxification, energetic metabolism and neurotransmission) of mussels was applied to relate biological responses with levels of petrochemical contamination along the NW coast of Portugal.

It is known that PAHs, one of the main components of petrochemical products, bind to the aryl hydrocarbon receptor (AhR) following cellular uptake in vertebrates^[13]. This binding may subsequently induce the expression of genes that code for enzymes involved in the metabolism and detoxification of PAHs, such as the cytochrome P450 mixed function oxidase system^[13]. In particular, the cytochrome P450 1A (CYP1A) has been reported to be dose-dependent of PAHs and PCBs exposure in fish^[6]. Therefore, the CYP1A has been used as a specific biomarker for petrochemical contamination when fish are used as bioindicator species. However, it has been reported that in mussels PAHs do not bind to the AhR receptor as easily, and as a consequence the activity of the CYP1A system is lower or non-existent in these organisms^[13, 14]. This suggests that the metabolism of PAHs in mussels may occur through a different pathway to that of vertebrates, and as such it can not be used as a specific biomarker of petrochemical contamination for these organisms^[13, 14]. Considering the limitations that the AhR and CYP1A systems of mussels present as environmental biomarkers, and regarding other toxicity mechanisms induced by petrochemical products (e.g. oxidative stress and carcinogenesis) in invertebrates, a significant effort should be dedicated to the

development of new tools that can be used as biomarkers to assess the effects of petrochemical contamination in such organisms, including at the transcriptional level.

THESIS AIMS

The global aim of this dissertation was to assess the ecotoxicological effects of petrochemical products on natural populations inhabiting rocky shores along the NW coast of Portugal. Considering the reasons already described the marine mussel *M. galloprovincialis* was selected as bioindicator. Moreover, considering limitations of the available biomarkers in mussels, an attempt was made to develop a novel molecular biomarker.

In particular this dissertation aimed to:

- i. Develop and evaluate the suitability of a monitoring program designed to assess the effects of petrochemical contamination based on a battery of biomarkers involved in key physiological process of mussels.
- ii. Investigate the spatial and temporal trends of petrochemical contamination along the NW coast of Portugal by implementing a long-term monitoring program.
- iii. Assess the chronic response of the selected biomarkers to petrochemical products by exposing mussels to a fuel-oil under laboratory conditions for 21 days.
- iv. Compare the enzymatic activity and the gene expression of the most responsive biomarkers following chronic exposure of mussels to petrochemical products, to better understand the toxicity mechanisms of these organisms.
- v. Develop a novel biomarker that could have a specific response to petrochemical products in mussels.

OUTLINE OF THE THESIS AND RATIONALE

The present dissertation is structured in four parts:

Part I – General introduction

In **Part I**, the current section, a general overview on the research assumptions, as well as the objectives and structure of the dissertation, is presented.

Part II – Evaluation of petrochemical contamination along the NW coast of Portugal

Some areas of the NW coast of Portugal are chronically exposed to petrochemical contamination due to the presence of maritime harbours and an oil refining industry. Considering the deleterious effects that these contaminants have in aquatic organisms, a long-term monitoring program was developed to assess the spatial and temporal trends of petrochemical contamination along the NW coast of Portugal. In **Part II** of the dissertation, the results of this long-term monitoring program are discussed.

Chapter 1. Biochemical responses of the marine mussel *Mytilus galloprovincialis* to petrochemical environmental contamination along the NW coast of Portugal

Chapter 1 represents the first stage of a monitoring program that was developed to evaluate the suitability of the selected monitoring strategy to assess petrochemical contamination. In this initial study, the levels of petroleum hydrocarbons quantified in mussels' tissues were correlated with the response of a battery of biomarkers involved in key physiological processes (antioxidant defences, detoxification, and energetic metabolism) of mussels. Moreover, to evaluate the possible effects of extrinsic factors on the biomarker response, abiotic parameters were quantified in water samples collected from each site.

Chapter 2. Multivariate and graphical analysis of biomarker responses as a tool for long-term monitoring: a study of petrochemical contamination along the NW coast of Portugal

The results obtained in Chapter 1 prior to the implementation of the long-term monitoring program, showed a good correlation between the levels of petroleum hydrocarbons and some of the selected biomarkers. These initial results allowed the classification of the sampling sites according to the levels of petrochemical contamination. As such, we concluded that the selected monitoring strategy appeared to be appropriate to assess the spatial and temporal trends of petrochemical contamination along the NW coast of Portugal. In **Chapter 2** the results of a long-term monitoring program were used to evaluate the effects of seasonality on the response of the battery of biomarkers selected for this study. Moreover, the potential of the selected biomarkers to discriminate trends in the levels to petrochemical contamination along the NW coast of Portugal throughout the year is also discussed. Finally, a multivariate and graphical analysis was used to integrate the comprehensive set of data obtained during this long-term monitoring program.

Part III – Development of new tools to assess the effects of petrochemical contamination considering mussels' toxicity mechanisms

It is known that the use of mussels' AhR and CYP1A systems as biomarkers of petrochemical contamination has some limitations. As such, knowing that other toxicity mechanisms (e.g. oxidative stress and carcinogenesis) can also be induced by petroleum hydrocarbons in invertebrates, in **Part III** of this dissertation an attempt was made to develop new tools that could be applied as specific biomarkers of petrochemical contamination at the transcriptional level in *M. galloprovincialis*.

Chapter 3. Integration of enzymatic activity and gene expression of antioxidant defences of *Mytilus galloprovincialis* chronically exposed to petrochemical contamination

In **Chapter 3**, the responsiveness of a battery of biomarkers was investigated to understand the toxicity mechanisms induced by petrochemical contaminants in marine mussels, in particular with respect to their antioxidant defence system. For this, the response of biomarkers was compared in mussels collected from the field with those

chronically exposed to fuel-oil in laboratorial bioassays. Regarding the biochemical results obtained during this study, which showed that the enzymes superoxide dismutase and catalase were the most responsive biomarkers, the gene expression of these antioxidant enzymes of *M. galloprovincialis* was also evaluated.

Chapter 4. *Ras* gene in marine mussels: a molecular level response to petrochemical exposure

Finally, considering that some components of petrochemical products are genotoxic and carcinogenic, the status of the *ras* proto-oncogene of *M. galloprovincialis*, as well as its potential to be used as a new biomarker of petrochemical contamination, was investigated in **Chapter 4**. In this study, changes in the gene expression, as well as the development of mutational damage, of mussels' *ras* gene were evaluated following chronic exposure to petrochemical products.

Part IV – General conclusions

In this final section the results of the studies undertaken are discussed, mainly focusing on long-term monitoring strategies, as well as on the potential use of new biomarkers to assess the effects of petrochemical contamination in wild populations of the marine mussel *M. galloprovincialis*.

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PART II

EVALUATION OF PETROCHEMICAL CONTAMINATION ALONG THE NW COAST OF PORTUGAL

CHAPTER 1

Biochemical responses of the marine mussel *Mytilus galloprovincialis* to petrochemical environmental contamination along the NW coast of Portugal

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ABSTRACT

Following the development of urban and industrial centres petrochemical products have become a widespread class of contaminants. The aim of this study was to investigate the effects of petrochemical contamination in wild populations of mussels (*Mytilus galloprovincialis*) along the NW Atlantic coast of Portugal, by applying antioxidant and energetic metabolism parameters as biomarkers. For that, mussels were collected at five sampling sites presenting different petrochemical contamination levels. To evaluate the mussels' antioxidant status, enzymatic activities of catalase, superoxide dismutase, glutathione peroxidase, glutathione reductase, glutathione S-transferases, as well as glutathione redox status, were evaluated in gills and digestive glands of mussels collected from the selected sites. Lipid peroxidation was determined in the same tissues to quantify cellular oxidative damage. Furthermore, to investigate how energetic processes may respond to these contaminants, the activity of NADP⁺-dependent isocitrate dehydrogenase was determined in mussels' digestive glands, and octopine dehydrogenase was determined in mussels' posterior adductor muscles. Furthermore, the concentrations of aliphatic hydrocarbons, unresolved complex mixture and polycyclic aromatic hydrocarbons (PAHs) were quantified in mussels' tissue, and abiotic parameters were quantified in water samples collected at each site. Several biomarkers showed statistically significant differences among sampling sites. The redundancy analysis (RDA) used to perform the integrated analysis of the data showed a clear separation of the sampling sites in three different assemblages, which are in agreement with the PAHs levels found in mussels' tissues. In addition, the RDA indicated that some of the selected biomarkers may be influenced by abiotic parameters (e.g. salinity, pH, nitrates and ammonia). The approach selected for this study seems to be suitable for monitoring petrochemical contamination.

Keywords: *Mytilus galloprovincialis*, oxidative stress, energetic metabolism, biomarkers, petrochemical products

1.1. INTRODUCTION

Petroleum products are a widespread class of environmental contaminants that may enter the marine environment through discharges of industrial and urban effluents, shipping activities, offshore oil production, oil spills, fossil fuel combustion, and natural seeps ^[1]. In recent decades, the development of industrial and urban centres has increased the levels of petrochemical products in the environment, particularly in estuaries and coastal areas. The NW Atlantic coast of Portugal is exposed to contamination by petrochemical products due to the presence of oil refining industry and two maritime harbours (located at Leixões and Viana do Castelo). Also, the proximity to important maritime traffic routes increases the risk of navigation accidents and oil spills ^[2]. In recent decades, spill accidents at oil terminals and those caused by navigation accidents, such as the “*Prestige*” oil spill that occurred in Galicia in 2002, have highlighted the ecological and socio-economic problems inherent to petrochemical contamination. Furthermore, the “*Prestige*” and previous accidents of lower dimension showed the necessity of obtaining baseline information on biological and chemical data for the Iberian Atlantic coast. In future, these data could be used as reference in situations of accidents involving petrochemical contamination. Petroleum products consist mainly of saturated non-cyclic hydrocarbons, cyclic hydrocarbons, oleofinic hydrocarbons, aromatic hydrocarbons, sulphur compounds, nitrogen-oxygen compounds and heavy metals. However, each crude oil or refined product widely varies in its chemical composition and physical properties, depending of its origin ^[3]. Following entry into the aquatic environment, these contaminants may suffer physical, chemical and biological alterations through weathering processes, which can be considered as one of the main factors influencing the toxicity and potential ecotoxicological effects of these environmental contaminants ^[4].

Polycyclic aromatic hydrocarbons (PAHs) are among the most toxic components of petroleum products. These hydrophobic compounds can be easily taken up by marine organisms due to their ability to interact with cellular molecules following binding to lipophilic sites. If the target is a key molecule of a cellular process, a toxic response may be induced, and, at the extreme, the integrity of the organism can be seriously compromised ^[5]. After being taken up by an organism, hydrocarbons and their metabolic products may enhance the production of reactive oxygen species (ROS) by several mechanisms that can lead to cellular damage through protein oxidation, lipid peroxidation (LPO) and DNA damage ^[6]. To prevent these injuries, enzymatic and non-enzymatic antioxidant systems are triggered to eliminate contaminant stimulated ROS, allowing the organism to overcome oxidative stress in polluted environments ^[7].

Bivalve molluscs, particularly marine mussels such as *Mytilus* spp., have been used as indicator organisms in environmental monitoring programmes due to their wide distribution, sedentary lifestyle, tolerance to a large range of environmental conditions and because they are filter-feeders with very low metabolism, which allows the bioaccumulation of many chemicals in their tissues ^[8].

The objective of this study was to investigate the effects of petrochemical contamination in wild populations of mussels (*Mytilus galloprovincialis*) along the NW Atlantic coast of Portugal. For that, antioxidant system and energetic metabolism of mussels were applied as biomarkers. In addition, the concentrations of aliphatic hydrocarbons (AH), unresolved complex mixture (UCM) and PAHs were quantified in mussels' tissue to investigate possible correlations between the response of the selected biochemical parameters and petroleum hydrocarbon levels. Furthermore, several abiotic parameters were quantified in water samples from each site to investigate possible effects of these environmental variables in the biomarkers. It is known that oxidative damage is an important mechanism of toxicity induced by petrochemical products, namely by PAHs ^[6]. Therefore, the activities of superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) were selected as biomarkers since they are important enzymatic antioxidant defences ^[7]. Glutathione reductase (GR), which regenerates reduced glutathione (GSH), oxidized by GPx during the elimination of peroxides to maintain cellular redox status was also assessed ^[7]. GSH, a ubiquitous non-protein thiol that plays a major role in the maintenance of intracellular redox balance and in the regulation of signalling pathways enhanced by oxidative stress was quantified as a non-enzymatic antioxidant defence ^[9]. In addition, the enzymatic activities of glutathione S-transferases (GST), a family of multi-functional enzymes involved in Phase II of biotransformation that are related to cellular antioxidant defences due to the conjugation of electrophilic xenobiotics and oxidized components with GSH, were also evaluated ^[10]. Furthermore, NADP⁺-dependent isocitrate dehydrogenase (IDH) one of the enzymes that has the ability to regenerate cellular NADPH was also evaluated due to its role in the antioxidant system, since NADPH, a cofactor of GR, needs to be regenerated during the maintenance of the cellular redox status ^[11,12]. Finally, octopine dehydrogenase (ODH) was investigated due to its importance for the energetic metabolism of intertidal bivalves such as *M. galloprovincialis* ^[13]. ODH maintains the redox balance of muscle tissue during periods of temporary anoxia by the regeneration of cytoplasmatic NADH to NAD, also allowing the energetic supply through the maintenance of the anaerobic glycolysis mechanism ^[14].

1.2. MATERIAL & METHODS

1.2.1. Sampling sites

In January 2005, mussels were retrieved from five sampling sites along the NW coast of Portugal (Figure 1.1). These sites were selected regarding possible differences in petrochemical contamination levels.

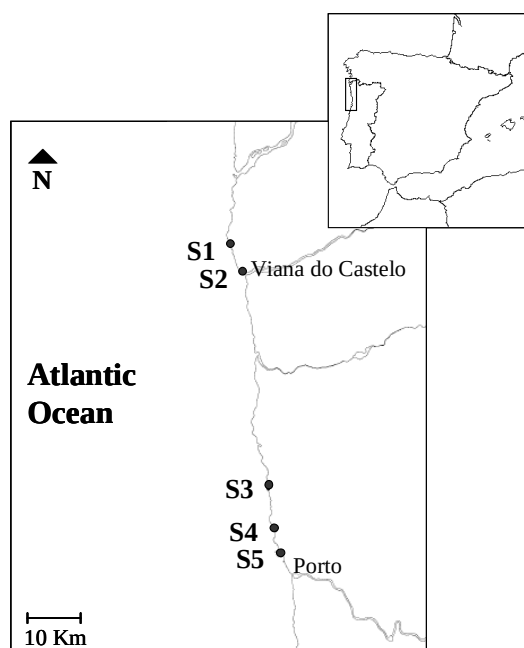


Figure 1.1 Map of the NW coast of Portugal, showing the location of the five sampling sites. S1: Carreço, S2: Viana do Castelo harbour, S3: Vila Chã, S4: Cabo do Mundo, S5: Leixões harbour.

S1 – Carreço (41°44'27"N; 08°52'40"W), is a rocky shore located 10 Km North of Viana do Castelo. Apparently it is free of significant contamination sources. However, it is relatively close to the region affected by the “*Prestige*” oil spill ^[15].

S2 – Viana do Castelo harbour (41°41'01"N; 08°50'40"W), is located at the mouth of Lima river. It is continuously subjected to petrochemical contamination through the activity of commercial and fishing vessels. Records exist of the constant release of untreated urban effluents into the river and estuary by several municipalities ^[16]. Additionally, in 2000, this harbour was severely affected by the “*Coral Bulker*” oil spill ^[17].

S3 – Vila Chã (41°17'45"N; 08°44'16"W), is a beach near a fishing village located 25 Km North of Porto. It was selected due to the absence of significant contamination sources, and because it has been used as reference site in previous studies of our laboratory ^[15, 18]. In addition, it has been described as having a high biodiversity of intertidal organisms, indicating low levels of anthropogenic pressure ^[19].

S4 – Cabo do Mundo (41°13'33"N; 08°43'03"W), is a rocky shore with a small watercourse located 14 Km North of Porto. Due to the presence of an oil refinery this site has been chronically exposed to petrochemical products, including PAHs ^[20] and heavy metals ^[21]. It has also been reported to be highly impacted in previous studies ^[15, 18].

S5 – Leixões harbour (41°10'58"N; 08°41'55"W), is located 10 Km North of Porto at the mouth of Leça river. It comprises the largest seaport infrastructure in the North of Portugal and is one of the most versatile multi-purpose harbours in the country. Due to intense vessel traffic and to oil terminal activity, the harbour is constantly subjected to petroleum hydrocarbon contamination ^[16]. During the summer 2004, an accident during maintenance activities caused a pipeline leak and subsequent oil spill to the surrounding shore.

1.2.2. Abiotic parameters

Salinity, conductivity, temperature (Wissenschaftlich Technische Werkstätten – WTW, LF 330 meter, Brüssel, Belgium), and pH (WTW, 537 meter) were measured *in situ* at the five sampling sites during low and high tide. At the same time, subsurface water samples were collected with 1.5 L polyethylene-terephthalate bottles and stored at 4°C for analysis. Water samples were filtered (64 µm) prior to nutrient analysis. Levels of ammonia, nitrates, nitrites and phosphates were measured using commercial photometer kits (Photometer 7000, Palintest, Kingsway, England).

1.2.3. Animal sampling

In January 2005, fifty adult mussels (mean anterior-posterior shell length of 3.5 ± 1.0 cm) were handpicked during low tide in the intertidal zone of the five sampling sites. Following collection, mussels were placed in thermally insulated boxes previously filled with water from the sampling site and immediately transported to the laboratory. Mussels were sacrificed two hours after collection to ensure equal sampling and transport

conditions among sites. From each sampling site, the whole tissue of thirty mussels was isolated for chemical analyses. Gills, digestive glands and posterior adductor muscles of the remaining twenty mussels were immediately isolated and pooled into ten groups for each tissue (one tissue portion of two mussels each) for biomarker analyses. Samples were frozen in liquid nitrogen and stored at -80°C until required for analysis.

1.2.4. Chemical analyses

A single analysis of petroleum hydrocarbon was performed in pooled tissues of thirty mussels collected at five sampling sites (S1-S5) along the NW coast of Portugal. The analytical procedures for extraction and purification of petroleum hydrocarbons were carried out using the method of CARIPOL/IOCARIBE/UNESCO (1986) ^[22] according to UNEP (1992) ^[23]. Each set of samples was accompanied by a complete blank and a spiked blank which was carried through the entire analytical scheme in identical conditions for all samples. Samples were extracted by homogenisation with a mixture of hexane:methyl chloride (1:1), and an internal standard was added before extraction. The aliphatic and aromatic fractions were purified and separated in three fractions by column chromatography with 10 g each of silica gel/alumina with hexane. The first fraction was eluted with *n*-hexane; the second fraction was eluted with *n*-hexane:methyl chloride (1:1) and the third fraction was eluted only with methyl chloride. The extracts concentrated containing fraction 1 (aliphatic) and fraction 2 and 3 (aromatics) were rotoevaporated to 1 mL and analysed by gas chromatography. Hydrocarbons were quantified using gas chromatography. Nitrogen was used as carrier gas (flow 1 mL mm⁻¹). The limit of detection for individual aromatic compounds was 0.01 µg g⁻¹ and recovery yields were up to 90%. The AH and UCM were quantified with an *n*-C28 standard. PAHs were identified by comparing their retention times with those from the aromatic analytical standards by Supelco 48743 according to the priority PAHs from method EPA 610.

1.2.5. Biomarkers

The following biochemical parameters were selected as indicators of key physiological functions of marine bivalves: GST for both detoxification and antioxidant defences; SOD, CAT, GPX, GR, total glutathione content (tGSx), GSH, oxidised glutathione (GSSG) and glutathione redox status (GSH/GSSG ratio) for antioxidant

defences; IDH for both antioxidant defences and energetic aerobic metabolism; and ODH for energetic anaerobic metabolism. Levels of LPO were measured as an indication of oxidative damage. All biochemical parameters used as indicators of detoxification and/or antioxidant defences were determined both in gills and digestive glands, except IDH that was only determined in digestive glands because previous studies indicated a very low activity of this enzyme in gill tissue (data not published). The tissue selected for ODH activity quantification was the posterior adductor muscle since previous studies indicated that this was the most suitable tissue for its quantification (data not published).

The activity of SOD was determined according to McCord and Fridovich (1969) ^[24] adapted to microplate. Tissues were homogenised (Ystral homogeniser, Ballrechten-Dottingen, Germany) in 50 mM sodium phosphate buffer (Merck 1.06579 and 1.06345, Darmstadt, Germany) with 1 mM ethylenediaminetetraacetic acid disodium salt dihydrate (Na₂-EDTA, Sigma E4884, Osterode, Germany) (pH 7.8) and centrifuged (Sigma 3K) at 15,000 *g* for 15 min at 4°C. The final concentrations of the assay chemicals were: 50 mM sodium phosphate buffer with 1 mM Na₂-EDTA (pH 7.8), 0.043 mM xanthine (Sigma X7375), 18.2 µM cytochrome *c* (Sigma C7752) and 0.3 U mL⁻¹ xanthine oxidase (Sigma X1875). One unit of SOD was defined as the amount of enzyme required to inhibit the rate of reduction of cytochrome *c* by 50%.

The activity of CAT was determined according to Aebi (1984) ^[25]. Tissues were homogenised in 50 mM potassium phosphate buffer (Merck 1.05101 and Merck 1.04873) (pH 7.0) and centrifuged at 15,000 *g* for 15 min at 4°C. The final concentrations of the assay chemicals were: 50 mM potassium phosphate buffer (pH 7.0) and 10 mM hydrogen peroxide (H₂O₂, Aldrich 21.676, Steinheim, Germany).

The activities of GPx and GR were determined according to Flohé and Günzler (1984) ^[26], and Carlberg and Mannervik (1975) ^[27], respectively. The two assays were adapted to microplate. The activity of GST was determined according to Habig et al. (1974) ^[28] adapted to microplate by Frasco et al. (2002) ^[29]. For these three enzymatic assays, tissues were homogenised using 100 mM potassium phosphate buffer with 2 mM Na₂-EDTA (pH 7.5) and centrifuged at 15,000 *g* for 15 min at 4°C. The final concentrations of the chemicals for the GPx assay were: 100 mM potassium phosphate buffer with 2 mM Na₂-EDTA, 1 mM dithiothreitol (DTT, Sigma D9779) and 1 mM of sodium azide (Sigma S8032) (pH 7.5), 2 mM GSH, 34 U mL⁻¹ glutathione reductase (GR, Sigma G3664), 0.24 mM β-nicotinamide adenine dinucleotide 2'-phosphate reduced tetrasodium salt (NADPH, Sigma N7505), and 0.6 mM H₂O₂. The final concentrations of the chemicals for the GR assay were: 100 mM potassium phosphate buffer with 2 mM Na₂-EDTA (pH 7.5), 0.5 mM GSSG (Sigma G4376) and 0.1 mM NADPH. The final concentrations of the

chemicals for the GST assay were: 100 mM potassium phosphate buffer (pH 6.5), 4 mM GSH and 1 mM 1-chloro-2,4-dinitrobenzene (Sigma C6396).

The levels of tGSx and GSSG were determined according to Baker et al. (1990)^[30]. Tissues were homogenised using 71.5 mM sodium phosphate buffer with 0.63 mM Na₂-EDTA (pH 7.5). Following homogenisation, 5% perchloric acid (Merck 0519) was added to the samples that were centrifuged at 15,000 *g* for 15 min at 4°C. Previous to the enzymatic assay, samples were neutralized with 760 mM potassium hydrogen carbonate (Sigma P4913). The final concentrations of the chemicals for the tGSx quantification were: 0.15 mM NADPH, 0.85 mM of 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB, Sigma D8130) and 7 U mL⁻¹ GR. A 5% solution of 2-vinylpyridine (Fluka 95040, Steinheim, Germany) was used to conjugate GSH for the GSSG determination. Glutathione concentrations were expressed as nmol of GSH equivalents (GSx) per mg of protein ($GSx = [GSH] + 2[GSSG]$). GSH/GSSG ratio was calculated as number of molecules: $GSH/GSSG = (tGSx - GSSG)/(GSSG/2)$, according to Peña-Llopis et al. (2001)^[31].

Levels of LPO were measured by the generation of thiobarbituric acid (TBARS)-malondialdehyde (MDA) reactive species, which were referred to MDA equivalents (Ohkawa et al., 1979)^[32]. Tissues were homogenised using 100 mM potassium phosphate buffer (pH 7.2) and centrifuged at 10,000 *g* for 5 min at 4°C. The reaction mixture contained: 11.4% of homogenate, 4.6% of 10.6 mM sodium dodecyl sulfate (Sigma D2525) with 0.1 mM butylated hydroxytoluene (Aldrich W218405), 40% of 20% acetic acid (Merck 1.00062) (pH 3.5), 40% of 22.2 mM thiobarbituric acid (Sigma T5500), and 4% of nanopure water in a final volume of 700 µL. The reaction mixture was heated in a 95°C water bath for 1 h. Once cold, 175 µL of nanopure water and 875 µL *n*-butanol (Merck 1.01990) and pyridine (Aldrich 270970) (15:1 v/v) were added and thoroughly mixed. Following centrifugation at 10,000 *g* for 5 min, the immiscible organic layer was removed and its absorbance measured at 530 nm.

The activity of IDH was determined according to Ellis and Goldberg (1971)^[33] adapted to microplate. Tissues were homogenised in 50 mM tris(hydroxymethyl)-aminomethane (Tris, Merck 1.08382) buffer (pH 7.8) and centrifuged at 15,000 *g* for 15 min at 4°C. The final concentrations of the assay chemicals were: 50 mM of Tris buffer (pH 7.8), 0.5 mM β-nicotinamide adenine dinucleotide phosphate (NADP, Sigma N0505), 7 mM DL-isocitric acid (Sigma I1252) and 4 mM manganese chloride tetrahydrate (Merck 1.05927).

The activity of ODH was determined according to Livingston et al. (1990) ^[34] adapted to microplate. Tissues were homogenised in 20 mM Tris buffer (pH 7.5) with 1 mM Na₂-EDTA and 1 mM DTT and centrifuged at 15,000 *g* for 15 min at 4°C. The final concentrations of the assay chemicals were: 100 mM imidazole hydrochloride (Sigma I3386) buffer (pH 7.0), 0.1 mM β-nicotinamide adenine dinucleotide (NADH, Sigma N8129), 10 mM L-arginine (Aldrich A9,240-6) and 2 mM pyruvic acid sodium salt (Sigma P2256). The protein content of the samples was determined by the Bradford method (Bradford, 1976) ^[35], using γ-bovine globulins (Sigma G5009) as standard.

1.2.6. Data analyses

The results of the biomarkers are presented as means ± standard deviation (SD). The comparison of the biomarkers among sampling sites was performed by one-way analysis of variance (one-way ANOVA), followed by a Tukey honestly significant difference (HSD) multiple comparison test whenever applicable ^[36]. The normality (Kolmogorov–Smirnov normality test) and homogeneity of variance (Hartley, Cochran C, and Barlett's test) of data was verified and data transformation was applied as required to fulfil ANOVA assumptions ^[36]. A Pearson correlation was performed to evaluate the degree of relationship between the biomarkers and abiotic parameters, as well as between the biomarkers and the petroleum hydrocarbon levels ^[36]. In addition, the ordination technique of redundancy analysis (RDA) was performed to evaluate the response of the biomarkers to abiotic parameters and petroleum hydrocarbons. Statistical analyses of data were performed using the software Statistica 6.0 (StatSoft, Tulsa, USA), with the exception of the RDA that was performed using the software CANOCO 4.52 for Windows (Biometris, Wageningen, The Netherlands).

1.3. RESULTS

1.3.1. Abiotic parameters

Temperature range midpoint values at all sites ranged from 12.4°C (S2) to 13.4°C (S1). The highest salinity range midpoint values (34.8 g L⁻¹ at S1 and 34.4 g L⁻¹ at S3) were found in sites located at open seashore, while the intermediate (32.3 g L⁻¹ at S4 and 32.6 g L⁻¹ at S5) and lowest values (28.3 g L⁻¹ at S2) were recorded at sites located near

the mouth of watercourses. The pH range midpoint values ranged from 7.23 (S3) to 7.89 (S4) at all stations. Ammonia concentrations were relatively higher at S2 (0.10 mg L⁻¹), S4 (0.49 mg L⁻¹) and S5 (0.82 mg L⁻¹), compared to the low levels of S1 (0.03 mg L⁻¹). Nitrite concentrations were relatively low at all sites (ranging from 0.01 mg L⁻¹ at S3 to 0.09 mg L⁻¹ at S2 and S4) except at S1 where a value of 0.17 mg L⁻¹ was found. Nitrate values ranged from 1.63 mg L⁻¹ at S2 to 3.20 mg L⁻¹ at S4. Phosphates concentrations were higher at S1 (0.21 mg L⁻¹), and S5 (0.30 mg L⁻¹), than at the remaining sites.

1.3.2. Chemical analyses

The results of chemical analyses, determined in single samples of pooled tissues of *M. galloprovincialis* collected at five sampling sites (S-S5) along the NW coast of Portugal, are presented in Table 1.1.

Table 1.1 Chemical analyses of petroleum hydrocarbons performed in whole tissue of *Mytilus galloprovincialis* collected at five sampling sites (S1-S5) along the NW coast of Portugal.

Petroleum hydrocarbons	Sampling Sites				
	S1	S2	S3	S4	S5
AH	101.06	45.67	62.20	39.65	168.67
UCM	545.03	840.18	788.27	360.81	2159.83
Σ PAHs	148.27	549.56	124.21	164.60	161.70
Acenaphthene	-	0.11	-	-	0.12
Acenaphthylene	1.10	0.07	-	0.29	-
Anthracene	0.17	0.16	0.18	0.33	0.30
Benzo[a]anthracene	-	0.13	-	0.11	0.43
Benzo[a]pyrene	53.72	239.90	46.65	49.55	71.54
Benzo[b]fluoranthene	0.67	0.28	-	0.37	0.40
Benzo[ghi]perylene	1.71	11.67	1.79	3.40	1.90
Benzo[k]fluoranthene	53.60	177.18	42.55	80.01	43.10
Chrysene	-	0.09	-	0.07	-
Dibenzo[ah]anthracene	1.31	8.22	0.83	-	16.63
Fluoranthene	-	0.06	-	-	0.15
Fluorene	0.11	-	0.06	0.17	0.43
Indeno[1,2,3-cd]pyrene	29.48	111.50	32.06	30.06	26.37
Naphthalene	7.17	0.06	0.05	0.03	0.05
Phenanthrene	0.03	0.13	0.04	0.21	0.27
Pyrene	0.20	-	-	-	-

AH – aliphatic hydrocarbons, UCM – unresolved complex mixture, Σ PAHs – total polycyclic aromatic hydrocarbons. Data are expressed in µg g⁻¹ dry weight.

The AH concentrations ranged from 168.67 $\mu\text{g g}^{-1}$ dry weight (dw) in S5 to 39.65 $\mu\text{g g}^{-1}$ dw in S4, in the following order: S5>S1>S3>S2>S4. The values of petroleum hydrocarbons present in the UCM ranged from 2159.83 $\mu\text{g g}^{-1}$ dw at S5 to 360.81 $\mu\text{g g}^{-1}$ dw at S4, in the following order: S5>S2>S3>S1>S4. For the total PAHs, the highest value (549.56 $\mu\text{g g}^{-1}$ dw) was found at S2, and presented the following order: S2>S4>S5>S1>S3. Regarding the results of the 16 priority PAHs, the major contributions for the total PAHs levels present in mussel tissues were given by benzo[a]pyrene, benzo[k]fluoranthene and indeno[1,2,3-cd]pyrene, corresponding approximately to 95% of this fraction. The pattern for total petroleum hydrocarbons in mussel tissues presented the following pattern: S5>S2>S3>S1>S4.

1.3.3. Biomarkers

The results of the biomarkers are presented in Figure 1.2 and Table 1.2. One-way ANOVA revealed significant differences among sampling sites for the following oxidative stress parameters determined in *M. galloprovincialis* digestive glands (SOD: $F_{4,45} = 29$, $p \leq 0.001$; CAT: $F_{4,45} = 24$, $p \leq 0.001$; GPx: $F_{4,45} = 60$, $p \leq 0.001$; GR: $F_{4,45} = 25$, $p \leq 0.001$; GST: $F_{4,45} = 3.2$, $p \leq 0.05$; LPO: $F_{4,45} = 19$, $p \leq 0.001$; tGSx: $F_{4,45} = 17$, $p \leq 0.001$; GSH: $F_{4,45} = 17$, $p \leq 0.001$; GSSG: $F_{4,45} = 29$, $p \leq 0.001$; GSH/GSSG: $F_{4,45} = 27$, $p \leq 0.001$) and gills (SOD: $F_{4,45} = 8.5$, $p \leq 0.001$; CAT: $F_{4,45} = 4.5$, $p \leq 0.05$; GPx: $F_{4,45} = 38$, $p \leq 0.001$; GR: $F_{4,45} = 13$, $p \leq 0.001$; GST: $F_{4,45} = 12$, $p \leq 0.001$; tGSx: $F_{4,45} = 3.2$, $p \leq 0.05$; GSH: $F_{4,45} = 3.7$, $p \leq 0.05$; GSH/GSSG: $F_{4,45} = 2.8$, $p \leq 0.05$). Nevertheless, the Tukey multi-comparison test did not provide evidence of significant differences among sampling sites for tGSx determined in mussels' gills. One-way ANOVA also revealed that levels of LPO ($F_{4,45} = 1.9$, $p > 0.05$) and GSSG ($F_{4,45} = 2.4$, $p > 0.05$) measured in the gills of *M. galloprovincialis* did not exhibit significant differences among sampling sites. Results of biochemical parameters related to energetic metabolism revealed significant differences among sampling sites (IDH: $F_{4,45} = 66$, $p \leq 0.001$, ODH: $F_{4,45} = 3.0$, $p \leq 0.05$).

The values of SOD activity recorded in digestive glands of mussels collected at S3-S5 were significantly higher than those recorded in mussels collected at S1 and S2. In gills, SOD activity values were significantly higher in mussels collected at S5 relatively to all the other sites except S1 (Figure 1.2).

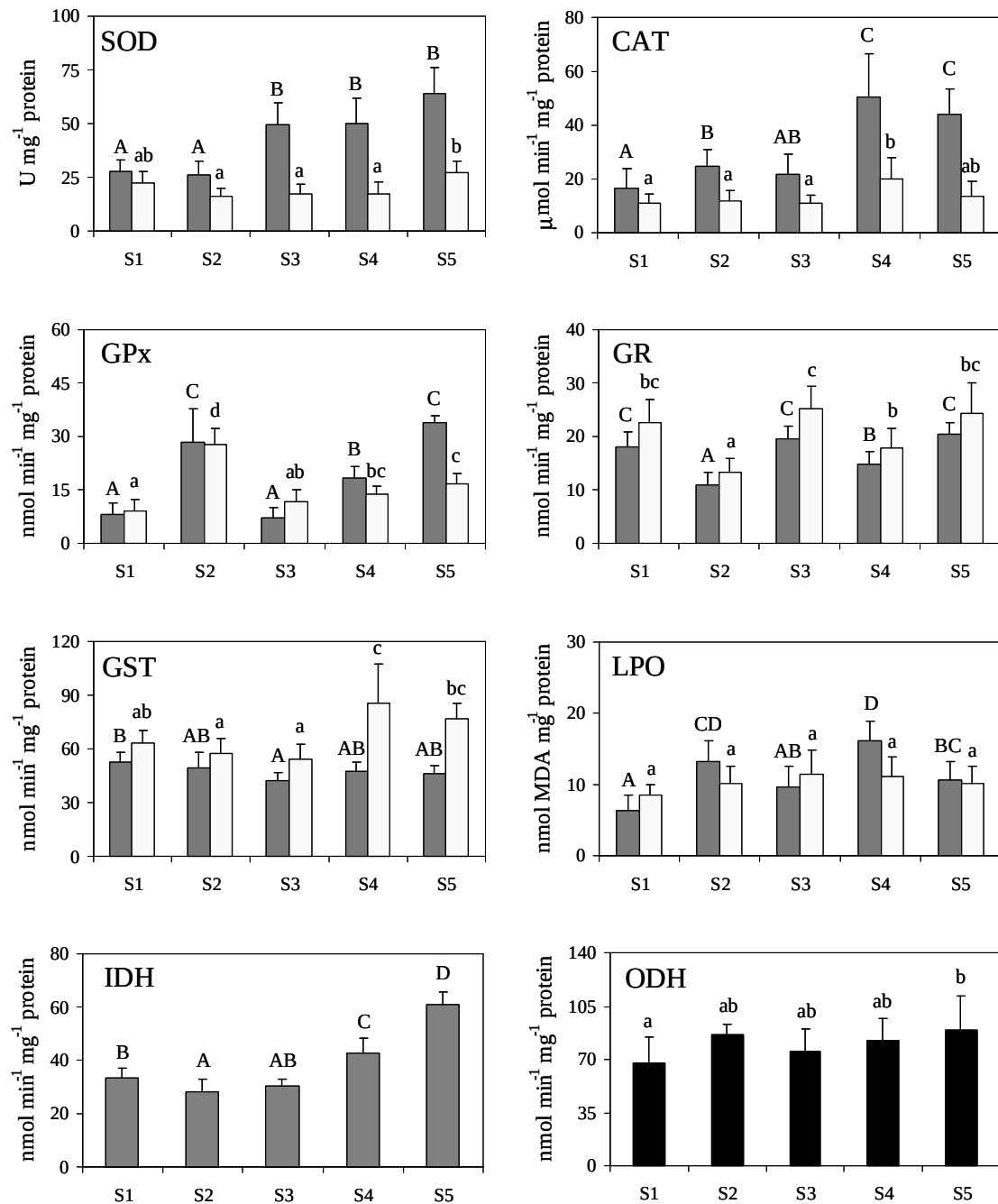


Figure 1.2 Biomarkers analysed in *Mytilus galloprovincialis* collected at five sampling sites (S1-S5) along the NW coast of Portugal. Values are presented as mean $\pm$ standard deviation ($n = 10$) of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GR), glutathione S-transferases (GST), lipid peroxides (LPO), NADP⁺-dependent isocitrate dehydrogenase (IDH), and octopine dehydrogenase (ODH). Different letters indicate significant differences among sampling sites by Tukey honestly significant difference multiple-comparison test ($p \leq 0.05$) for each biomarker. Capital letters indicate differences in the digestive gland (■) and small letters indicate differences in gills (□) for SOD, CAT, GPx, GR, GST and LPO. Capital letters also indicate differences in digestive glands (■) for IDH, and small letters also indicate differences in posterior adductor muscle (■) for ODH.

The values of CAT activity recorded in digestive glands of mussels collected at S4 and S5 were significantly higher than those recorded in mussels from the remaining sampling sites. Mussels from S2 exhibited significantly higher activity levels than those from S1, but similar to those collected in S3. In gills, significantly higher levels of activity were found in specimens collected at S4 compared to the remaining stations, except S5 (Figure 1.2).

The values of GPx activity in digestive glands of mussels from sites S2, S4, and S5 were significantly higher than those from S1 and S3, with S2 and S5 showing the most significant values. In gills, significantly higher GPx activity values were found in mussels from S2 when compared with animals from the remaining sites. Mussels collected at S4 showed GPx activity levels similar to those collected at S3 and S5, and significantly higher activity values than those collected at S1 (Figure 1.2).

The GR activity values found in mussels' digestive glands were significantly lower in animals from S2, while those from S1, S3 and S5 presented the higher significant values. In gills, the GR activity values were significantly lower in mussels collected at S2 relatively to those from the remaining sites, being the significantly higher activity levels found at S3 (Figure 1.2).

Significantly higher GST activity values were found in mussels' digestive glands collected at S1 relatively to mussels from S3, with no significant differences found among the remaining sites. In gills, the significantly higher GST activity values were found in mussels from S4. Mussels collected at S5 exhibited significantly higher activity levels compared to those from S2 and S3, but not from S1 (Figure 1.2).

Cell redox status is regulated by the equilibrium between the levels of GSH and GSSG. The values for the glutathione quantification are presented in Table 1.2. The significantly lower values of GSH/GSSG ratio were found in the digestive glands of mussels collected at S4 (as a result of lower levels of GSH) and S5. In gills, no significant differences were found in cellular GSH/GSSG equilibrium among sampling sites, despite the significantly higher values of GSH in mussels from S3 (Table 1.2).

The significantly higher LPO levels in mussels' digestive glands were found at S4. Mussels from S2 presented significantly higher LPO levels relatively to S1 and S3, but not relatively to S5. In gills, no significant differences were found in LPO levels among sampling sites (Figure 1.2).

The IDH activity values recorded in mussels collected at S5 were significantly higher than those found in animals from the remaining sites. Mussels from S4 presented IDH activity values significantly higher than S1-S3. In addition, mussels from S1

demonstrated significantly higher activity values than those from S2, but not than those from S3 (Figure 1.2).

Activity levels of ODH found in mussels collected at S5 were significantly higher than those collected from S1, but not significantly higher than the remaining sampling sites (Figure 1.2).

Table 1.2 Total glutathione content, reduced glutathione, oxidised glutathione, and glutathione redox status analysed in *Mytilus galloprovincialis* collected at five sampling sites (S1-S5) along the NW coast of Portugal.

Sites	Tissue	Biomarkers			
		tGSx	GSH	GSSG	GSH/GSSG
S1	Digestive gland	16.8 ± 2.64 ^B	13.8 ± 2.43 ^{BC}	2.98 ± 0.37 ^A	9.26 ± 1.45 ^B
	Gill	6.89 ± 2.84 ^a	4.84 ± 1.68 ^a	2.05 ± 1.21 ^a	6.10 ± 3.37 ^a
S2	Digestive gland	20.4 ± 4.86 ^B	16.8 ± 4.26 ^C	3.57 ± 0.96 ^A	9.70 ± 2.19 ^B
	Gill	7.11 ± 2.84 ^a	4.58 ± 1.77 ^a	2.53 ± 1.10 ^a	3.70 ± 0.54 ^a
S3	Digestive gland	16.2 ± 2.21 ^B	12.8 ± 2.07 ^B	3.43 ± 0.74 ^A	7.75 ± 1.83 ^B
	Gill	10.2 ± 3.63 ^a	7.49 ± 2.38 ^b	2.69 ± 1.26 ^a	6.07 ± 1.37 ^a
S4	Digestive gland	10.0 ± 2.83 ^A	6.16 ± 3.06 ^A	3.86 ± 1.04 ^A	3.65 ± 2.46 ^A
	Gill	7.76 ± 2.04 ^a	5.00 ± 2.03 ^{ab}	2.67 ± 1.22 ^a	4.59 ± 2.46 ^a
S5	Digestive gland	20.4 ± 3.28 ^B	12.7 ± 2.52 ^B	7.74 ± 1.73 ^B	3.37 ± 0.81 ^A
	Gill	9.97 ± 2.31 ^a	6.17 ± 1.97 ^{ab}	3.80 ± 1.98 ^a	3.99 ± 2.06 ^a

Values are presented as mean ± standard deviation ($n = 10$) for total glutathione content (tGSx), reduced glutathione (GSH), oxidised glutathione (GSSG), and glutathione redox status (GSH/GSSG ratio). Different letters indicate significant differences among sampling sites identified by Tukey honestly significant difference multiple-comparison test ($p \leq 0.05$) for each biomarker. Capital letters indicate differences in the digestive gland and small letters indicate differences in gills. Data are expressed in nmol glutathione equivalents mg^{-1} protein.

1.3.4. Effects of petroleum hydrocarbons and abiotic parameters on biomarkers

Significant Pearson correlation values ($p \leq 0.01$) were found between some biomarkers and petroleum hydrocarbons levels in mussels' tissue, as well as between some biomarkers and the abiotic parameters quantified in water samples from the selected sampling sites (Table 1.3 and 1.4).

The most significant positive correlations ($r > 0.50$) between biomarkers and petroleum hydrocarbons were found between AH levels and the activities of SOD in gills, GR in digestive glands and IDH also in digestive glands; between UCM levels and the activities of GPx and IDH in digestive glands, and SOD both in mussels' gills and digestive glands; and between PAHs levels and the activities of GPx in mussels' gills. Significant negative correlations were found between the PAHs levels and the activities of GR both in mussels' gills and digestive glands (Table 1.3).

Table 1.3 Significant Pearson correlation values ($p \leq 0.01$) between petroleum hydrocarbon levels and biomarkers quantified in *Mytilus galloprovincialis* collected at five sampling sites (S1-S5) along the NW coast of Portugal.

Petroleum hydrocarbons	Biomarkers			
	SOD	GPx	GR	IDH
AH	0.646 ^b	-	0.579 ^a	0.731 ^a
UCM	0.524 ^a	0.641 ^a	-	0.728 ^a
	0.514 ^b			
Σ PAHs	-	0.833 ^b	-0.733 ^a	-
			-0.664 ^b	

AH – aliphatic hydrocarbons, UCM – unresolved complex mixture, Σ PAHs – total polycyclic aromatic hydrocarbons, SOD – superoxide dismutase, GPx – glutathione peroxidase, GR – glutathione reductase, IDH – NADP⁺-dependent isocitrate dehydrogenase. ^a Digestive glands; ^b gills.

Regarding the correlations between the biomarkers and the abiotic parameters, significant positive correlations were found between salinity and GR activity in both tissues; between pH and GPx activities in digestive glands; between nitrates and CAT, SOD and IDH activities in digestive glands, and GST activities in gills; between ammonia and CAT, SOD, GPX and IDH activities in mussels' digestive glands and in GST in mussels' gills; and between phosphates and the activities of SOD in gills and IDH in digestive glands. Significant negative correlations were found between temperature and GPx activity in gills; between salinity and GPx activity in both tissues; between pH and GR activity in mussels' gills; between nitrites and SOD activity in digestive glands; between nitrate and ammonia levels and the GSH/GSSG ration in mussels' digestive glands (Table 1.4).

Table 1.4 Significant Pearson correlation values ($p \leq 0.01$) between abiotic parameters quantified in water samples and biomarkers determined in *Mytilus galloprovincialis* collected at five sampling sites (S1-S5) along the NW coast of Portugal.

Abiotic parameters	Biomarkers						
	CAT	SOD	GPx	GR	GST	GSH/GSSG	IDH
T	-	-	-0.617 ^b	-	-	-	-
S	-	-	-0.636 ^a	0.697 ^a	-	-	-
			-0.869 ^b	0.655 ^b			
pH	-	-	0.574 ^a	-0.505 ^b	-	-	-
NH ₄	0.707 ^a	0.658 ^a	0.654 ^a	-	0.579 ^b	-0.759 ^a	0.907 ^a
NO ₃	0.647 ^a	0.513 ^a	-	-	0.685 ^b	-0.727 ^a	0.683 ^a
NO ₂	-	-0.578 ^a	-	-	-	-	-
PO ₄	0.601 ^b	-	-	-	-	-	0.566 ^a

T – temperature, S – salinity, NH₄ – ammonia, NO₃ – nitrate, NO₂ – nitrite, PO₄ – phosphates, CAT – catalase, SOD - superoxide dismutase, GPx – glutathione peroxidase, GR – glutathione reductase, GST - glutathione S-transferases, GSH/GSSG – glutathione redox status, IDH – NADP⁺-dependent isocitrate dehydrogenase.

^a Digestive glands; ^b gills.

1.3.5. Integrated data analysis

The results of the RDA analysis are presented in the tri-plot ordination diagram of Figure 1.3. The first two axes of the RDA analysis accounted for 82.2% of the overall variability of the data. Therefore, the other axes were neglected because they did not provide significant additional information. The first RDA axis (horizontal) accounted for 63.4% of the total variability and was responsible for a clear separation of the sampling sites S1-S3 from the sites S4 and S5 (Figure 1.3). The environmental factors that most contributed for this separation were nitrates, ammonia and UCM, which seem to have a major influence in the activities of IDH in mussels' digestive glands, GST in gills, as well as CAT and SOD quantified in both tissues. Furthermore, the levels of nitrates, ammonia and UCM seem to influence negatively the GSH/GSSH ratio of mussels' digestive glands. The second RDA axis (vertical) accounted for 18.8% of the total variability of the data and was responsible for the clear separation of the sampling site S2 from the sites S1 and S3 (Figure 1.3). This separation is clearly defined by the levels of PAHs, salinity, and in less extent by the pH. The activity of GPx in mussels' gills seem to be strongly influenced by the PAHs levels, while the activity of this enzyme measured in mussels'

digestive glands seem to be mainly influenced by the pH. In addition, the activity of ODH and the LPO levels in mussels' digestive glands also seem to be related with the pH.

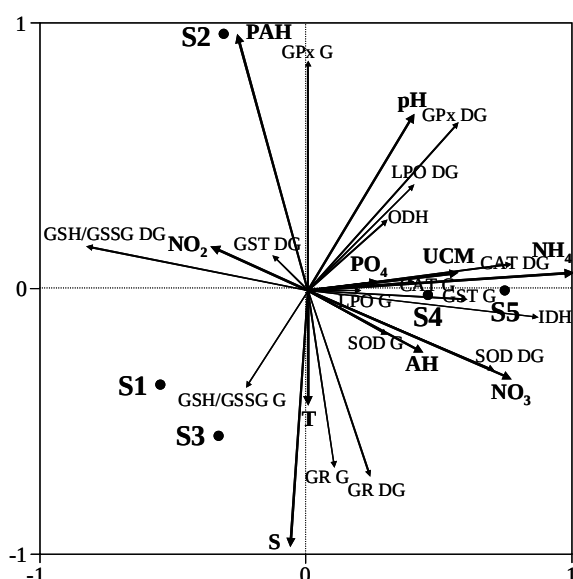


Figure 1.3 Redundancy analysis (RDA) ordination diagram with sampling sites (●), environmental parameters (thick arrows), and biomarkers (thin arrows); first axis is horizontal, second axis is vertical. The environmental parameters measured in five sampling sites (S1-S5) along the NW coast of Portugal are T – temperature, S - salinity, NH₄ - ammonia, NO₃ – nitrates, NO₂ – nitrites, PO₄ – phosphates, AH – aliphatic hydrocarbons, UCM – unresolved complex mixture, and PAH – polycyclic aromatic hydrocarbons. The biomarkers quantified in *Mytilus galloprovincialis* digestive glands (DG) and gills (G) are SOD – superoxide dismutase, CAT – catalase, GPx - glutathione peroxidase, GR – glutathione reductase, GST – glutathione S-transferases, LPO – lipid peroxides, IDH – NADP⁺-dependent isocitrate dehydrogenase, ODH – octopine dehydrogenase, and GSH/GSSG – glutathione redox status.

1.4. DISCUSSION

In recent decades, several monitoring programs have been undertaken using *M. galloprovincialis* as a sentinel organism to investigate the exposure to petrochemical products. A considerable number of these studies have focused mainly on the accumulation and distribution of petroleum hydrocarbons within mussel tissues [37, 38], as opposed to the deleterious effects of contaminants on the biota [39]. The recognition that free radical reactions are important both in normal biological processes, as well as in toxicity mechanisms induced by contaminants, has lead to a considerable increase in the

application of oxidative stress biomarkers in several aquatic organisms ^[7]. Using wild specimens of *M. galloprovincialis* collected at five sampling sites along the NW coast of Portugal, this study aimed to assess several enzymatic and non-enzymatic antioxidant defences in order to evaluate the antioxidant status of mussels potentially exposed to different sources of petrochemical contamination, and to evaluate their applicability as biomarkers. Similar approaches have been used by other authors considering the evaluation of the response of antioxidant defences in natural populations of *Mytilus* spp. exposed to metals ^[40, 41], organic contaminants ^[42, 43, 9], and complex mixtures of contaminants ^[44, 45].

Chemical analyses of petroleum hydrocarbon have not been monitored regularly in the NW coast of Portugal, thus, it is not possible to establish temporal trends for these contaminants. Nevertheless, the levels of PAHs quantified in *M. galloprovincialis* tissues during the present study (124.21 $\mu\text{g g}^{-1}$ S3 to 549.56 $\mu\text{g g}^{-1}$ in S2) were higher than those determined in 1998 in mussels collected in the region between S3 and S5 (0.60 $\mu\text{g g}^{-1}$ to 40.00 $\mu\text{g g}^{-1}$) ^[20]. The petroleum hydrocarbon levels found in the present work are also in the same range of values determined in *M. galloprovincialis* collected along the NW Mediterranean coast ^[46, 37]. In the present study, results of chemical analyses performed in mussel tissues showed that sampling sites S2 and S5, located in Viana do Castelo and Leixões harbour respectively, presented the highest levels of total petroleum hydrocarbons, with S2 presenting the highest levels of PAHs, and S5 presenting the highest levels of AH and UCM. UCM is a fraction of petrochemical hydrocarbons commonly found in mussel tissues, and comprises both aromatic and non aromatic compounds ^[47], however the toxicity of UCM has not been extensively studied ^[47, 48]. It is known that non-aromatic hydrocarbons present in UCM have low toxicity to mussels; nevertheless, the oxidation of these compounds can enhance toxicity mechanisms in aquatic organisms ^[47]. Moreover, it has been reported by Rowland et al. (2001) ^[48] and Donkin et al. (2003) ^[47] that aromatic hydrocarbons present in UCM enhanced non-specific narcotic responses in *M. edulis* exposed to this petroleum fraction.

Sampling sites S1 and S3, located in open shore, presented the lowest levels of PAHs compared to the remaining sampling sites, and presented lower levels of total petroleum hydrocarbons than S2 and S5. Finally, sampling site S4, that is located in the vicinity of an oil refinery, surprisingly presented the lowest levels of total petroleum hydrocarbons. However this is due to low levels of AH and UCM, since S4, with the exception of S2, is the sampling site that presented the highest PAHs levels. The PAHs levels found in S4 during the present work (164 $\mu\text{g g}^{-1}$ dw) were higher than those found in mussels collected in 1998 (0.60-40.00 $\mu\text{g g}^{-1}$ dw) in the surrounding area of the oil

refinery^[20]. In accordance to Villeneuve et al. (1999)^[46], a low proportion of UCM relatively to the total petroleum hydrocarbons, as found in mussels from S4, may suggest recent discharges of petrochemical products to the environment. In fact, and as suggested by Wetzel and Van Vellet (2004)^[37] high levels of UCM are indicative of weathering processes. Considering the toxicity of PAHs to animals, it seems important to rank the sampling sites according to the concentrations of these compounds. Therefore, based on the PAHs concentration determined in whole body of local mussels, the ranking is: S2 (high contamination) > S4 and S5 (moderate contamination) > S1 and S3 (low contamination).

The present study illustrated that mussels collected at S4 and S5 presented significantly higher levels of CAT and SOD activity in the digestive glands than those collected at the remaining sites. Previous studies showed that antioxidant enzymes of *Mytilus* spp.^[49], *Perna viridis*^[50], and *Chamaelea gallina*^[51] also demonstrated higher activity values in response to organic contaminants. Considering that the induction of antioxidant enzymes represents a protective response to eliminate ROS resulting from contamination exposure, it has been hypothesised that such increase may be related to adaptations to contaminant induced stress^[50, 7]. However, the induction of antioxidant enzyme activity due to the presence of high levels of contaminants in the environment should not be considered as being a general rule, since a considerable variation of responses has been found among different species, following exposure to single or complex mixture of contaminants^[7]. For example, under laboratory conditions, some authors have reported a decrease in antioxidant enzyme activities following short-term exposure of *M. galloprovincialis* to resin acids^[52] and to metals^[41]. Some authors suggested that relatively short exposure periods, normally no more than seven days, may induce a transient decrease in antioxidant enzyme activities, which can be followed by the induction of the antioxidant system. Thus, an increase in the activity of antioxidant enzymes may reflect an adaptation to the chronic exposure to high levels of contamination, since this would confer increased protection from oxidative stress^[50, 53]. SOD and CAT, as the first lines of antioxidant defences, are very responsive to increasing levels of contaminant stimulated ROS production. For example, Porte et al. (1991)^[49] demonstrated increasing SOD and CAT activity values due to petroleum hydrocarbon accumulation in mussel tissues. In the present work significant correlations were found between SOD activities determined in mussel gills and AH levels, as well as between SOD activity in both tissues and UCM levels. However, no significant correlation values were found between CAT activities and petroleum hydrocarbons.

This study also demonstrated that mussels collected at sites S4 and S5 presented significant higher levels of GST activity in gills than those collected at the remaining sites. Several other field studies have demonstrated a similar relationship between environmental contamination and GST activity in mussels ^[50, 17]. In the present work, although GST activity levels in mussels' gills increased at S4 and S5, no significant changes in GST activity were found in the digestive gland. This result may be due to the fact that toxic intermediates produced in the digestive gland during contaminant metabolism may inactivate the enzyme, resulting in reduced GST activity levels in this organ, as previously discussed by Cheung et al. (2001) ^[50] in studies with *P. viridis*; however, further studies need to be performed to confirm this hypothesis. Additionally, since gills experience higher exposure to environmental contaminants than digestive gland, they may present higher detoxification rates, and consequently higher GST activities ^[50]. The high GST activity in gills may also compensate the low CAT activity levels found in this organ since GST also presents peroxidase activity ^[54].

Both the detoxification of contaminants, through the action of GST, and the detoxification of ROS, through the action of some antioxidant enzymatic defences, may lead to depletion of GSH. For example, GPx promotes the oxidation of GSH to GSSG to eliminate organic and inorganic peroxides from the organism. As GSSG accumulates, and to maintain the cellular redox balance, it must be reduced to GSH by GR at the expense of NADPH, which needs to be regenerated by the pentose phosphate pathway or by NADP⁺-dependent IDH ^[31, 55, 56]. If the production of GSSG is higher than the regeneration of GSH, GSSG accumulates and is translocated outside the cell by specific transporters to avoid NADPH exhaustion. This may cause the depletion of cellular GSH and the disruption of the cellular redox balance ^[31, 55, 56]. A similar situation was observed in the present work, with mussels collected at the sites S4 and S5 demonstrating significantly lower GSH/GSSG rates in digestive glands. These low GSH/GSSG rates are due to low levels of GSH and may be explained, to some extent, by the high GPx and low GR activity levels found in the same mussels. However, for a complete understanding of the cellular mechanisms that regulate GSH/GSSG cellular balance, further studies concerning the activities of the enzymes involved in the GSH synthesis (γ -glutamylcystein synthetase and GSH synthetase) and GSH cellular transport (γ -glutamyl transpeptidase) should be performed. As previously referred, the low GSH/GSSG rates found in the digestive glands of mussels collected at S4 and S5, may also be related to the low GST activity levels found in this organ. Similar results were also found in *M. galloprovincialis* ^[52] and *Perna viridis* ^[50].

In the present work, significant correlations were found between PAHs levels and the enzymatic activities of GPx in mussels' gills, as well as with GR activities both in gills and digestive glands. A significant correlation was also found between the UCM levels and the activities of GPx determined in mussels' digestive gland, as well as between the levels of AH and the GR activity in mussels' digestive glands. Both GPx and GR seem to be suitable biomarkers to assess petrochemical contamination.

Oxidative damage, such as that induced by LPO, may be associated with some aspects of impaired cellular or higher biological function, including disease ^[7]. Significantly higher LPO levels were found in digestive glands of mussels collected at S2, S4 and S5, despite the high activity levels of CAT, SOD and GPx. This may be due to the fact that low levels of contaminant-stimulated ROS can have a significant toxic effect, particularly upon the cell membrane and DNA, even when antioxidant enzymatic defences are responding ^[52]. Cell membrane damage induced by LPO ^[57] may be related to the depletion of GSH, since changes in membrane permeability can decrease GSH cellular levels by allowing faster ROS intake and GSH loss ^[55].

Presently, the biochemical role of NADP⁺-dependent IDH is not completely understood. Recent studies suggested that it may function as regulator of cellular defences against oxidative stress, mainly by the regeneration of NADPH oxidised by GR during the reduction of GSSG to GSH ^[11, 12, 58]. Consequently, this enzyme may have an important role in antioxidant defence regulation since it is directly related with the maintenance of the cellular redox balance. The results obtained in the present work showed that mussels collected from S4 and S5 exhibited significant higher IDH activity levels than those collected at the remaining sampling sites. In contrast, mussels from S2 had lower IDH activity levels than those from S1, indicating lower levels of NADPH regeneration, which is in accordance with the lower GR activity levels found in digestive glands of mussels collected at S2. However, high IDH activity levels found in mussels from S4 are not in accordance with the low GR activity levels found in the digestive glands of mussels collected at that site, and further studies need to be performed in order to understand the relationship between these two antioxidant enzymes. Significant correlation levels were found between the IDH activity levels and the levels of both AH and UCM. Prior to the application of this biochemical parameter as a biomarker for petrochemical contamination, further studies need to be performed to investigate its responsiveness to other contaminants, such as PCBs or metals.

ODH is a pyruvate oxidoreductase enzyme involved in the anaerobic metabolism of several invertebrates, with a function similar to lactate dehydrogenase in vertebrates, which regenerates NAD⁺ during anaerobic glycolysis ^[59]. The study of this respiratory

enzyme is of significance, since impairment in the energetic metabolism of marine bivalves in the presence of petroleum hydrocarbons has been reported ^[60]. Under stressful conditions, such as environmental contamination by petrochemical products, mussels reduce cellular respiration as an attempt to conserve energy ^[59, 60]. Thus, the rate of cellular oxygen uptake may be insufficient and, as such, anaerobic metabolism may be enhanced to cope with this respiratory deficit and to supply extra ATP ^[59, 60]. Significant differences in ODH activity were only found between mussels collected at S5 and S1. Considering that IDH may have a function in the regulation of the citric acid cycle, and since both IDH and ODH activity levels were higher in mussels collected from the contaminated site S5, we hypothesise that mussels may employ both aerobic and anaerobic metabolism to obtain more energy levels to cope with contaminant induced stress. To date, the studies involving IDH and ODH have only focused upon their biological function ^[11, 12, 34, 61, 62, 63] and to our knowledge, this was the first time that they were applied as biomarkers in field studies with mussels. Due to the current lack of data, additional studies are required prior to their general application in monitoring programs.

The ordination diagram obtained by the RDA analysis clearly distinguished three sampling site assemblages that are related with nitrates, ammonia and UCM (first axis) and with the salinity, pH, and PAHs levels (second axis). The sampling site S2 appears isolated from the remaining, S4 and S5 appear in one group, and S1 and S3 appear in a second group (Figure 1.3). The separation of the sampling sites in these three groups is in agreement with the PAHs levels found in mussels' tissues (Table 1.1). The RDA analysis and the Pearson correlation also indicated that some of the selected biomarkers may be influenced by abiotic factors (Figure 1.3 and Table 1.4). The IDH activity presents a positive correlation with the AH and UCM levels, however, it also presents a positive correlation with nitrates, ammonia and phosphates. Therefore, an increase in IDH activity due to the presence of petroleum hydrocarbons should be well analysed when high concentrations of the previous nutrients are present in the environment. Likewise, the activities of CAT, SOD, GPx and GSH/GSSG ratio in digestive glands, as well as GST in gills seem to be influenced by the nitrate and ammonia levels present in the environment. Moreover the effects of salinity and pH on the activities of GPx and GR in both mussels' gills and digestive glands should be considered since the salinity has an opposite effect, and pH has a similar effect, as the PAHs levels have in these enzymes. Therefore, the influence of abiotic factors should be taken into consideration in studies where the selected biochemical parameters are applied as biomarkers.

1.5. CONCLUSIONS

In conclusion, the battery of biochemical parameters applied as biomarkers in the present work, including mussels' antioxidant defences measured in two distinct tissues and energetic metabolism enzymes, as well as petroleum hydrocarbon quantified in mussels' tissue and abiotic parameters determined in water samples, provided a discrimination of sites with different levels of petrochemical contamination after redundancy analysis. Significant correlations between some of the biomarkers and abiotic parameters were found suggesting that further studies on this question, namely with nitrates and ammonia, should be performed. This work represents the first stage of a monitoring program that is being developed in wild populations of *M. galloprovincialis* along the NW coast of Portugal to evaluate the effects of petroleum products on biochemical parameters involved in physiological functions determinant for the survival and performance of the animals. Future surveys will evaluate seasonal variation in these biochemical parameters and allow the determination of basal enzymatic activity levels in wild populations of *M. galloprovincialis* along the NW coast of Portugal over more than a year. This approach constitutes a research strategy that has been recommended [64, 65] and that is important to separate effects due to chemical contamination from those due to natural fluctuations of both water physicochemical parameters and mussels' annual physiological cycle.

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CHAPTER 2

Multivariate and graphical analysis of biomarker responses as a tool for long-term monitoring: a study of petrochemical contamination along the NW coast of Portugal

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ABSTRACT

An environmental monitoring program was conducted for twelve months to assess the spatial and temporal trends of petrochemical contamination along the NW coast of Portugal and its effects on wild populations of the mussel *Mytilus galloprovincialis*. During this period mussels were collected every three months at five sampling sites for analysis of petroleum hydrocarbon content. Likewise, biochemical parameters involved in key physiological processes of mussels (antioxidant defences, detoxification, energetic metabolism and neurotransmission) were used as biomarkers. The implementation of this monitoring program followed a pilot survey conducted to evaluate the suitability of the selected monitoring strategy to assess petrochemical contamination (see Chapter 1). In this first survey, a good correlation was found between the levels of petroleum hydrocarbons and some of the selected biomarkers. These results led us to investigate the effect of seasonality on the biomarkers response by correlating them with abiotic parameters quantified in water samples collected at each sampling site and sampling season. Multidimensional scaling and cluster analysis illustrated a clear separation of the sampling sites as function of the biomarker response. Biomarkers quantified in mussels sampled from sites which were less impacted exhibited significant differences in their response throughout the sampling period, while those quantified in mussels sampled from sites which were more impacted did not exhibit these seasonal fluctuations. This suggests that the effect of high levels of contamination may overlap those of abiotic factors. Additionally, the results of the principal component analysis and the BIOENV test showed that the response of the selected biomarkers over time was more correlated with the levels of unresolved complex mixture (UCM) than with individual polycyclic aromatic hydrocarbons. In particular, the activity of octopine dehydrogenase presented a significant positive correlation with the levels of UCM and apparently was not significantly influenced by seasonality. Herein, the use of multivariate and graphical analysis is demonstrated to be a good approach to integrate environmental monitoring data.

Keywords: *Mytilus galloprovincialis*, biomarkers, petrochemical hydrocarbons, multivariate analysis, environmental monitoring

2.1. INTRODUCTION

As a result of the degradation of the marine environment due to the chronic release of contaminants there is a need to develop internationally accepted long-term monitoring programs to assess the impact of contaminants on the marine environment ^[1].

The NW coast of Portugal is particularly exposed to chronic petrochemical contamination due to the presence of maritime harbours and oil refining industry. Moreover, the proximity to important maritime traffic routes also increases the risk of navigation accidents and oil spills ^[2]. Recently, the field works developed by Salgado and Serra (2001) ^[3], Moreira et al. (2004) ^[4] and Lima et al. (2007) ^[5] with *Mytilus galloprovincialis*, as well as Lima et al. (2008) ^[6] with *Lipophrys pholis*, highlighted for the levels and effects of petrochemical contamination in the NW coast of Portugal. Nevertheless, even considering the quoted studies, there is a recognised scarceness of biological and chemical data regarding petrochemical contamination in this area of the Iberian Peninsula, particularly concerning seasonal variation ^[5]. In an effort to address the current need for ecotoxicological data, an environmental monitoring program was established during twelve months to assess the spatial and temporal trends of petrochemical contamination along the NW coast of Portugal using the marine mussel *M. galloprovincialis* as bioindicator. Bivalve molluscs, particularly marine mussels such as *M. galloprovincialis*, have been used as indicator organisms in environmental monitoring programmes since the “Mussel Watch” program established in the mid 1970s ^[7]. These organisms are suitable indicators in environmental monitoring programs mainly because they are filter-feeders with very low metabolism, which results in the bioaccumulation of many chemicals in their tissues ^[8]. Moreover, mussels appear to be better bioindicators than fish when assessing the effects of chronic petrochemical contamination because certain compounds of petrochemical products, such as polycyclic aromatic hydrocarbons (PAHs) are highly biodegradable by vertebrates and tend not to accumulate in their tissues in concentrations that reflect long-term exposure ^[9].

In the present work, mussels were collected periodically from five sampling sites for analysis of petroleum hydrocarbon content, namely aliphatic hydrocarbons (AH), unresolved complex mixture (UCM) and PAHs. Likewise, several biochemical parameters involved in key physiological processes of mussels were applied as biomarkers. The enzymatic activities of total superoxide dismutase (SOD), catalase (CAT), selenium-dependent glutathione peroxidase (GPx), glutathione reductase (GR), and glutathione S-transferases (GST) were quantified to evaluate the mussels' antioxidant status and/or

detoxification processes. Moreover, levels of reduced and oxidised glutathione (GSH and GSSG respectively) were assessed to quantify cellular redox status (GSH/GSSG), and lipid peroxides (LPO) were determined to provide an indication of cellular oxidative damage induced by petrochemical contamination. The activity of NADP⁺-dependent isocitrate dehydrogenase (IDH) was determined as part of the mussels' antioxidant defence system and energetic aerobic metabolism, while octopine dehydrogenase (ODH) was determined to investigate the response of mussels' energetic anaerobic processes to this class of contaminants. Finally, acetylcholinesterase (AChE) activity was quantified to assess mussels' neurotransmission levels. The implementation of this monitoring program followed a pilot survey conducted to obtain preliminary data used to evaluate the suitability of the selected monitoring strategy to assess petrochemical contamination, which is a research strategy that has been recommended by Clarke and Green (1988) ^[10]. In this first survey, a good correlation was found between the levels of petroleum hydrocarbons and some of the selected biomarkers, indicating the suitability of the selected monitoring program (see Chapter 1).

In general, the aquatic environment is exposed to numerous stressors which make the establishment of causal links between the levels of a specific contaminant and the response of biomarkers rather complex ^[11]. One of the strategies employed to investigate the existence of these relationships is the application of long-term biomarker-based monitoring programs ^[11]. However, the lack of an appropriate statistical analysis that integrates the complete set of data (biological, chemical and abiotic) and that allows a clear and easy visual interpretation of the results can limit the full potential of such monitoring strategy ^[11, 12]. The first biomarker-based monitoring programs were mainly designed to assess the existence of biological impairments in wild organisms. However, causal agents were not usually identified ^[11]. As such, monitoring data were mainly analysed using univariate statistical analysis to differentiate impacted from non-impacted sites ^[10]. Technological advances and the need for chemical analysis during the last two decades have increased the complexity of biomarker-based monitoring programs and subsequent analysis ^[11, 12, 13, 14]. Clarke and Green (1988) ^[10] have suggested a statistical design and analysis to study the biological effects of contaminants which is widely used in ecological research, particularly in community studies, which, like in current biomarker-based monitoring programs, account for a large range of variables (biological, chemical and abiotic). The suggested approach consists of a set of multivariate and graphical methods that allow a clearer integration and interpretation of such comprehensive set of data ^[10]. Moreover, this analysis allows the recognition of relationships between contaminant levels and biological responses while accounting for the possible influence of

other factors such as season or abiotic parameters, which is fundamental for the proper interpretation of seasonality in biomarker-based monitoring programs ^[11]. This strategy has been widely applied in ecological research including some works published by our research group ^[15]. However, few examples exist where this approach has been applied to biomarkers ^[16, 17]. The aim of the present work was to establish a long-term monitoring program to assess the spatial and temporal trends of petrochemical contamination along the NW coast of Portugal using *M. galloprovincialis* as a bioindicator, and to propose the application of a set of multivariate and graphical analysis widely used in ecological studies to a biomarker-based monitoring program.

2.2. MATERIAL & METHODS

2.2.1. Sampling sites

The sites selected for the present study are located along the NW coast of Portugal and were chosen according to the level and distinct sources of petrochemical contamination (Figure 2.1).

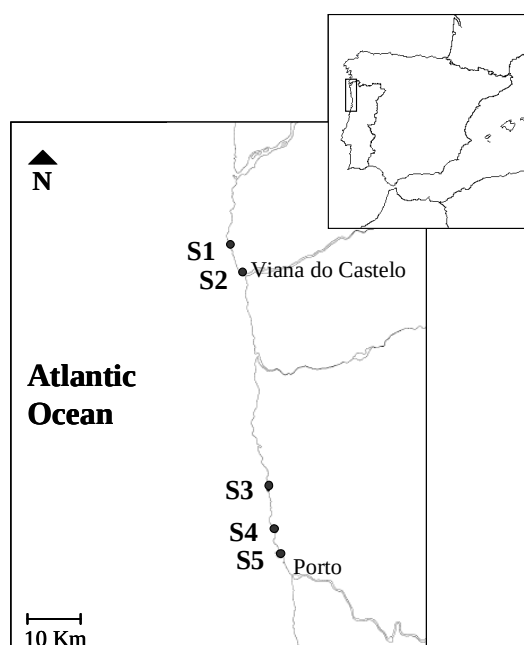


Figure 2.1 Map of the NW coast of Portugal, showing the location of the five sampling sites. S1: Carreço, S2: Viana do Castelo harbour, S3: Vila Chã, S4: Cabo do Mundo, S5: Leixões harbour.

S1 – Carreço (41°44'27"N; 08°52'40"W), is a rocky shore located 10 Km North of Viana do Castelo. Apparently it is free of significant contamination sources. Nevertheless, it is relatively close to the region affected by the “*Prestige*” oil spill ^[18].

S2 – Viana do Castelo harbour (41°41'01"N; 08°50'40"W), is located at the mouth of Lima river. It is continuously subjected to petrochemical contamination through the activity of commercial and fishing vessels. Records exist of the constant release of untreated urban effluents into the river and estuary by several municipalities ^[19]. Additionally, in 2000, this harbour was severely affected by the “*Coral Bulker*” oil spill ^[4].

S3 – Vila Chã (41°17'45"N; 08°44'16"W), is a beach near a fishing village located 25 Km north of Porto. It was selected due to the absence of significant contamination sources, and because it has been used as reference site in previous studies of our laboratory ^[18, 20]. In addition, it has been described as having a high biodiversity of intertidal organisms, indicating low levels of anthropogenic pressure ^[21].

S4 – Cabo do Mundo (41°13'33"N; 08°43'03"W), is a rocky shore with a small watercourse located 14 Km North of Porto. Due to the presence of an oil refinery industry this site has been chronically exposed to petrochemical products, including PAHs ^[3] and heavy metals ^[22]. It has also been reported to be highly impacted in previous studies ^[18, 20].

S5 – Leixões harbour (41°10'58"N; 08°41'55"W), is located 10 Km North of Porto at the mouth of Leça river. It comprises the largest seaport infrastructure in the North of Portugal and is one of the most versatile multi-purpose harbours in the country. Due to intense vessel traffic and to oil terminal activity, the harbour is constantly subjected to petroleum hydrocarbon contamination ^[19]. During the summer 2004, an accident during maintenance activities caused a pipeline leak and subsequent oil spill to the surrounding shore.

2.2.2. Abiotic parameters

Following each mussel sampling, temperature and salinity (Wissenschaftlich Technische Werkstätten –WTW, LF 330 meter, Brüssel, Belgium), as well as pH (WTW, 537 meter) were measured *in situ* at the five sampling sites during low tide. At the same time, subsurface water samples were collected with 1.5 L polyethylene-terephthalate bottles and stored at 4°C for analysis. Prior to nutrient analysis the water samples were vacuum filtered (64 µm) to eliminate any suspension particles that could interfere with the analytical procedure. Levels of ammonia, nitrates, nitrites and phosphates were measured using commercial photometer kits (Photometer 7000, Palintest, Kingsway, England).

2.2.3. Animal sampling

At three monthly intervals, between the autumn of 2005 and the autumn of 2006, fifty adult mussels (mean anterior-posterior shell length of 3.5 ± 1.0 cm) were handpicked during low tide in the intertidal zone of the five sampling sites (Figure 2.1). Following collection, mussels were placed in thermally insulated boxes previously filled with water from the sampling site and immediately transported to the laboratory. Mussels were sacrificed two hours after collection to ensure equal sampling and transport conditions among sites. From each sampling site, the whole tissue of thirty mussels was isolated for chemical analyses. Moreover, the haemolymph of twenty mussels retrieved from each site, was collected with a 2 mL syringe (0.8 × 40 mm needle; Braun, Melsungen, Germany) from the posterior adductor muscle and diluted (1:2) with ice-cold 100 mM potassium phosphate buffer (pH 7.2) (Merck 5101 and 4873) as described in Moreira et al. (2001) ^[23]. From the same mussels, gills, digestive glands and posterior adductor muscles were immediately isolated and pooled into ten groups for each tissue (one tissue portion of two mussels each) for biomarker determinations. Tissue samples, except haemolymph that was used immediately, were frozen in liquid nitrogen and stored at -80°C for a period not exceeding 2 months.

2.2.4. Chemical analyses

A single analysis of petroleum hydrocarbon was performed in pooled tissues of thirty mussels collected at five sampling sites (S1-S5) along the NW coast of Portugal. The analytical procedures for extraction and purification of petroleum hydrocarbons were carried out using the method of CARIPOL/IOCARIBE/UNESCO (1986) ^[24] according to UNEP (1992) ^[25]. Each set of samples was accompanied by a complete blank and a spiked blank which was carried through the entire analytical scheme in identical conditions for all samples. Samples were extracted by homogenisation with a mixture of hexane:methyl chloride (1:1), and an internal standard was added before extraction. The aliphatic and aromatic fractions were purified and separated in three fractions by column chromatography with 10 g each of silica gel/alumina with hexane. The first fraction was eluted with *n*-hexane; the second fraction was eluted with *n*-hexane: methyl chloride (1:1) and the third fraction was eluted only with methyl chloride. The extracts concentrated containing fraction 1 (aliphatic) and fraction 2 and 3 (aromatics) were rotoevaporated to 1 mL and analysed by gas chromatography. Hydrocarbons were quantified using gas

chromatography. Nitrogen was used as carrier gas (flow 1 mL mm⁻¹). The limit of detection for individual aromatic compounds was 0.01 µg g⁻¹ and recovery yields were up to 90%. The aliphatic hydrocarbons (AH) and unresolved complex mixture (UCM) was quantified with an *n*-C28 standard. PAHs were identified by comparing their retention times with those from the aromatic analytical standards by Supelco 48743 according to the priority PAHs from method EPA 610.

2.2.5. Biomarkers

All the biochemical parameters used as biomarkers of antioxidant defence and/or detoxification, as well as oxidative cell damage were determined in mussels' gills and digestive glands. Additionally, IDH was only quantified in mussels' digestive glands because previous studies indicated a very low activity of this enzyme in gill tissue (data not published). The posterior adductor muscle was selected for the quantification of ODH due to the importance of this enzyme on the maintenance of the redox balance of invertebrate muscle tissue during periods of temporary anoxia ^[26]. Finally, AChE was quantified in mussels' haemolymph because this is the tissue in which mussels' AChE presents a higher specific activity when compared with other tissues ^[27].

The activity of SOD was determined according to McCord and Fridovich (1969) ^[28] adapted to microplate. Tissues were homogenised (Ystral homogeniser, Ballrechten-Dottingen, Germany) in 50 mM sodium phosphate buffer (Merck 1.06579 and 1.06345, Damstadt, Germany) with 1 mM ethylenediaminetetraacetic acid disodium salt dihydrate (Na₂-EDTA, Sigma E4884, Osterode, Germany) (pH 7.8) and centrifuged (Sigma 3K) at 15,000 *g* for 15 min at 4°C. The final concentrations of the assay chemicals, in a final volume of 300 µL, were: 50 mM sodium phosphate buffer with 1 mM Na₂-EDTA (pH 7.8), 0.043 mM xanthine (Sigma X7375), 18.2 µM cytochrome *c* (Sigma C7752) and 0.3 U mL⁻¹ xanthine oxidase (XO, Sigma X1875). The reaction was initiated with the addition of the XO solution, and the reduction of the cytochrome *c* was assessed by the increase of absorbance at 550 nm, using a microplate reader (Bio-Tek®, model Power Wave 340, Winooski, USA). One unit of SOD was defined as the amount of enzyme required to inhibit the rate of reduction of cytochrome *c* by 50%.

The activity of CAT was determined according to Aebi (1984) ^[29]. Tissues were homogenised in 50 mM potassium phosphate buffer (Merck 1.05101 and Merck 1.04873) (pH 7.0) and centrifuged at 15,000 *g* for 15 min at 4°C. The final concentrations of the assay chemicals, in a final volume of 600 µL, were: 50 mM potassium phosphate buffer

(pH 7.0) and 10 mM hydrogen peroxide (H_2O_2 , Aldrich 21.676, Steinheim, Germany). The reaction was initiated with the addition of the H_2O_2 solution, and its decomposition was assessed by the decrease of absorbance at 240 nm, using a spectrophotometer (Jenway 6405 UV/Vis, Dunmow, England).

The activities of GPx and GR were determined according to Flohé and Günzler (1984)^[30], and Carlberg and Mannervik (1975)^[31], respectively. The two assays were adapted to microplate. The activity of GST was determined according to Habig et al. (1974)^[32] adapted to microplate by Frasco et al. (2002)^[33]. For these three enzymatic assays, tissues were homogenised using 100 mM potassium phosphate buffer with 2 mM $\text{Na}_2\text{-EDTA}$ (pH 7.5) and centrifuged at 15,000 g for 15 min at 4°C. The final concentrations of the chemicals for the GPx assay, in a final volume of 300 μL , were: 100 mM potassium phosphate buffer with 2 mM $\text{Na}_2\text{-EDTA}$, 1 mM dithiothreitol (DTT, Sigma D9779) and 1 mM of sodium azide (Sigma S8032) (pH 7.5), 2 mM GSH, 34 U mL^{-1} GR (Sigma G3664), 0.24 mM β -nicotinamide adenine dinucleotide 2'-phosphate reduced tetrasodium salt (NADPH, Sigma N7505), and 0.6 mM H_2O_2 . The reaction was initiated with the addition of the H_2O_2 solution, and the oxidation of NADPH was assessed by the decrease of absorbance at 340 nm, using a microplate reader. The final concentrations of the chemicals for the GR assay, in a final volume of 300 μL , were: 100 mM potassium phosphate buffer with 2 mM $\text{Na}_2\text{-EDTA}$ (pH 7.5), 0.5 mM GSSG (Sigma G4376) and 0.1 mM NADPH. The reaction was initiated with the addition of the NADPH solution, and the oxidation of NADPH was assessed by the decrease of absorbance at 340 nm, using a microplate reader. The final concentrations of the assay chemicals for the GST assay, in a final volume of 300 μL , were: 100 mM potassium phosphate buffer (pH 6.5), 4 mM GSH and 1 mM 1-chloro-2,4-dinitrobenzene (CDNB, Sigma C6396). The activity of GST was determined by measuring the formation of a thioether by the conjugation of CDBN with GSH. This conjugation is followed by an increase in absorbance at 340 nm, using a microplate reader.

Total glutathione (tGSx) and GSSG were determined according to Baker et al. (1990)^[34]. Tissues were homogenised using 71.5 mM sodium phosphate buffer with 0.63 mM $\text{Na}_2\text{-EDTA}$ (pH 7.5). Following homogenisation, 5% perchloric acid (Merck 0519) was added to the samples that were centrifuged at 15,000 g for 15 min at 4°C. Previous to the enzymatic assay, samples were neutralized with 760 mM potassium hydrogen carbonate (Sigma P4913). The final concentrations of the chemicals for the tGSx quantification, in a final volume of 205 μL , were: 0.15 mM NADPH, 0.85 mM of 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB, Sigma D8130) and 7 U mL^{-1} GR. A 5% solution of 2-vinylpyridine (Fluka 95040, Steinheim, Germany) was used to conjugate GSH for the

GSSG determination. Glutathione equivalents were quantified by monitoring the formation of 5-thio-2-nitrobenzoic acid formed by the conjugation of the SH- group of glutathione and the DTNB at 414 nm, using a microplate reader. Glutathione concentrations were expressed as nmol of GSH equivalents (GSx) per mg of protein ($GSx = [GSH] + 2[GSSG]$). GSH/GSSG ratio was calculated as number of molecules: $GSH/GSSG = (tGSx - GSSG)/(GSSG/2)$, according to Peña-Llopis et al. (2001) ^[35].

Levels of LPO were measured by the generation of thiobarbituric acid (TBARS)-malondialdehyde (MDA) reactive species, which were referred to MDA equivalents (Ohkawa et al., 1979) ^[36]. Tissues were homogenised using 100 mM potassium phosphate buffer (pH 7.2) and centrifuged at 10,000 *g* for 5 min at 4°C. The reaction mixture contained: 11.4% of homogenate, 4.6% of 10.6 mM sodium dodecyl sulfate (Sigma D2525) with 0.1 mM butylated hydroxytoluene (Aldrich W218405), 40% of 20% acetic acid (Merck 1.00062) (pH 3.5), 40% of 22.2 mM thiobarbituric acid (Sigma T5500), and 4% of nanopure water in a final volume of 700 μ L. The reaction mixture was heated in a 95°C water bath for 1 h. Once cold, 175 μ L of nanopure water and 875 μ L *n*-butanol (Merck 1.01990) and pyridine (Aldrich 270970) (15:1 v/v) were added and thoroughly mixed. Following centrifugation at 10,000 *g* for 5 min, the immiscible organic layer was removed and its absorbance measured at 530 nm, using a microplate reader.

The activity of IDH was determined according to Ellis and Goldberg (1971) ^[37] adapted to microplate. Tissues were homogenised in 50 mM tris(hydroxymethyl)-aminomethane (Tris, Merck 1.08382) buffer (pH 7.8) and centrifuged at 15,000 *g* for 15 min at 4°C. The final concentrations of the assay chemicals, for a final volume of 300 μ L, were: 50 mM of Tris buffer (pH 7.8), 0.5 mM β -nicotinamide adenine dinucleotide phosphate (NADP, Sigma N0505), 7 mM DL- isocitric acid (Sigma I1252) and 4 mM manganese chloride tetrahydrate (Merck 1.05927). The reaction was initiated with the addition of the DL-isocitric acid solution, and the reduction of NADP was assessed by the increase of absorbance at 340 nm, using a microplate reader.

The activity of ODH was determined according to Livingston et al. (1990) ^[38] adapted to microplate. Tissues were homogenised in 20 mM Tris buffer (pH 7.5) with 1 mM Na₂-EDTA and 1 mM DTT and centrifuged at 15,000 *g* for 15 min at 4°C. The final concentrations of the assay chemicals, in a final volume of 300 μ L, were: 100 mM imidazole hydrochloride (Sigma I3386) buffer (pH 7.0), 0.1 mM β -nicotinamide adenine dinucleotide (NADH, Sigma N8129), 10 mM L-arginine (Aldrich A9,240-6) and 2 mM pyruvic acid sodium salt (Sigma P2256). The reaction was initiated with the addition of the pyruvic acid solution, and the enzyme activity was determined by monitoring the decrease in absorbance due to oxidation of NADH at 340 nm, using a microplate reader.

The activity of AChE was determined according to Ellman et al. (1961) ^[39], adapted to microplate by Guilhermino et al. (1996) ^[40]. The AChE assay was performed directly in mussels' haemolymph diluted (1:2) in ice-cold 100 mM potassium phosphate buffer (pH 7.2), immediately after its collection. The final concentrations of the assay, in a final volume of 300 μ L, were: 100 mM potassium phosphate buffer (pH 7.2), 0.40 mM acetylthiocholine iodide (ATCh, Sigma A5751, Steinheim, Germany) and 0.27 mM DTNB. In this assay the AChE hydrolyses the substrate ATCh in thiocholine and acetate. Following this reaction, the thiocholine reacts with DTND forming a mixed disulphide and the yellow chromophore 5-thio-2-nitrobenzoic acid (TNB). The TNB formation is followed by an increase in absorbance at 412 nm, using a microplate reader. Cholinesterase activity detected in *M. galloprovincialis* haemolymph was previously shown to have properties of true AChE ^[23].

The protein content of the samples was determined by the Bradford method (Bradford, 1976) ^[41], using γ -bovine globulins (Sigma G5009) as standard. All enzymatic assays were performed at 25°C.

2.2.6. Data analyses

The results of the biomarkers are presented as means $\pm$ standard deviation (SD). Prior to the analysis of variance (ANOVA), data was checked for normality (Kolmogorov–Smirnov normality test) and homogeneity of variance (Hartley, Cochran C, and Bartlett's tests), and data transformation was done as required to fulfil ANOVA assumptions ^[42]. For parametric data, the effects of sampling season and sampling site, as well as their interactions, were studied for the selected biomarkers by performing a two-way ANOVA, followed by a Tukey honestly significant difference (HSD) multiple comparison test whenever applicable ^[42]. For non-parametric data, the effects of sampling season and sampling site were studied for the biomarkers by performing a Kruskal–Wallis nonparametric ANOVA followed by a Dunn's test (pair-wise multiple comparison) ^[42]. Furthermore, a Spearman correlation was performed to evaluate the degree of relationship between biomarkers and petroleum hydrocarbon levels, as well as biomarkers and abiotic parameters ^[42]. Seasonality in the response of the biomarkers to petrochemical contamination was evaluated by multivariate analyses. For the annual data, as well as for each sampling season, triangular similarity matrices were calculated for the biomarkers using the Bray-Curtis similarity coefficient, following a Log (x+1) transformation of the data ^[43]. Using these correlation matrixes, a two dimensional non-metric multidimensional scaling (MDS) and a cluster analysis were performed to

discriminate the similarities of each sampling season and sampling site for the annual data, as well as to discriminate the similarities of each sampling sites within each sampling season ^[44]. In addition, a pair-wise comparisons test ANOSIM, which was performed in pre defined sets of sampling sites and sampling seasons, confirmed the existence of significant differences between the groups obtained by the MDS and Cluster analysis for the annual data ^[44]. A similarity percentages test (SIMPER) was performed to discriminate which biomarkers had the greatest influence on the similarities within groups and dissimilarities among groups obtained by the MDS and cluster analysis ^[44]. In addition, principal component analysis (PCA) was performed to discriminate the similarities of each sampling season and sampling site for the annual data, as well as to discriminate the similarities of each sampling sites within each sampling season, as a function of the petroleum hydrocarbon levels measured in mussels' tissue ^[43]. Finally, the biota and/or environment matching (BIOENV) procedure were performed to evaluate which petroleum hydrocarbons better relate with the biomarkers ^[45]. Complementary to this analysis, a graphical comparison was performed between the MDS and PCA plots of the distribution of the sampling season and sampling site for the annual data, as well as between the sampling sites within each sampling season ^[46]. Statistical analyses of data were performed using Statistica 6.0 (StatSoft, Tulsa, USA), with the exception of the Dunn's test that was performed using Sigma Stat 3.5 (Systat Software Inc, California, USA). Finally, multivariate analyses of the data were performed using PRIMER 5 package for Windows (PRIMER-E Ltd., Plymouth, UK).

2.3. RESULTS

2.3.1. Abiotic parameters

Seasonal variation of abiotic parameters measured at five sampling sites (S1-S5) along the NW coast of Portugal from the autumn 2005 to the autumn 2006 are presented in Table 2.1.

Temperature values ranged from 10.5 °C at S4 during the winter to 21.6 °C at S1 during the autumn 2005. All sites exhibited comparable seasonal temperature fluctuations with the highest values measured during autumn 2005 and the lowest during winter.

Table 2.1 Seasonal variation of abiotic parameters quantified in water samples collected at five sampling sites (S1-S5) along the NW coast of Portugal, from the autumn 2005 to the autumn 2006.

Abiotic parameters	Site	Sampling season				
		Autumn 05	Winter 05/06	Spring 06	Summer 06	Autumn 06
Temperature (°C)*	S1	21.6	12.3	14.6	17.2	17.2
	S2	20.1	10.8	13.9	16.5	17.6
	S3	18.6	12.0	16.2	18.9	17.8
	S4	17.4	10.5	16.5	20.0	17.4
	S5	20.6	13.3	15.0	17.7	17.2
Salinity (g L ⁻¹)*	S1	36.3	35.1	35.5	34.2	33.1
	S2	31.7	22.1	17.1	32.6	17.8
	S3	35.7	32.5	35.2	34.5	33.0
	S4	35.4	21.8	27.0	34.6	28.3
	S5	32.6	31.9	27.0	34.3	30.1
pH*	S1	7.80	8.13	8.05	7.89	7.83
	S2	7.74	8.11	7.74	7.87	7.98
	S3	7.83	8.04	8.14	7.45	7.76
	S4	8.00	8.09	8.15	7.96	8.01
	S5	8.03	8.04	7.73	7.48	8.04
Ammonia (mg L ⁻¹)**	S1	0.12	0.14	0.26	0.05	0.06
	S2	0.14	0.13	0.13	0.13	0.05
	S3	0.06	0.22	0.59	0.12	0.05
	S4	0.98	2.38	0.41	0.09	0.14
	S5	1.75	0.8	1.61	0.62	0.89
Nitrate (mg L ⁻¹)**	S1	0.36	0.86	1.54	0.92	1.60
	S2	0.68	1.08	0.74	0.98	1.34
	S3	0.64	0.74	1.02	0.62	1.20
	S4	1.06	1.22	0.98	0.44	1.34
	S5	1.24	1.34	1.20	1.62	1.38
Nitrite (mg L ⁻¹)**	S1	0.01	0.01	0.01	0.00	0.06
	S2	0.01	0.03	0.01	0.00	0.02
	S3	0.00	0.00	0.01	0.01	0.06
	S4	0.09	0.25	0.41	0.02	0.17
	S5	0.11	0.13	0.32	0.06	0.35
Phosphate (mg L ⁻¹)**	S1	0.03	0.09	0.06	0.08	0.08
	S2	0.07	0.12	0.03	0.08	0.07
	S3	0.04	0.10	0.03	0.08	0.38
	S4	0.34	0.99	0.21	0.14	0.12
	S5	0.49	0.27	0.44	0.18	0.31

* measured *in situ*, ** measured in subsurface water samples.

Salinity levels ranged from 17.1 g L^{-1} at S2 during the spring to 36.3 g L^{-1} at S1 during the autumn 2005. As expected, sites located near river mouths (S2, S4 and S5) exhibited similar seasonal patterns with the highest salinity levels during the autumn 2005 and summer and the lowest during winter and spring. Sites located in open seashore (S1 and S3) did not exhibit major variation in salinity over the year. The pH was relatively constant, ranging from 7.45 at S3 during summer to 8.15 at S4 during the spring.

The lowest ammonia levels (0.05 mg L^{-1}) were found at S1 in the summer, as well as at S2 and S3 during the autumn 2006. The highest ammonia levels (2.38 mg L^{-1}) were found at S4 during the winter. Sites S1, S2 and S3 had low levels of ammonia compared to the remaining sites and did not exhibit any accentuated seasonality. Site S4 had an increase in ammonia levels during the winter, followed by an accentuated decrease to levels similar to those found at S1, S2 and S3. Site S5 had the highest levels of ammonia during the autumn 2005 and spring.

Nitrate levels ranged from 0.36 mg L^{-1} at S1 during the autumn 2005 to 1.62 mg L^{-1} at S5 during the summer. Sites S1 and S3 exhibited an increase in nitrate levels throughout the year with the exception of the summer when a decrease was registered. The remaining sites exhibited an increase in nitrate levels from autumn 2005 to winter, followed by a decrease during spring. Between spring and autumn 2006 the nitrate levels quantified at these sites did not follow a discernable pattern.

Low nitrite levels (0.01 to 0.06 mg L^{-1}) were found at S1, S2 and S3 throughout the year. Site S4 and S5, which presented the highest nitrite levels, exhibited similar fluctuation patterns, with an increase from autumn 2005 to the spring followed by a decrease in summer and a second increase in autumn 2006. No nitrite was detected at S3 during the autumn 2005 and winter or at S1 and S2 in the summer.

The lowest phosphate levels (0.03 mg L^{-1}) were found at S1 during the autumn 2005, as well as at S2 and S3 during the spring. The highest phosphate levels (0.99 mg L^{-1}) were found at S4 during the winter. Sampling sites S1, S2 and S3 did not exhibit major seasonal fluctuations, with the exception of site S3 where phosphate levels increased severely in the autumn 2006. Site S4 exhibited an accentuated increase of the phosphate levels in the winter, followed by a decrease during the subsequent periods. Site S5 exhibited slight decrease in the phosphate levels in the winter and summer.

2.3.2. Chemical analyses

The results of the seasonal variation of petroleum hydrocarbon levels analysed in the whole tissue of *M. galloprovincialis* collected at five sampling sites along the NW coast of Portugal, from the autumn 2005 to the autumn 2006 are presented in Table 2.2.

The levels of AH ranged from $0.88 \mu\text{g g}^{-1}$ dry weight (dw) in mussels collected at S4 during the autumn 2006 to $22.55 \mu\text{g g}^{-1}$ dw in mussels collected S3 during the autumn 2005. The lowest AH levels were quantified in mussels from S4 ($0.88\text{-}3.99 \mu\text{g g}^{-1}$ dw) and S5 ($2.62\text{-}3.57 \mu\text{g g}^{-1}$ dw) and did not exhibit major seasonal fluctuations throughout the year. The highest AH levels were quantified in mussels from S1 ($18.48 \mu\text{g g}^{-1}$ dw) and S3 ($22.55 \mu\text{g g}^{-1}$ dw) collected during the autumn 2005. However, by the summer AH levels quantified in mussels from S1 and S3 had reached levels similar to those found at S4 and S5.

The levels of petroleum hydrocarbons present in UCM, which represents the main fraction of the total petroleum hydrocarbon, ranged from $364.59 \mu\text{g g}^{-1}$ dw at S2 during the autumn 2006 to $2146.95 \mu\text{g g}^{-1}$ dw at S5 during the winter. The levels of UCM measured at site S1 decreased from the autumn 2005 until the summer, increasing in the autumn 2006. Site S2 showed an increase in the UCM levels from the autumn 2005 to the spring, followed by a decrease in the following periods. Mussels collected at S3 and S4 showed similar seasonal fluctuation patterns of UCM, except during the final sampling season, when the levels found at S3 decreased while those found at S4 increased in comparison to the levels measured in the summer. Mussels collected from S5 showed the highest levels of UCM, exhibiting an increase from the autumn 2005 to the winter, followed by a decrease in the spring, maintaining similar levels onwards.

The levels of total PAHs ranged from $0.32 \mu\text{g g}^{-1}$ dw in mussels collected at S3 during the summer to $7.32 \mu\text{g g}^{-1}$ dw in mussels collected at S4 during the autumn 2006. The levels of PAHs quantified in mussels collected at S1 increased from the autumn 2005 to spring, decreasing again in the following sampling period. In mussels from S3, PAHs levels decreased until the summer increasing again in the autumn 2006. PAHs levels quantified in mussels collected from S2, S4 and S5 exhibited a similar seasonal pattern, decreasing between autumn 2005 and winter, increasing in spring, decreasing again in summer and finally increasing towards the final sampling period. Regarding the results of the 16 priority PAHs, the major contributors to the total PAHs levels present in mussel tissues were naphthalene, anthracene, acenaphthylene and indeno(1,2,3-cd)pyrene, corresponding to approximately 93% of this fraction.

Table 2.2 Seasonal variation of petroleum hydrocarbon levels analysed in whole tissue of *Mytilus galloprovincialis* collected at five sampling sites (S1-S5) along the NW coast of Portugal, from the autumn 2005 to the autumn 2006.

Petroleum hydrocarbons	Sampling Site									
	S1					S2				
	Aut 05	Win 06	Spr 06	Sum 06	Aut 06	Aut 05	Win 06	Spr 06	Sum 06	Aut 06
AH	18.48	5.20	10.28	2.79	3.47	6.30	8.70	3.48	4.60	3.20
UCM	889.66	705.89	479.11	429.41	663.93	483.17	826.97	922.28	881.38	364.59
ΣPAHs	3.57	5.94	6.32	1.65	1.45	1.86	0.89	5.65	1.04	2.02
Acenaphthene	0.10	0.10	-	0.03	-	0.09	0.03	-	0.02	0.05
Acenaphthylene	0.18	0.20	1.81	0.08	0.11	0.11	0.07	-	0.03	0.13
Anthracene	0.36	1.77	-	0.06	0.09	0.19	-	0.91	0.06	1.19
Benzo[a]anthracene	0.17	-	-	-	-	-	-	-	-	-
Benzo[a]pyrene	-	0.58	-	-	-	-	-	-	-	-
Benzo[b]fluoranthene	-	-	-	-	-	-	-	-	-	-
Benzo[ghi]perylene	-	-	-	-	-	-	-	-	-	-
Benzo[k]fluoranthene	-	-	-	-	-	-	-	-	-	-
Chrysene	0.26	-	-	-	-	0.05	-	-	-	-
Dibenzo[ah]anthracene	0.16	0.17	-	-	-	-	-	-	-	-
Fluoranthene	0.16	0.15	-	-	-	-	0.01	-	-	-
Fluorene	-	-	-	-	-	-	-	-	-	-
Indeno[1,2,3-cd]pyrene	0.82	0.06	0.68	0.02	-	0.07	-	0.26	-	-
Naphthalene	1.36	2.82	3.83	1.43	1.25	1.35	0.71	4.47	0.93	0.59
Phenanthrene	-	0.09	-	0.03	-	-	0.07	-	-	0.06
Pyrene	-	-	-	-	-	-	-	-	-	-

AH – aliphatic hydrocarbons, UCM – unresolved complex mixture, ΣPAHs – total polycyclic aromatic hydrocarbons. Data are expressed in $\mu\text{g g}^{-1}$ dry weight.

Table 2.2 (continued).

Petroleum hydrocarbons	Sampling Site									
	S3					S4				
	Aut 05	Win 06	Spr 06	Sum 06	Aut 06	Aut 05	Win 06	Spr 06	Sum 06	Aut 06
AH	22.55	3.72	1.48	5.41	2.57	3.99	2.94	1.88	2.45	0.88
UCM	650.45	1172.63	638.41	958.50	694.20	477.86	1091.87	782.09	1033.64	1231.61
ΣPAHs	3.95	2.92	2.53	0.32	2.44	1.91	1.44	3.18	1.90	7.32
Acenaphthene	-	0.06	-	0.16	0.04	0.06	0.23	-	0.02	-
Acenaphthylene	0.32	0.08	0.64	0.03	0.18	0.09	0.06	-	0.04	-
Anthracene	0.28	0.16	-	0.09	0.24	0.13	-	0.52	0.04	1.00
Benzo[a]anthracene	-	-	-	-	-	-	-	-	-	-
Benzo[a]pyrene	-	-	-	-	-	-	-	-	-	-
Benzo[b]fluoranthene	-	-	-	-	-	-	-	-	-	-
Benzo[ghi]perylene	-	-	-	-	-	-	-	-	-	-
Benzo[k]fluoranthene	-	-	-	-	-	-	-	-	-	-
Chrysene	0.35	-	0.22	-	-	0.04	-	-	-	-
Dibenzo[ah]anthracene	-	-	-	-	0.04	-	-	-	-	-
Fluoranthene	-	-	0.13	-	-	-	0.04	-	-	-
Fluorene	-	-	-	-	-	-	-	-	0.01	-
Indeno[1,2,3-cd]pyrene	0.95	0.03	0.31	-	0.06	0.11	0.02	0.19	-	0.29
Naphthalene	2.05	2.59	1.23	0.04	1.84	1.48	1.09	2.47	1.76	6.03
Phenanthrene	-	-	-	-	0.04	-	-	-	0.03	-
Pyrene	-	-	-	-	-	-	-	-	-	-

AH – aliphatic hydrocarbons, UCM – unresolved complex mixture, ΣPAHs – total polycyclic aromatic hydrocarbons. Data are expressed in $\mu\text{g g}^{-1}$ dry weight.

Table 2.2 (continued).

Petroleum hydrocarbons	Sampling site				
	S5				
	Aut 05	Win 06	Spr 06	Sum 06	Aut 06
AH	2.65	3.40	2.62	3.57	3.31
UCM	1201.74	2146.95	1664.10	1683.68	1880.44
ΣPAHs	1.21	0.99	1.77	0.81	1.97
Acenaphthene	-	0.01	0.02	0.02	0.02
Acenaphthylene	0.11	0.05	0.10	0.12	0.11
Anthracene	0.10	0.19	0.48	0.10	0.98
Benzo[a]anthracene	-	-	-	-	-
Benzo[a]pyrene	-	-	-	-	-
Benzo[b]fluoranthene	-	-	-	-	-
Benzo[ghi]perylene	-	-	-	-	-
Benzo[k]fluoranthene	-	-	-	-	-
Chrysene	0.05	-	-	-	-
Dibenzo[ah]anthracene	0.03	0.04	0.14	-	0.08
Fluoranthene	0.02	0.01	0.02	0.01	0.01
Fluorene	-	-	-	-	-
Indeno[1,2,3-cd]pyrene	0.27	0.02	0.11	-	0.05
Naphthalene	0.61	0.64	0.90	0.53	0.70
Phenanthrene	0.02	0.03	-	0.03	0.02
Pyrene	-	-	-	-	-

AH – aliphatic hydrocarbons, UCM – unresolved complex mixture, ΣPAHs – total polycyclic aromatic hydrocarbons.

Data are expressed in $\mu\text{g g}^{-1}$ dry weight.

2.3.3. Biomarkers

The results of the biomarkers are presented in Table 2.3 and 2.4, as well as in Figures 2.2 to 2.6. Regarding seasonality, the levels of SOD activity measured in digestive glands of mussels collected during the winter and spring were significantly higher than those collected during the remaining periods. Likewise, levels of SOD activity measured in digestive glands of mussels collected during the autumn 2005 were significantly higher than in mussels collected during the autumn 2006, but no significant differences were found with those collected during the summer. There were no significant differences in the levels of SOD activity measured in gills of mussels collected throughout the year (Table 2.3 and Figure 2.2).

The levels of CAT activity quantified in digestive glands of mussels collected during the winter were significantly higher than those collected during the autumn 2005 and significantly lower than those collected during spring and autumn 2006, but no significant differences were found in mussels collected during the summer. The levels of CAT activity quantified in gills of mussels collected during the autumn 2006 were significantly higher than in mussels collected during the remaining periods. The levels of CAT activity quantified in gills of mussels collected during the spring were significantly higher than in mussels collected during the autumn 2005 and significantly lower than in mussels collected during the summer; however no significant differences were found with those collected during the winter (Table 2.3 and Figure 2.2).

The levels of GPx activity measured in digestive glands of mussels collected during both autumn periods and winter were significantly higher than in mussels collected during the spring, but significantly lower than in mussels collected during the summer. The levels of GPx activity measured in gills of mussels collected during both autumn periods and summer were significantly higher than in mussels collected during the spring, but not significantly different from those collected during the winter (Table 2.3 and Figure 2.2).

The levels of GR activity quantified in digestive glands of mussels collected during the autumn 2005 and winter were significantly higher than in mussels collected during the spring and summer, but no significant differences were found with those collected during the autumn 2006 (Table 2.4 and Figure 2.3).

Table 2.3 Summary of the results of the two-way ANOVA and Tukey honestly significant difference multi-comparison test performed to assess the effects of the sampling season, sampling site, as well as their interactions, on biomarkers quantified in *Mytilus galloprovincialis* collected at five sampling sites (S1-S5) along the NW coast of Portugal.

Biomarkers	Tissue	Factor	d.f.	F	p		Tukey test				
							1	2	3	4	5
SOD	DG	Season	4	79.05	≤ 0.001	**	B	C	C	AB	A
		Site	4	36.83	≤ 0.001	**	a	a	a	b	c
		Se x Si	16	3.03	≤ 0.001	**					
	GL	Season	4	1.64	0.165	n.s.	A	A	A	A	A
		Site	4	8.93	≤ 0.001	**	ab	a	a	a	b
		Se x Si	16	6.05	≤ 0.001	**					
CAT	DG	Season	4	25.05	≤ 0.001	**	A	B	C	BC	C
		Site	4	129.65	≤ 0.001	**	a	ab	b	c	d
		Se x Si	16	15.91	≤ 0.001	**					
	GL	Season	4	24.76	≤ 0.001	**	A	BC	B	C	D
		Site	4	40.48	≤ 0.001	**	a	c	b	c	c
		Se x Si	16	15.74	≤ 0.001	**					
GPx	DG	Season	4	46.94	≤ 0.001	**	B	B	A	C	B
		Site	4	43.93	≤ 0.001	**	a	b	b	c	c
		Se x Si	16	20.34	≤ 0.001	**					
	GL	Season	4	6.39	≤ 0.001	**	B	AB	A	B	B
		Site	4	17.74	≤ 0.001	**	ab	c	d	bc	a
		Se x Si	16	12.42	≤ 0.001	**					
GR	DG	Season	4								
		Site	4	n.p.	n.p.	n.p.			n.p.		
		Se x Si	16								
	GL	Season	4	30.43	≤ 0.001	**	D	C	A	AB	B
		Site	4	79.83	≤ 0.001	**	c	a	c	b	a
GST	DG	Season	4								
		Site	4	n.p.	n.p.	n.p.			n.p.		
		Se x Si	16								
	GL	Season	4	85.97	≤ 0.001	**	D	B	B	C	A
		Site	4	102.09	≤ 0.001	**	a	b	b	c	d
		Se x Si	16	14.70	≤ 0.001	**					
LPO	DG	Season	4	96.35	≤ 0.001	**	C	C	A	A	B
		Site	4	20.08	≤ 0.001	**	a	a	a	b	b
		Se x Si	16	4.34	≤ 0.001	**					
	GL	Season	4	31.95	≤ 0.001	**	C	C	AB	A	B
		Site	4	4.34	≤ 0.05	*	ab	b	ab	a	ab
		Se x Si	16	11.90	≤ 0.001	**					

SOD – total superoxide dismutase, CAT – catalase, GPx – selenium-dependent glutathione peroxidase, GR – glutathione reductase, GST – glutathione S-transferases, LPO – lipid peroxides, DG – digestive glands, GL – gills, d.f. – degrees of freedom, F – Fisher's F ratio, p – probability of F, * significant ($p \leq 0.05$), ** significant ($p \leq 0.001$), n.s. – non-significant ($p > 0.05$), n.p. – non-parametric (see Table 2.4). Numbers 1-5 refer to autumn 2005, winter, spring, summer and autumn 2006 respectively when referred to sampling season (Se), or to S1-S5 when referred to sampling site (Si). Different capital letters indicate significant differences among sampling seasons and small letters indicate significant differences among sampling sites by Tukey honestly significant difference multiple-comparison test ($p \leq 0.05$) for each biomarker.

Table 2.3 (continued).

Biomarkers	Tissue	Factor	d.f.	<i>F</i>	<i>p</i>		Tukey test				
							1	2	3	4	5
tGSx	DG	Season	4	19.24	≤ 0.001	**	A	AB	D	CD	BC
		Site	4	124.68	≤ 0.001	**	c	b	c	a	c
		Se x Si	16	6.20	≤ 0.001	**					
	GL	Season	4	3.36	≤ 0.05	*	A	AB	AB	AB	B
		Site	4	29.33	≤ 0.001	**	b	b	b	a	a
		Se x Si	16	1.14	0.320	n.s.					
GSH	DG	Season	4	12.43	≤ 0.001	**	A	AB	C	C	BC
		Site	4	61.74	≤ 0.001	**	c	b	c	a	a
		Se x Si	16	3.80	≤ 0.001	**					
	GL	Season	4	4.81	≤ 0.001	**	A	AB	AB	AB	C
		Site	4	13.33	≤ 0.001	**	b	b	b	a	a
		Se x Si	16	0.95	0.511	n.s.					
GSSG	DG	Season	4	7.98	≤ 0.001	**	A	A	B	AB	A
		Site	4	79.14	≤ 0.001	**	b	b	b	a	a
		Se x Si	16	5.89	≤ 0.001	**					
	GL	Season	4	1.29	0.275	n.s.	A	A	A	A	A
		Site	4	33.13	≤ 0.001	**	b	b	b	a	a
		Se x Si	16	1.56	0.081	n.s.					
GSH/GSSG	DG	Season	4	2.81	≤ 0.05	*	A	A	A	A	A
		Site	4	2.81	≤ 0.05	*	ab	a	ab	b	ab
		Se x Si	16	2.43	≤ 0.05	*					
	GL	Season	4	2.75	≤ 0.05	*	A	AB	AB	AB	B
		Site	4	4.16	≤ 0.05	*	a	a	a	b	ab
		Se x Si	16	1.11	0.347	n.s.					
IDH	DG	Season	4	33.11	≤ 0.001	**	A	C	C	AB	B
		Site	4	65.73	≤ 0.001	**	b	a	b	b	c
		Se x Si	16	2.58	≤ 0.05	*					
ODH	PAM	Season	4	48.84	≤ 0.001	**	A	B	AB	C	A
		Site	4	90.33	≤ 0.001	**	a	a	a	a	b
		Se x Si	16	5.45	≤ 0.001	**					
AChE	HMLP	Season	4	20.72	≤ 0.001	**	B	C	B	B	A
		Site	4	94.13	≤ 0.001	**	b	a	ab	c	b
		Se x Si	16	5.95	≤ 0.001	**					

tGSx – total glutathione content, GSH – reduced glutathione, GSSG – oxidised glutathione, GSH/GSSG – glutathione redox status, IDH – NADP⁺-dependent isocitrate dehydrogenase, ODH – octopine dehydrogenase, AChE – acetylcholinesterase, DG – digestive glands, GL – gills, PAM – posterior adductor muscle, HMLP – haemolymph, d.f. – degrees of freedom, *F* – Fisher's *F* ratio, *p* – probability of *F*, * significant ($p \leq 0.05$), ** significant ($p \leq 0.001$), n.s. – non-significant ($p > 0.05$), n.p. – non-parametric (see Table 2.4). Numbers 1-5 refer to autumn 2005, winter, spring, summer and autumn 2006 respectively when referred to sampling season (Se), or to S1-S5 when referred to sampling site (Si). Different capital letters indicate significant differences among sampling seasons and small letters indicate significant differences among sampling sites by Tukey honestly significant difference multiple-comparison test ($p \leq 0.05$) for each biomarker.

Table 2.4 Summary of the results of the Kruskal-Wallis one-way ANOVA and Dunn's test performed to assess the effects of the sampling season and sampling site on biomarkers quantified in *Mytilus galloprovincialis* collected at five sampling sites (S1-S5) along the NW coast of Portugal.

Biomarkers	Tissue	Factor	d.f.	<i>H</i>	<i>p</i>	Dunn test					
						1	2	3	4	5	
GR	DG	Season	4	44.98	≤ 0.001	**	B	B	A	A	AB
		Site	4	77.83	≤ 0.001	**	c	a	c	bc	b
GST	DG	Season	4	20.07	≤ 0.001	**	B	A	AB	A	A
		Site	4	50.70	≤ 0.001	**	b	ab	a	a	bc

GR – glutathione reductase, GST – glutathione S-transferases, DG – digestive glands, d.f. – degrees of freedom, *H* –Kruskal-Wallis statistic, * significant ($p \leq 0.05$), ** significant ($p \leq 0.001$), n.s. – non-significant ($p > 0.05$). Numbers 1-5 refer to autumn 2005, winter, spring, summer and autumn 2006 respectively when referred to sampling season, or to S1-S5 when referred to sampling site. Different capital letters indicate significant differences among sampling seasons and small letters indicate significant differences among sampling sites by Dunn test ($p \leq 0.05$).

The levels of GR activity quantified in gills of mussels collected during the autumn 2005 were significantly higher than those collected during the remaining periods. The levels of GR activity quantified in gills of mussels collected during the autumn 2006 were significantly higher than in those collected during the spring and significantly lower than in mussels collected during the winter, however no significant differences were found with those collected during the summer (Table 2.3 and Figure 2.3).

The levels of GST activities measured in digestive glands of mussels collected during the autumn 2005 were significantly higher than those collected during the remaining periods except with those collected during spring (Table 2.4 and Figure 2.3). The significantly highest values of GST activities measured in gills were found in mussels collected during the autumn 2005. Mussels collected during winter and spring had significantly higher levels of GST activities in gills than those collected during autumn 2006, but had significantly lower levels than those collected during summer (Table 2.3 and Figure 2.3).

Mussels collected during the autumn 2006 presented significantly higher levels of LPO in digestive glands than those collected during the spring and summer, but significantly lower than those collected during the autumn 2005 and winter. Mussels collected during the autumn 2006 presented levels of LPO in gills significantly higher than those collected during summer and significantly lower than those collected during autumn 2005 and winter, however no significant differences were found with mussels collected during the spring (Table 2.3 and Figure 2.3).

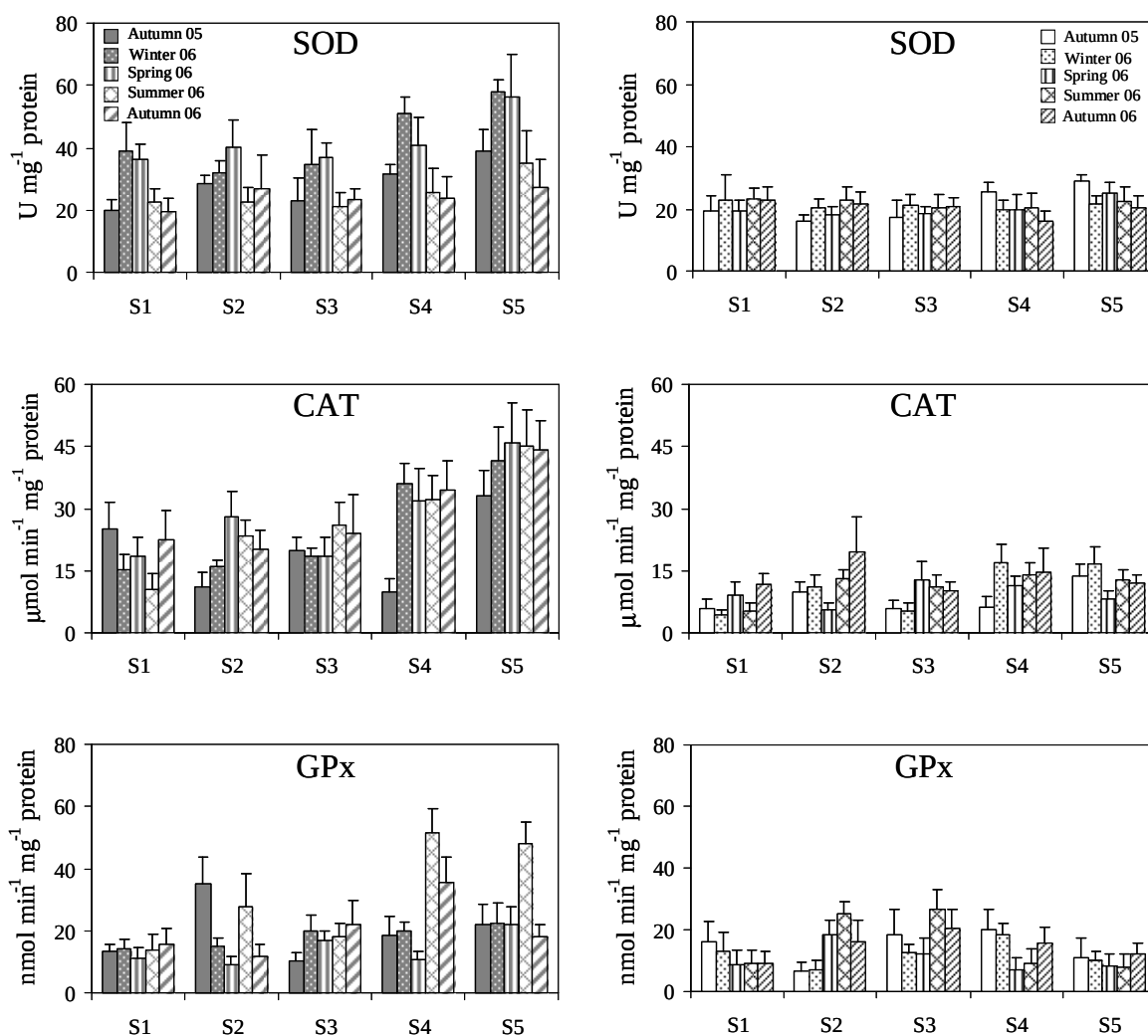


Figure 2.2 Seasonal variation of biomarkers analysed in *Mytilus galloprovincialis* collected at five sampling sites (S1-S5) along the NW coast of Portugal from the autumn 2005 to the autumn 2006. Values are presented as mean $\pm$ standard deviation ($n = 10$) of total superoxide dismutase (SOD), catalase (CAT) and selenium-dependent glutathione peroxidase (GPx) quantified in mussels' digestive glands (left column) and gills (right column). Legend regarding sampling seasons presented in the graphs of SOD should be considered for the subsequent graphs.

Mussels collected during spring exhibited levels of tGSx in digestive glands significantly higher than those collected in the remaining period except summer, while mussels collected during the autumn 2005 exhibited the significantly lowest levels except when compared with those collected during the winter. Mussels collected during the autumn 2006 had significantly higher levels of tGSx in gills from mussels collected during the autumn 2005, however no significant differences were found to those collected during the remaining periods (Table 2.3 and Figure 2.4).

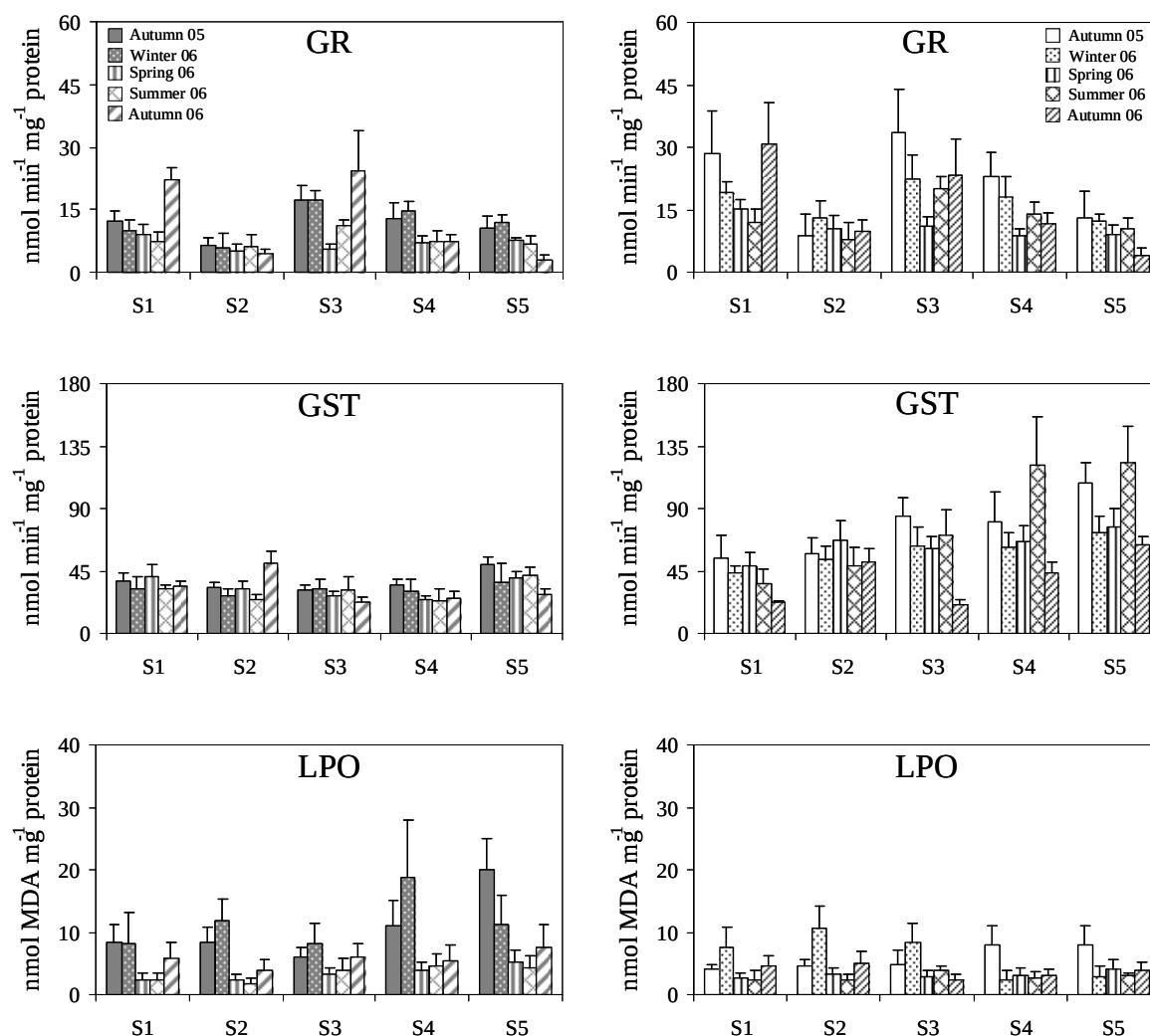


Figure 2.3 Seasonal variation of biomarkers analysed in *Mytilus galloprovincialis* collected at five sampling sites (S1-S5) along the NW coast of Portugal from the autumn 2005 to the autumn 2006. Values are presented as mean $\pm$ standard deviation ($n = 10$) of glutathione reductase (GR), glutathione S-transferases (GST) and lipid peroxides (LPO) quantified in mussels' digestive glands (left column) and gills (right column). Legend regarding sampling seasons presented in the graphs of GR should be considered for the subsequent graphs.

Mussels collected during the spring and summer exhibited significantly higher levels of GSH in digestive glands than those collected in the remaining periods, except to those collected during the autumn 2006. Mussels collected during the autumn 2005 had the significantly lowest levels of GSH in digestive glands, however no significant differences were found with mussels collected during the winter. The levels of GSH in gills exhibited the significantly highest values in mussels collected during the autumn 2006. Mussels collected during the remaining periods did not show any significant differences among them (Table 2.3 and Figure 2.4).

The levels of GSSG in digestive glands of mussels collected during the spring were significantly higher than in mussels collected during both autumn periods and winter. No significant differences were found in GSSG levels quantified in digestive glands of mussels collected during the summer and those collected during the remaining periods. No significant differences were found among sampling seasons for the levels of GSSG quantified in mussels' gills (Table 2.3 and Figure 2.4).

No significant differences were found in the GSH/GSSG ratio in digestive glands of mussels collected throughout the sampling period. The GSH/GSSG ratio quantified in gills of mussels collected during the autumn 2006 were significantly higher than in mussels collected during the autumn 2005, but no significant differences were found among the remaining periods (Table 2.3 and Figure 2.4).

The levels of IDH activity in digestive glands of mussels collected during the autumn 2006 were significantly higher than in mussels collected during the autumn 2005 and significantly lower than in mussels collected during the winter and spring, but no significant differences were found with those collected during the summer (Table 2.3 and Figure 2.5).

The levels of ODH quantified in mussels collected during the winter were significantly higher than those collected both autumn periods and significantly lower than those collected during summer, but no significant differences were found with those collected during spring (Table 2.3 and Figure 2.5).

Finally, the levels of AChE activity the haemolymph of mussels collected during the autumn 2005, spring and summer exhibited significant higher values than those collected during the autumn 2006, but significantly lower than those collected during the winter (Table 2.3 and Figure 2.6).

Regarding the sampling sites, the levels of SOD activity quantified in digestive glands of mussels collected at S4 were significantly higher than those collected at S1- S3, but significantly lower than those collected at S5. The levels of SOD activity in gills of mussels collected at S5 were significantly higher than those collected in the remaining sites with the exception of S1 (Table 2.3 and Figure 2.2).

The levels of CAT activity quantified in digestive glands of mussels from S5 were significantly higher than those from the remaining sampling sites. Digestive glands of mussels from S3 presented levels of CAT activity significantly higher from those collected at S1 and significantly lower than those from S4; however, no significant differences were found with mussels from S2 (Table 2.3 and Figure 2.2).

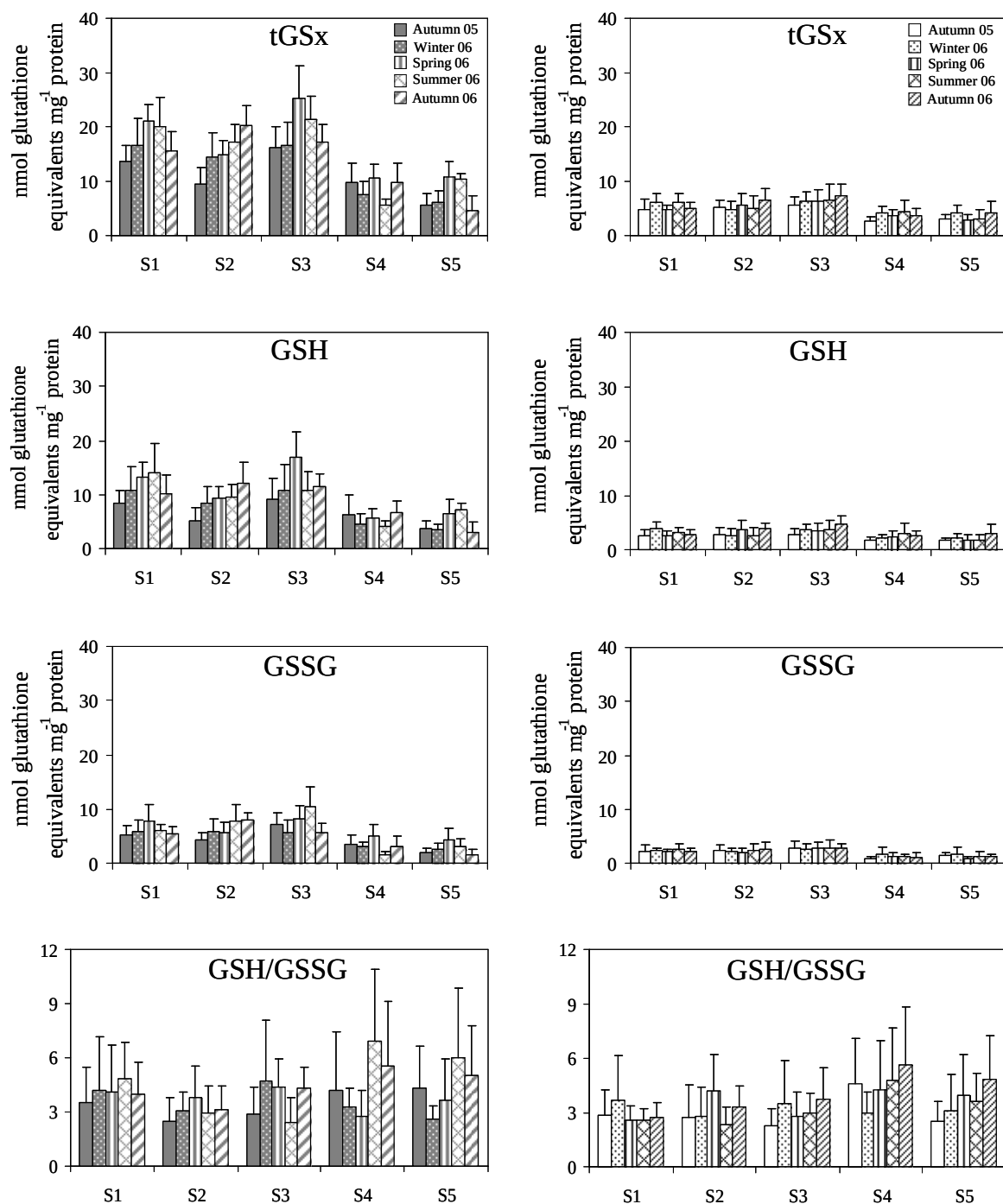


Figure 2.4 Seasonal variation of biomarkers analysed in *Mytilus galloprovincialis* collected at five sampling sites (S1-S5) along the NW coast of Portugal from the autumn 2005 to the autumn 2006. Values are presented as mean $\pm$ standard deviation ($n = 10$) of total glutathione content (tGSx), reduced glutathione (GSH), oxidised glutathione (GSSG) and glutathione redox status (GSH/GSSG ratio) quantified in mussels' digestive glands (left column) and gills (right column). Legend regarding sampling seasons presented in the graphs of tGSx should be considered for the subsequent graphs.

The levels of CAT activity quantified in gills of mussels collected at S3 were significantly higher than those from S1, but significantly lower than those collected from the remaining sites (Table 2.3 and Figure 2.2).

The levels of GPx activity in digestive glands of mussels collected from S2 and S3 were significantly higher than in those from S1 but significantly lower than in those from S4 and S5. Levels of GPx activity in the gills of mussels from S2 were significantly higher than those from S1 and S5 and significantly lower than those from S3, but no significant differences were found with mussels from S4. Likewise, mussels collected at S5 did not exhibit significant differences in the levels of GPx activity quantified in gills of mussels collected at S1 (Table 2.3 and Figure 2.2).

The levels of GR activity quantified in digestive glands of mussels from S5 were significantly higher than those from S2 but significantly lower than those from S1 and S3. Mussels from S4 did not exhibit significant differences in GR activity levels in digestive gland from those collected at S3 and S5 (Table 2.4 and Figure 2.3).. The levels of GR activity in gills of mussels from S4 were significantly higher than those collected from S2 and S5, but significantly lower than those from S1 and S3 (Table 2.3 and Figure 2.3).

The levels of GST activities quantified in digestive glands of mussels from S5 were significantly higher than those collected at the remaining sites except those from S1. Mussels from S1 presented significantly higher values of GST activities in digestive glands than those from S3 and S4, but not from S2 (Table 2.4 and Figure 2.3). Mussels from S5 presented the significantly highest values of GST activities in gills. Mussels from S2 and S3 had levels of GST activities in gills significantly higher than those from S1 but significantly lower than those from S4 (Table 2.3 and Figure 2.3).

The levels of LPO quantified in digestive glands of mussels from S4 and S5 were significantly higher than those collected from the remaining sites. The levels of LPO in gills of mussels from S2 were significantly higher than those from S4 but no significant differences were found with those from S1, S3 and S5 (Table 2.3 and Figure 2.3).

Levels of tGSx quantified in digestive glands of mussels from S2 were significantly higher than those collected from S4 and S5, but significantly lower than those from S1 and S3. Levels of tGSx in gills of mussels from S1-S3 were significantly higher than those collected from S4 and S5 (Table 2.3 and Figure 2.4).

The levels of GSH quantified in both digestive glands and gills of mussels followed a similar pattern of the tGSx levels (Table 2.3 and Figure 2.4).

The levels of GSSG quantified in the digestive glands of mussels from S1-S3 exhibited significant higher values than those from S4 and S5. The levels of GSSG

quantified in gills followed a similar pattern of those quantified in digestive glands (Table 2.3 and Figure 2.4).

The GSH/GSSG ratio quantified in digestive glands of mussels from S4 were significantly higher than those collected at S2, but did not exhibit significant differences to those from the remaining sites. The GSH/GSSG ratio quantified in gills of mussels from S4 were significantly higher than those collected at the remaining sampling sites with the exception of those collected at S5 (Table 2.3 and Figure 2.4).

The levels of IDH activity quantified in digestive glands of mussels from S1, S3 and S4 were significantly higher than those from S2, but significantly lower than those from S5 (Table 2.3 and Figure 2.5).

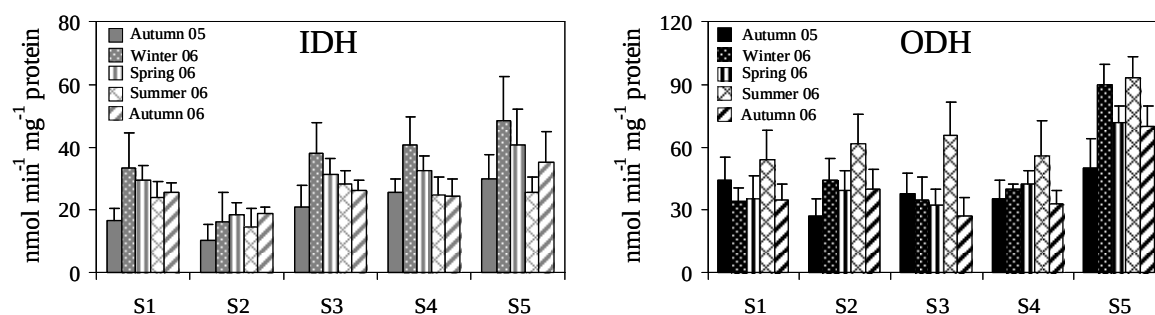


Figure 2.5 Seasonal variation of biomarkers analysed in *Mytilus galloprovincialis* collected at five sampling sites (S1-S5) along the NW coast of Portugal from the autumn 2005 to the autumn 2006. Values are presented as mean $\pm$ standard deviation ($n = 10$) of NADP⁺-dependent isocitrate dehydrogenase (IDH) quantified in mussels' digestive glands (left column), and octopine dehydrogenase (ODH) quantified in mussels' posterior adductor muscle (right column).

The levels of ODH activity measured in the posterior adductor muscle of mussels from S5 were significantly higher than those from the remaining sites (Table 2.3 and Figure 2.5).

The levels of AChE activity quantified in the haemolymph of mussels from S1 and S5 were significantly higher than those from S2 and significantly lower than those from S4. No significant differences were found between AChE activity levels in mussels from S3 with those collected from S1, S2 and S5 (Table 2.3 and Figure 2.6).

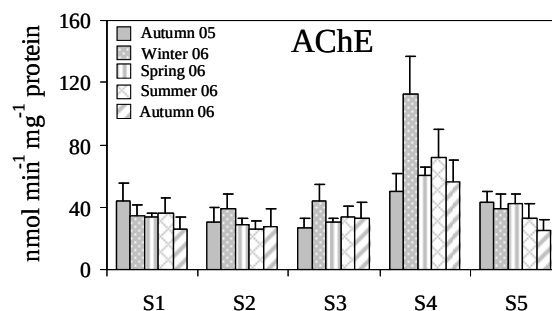


Figure 2.6 Seasonal variation of acetylcholinesterase activity analysed in *Mytilus galloprovincialis* collected at five sampling sites (S1-S5) along the NW coast of Portugal from the autumn 2005 to the autumn 2006. Values are presented as mean $\pm$ standard deviation ($n = 20$) of acetylcholinesterase quantified in mussels' haemolymph.

2.3.4. Effects of petroleum hydrocarbons and abiotic parameters on biomarkers

Significant Spearman correlation values ($p \leq 0.01$) were found between the biomarkers and some of the petroleum hydrocarbon levels quantified in mussels' tissue, as well as between the biomarkers and some of the physicochemical parameters quantified in water samples (Table 2.5 and Table 2.6). Regarding petroleum hydrocarbons, the most significant positive correlations ($r \geq 0.50$) were found between the UCM levels and the activities of CAT in mussels' digestive gland along with ODH in mussels' posterior adductor muscle. Significant negative correlations were found between the UCM levels and the levels of tGSx and GSSG quantified in mussels' digestive glands, as well as between the levels of total PAHs and the activity of ODH (Table 2.5).

Table 2.5 Significant Spearman correlation coefficients ($p \leq 0.01$) between petroleum hydrocarbon levels and biomarkers quantified in *Mytilus galloprovincialis* collected at five sampling sites (S1-S5) along the NW coast of Portugal from the autumn 2005 to the autumn 2006.

Petroleum hydrocarbons	Biomarkers			
	CAT	tGSx	GSSG	ODH
UCM	0.759 ^a	-0.544 ^a	-0.502 ^a	0.510 ^c
Σ PAHs	-	-	-	-0.510 ^c

UCM – unresolved complex mixture, Σ PAHs – total polycyclic aromatic hydrocarbons, CAT – catalase, tGSx – total glutathione content, GSSG – oxidized glutathione, ODH – octopine dehydrogenase. ^a Digestive glands; ^c posterior adductor muscle.

Regarding abiotic parameters, the most significant positive correlations ($r \geq 0.50$) were found between the levels of ammonia and the activity of SOD in mussels' digestive glands, as well as between the levels of nitrites and the activity of CAT in mussels' digestive glands. Significant negative correlations were found between temperature and the activity of SOD in mussels' digestive glands, as well as between the levels of nitrite and phosphates and the levels of tGSx, GSSG and GSH quantified in mussels' digestive glands (Table 2.6).

Table 2.6 Significant Spearman correlation coefficients ($p \leq 0.01$) between abiotic parameters quantified in water samples and biomarkers determined in *Mytilus galloprovincialis* collected at five sampling sites (S1-S5) along the NW coast of Portugal from the autumn 2005 to the autumn 2006.

Abiotic parameters	Biomarkers				
	CAT	SOD	tGSx	GSSG	GSH
T	-	-0.507 ^a	-	-	-
NH ₄	-	0.635 ^a	-	-	-
NO ₂	0.567 ^a	-	-0.591 ^a	-0.527 ^a	-0.520 ^a
PO ₄	-	-	-0.570 ^a	-0.552 ^a	-0.505 ^a

T – temperature, NH₄ – ammonia, NO₂ – nitrite, PO₄ – phosphates, CAT – catalase, SOD – superoxide dismutase, tGSx – total glutathione content, GSSG – oxidized glutathione, GSH – reduced glutathione. ^a Digestive glands.

2.3.5. Seasonality of the response of biomarkers to petrochemical contamination

The seasonality of the response of the biomarkers to petrochemical contamination was assessed by performing multivariate and graphical analysis. The results of these analyses are presented in Figure 2.7 to 2.9, as well as Tables 2.7 and 2.8.

The results of the MDS and cluster analysis, based on the similarity matrix calculated for the biomarkers using the Bray-Curtis similarity coefficient, showed a clear separation of these parameters into two distinct groups: group A, which corresponds to the biomarkers quantified in mussels collected at the sampling sites S1-S3, and group B, which corresponds to the biomarkers quantified in mussels collected at sampling sites S4 and S5 (Figure 2.7). Moreover, the ANOSIM test based on the similarity of the biomarkers revealed significant differences between Group A and Group B ($R = 0.835$; $p \leq 0.001$).

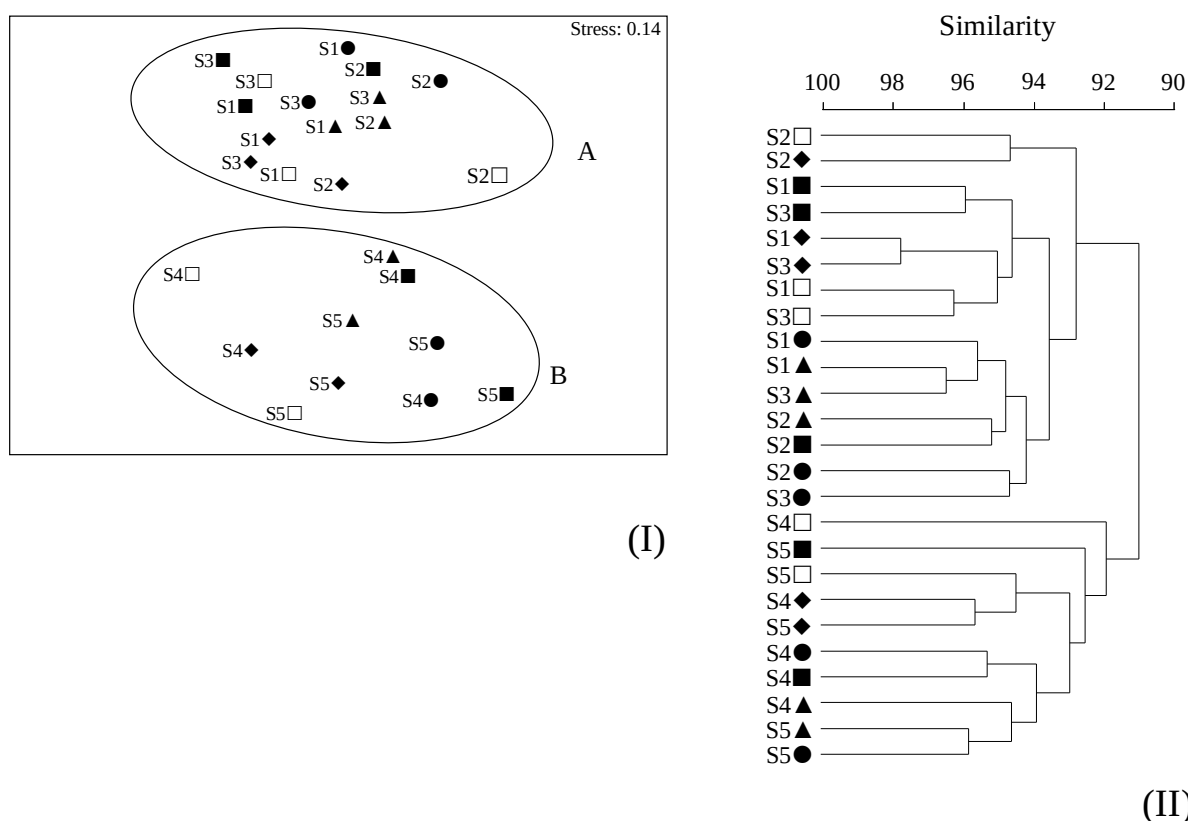


Figure 2.7 Two dimensional non-metric multidimensional scaling (MDS) ordination plot of the biomarkers analysed in *Mytilus galloprovincialis* collected at five sampling sites (S1-S5) along the NW coast of Portugal from the autumn 2005 to the autumn 2006, discriminating the distribution of the sampling sites into two distinct groups (A and B) (I). Dendrogram of the cluster analysis for biomarkers quantified in *Mytilus galloprovincialis* collected at five sampling sites (S1-S5) along the NW coast of Portugal during the autumn 2005 (□), winter (◆), spring (▲), summer (●) and autumn (■) 2006 (II).

The results of the SIMPER analysis indicated that the biomarkers that were most responsible for the assemblage of the sampling sites S1-S3 in the Group A, as well as S4 and S5 in the group B, were ODH in the posterior adductor, AChE in the haemolymph, along with GST and SOD in both gills and digestive glands. These biomarkers explained 32% of the similarity within the Group A and 34% within the Group B. Moreover, this analysis indicated that the biomarkers that explained 32% of the dissimilarities between Group A and Group B were the levels of LPO, tGSx, and consequently of GSSG and GSH, as well as the activity of CAT in the mussels' digestive gland (Table 2.7).

Table 2.7 Results of SIMPER analysis indicating which biomarkers contributed most to the overall similarities within each group, and overall dissimilarities between groups of sampling sites.

% Similarity			% Dissimilarity		
Group A	Individual contribution	Cumulative contribution	Group A-B	Individual contribution	Cumulative contribution
GST ^b	6.77	6.77	tGSx ^a	6.82	6.82
ODH ^c	6.49	13.26	GSSG ^a	6.41	13.23
AChE ^d	6.24	19.50	GSH ^a	6.29	19.52
GST ^a	6.19	25.69	CAT ^a	6.23	25.75
SOD ^a	5.87	31.56	LPO ^a	6.18	31.93
Average similarity of Group A		93.79	Average dissimilarity Group A-B		9.02
Group B					
GST ^b	7.68	7.68			
ODH ^c	6.98	14.66			
AChE ^d	6.75	21.41			
SOD ^a	6.34	27.75			
GST ^a	6.18	33.93			
Average similarity of Group B		93.09			

SOD – total superoxide dismutase, CAT – catalase, GST – Glutathione S-transferases, LPO – lipid peroxides, tGSx, total glutathione content, GSH – reduced glutathione, GSSG – oxidised glutathione, ODH – octopine dehydrogenase, AChE - acetylcholinesterase. ^a Digestive glands; ^b gills, ^cposterior adductor muscle, ^dhaemolymph. Group A – sampling sites S1-S3, Group B – sampling sites S4-S5. All values are presented in percentages.

Regarding seasonal variation of the biomarkers, the cluster analysis correspondent to the Group A exhibited two main branches that showed a clear separation of the mussels collected at S2 during the Autumn 2005 and winter from the remaining mussels. The second branch of group A separates the mussels collected at S1 and S3 during both autumn periods and winter from the mussels collected at the three sampling sites during the spring and summer; however, mussels collected at S2 during the autumn 2006 were also included in this branch (Figure 2.7). The cluster analysis correspondent to the Group B isolates the mussels collected at S4 during the autumn 2005 and at S5 during the autumn 2006 into two separate branches, and forms a third branch that divides the mussels at S5 during the autumn 2005 and the mussels collected at S4 and S5 during the winter from those collected S4 and S5 during the spring and summer; however, mussels collected at S4 during the autumn 2006 were also included in this branch (Figure 2.7).

The results of the ANOSIM test based on the similarity of the biomarkers revealed significant differences in the autumn and winter with the spring and summer ($R = 0.285$; $p \leq 0.05$) for the sampling sites S1-S3 correspondent to the Group A; however, no

significant differences were found among sampling seasons for the sampling sites S4 and S5 correspondent to the Group B ($R = 0.103$; $p = 0.295$). The results of the SIMPER analysis indicated that the biomarkers that were responsible for the assemblage of the sampling season autumn and winter as well as spring and summer for the sites S1-S3 in the Group A were GST in gills, ODH in posterior adductor muscles, AChE in the haemolymph, as well as GST and SOD in mussels' digestive glands, explaining about 32% of the similarity within autumn/winter and spring/summer groups. Moreover, this analysis indicated that the biomarkers that explained 40% of the dissimilarities between these two seasonal groups were the levels of LPO in mussels' gills and digestive glands, as well as the activities of GR in gills and digestive glands and GPx in gills (Table 2.8).

Table 2.8 Results of SIMPER analysis indicating which biomarkers contributed most to the overall similarities within each group, and overall dissimilarities between sampling seasons for *Mytilus galloprovincialis* collected at S1-S3.

% Similarity			% Dissimilarity		
Group I	Individual contribution	Cumulative contribution	Group I-II	Individual contribution	Cumulative contribution
GST ^b	6.53	6.53	LPO ^a	10.78	10.78
ODH ^c	6.34	12.86	GR ^a	8.02	18.08
AChE ^d	6.21	19.07	GR ^b	7.47	26.27
GST ^a	6.17	25.24	LPO ^b	6.96	33.23
SOD ^a	5.77	31.02	GPx ^b	6.40	39.63
Average similarity of Group I		93.91	Average dissimilarity Group I-II		6.51
Group II					
GST ^b	7.05	7.05			
ODH ^c	6.71	13.76			
AChE ^d	6.21	19.98			
GST ^a	6.14	26.11			
SOD ^a	5.92	32.03			
Average similarity of Group II		94.57			

SOD – total superoxide dismutase, GPx – selenium-dependent glutathione peroxidase, GR – glutathione reductase, GST – Glutathione S-transferases, LPO – lipid peroxides, ODH – octopine dehydrogenase, AChE – acetylcholinesterase. ^a Digestive glands; ^b gills, ^c posterior adductor muscle, ^d haemolymph. Group I – winter and autumn, Group II – spring and summer, All values are presented in percentages.

The results of the PCA analysis, performed to discriminate the similarities of each sampling season and sampling site as a function of the petroleum hydrocarbon levels

measured in mussels' tissue that exhibited significant Spearman correlation values with biomarkers, indicated that the two principal components accounted for 86.9% of the overall variability of the data (Figure 2.8). The other components were neglected because they did not provide significant additional explanation to the data. The first principal component, corresponding to the horizontal axis of the plot diagram, accounted for 52.6% of the variability of the data and was clearly associated with the levels of the total PAHs. The second principal component, corresponding to the vertical axis of the plot diagram, accounted for 34.3% of the variability of the data and was clearly associated with the levels of UCM (Figure 2.8). The BIOENV analysis indicated that the best correlations between the levels of petroleum hydrocarbons and the biomarkers (using the Spearman rank correlations) occurred with the UCM fraction ($r = 0.361$).

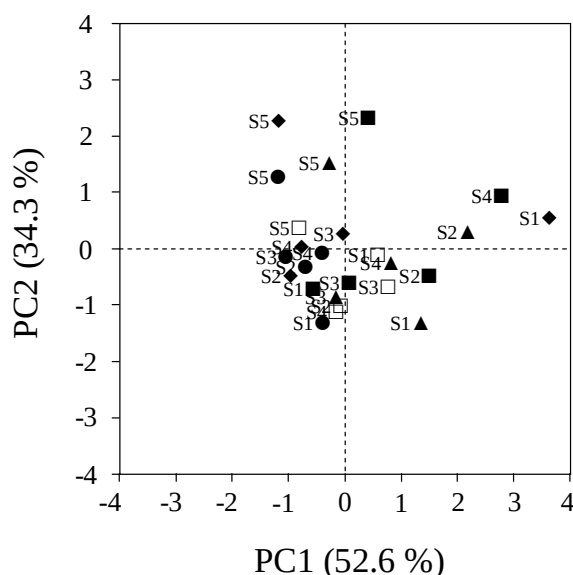


Figure 2.8 Principal component analysis (PCA) score plot for the five sampling sites as a function of the petroleum hydrocarbon levels measured in mussels' tissue. The first two principal components (PC1 and PC2) account for 52.6 % and 34.3 % of the variability in the data set, respectively. The sampling seasons are: autumn 2005 (□), winter (◆), spring (▲), summer (●) and autumn (■) 2006.

A multivariate analysis was also performed for each sampling season. The MSD analysis separated the five sampling sites into three distinct assemblages: Group A, B and C (Figure 2.9).

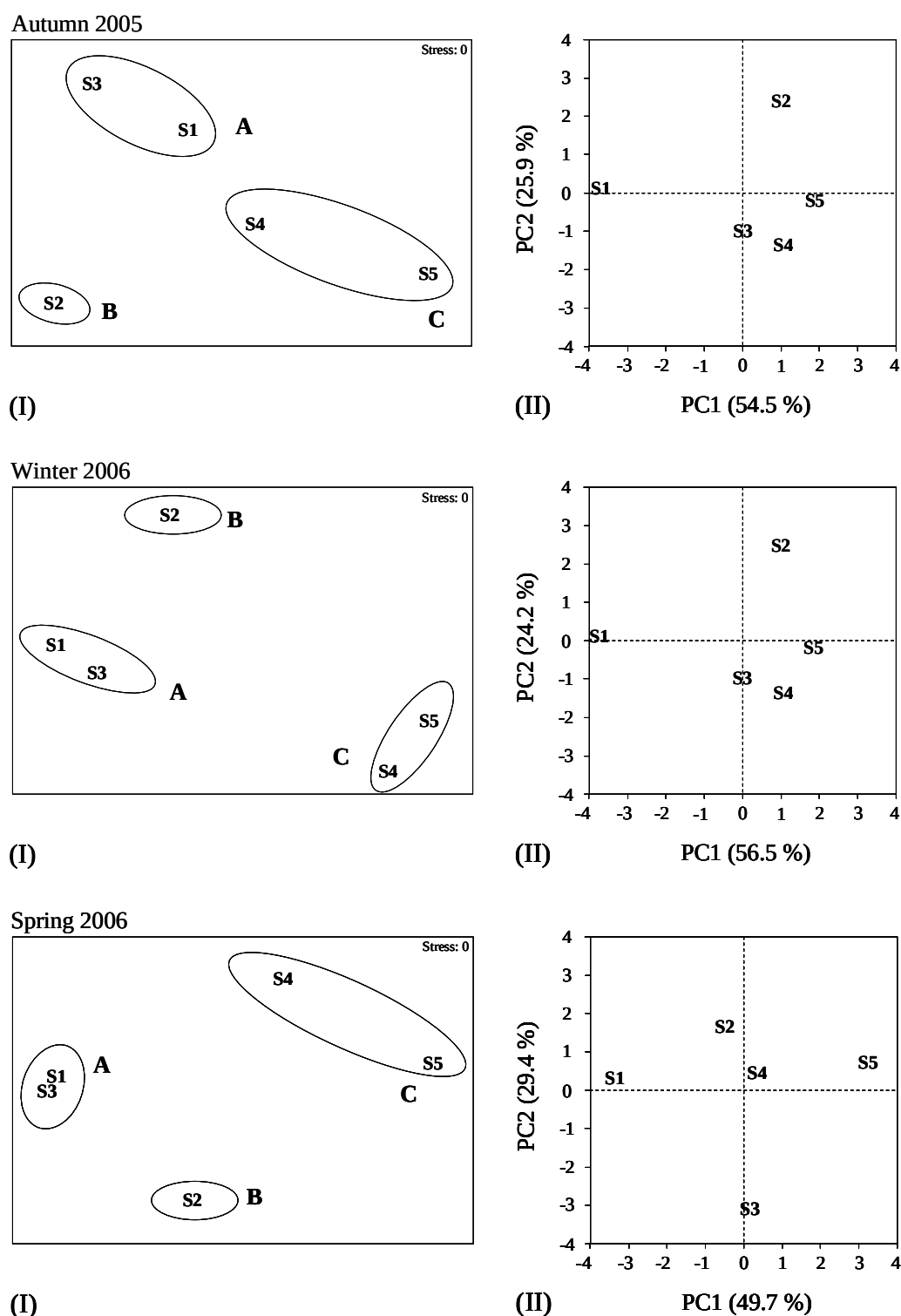


Figure 2.9 Two dimensional non-metric multidimensional scaling (MDS) ordination plot of the biomarkers analysed in *Mytilus galloprovincialis* collected at five sampling sites (S1-S5) along the NW coast of Portugal for each sampling season, discriminating the distribution of sampling sites (I). Principal component analysis (PCA) score plot for the five sampling sites as a function of the petroleum hydrocarbon levels measured in mussels' tissue for each sampling season (II). The percentage of variability explained by the two first principal components (PC1 and PC2) is indicated in the axis of the graph for each sampling season: autumn 2005, winter, spring, summer and autumn 2006.

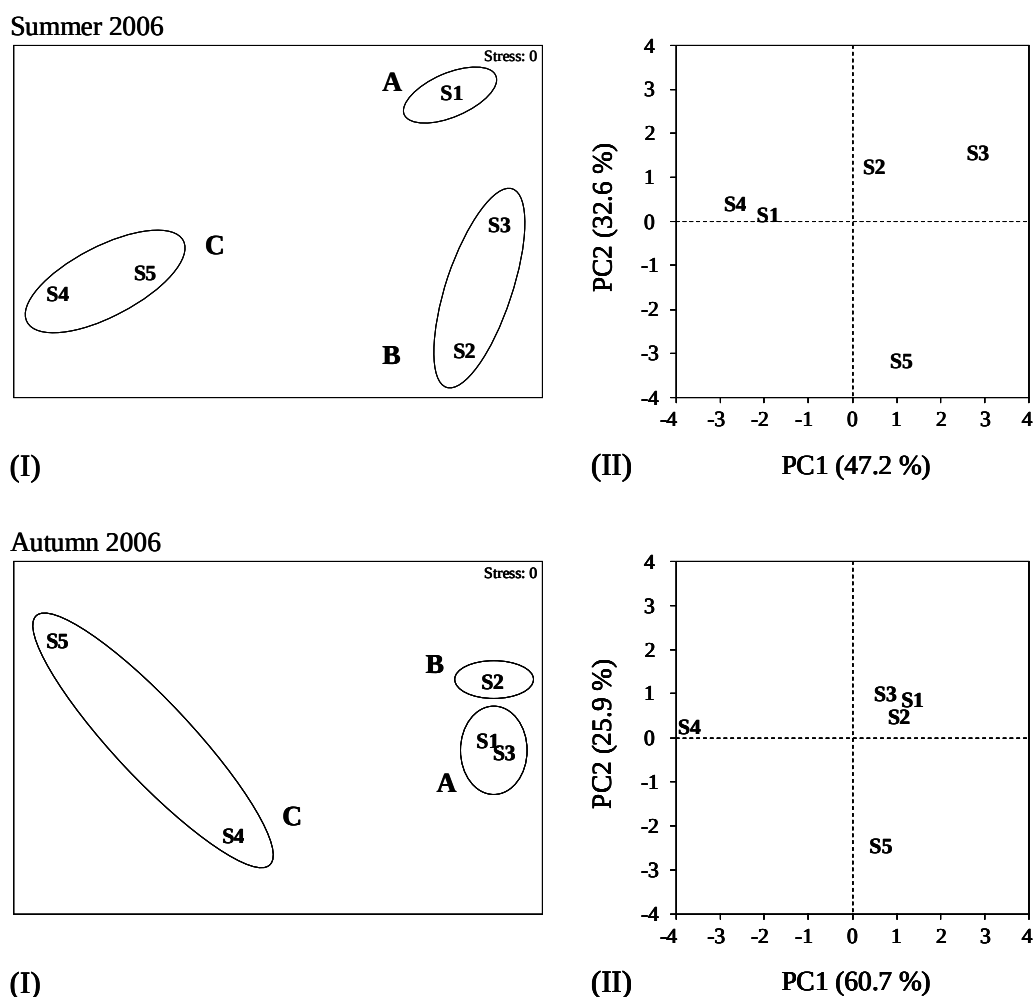


Figure 2.8 (continued).

A clear separation of sampling sites S4 and S5, which were assembled in Group C, was found in relation to the remaining sampling sites and was clearly evident throughout the entire sampling period. Sampling sites S1 and S3 were assembled in Group A and sampling site S2 in Group B throughout the sampling period with the exception of the summer period during which the MDS analysis showed a higher similarity between sampling sites S3 and S2. Similarly to the results of the annual data, the SIMPER analysis showed that the biomarkers that were mainly responsible for the assemblage of S1 and S3 in group A and S4 and S5 in group C in each sampling season were ODH, AChE and GST in mussels' gills. Though, the biomarkers that mainly contribute to the separation of the three groups are related with the glutathione metabolism either in gills or digestive glands of mussels.

The results for the PCA analysis indicate that two principal components accounted for 80% of the overall variability of the data from the autumn 2005 to the summer, and

87% in the autumn 2006. The other components were neglected because they did not provide significant additional explanation of the data.

The first principal component, which corresponds to the horizontal axis of the plot diagram, is mainly related with the levels of PAHs and AH, which accounted for 47% of the variability of the data during the summer to 60.7% during the autumn 2006 (Figure 2.9). The second principal component, which corresponds to the vertical axis of the plot diagram, is mainly related with the levels of UCM, which accounted for 24.2% of the variability of the data during the spring to 32.6% during the summer (Figures 2.9).

2.4. DISCUSSION

Since the establishment of the “Mussel Watch” program in mid 1970s ^[7], the marine mussel *M. galloprovincialis* has been widely selected as a suitable bioindicator for long-term environmental monitoring programs to assess the deleterious effects of contaminants such as heavy metals ^[47, 48], PCBs ^[49, 50], and PAHs ^[49, 50, 51]. In our research group, *M. galloprovincialis* has been applied as a bioindicator since mid 1990s. The first studies performed by our research group using *M. galloprovincialis* as a bioindicator were developed by Moreira and co-workers to assess the suitability of mussels’ AChE and GST activities as biomarkers of environmental contamination ^[20]. *M. galloprovincialis* was then used to assess the effects of the “Coral Bulker” oil spill in Viana do Castelo harbour in 2000 ^[4], and to estimate the levels of contamination by perfluorooctane sulfonates in the North-central Portuguese estuaries ^[52]. Since, January 2005 *M. galloprovincialis* has been applied as bioindicator to assess the effects of petrochemical contamination along the NW coast of Portugal ^[5, 53] (see Chapter 1 and Chapter 4). Herein, we present the results of a long-term monitoring programme to assess the spatial and temporal trends of petrochemical contamination along the NW coast of Portugal using the marine mussel *M. galloprovincialis* as bioindicator.

Prior to this work, levels of PAHs in the NW coast of Portugal have not been monitored regularly with the analysis of Serra and Salgado in 1998 ^[3] being one of the few references available for comparison. Likewise, there is a scarceness of data regarding spatial and temporal trends in the levels of AH and UCM for this region of Portugal.

The levels of PAHs quantified in *M. galloprovincialis* tissues during the present study ($0.32 \mu\text{g g}^{-1} \text{ dw}$ in S3 during summer to $7.32 \mu\text{g g}^{-1} \text{ dw}$ in S4 during autumn 2006) were considerably lower than those determined in a previous survey conducted at the same sampling sites during January 2005 ($124.21 \mu\text{g g}^{-1} \text{ dw}$ in S3 to $549.56 \mu\text{g g}^{-1} \text{ dw}$ in

S2) ^[5] (see Chapter 1). However, the PAHs results obtained in this long-term monitoring program were similar to the range of values determined during 1998 in mussels collected in the region between S3 and S5 (0.60-40.00 $\mu\text{g g}^{-1}$ dw) ^[3]. Occasional increases in the levels of PAHs have been reported in areas of Galicia affected by the “*Prestige*” oil spill, which have been caused by the remobilisation of crude oil residues from contaminated intertidal areas or sediments following rough winter weather conditions ^[54]. A similar situation might explain the high levels of PAHs found in mussels collected along the NW coast of Portugal during January 2005.

The sampling sites selected for this long-term monitoring program have been previously ranked according to the levels of PAHs quantified in mussels’ tissues during January 2005 ^[5] (see Chapter 1). Sampling sites located in the vicinity of commercial harbours (S2: Viana do Castelo harbour, S5: Leixões harbour) and oil refinery industry (S4: Cabo do Mundo) were classified as having high to moderate levels of contamination, while those located in open seashore (S1: Carreço, S3: Vila Chã) were classified as having low levels of contamination ^[5]. However, because during this study the levels of PAHs in all sampling sites were much lower than those quantified during the previous survey, and since high levels of PAHs were found in Carreço (S1) between the autumn 2005 and summer 2006, when compared to sites classified as having high to moderate levels of PAHs, the ranking of the sampling sites as function of petrochemical contamination will be further discussed upon the integration of these results with the response of the selected biomarkers.

As for the levels of PAHs, the levels of AH quantified in mussels’ tissue during the present study (0.88 $\mu\text{g g}^{-1}$ dw in S4 during autumn 2006 to 22.55 $\mu\text{g g}^{-1}$ dw in S3 during autumn 2005) were also considerably lower than those determined in January 2005 in the same area (39.65 $\mu\text{g g}^{-1}$ dw in S4 to 168.67 in S5) ^[5] (see Chapter 1). In particular, low levels of PAHs and AH were found in all sampling sites during the summer. Low levels of PAHs were also found during the summer period in *Mytilus edulis* collected in the Baltic Sea when compared to PAHs levels measured during winter and spring ^[55]. These low levels of PAHs and AH found during the summer might be explained by seasonal variations in feeding rates, lipid content and the reproductive cycle of mussels ^[55, 56]. Feeding rates increase following algal blooms which occur in spring and autumn, increasing the exposure of filter feeding organisms, such as mussels, to contaminants. Likewise, spawning episodes, which might occur from late spring to late summer, can release lipids as well as hydrophobic compounds, such as PAHs accumulated in mussels’ gonads ^[8].

The levels of UCM quantified in mussels' tissue throughout this monitoring program ($364.59 \mu\text{g g}^{-1}$ dw in S2 during autumn 2006 to $2146.95 \mu\text{g g}^{-1}$ dw in S5 during winter) were in the same range of the values found previously ($360.81 \mu\text{g g}^{-1}$ dw in S4 to $2159.83 \mu\text{g g}^{-1}$ dw in S5) ^[5] (see Chapter 1). In accordance to the results of January 2005, the highest values of UCM were found at Leixões harbour (S5) ^[5]. It is known that high ratios of unresolved to resolved petroleum hydrocarbons (UCM/total petroleum hydrocarbons) are result of degradation of petrogenic products by weathering processes ^[57, 58]. This may reflect long-term contamination of S5 by petrogenic products, either from occasional fuel spills from fishing vessels or by maintenance activities in the harbour's oil terminals. The toxicity of UCM has not been extensively studied. However, it is known that the oxidation of non-aromatic hydrocarbons, as well as some aromatic hydrocarbons, present in this petroleum fraction can enhance toxicity mechanisms in aquatic organisms ^[59, 60]. Lima et al. (2007) ^[5] found significant correlations between the levels of UCM quantified in mussels' tissues and the activities of SOD, GPx and IDH quantified in mussels' digestive glands in January 2005 within the same study area (see Chapter 1).

One way of assessing the deleterious effects of petrochemical contamination in aquatic ecosystems is through the implementation of biomarker based monitoring programs ^[61]. In particular, biomarkers have been extensively used to assess environmental impacts following the oil spills of "Exxon Valdez" in Prince William Sound (Alaska, USA) ^[62, 63], "Aegean Sea" in the coast of Galicia (NW Spain) ^[57], "Coral Bulker" in Viana do Castelo harbour (NW Portugal) ^[4], and more recently the "Prestige" affecting Galicia and the Cantabric coasts ^[51]. In the present study, several enzymatic and non-enzymatic parameters involved in key physiological processes of marine invertebrates (antioxidant defences, detoxification, energetic metabolism and neurotransmission) were applied as biomarkers to assess the effects of chronic petrochemical contamination in *M. galloprovincialis* collected along the NW coast of Portugal.

Generally, molecular and biochemical biomarkers have been used in ecotoxicology as early warning indicators of contamination. Since the deleterious effects of some chemicals are usually first displayed at lower levels of biological organisation, it is possible to predict effects that may occur later at population, community and ecosystem levels, allowing enough time for the development of preventive measures ^[64]. As previously discussed, feeding rates, lipid content and the reproductive cycle might affect levels of PAHs accumulated in mussels' tissues ^[55, 56]. Likewise, to produce an accurate interpretation of a biomarkers response to petrochemical contamination, natural seasonality of the mussels' biochemical processes needs to be considered ^[65]. In

particular, the levels of some oxidative stress parameters, broadly used as biomarkers of petrochemical contamination, may fluctuate considerably throughout the year due to the influence of abiotic factors (e.g. temperature, salinity, dissolved oxygen concentrations) ^[66, 67]. Reactive oxygen species (ROS), including superoxide ($O_2^{\cdot-}$) and hydroxyl ($\cdot OH$) radicals, as well as H_2O_2 , are produced as by-products of normal cellular functions such as the mitochondrial electron transport chain, the microsomal system of the endoplasmatic reticulum, and enzymatic oxidase reactions ^[66]. When normal environmental conditions change (e.g. temperature, salinity, dissolved oxygen concentrations), the production of ROS might be enhanced as a natural response to these environmental fluctuations ^[66, 67]. However, several studies have shown that cellular production of ROS may also be enhanced when aquatic organisms are exposed to some organic contaminants (e.g. PAHs, PCBs, dioxins, quinines, nitroamines) ^[68], and metals (e.g. iron, copper, chromium, mercury, lead) ^[69]. In the absence of stress conditions, the mussels' normal metabolism maintains a balance between the generation of ROS and their detoxification and removal by enzymatic and non-enzymatic antioxidant defence mechanisms. However, when an imbalance occurs and ROS production increases, the activity of antioxidant enzymes such as SOD, CAT and GPx is enhanced to eliminate ROS, which in high concentrations can be highly toxic to aquatic organisms due to lipid peroxidation of cell membranes, protein oxidation and DNA damage ^[70, 71]. As such, seasonal fluctuations in the levels of oxidative stress parameters may cause significant limitations upon the interpretation of biomarker results *per se* as increased levels of some antioxidant enzymes may simply be related with normal physiological responses of mussels to abiotic cyclic conditions and not with contaminant exposure ^[65]. To overcome this problem in the present study, the seasonality of biomarker response to petrochemical contamination was assessed by correlating them with abiotic parameters quantified in water samples collected during each survey. This procedure follows the monitoring strategy suggested previously by Lima et al. (2007) ^[5] upon the survey of January 2005, when significant correlations were found between the response of some biomarkers and abiotic parameters, namely ammonia and nitrates (see Chapter 1).

The function of the antioxidant enzymes SOD and CAT is to neutralise ROS before they initiate radical chain reactions. While SOD detoxifies $O_2^{\cdot-}$ radicals, generating H_2O_2 , CAT reduces the environmental and internally produced H_2O_2 ^[68, 70]. Some studies suggest that the activities of mussels' SOD and CAT enzymes are under strict seasonal control, normally exhibiting the lowest baseline activity values during winter ^[65]. The results obtained in the present study illustrated that mussels' digestive glands exhibited significantly high levels of SOD and CAT activities during spring. Surprisingly, significantly

high levels of SOD activity in mussels' digestive glands were also found during winter, which could indicate ROS formation enhanced by contaminants. However, no significant correlations were found between levels of SOD activity in mussels' digestive glands and petroleum hydrocarbons. This suggests that other classes of contaminants present in the environment may be inducing the production of ROS, and consequently increasing the levels of SOD activity in mussels' digestive glands as a compensation mechanism to prevent cellular damage. A significant negative correlation was found between the levels of SOD activity in mussels' digestive glands and temperature, backing the previous statement. Studies carried out with *M. galloprovincialis* exposed to high levels of metal contamination in the Mediterranean Sea also exhibited increased levels of SOD activity during the winter period, decreasing later during spring ^[47]. In these studies, mussels collected from polluted sites also exhibited higher levels of SOD activity than mussels from unpolluted sites ^[47]. This is in agreement with our data, which in keeping with the survey conducted in January 2005, showed that mussels collected at near an oil refinery industry (S4) and Leixões harbour (S5) presented significantly higher levels of SOD and CAT activities in digestive glands than in those collected at the remaining sites ^[5] (see Chapter 1). Finally, a significant positive correlation was found between the levels of SOD activity in mussels' digestive glands and ammonia levels. Likewise, significant positive correlations were also found between CAT activity in mussels' digestive glands and the levels of UCM and nitrites.

The GST, a family of multi-functional enzymes involved in Phase II of biotransformation processes, has an important role in the detoxification processes of molluscs and is known to be linked to their antioxidant defence system. Besides having an active role in the conjugation of electrophilic xenobiotics with GSH, it has been reported that GST enzymes (GST1) in *M. edulis* also present a distinct GSH peroxidase activity ^[65]. The present study showed that significantly high levels of GST activity were found in both digestive glands and gills of mussels collected during autumn 2005. The autumn of 2005 was the period in which mussels presented significantly low levels of CAT activity in both digestive glands and gills. This situation, which was also verified in the survey performed in January 2005 in mussels' gills, may suggest that an increase in the levels of GST activity might act as a cellular compensation mechanism when CAT activity is low, in order to protect against ROS induced damage ^[5] (see Chapter 1). A similar situation has been reported for the GST levels in gills of *M. edulis* from Le Havre harbour in France during winter, when the baseline levels of antioxidant defences in molluscs should be at their lowest levels ^[65, 72]. In addition, high levels of GST activity were also found in mussels collected from Leixões harbour (S5), as happened during 2005 ^[5] (see Chapter 1).

However, high levels of GST activity were also found in gills of mussels from Carreço (S1), previously classified as having low levels of petrochemical contamination. Abnormal high levels of PAHs measured in the tissues of mussels collected at this site could explain these high levels of GST, as reported by Moreira et al. (2004) ^[4] in *M. galloprovincialis* collected following the “*Coral Bulker*” oil spill. However, herein no significant correlations have been found between GST enzymes and the levels of PAHs.

The GST is one of many enzymes involved in GSH metabolism in mussels ^[73]. GSH is the most abundant cellular non-protein thiol, which plays a major role in the maintenance of intracellular redox balance, as well as in the regulation of signalling pathways enhanced by oxidative stress ^[74]. When high levels of ROS are detected, in particular organic and inorganic peroxides, GSH is oxidised to its disulfide form (GSSG) by the activity of GPx. However, the GSH/GSSG ratio needs to remain high in order to maintain the redox homeostasis of the cell ^[75]. It is known that low GSH/GSSG ratios may impair the structure and function of cellular membranes, the maintenance and polymerization of microtubules, and the metabolism of proteins and electrophilic agents ^[75]. Consequently, when the organism is under oxidative stress, cellular levels of GSH can be maintained by the enzyme GR which converts GSSG back into GSH at the expense of NADPH, which is posteriorly regenerated by pentose phosphate pathway or by NADP⁺-dependent IDH ^[75, 76]. In the present work, significantly high levels of GPx activity were found in digestive glands and gills of mussels collected during the summer, and significantly low levels of GPx activity were found during the spring. However, significantly low levels of GR activity were found in digestive glands and gills of mussels collected during spring and summer. This is in agreement with high levels of GSSG found in digestive glands of mussels collected during the summer. However, high levels of GSSG were also found during the spring, and no seasonal fluctuations were found in the GSSG levels quantified in mussels' gills. It is important to mention that in the present work the GPx quantifications were limited to selenium-dependent enzymes, which only reduce H₂O₂ molecules to water, as well as organic hydroperoxides to their matching hydroxy compounds ^[77]. High levels of GSSG during the spring might have been generated by high activity of other forms of GPx enzymes, which are selenium independent and only reduce organic hydroperoxides ^[77]. For a correct interpretation of GSH metabolism we recommend that both families of GPx enzymes are quantified in future work. Moreover, considering the previous results it is unlikely that significantly high levels of GSH will be found during the summer, since low levels of GR activity seem to indicate that GSH was not being regenerated. However, for a full understanding of GSH results, the activity of enzymes involved in GSH synthesis (γ -glutamylcystein synthetase and GSH synthetase)

and GSH cellular transport (γ -glutamyl transpeptidase) should also be considered ^[76]. No significant differences were found in the GSH/GSSG ratio quantified in mussels' digestive glands throughout the year, which seems to indicate that the antioxidant defences in this organ were operating effectively regardless of any seasonal fluctuations in environmental parameters. However, in gills, significant differences in the GSH/GSSG ratio were found among sampling periods indicating that this organ may be more susceptible to seasonal fluctuations of environmental parameters. Mussels' gills are more exposed to environmental stressors than digestive glands, and overall present lower levels of antioxidant enzyme activities ^[65]. The results obtained during this monitoring program also showed that significantly high levels of GPx activity were found in the digestive glands of mussels collected at near an oil refinery industry (S4) and Leixões harbour (S5). However, significantly high levels of GR activity were found in digestive glands of mussels from sites previously classified as having low levels of petrochemical contamination (S1 and S3). These results are in agreement with the results found by Lima et al. (2007) ^[5] during January 2005 (see Chapter 1). Surprisingly, low GSH/GSSG ratios were found in S1 and S3 when compared to S4, which is in agreement with low levels of GR activity and high levels of GSSG found at S1 and S3. In the present work significant correlations were found between the levels of GSSG and UCM, as well as between the levels of GSH and GSSG and the levels of nitrites and phosphates. It is important to mention that no significant correlation was found between the levels of GPx and GR activities, and petroleum hydrocarbon levels as reported in January 2005 ^[5] (see Chapter 1). This might indicate that GPx and GR are not as suitable as initially thought for use *per se* in long-term monitoring programs to assess the effects of petrochemical contamination.

When antioxidant defences are unable to overcome the production of ROS, oxidative damage such as LPO may occur impairing the cellular integrity of the organism ^[68]. In the present study high levels of cellular impairment were detected during autumn 2005 and winter for both tissues as indicated by high levels of LPO. These results are in agreement with low levels of SOD and CAT activities quantified during the autumn 2005. Moreover, high levels of SOD activity quantified in mussels' digestive glands during the winter were not sufficient to avoid cellular damage induced by LPO. In agreement with the results found in the present work, high levels of LPO are expected to appear during winter periods when the baseline activities of antioxidant enzymes are at their lowest levels ^[65]. High levels of LPO were also found in digestive glands of mussels collected near an oil refinery industry (S4) and Leixões harbour (S5), as well as in gills of mussels from Viana do Castelo harbour (S2). These results were expected considering the preliminary survey conducted in January 2005 ^[5] (see Chapter 1).

As for antioxidant defences, the activity of enzymes involved in the aerobic and anaerobic energetic metabolism of molluscs is strongly influenced by seasonal fluctuations of environmental parameters, namely temperature and food abundance ^[65]. For this study we selected the enzymes NADP⁺-dependent IDH and ODH to assess the effects of petrochemical contamination in the mussels' energetic metabolism. Studies involving NADP⁺-dependent IDH and ODH have mainly focused upon their biological function and have not been applied regularly as biomarkers in monitoring programs ^[78, 79, 80, 81, 82, 83, 84]. As such, interpretation of the results obtained for these enzymes during this long-term monitoring program may be hampered by the lack of data regarding the use of these enzymes as biomarkers. Presently, the biochemical role of NADP⁺-dependent IDH is not fully elucidated. While NAD⁺-dependent IDH is one of the enzymes involved in the citric acid cycle, NADP⁺-dependent IDH seems to act more as a regulator of cellular defences against oxidative stress, mainly by the regeneration of NADPH oxidised by GR during the reduction of GSSG to GSH ^[76, 83, 84]. In the present work, high levels of IDH activity were found during winter and spring, illustrating that high levels of NADPH were available for GR to regenerate GSH using GSSG produced by GPx upon the reduction of peroxides. During winter relatively high levels of GPx and GR activity were found in mussels' digestive glands, which coincided with low levels of GSH. Since this is the time of the year during which mussels' exhibit low baseline levels of antioxidant defences, this increase in the activity of GPx, GR and subsequently IDH, seem to indicate that ROS had been produced due to the presence of contaminants (a similar situation has been discussed for the results of SOD). Moreover, if the production of GSSG exceeds the regeneration rate of GSH, in order to maintain the normal levels of cellular redox status, the excess GSSG produced during the elimination of ROS is translocated outside the cell by specific transporters to avoid NADPH exhaustion ^[35, 75]. This is in agreement with the low levels of GSSG found during this time of the year, which might have been transported outside the cell to maintain its redox status. By spring the activities of these enzymes were much lower and GSH levels had been re-established. Regarding sampling sites, significantly high levels of IDH activity were found in mussels from Leixões harbour (S5), while significantly low levels of IDH activity were found at Viana do Castelo harbour (S2), as had occurred previously in January 2005 ^[5] (see Chapter 1). However, when evaluating the annual data of IDH activity, no significant correlations were found with petroleum hydrocarbons, as verified during January 2005 with AH and UCM levels ^[5] (see Chapter 1).

ODH is a pyruvate oxidoreductase enzyme involved in the anaerobic metabolism of several invertebrates, with a function similar to lactate dehydrogenase in vertebrates, which regenerates NAD⁺ during anaerobic glycolysis ^[85]. Since this enzyme is involved in

the anaerobic metabolism of mussels we may deduce that during the summer, when temperature is higher and levels of dissolved oxygen in water are lower, it will exhibit higher levels of activity to compensate for the lack of oxygen available for aerobic respiration. The high levels of ODH that were found in mussels collected during the summer seem to support this. However, no significant correlations were found between ODH and the environmental parameters measured during this study. Moreover, significantly high levels of ODH activity were found in mussels from Leixões harbour (S5) as previously reported during January 2005 ^[5] (see Chapter 1). In the present study significant correlations were found between the levels of ODH activity, and the levels of UCM and PAHs (particularly anthracene). It is known that impairment of the energetic metabolism of marine bivalves has occurred in the presence of petroleum hydrocarbons ^[86]. When mussels are exposed to certain environmental contaminants, such as petrochemical products, they are able to reduce cellular respiration to conserve energy ^[85, 86]. If, as a result, the rate of cellular oxygen uptake is insufficient anaerobic metabolism may be enhanced to supply extra ATP ^[85, 86]. The results obtained for ODH during this long-term monitoring program seem to indicate that this enzyme is a suitable biomarker to assess the effects of chronic petrochemical contamination. Unexpectedly, no correlation was found between this enzyme and environmental parameters indicating that the effects of petrochemical contamination may overcome those of seasonal fluctuations of environmental parameters.

Finally the enzyme AChE, which is involved in breakdown of the neurotransmitter acetylcholine during the transmission of nerve impulses across cholinergic synapses, has been widely used as an indicator of neurotoxicity in marine invertebrates ^[87]. Its inhibition has been widely used as a specific biomarker for organophosphate and carbamate pesticides, but significant inhibitions in AChE activity have also been reported in *M. galloprovincialis* exposed to petrochemical contamination ^[4, 88]. This is in agreement with our results. In particular, significantly low levels of AChE activity were found in mussels collected at Viana do Castelo harbour (S2), which was affected by an oil spill in 2000. Moreira et al. (2004) ^[4] reported low levels of AChE activity in mussels collected near the site of the oil spill when compared to mussels collected at a distance of 10 Km. However, after a year this effect on the mussels' AChE activity was no longer found. The low levels of AChE activity reported herein did not present any significant correlation with the petroleum hydrocarbons quantified in mussels' tissues. As such other classes of contaminants might be responsible for these results. Anticholinergic products, such as insecticides, can easily affect this location in the form of runoff from agriculture fields, as well as domestic and industrial effluents. Unexpected significantly high levels of AChE

activity were quantified in mussels collected during the winter. These results might have been influenced by abnormal values quantified in mussels collected near an oil refinery industry (S4) during this time of the year. High levels of AChE activity have been detected in mussels collected at S4 throughout the year when compared to those collected at the remaining sites. Moreover, during the winter extremely high levels of AChE activity were detected in mussels from S4 over three consecutive winter periods (data not published). It has been reported that high levels of AChE activity and/or expression are related to the apoptosis of neurons in neurodegenerative disorders, tumorigenesis and abnormal megakaryocytopoiesis ^[89, 90]. In addition, Small et al. (1996) ^[89] have reported that AChE can be expressed in tissues that are not directly innervated by cholinergic nerves. In future surveys, in addition to the quantification of AChE, we recommend the use of a biomarker for apoptosis (e.g. caspase-3) to better understand this increase in the levels of AChE activity in mussels collected near an oil refinery industry.

Despite being well established, the use of biomarkers in ecotoxicology studies has been the target of some controversy regarding their suitability as a tool for risk assessment studies ^[91]. Normally, biomarkers are molecular/biochemical endpoints which correspond to low levels of biological organization ^[64]. Therefore, at population and/or community levels, the biological significance of the biomarker response may not be relevant, which limits its use in risk assessment studies ^[4, 91]. Nevertheless, the use of biomarkers may be a valuable tool to provide information about toxicity mechanisms enhanced by contaminants, as well as deleterious effects that may impair the performance of the organism ^[4, 68]. As such, alongside chemical analysis, biomarkers can be included in environmental monitoring programs as a fast-screening tool of the biological effects of contaminants, prior the implementation of preventative bioremediation strategies ^[4, 64]. Another limitation that needs to be considered when implementing biomarker-based monitoring programs is the fact that the response of a single biomarker may be impaired due to its sensitivity to seasonal fluctuations of abiotic factors and physiological cycles ^[55, 56]. Moreover, field sites are normally exposed to a complex mixture of contaminants, making it difficult to correlate biomarker responses with a particular class of contaminants ^[11]. Therefore, examining biomarkers singly to show an effect of contaminant exposure is insufficient, and unlikely to yield useful predictions of effects at higher organisational levels ^[11, 16]. Instead it is recommended to study patterns of several biomarker responses to obtain a holistic view of the effects of contaminants on the biological system, since each biomarker will highlight the influence of a specific class of contaminant ^[11, 16]. However, the interpretation of such comprehensive set of data may be rather difficult.

In this study we implemented a multivariate and graphical analysis, initially recommended by Clarke and Green (1988) ^[10] for the study of biological effects of contaminants, to assess its suitability as a tool for the analysis of chemical analysis and biomarker response obtained during long-term monitoring programs. Other multivariate analyses have been applied in biomarker-based monitoring programs. However, the statistical and graphical interpretation of some results can be somewhat complex (e.g. star plots of integrated biomarker response) ^[12]. In the present study the results of the MDS and cluster analysis made a clear separation of the sampling sites according to the biomarker response. Sampling sites S1-S3 were assembled in one group, while sampling sites S4 and S5 were assembled in a second group. In agreement with these results, Cabo do Mundo (S4), located near an oil refinery industry, and Leixões harbour (S5) have been previously grouped together according to the levels of PAHs quantified in mussels' tissues upon the survey conducted during January 2005 ^[5] (see Chapter 1). As such, the multivariate analysis herein implemented seems to be a suitable tool for the discrimination of contamination levels according to biomarker response. Astley et al. (1999) ^[16] also reported that the integration of biomarker response according to MDS and cluster analysis was more sensitive in the discrimination of a contamination gradient in the Tees Estuary (UK) than the conventional toxicity tests, *Tisbe battagliai* and MicrotoxTM. In the present study Cabo do Mundo (S4) and Leixões harbour (S5) were grouped according to the response of ODH, and AChE, as well as GST and SOD quantified in mussels' gills and digestive glands. From these, only ODH had significant correlation with petroleum hydrocarbons (UCM and PAHs). This may indicate that the response of AChE and GST, which presented no significant correlations with abiotic parameters, might be related with the exposure to other classes of contaminants. The levels of SOD activity quantified in mussels' digestive glands correlated positively with ammonia. These biomarkers were also responsible for the assemblage of S1-S3 in a separate group. Moreover, it was the response of the biomarkers LPO, GSH, GSSG, as well as CAT quantified in mussels' digestive glands that were responsible for the dissimilarities found between mussels collected from S1-S3 from those collected from S4 and S5. It is important to point out that these biomarkers, with the exception of LPO, presented significant correlations with abiotic parameters, namely the levels of nitrites and phosphates. Only CAT showed a significant correlation with the levels of UCM. Regarding seasonality in the biomarkers response, the cluster analysis indicated that the biochemical parameters quantified in mussels sampled from S1-S3, which apparently were less impacted by petrochemical contamination, exhibited significant differences in the biomarker response quantified during autumn and winter periods, to those quantified during spring and summer. However, biomarker response quantified in mussels sampled from S4 and S5, sites which

were potentially more impacted, did not exhibit these seasonal fluctuations. This suggests that the effect of high levels of contamination may overlap those of abiotic factors. The biomarkers responsible for the differences detected between the autumn/winter and spring/summer periods were LPO, GR, and GPx quantified in mussels' in gills.

Moreover, the PCA analysis performed with the levels of petroleum hydrocarbons quantified in mussels' tissues explained 86.9% of the variability of the data. While the levels of PAHs explained 52.6% of the distribution of sampling sites according to the levels of petrochemical contamination, the levels of UCM explained 34.3% of that distribution. However, for a more accurate interpretation of the data in future work we recommend chemical analysis of other classes of contaminants such as metals and PCBs. By performing these additional chemical analysis, and by conducting MDS and PCA statistical tests for each sampling period it will be easier to check which type of contaminant is responsible for the overall response of biomarkers and for the assemblage of sampling sites according to levels of contamination. Upon the graphical analysis of the data obtained in the present work for each sampling period we observe almost a perfect match between the biomarker response analysed by MDS and the levels of petrochemical contamination analysed by PCA during the autumn 2006, indicating that during this period this was the class of contaminants that was influencing the response of the selected biomarkers. However, such a good match between the results of MDS and PCA analysis might indicate the influence of other type of contaminants in the biomarker response. As expected, the results of the BIOENV analysis indicated that the UCM petroleum fraction was more closely related with the response of biomarkers. Future work regarding the toxicity mechanisms enhanced by this petroleum fraction to invertebrates should be preformed, including whole-organism responses such as post-exposure feeding, growth rates and survival.

2.5. CONCLUSIONS

In conclusion, it was recognised that the multivariate and graphical analyses used in this work are valuable tools for the interpretation of complex sets of chemical and biomarker data obtained during long-term monitoring programs, as previously reported by Astley et al. (1999) ^[16] for data obtained in the Tees Estuary. These analyses illustrated that some of the selected biomarkers were able to discriminate the selected sampling sites according to the levels of contamination. Biomarkers involved in the mussels' anaerobic metabolism (ODH), neurotransmission (AChE), and detoxification (GST)

processes, as well as some oxidative stress parameters (SOD, CAT, LPO, GSH, and GSSG) were shown to have the greatest influence upon sampling site discrimination. Moreover, these multivariate and graphical analyses also illustrated that biomarkers quantified in mussels sampled from sites which were potentially less impacted exhibited significant differences in their response throughout the year, while those quantified in mussels sampled from sites which were potentially more impacted did not demonstrate seasonal fluctuations. This suggests that the effects of high levels of contamination may overlap those of abiotic factors. In particular, Anderson and Lee (2006) ^[61] stated that for a biomarker to be used to monitor petrochemical contamination, its response needs to be exclusively linked to petroleum exposure and not be strongly influenced by internal and external confounding factors. Herein, we observed that the activity of ODH presented a significant positive correlation with the levels of UCM and apparently was not influenced by seasonality indicating its suitability as biomarker. We suggest that the monitoring strategy implemented in the present work to assess the spatial and temporal trends of petrochemical contamination along the NW coast of Portugal is suitable since it was possible to discriminate the levels of petroleum hydrocarbon contamination present in each sampling site according to biomarker responses quantified in *M. galloprovincialis*. This strategy is therefore recommended for future work.

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PART III

DEVELOPMENT OF NEW TOOLS TO ASSESS THE EFFECTS OF PETROCHEMICAL CONTAMINATION CONSIDERING MUSSELS' TOXICITY MECHANISMS

CHAPTER 3

Integration of enzymatic activity and gene expression of antioxidant defences of *Mytilus galloprovincialis* chronically exposed to petrochemical contamination

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ABSTRACT

It is known that petrochemical products induce a variety of toxicity mechanisms in aquatic organisms. The present work aimed to study the response of the marine mussel *Mytilus galloprovincialis* to chronic exposure of petrochemical products under natural scenarios and laboratory conditions. For this, key physiological processes (antioxidant defences, detoxification, energetic metabolism and neurotransmission) of mussels were used as biomarkers. Wild mussels were collected along the NW coast of Portugal from sites with different levels of petrochemical contamination. The multidimensional scaling (MDS) analysis assembled these sites into three groups (A, B and C) as function of the biomarker response. The separation of Group A (low levels of contamination) and Group C (high levels of contamination) was mainly due to differences in the activities of catalase (CAT) and superoxide dismutase (SOD) in mussels' digestive glands. The MDS grouping corresponded well with the principal component analysis ordination diagram, which assembled the sampling sites as function of petroleum hydrocarbon levels measured in mussels' tissues. An exception was found in a site located near an oil refinery, which may be under the influence of different classes of contaminants. The effects of petrochemical products were then evaluated in mussels chronically exposed to water-accommodated fraction of #4 fuel-oil (WAF) under laboratory conditions. Results showed that the activity of the enzymes CAT and SOD measured in mussels' digestive glands exhibited an induction of 65% and 138% respectively for the 50% WAF. In light of these results, the gene expression of Cu/Zn-SOD and CAT on mussels' digestive glands was investigated. Levels of gene expression were compared with enzymatic activities to elucidate oxidative stress mechanisms in invertebrates at the transcriptional level. Results showed that gene expression of CAT corresponded well with its enzymatic activity in mussels chronically exposed to petrochemical products, showing its role as a major defence against oxidative stress induced by contaminants. The use of gene expression of CAT as a biomarker for petrochemical contamination is further discussed.

Keywords: *Mytilus galloprovincialis*, oxidative stress, biomarkers, gene expression, petrochemical contamination

3.1. INTRODUCTION

The input of petrochemical products into estuaries and coastal areas has been increasing considerably due to the growing demands for fossil fuels. As a consequence, the number of monitoring programs developed to assess the deleterious effects of this class of contaminants in aquatic ecosystems has been increasing worldwide. To date, the majority of environmental monitoring programs have focussed on the integration of chemical data and/or biomarker responses at the individual (e.g. scope for growth and feeding rate) ^[1, 2], cellular (e.g. lisosomal membrane stability and neutral lipid retention) ^[3, 4], and biochemical level (e.g. activity of enzymes involved in detoxification and oxidative stress processes) ^[5, 6, 7]. However, recent technological advances have allowed the development and implementation of new tools to assess the adverse effects of contaminants at the transcriptional level ^[8, 9]. The genomes of some aquatic vertebrates, such as the zebrafish (*Danio rerio*), are already available, allowing a fast and easy identification of gene sequences from fish used as bioindicators in monitoring programs ^[10]. However, until recently such information was seldom available for invertebrates ^[8]. This limitation impaired the development of new tools that could allow the identification of contaminant induced damage at the transcriptional level in organisms such as the marine mussel *Mytilus galloprovincialis* ^[8]. Consequently, the development of protocols that allowed the study of gene expression in *M. galloprovincialis* under normal or stressing conditions was only feasible following work such as that by Vernier and co-workers (2003), which created one of the first catalogues of genes for mussels ^[11]. Marine mussels, such as *M. galloprovincialis*, have been commonly used in monitoring programs due to their ecological and economic importance. Mussels are filter-feeders with low metabolism that accumulate chemicals in their tissues in concentrations above those existent in the environment, which may reflect long-term exposure to contaminants such as petrochemical products ^[12]. A biomarker that has been widely applied to assess the effects of petrochemical contamination is the aryl hydrocarbon receptor (AhR) and the cytochrome P450 1A (CYP1A) ^[13, 14]. It has been reported for field and laboratory studies that these biochemical parameters have a dose-dependent response to polycyclic aromatic hydrocarbons (PAHs) ^[13, 14]. However, as previously mentioned such dose-dependent response occurs primarily in aquatic vertebrates. In mussels, PAHs tend not to bind to the AhR receptor as easily and as a consequence the activity of the CYP1A system is lower or non-existent in these organisms ^[13, 14]. As such, the application of AhR and CYP1A as biomarkers is rather limited in studies that use mussels as bioindicators. To our knowledge there is none specific biomarker of effect for petrochemical

contamination in mussels. Regarding these limitations a significant effort should be dedicated to the development of new tools that can be used as biomarkers to assess the effects of petrochemical contamination in such organisms, especially knowing that other toxicity mechanisms (e.g. oxidative stress) are also induced by petrochemical products in aquatic invertebrates.

With the aim of developing new tools to assess the effects of this class of contaminants using *M. galloprovincialis* as a bioindicator, and to better understand the toxicity mechanisms induced by this class of contaminants in marine mussels, the present study integrated the enzymatic activity and gene expression of two enzymes involved in the antioxidant defence system of *M. galloprovincialis* chronically exposed to petrochemical products under natural exposure scenarios and controlled laboratory conditions. Three specific issues were investigated. First, as part of a long-term monitoring program developed to assess the levels of petrochemical contamination along the NW coast of Portugal, wild mussels were collected from five sampling sites with different levels of petrochemical contamination. For that, key physiological processes (antioxidant defences, detoxification, energetic metabolism and neurotransmission) of mussels were applied as biomarkers. In addition, the levels of aliphatic hydrocarbons (AH), unresolved complex mixture (UCM), and PAHs were quantified in mussels' tissue to investigate possible correlations between biomarker responses and the levels of petrochemical contamination of each site. Abiotic parameters were also quantified in water samples from each site to investigate possible effects on the biomarkers response to petrochemical contamination. Second, the effects of petrochemical products were further evaluated in mussels chronically exposed to water-accommodated fraction of #4 fuel-oil (WAF) under controlled laboratory conditions to determine the specific response of the selected biomarkers to such products. The results of field and laboratory studies indicated that the enzymes superoxide dismutase (SOD) and catalase (CAT) quantified in mussels' digestive glands were the most responsive biomarkers to petrochemical exposure. These enzymes, which are highly responsive to increasing levels of contaminant stimulated reactive oxygen species (ROS), are the first lines of antioxidant defences to act in order to protect the organisms from cellular oxidative damage ^[15]. As such, in light of the biomarker results obtained in the field and laboratory studies, and with the aim of developing new tools to diagnose contaminant induced damage at the transcriptional level in *M. galloprovincialis*, the third issue to be investigated in the present study was the response of the gene expression of Cu/Zn-SOD and CAT to petrochemical exposure on mussels' digestive glands.

3.2. MATERIAL & METHODS

3.2.1. Sampling sites

The sites selected for the present study are located along the NW coast of Portugal and were chosen according to the level and distinct sources of petrochemical contamination (Figure 3.1).



Figure 3.1 Map of the NW coast of Portugal, showing the location of the five sampling sites. S1: Carreço, S2: Viana do Castelo harbour, S3: Vila Chã, S4: Cabo do Mundo, S5: Leixões harbour.

S1 – Carreço (41°44'27"N; 08°52'40"W), is a rocky shore located 10 Km North of Viana do Castelo. Apparently it is free of significant contamination sources. Nevertheless, it is relatively close to the region affected by the “*Prestige*” oil spill ^[16].

S2 – Viana do Castelo harbour (41°41'01"N; 08°50'40"W), is located at the mouth of Lima river. It is continuously subjected to petrochemical contamination through the activity of commercial and fishing vessels. Records exist of the constant release of

untreated urban effluents into the river and estuary by several municipalities ^[17]. Additionally, in 2000, this harbour was severely affected by the “*Coral Bulker*” oil spill ^[18].

S3 – Vila Chã (41°17'45"N; 08°44'16"W), is a beach near a fishing village located 25 Km north of Porto. It was selected due to the absence of significant contamination sources, and because it has been used as reference site in previous studies of our laboratory ^[16, 19]. In addition, it has been described as having a high biodiversity of intertidal organisms, indicating low levels of anthropogenic pressure ^[20].

S4 – Cabo do Mundo (41°13'33"N; 08°43'03"W), is a rocky shore with a small watercourse located 14 Km North of Porto. Due to the presence of an oil refinery industry this site has been chronically exposed to petrochemical products, including PAHs ^[21] and heavy metals ^[22]. It has also been reported to be highly impacted in previous studies ^[16, 19].

S5 – Leixões harbour (41°10'58"N; 08°41'55"W), is located 10 Km North of Porto at the mouth of Leça river. It comprises the largest seaport infrastructure in the North of Portugal and is one of the most versatile multi-purpose harbours in the country. Due to intense vessel traffic and to oil terminal activity, the harbour is constantly subjected to petroleum hydrocarbon contamination ^[17]. During the summer 2004, an accident during maintenance activities caused a pipeline leak and subsequent oil spill to the surrounding shore.

3.2.2. Abiotic parameters

At the time of sampling, temperature and salinity (Wissenschaftlich Technische Werkstätten –WTW, LF 330 meter, Brüssel, Belgium), as well as pH (WTW, 537 meter) were measured *in situ* at the five sampling sites during low and high tide. At the same time, subsurface water samples were collected with 1.5 L polyethylene-terephthalate bottles and stored at 4°C for analysis. Prior to nutrient analysis the water samples were vacuum filtered (64 µm) to eliminate any suspension particles that could interfere with the analytical procedure. Levels of ammonia, nitrates, nitrites and phosphates were measured using commercial photometer kits (Photometer 7000, Palintest, Kingsway, England).

In addition, during the laboratorial exposure of *M. galloprovincialis* to WAF, temperature, salinity, pH and dissolved oxygen concentration (WTW, 340 meter) were measured before and after each media change to monitor water quality.

3.2.3. Animal sampling

In April 2005, 58 adult mussels (mean anterior-posterior shell length of 3.5 ± 1.0 cm) were handpicked during low tide in the intertidal zone of the five sampling sites (Figure 3.1). Following collection, mussels were placed in thermally insulated boxes previously filled with water from the sampling site and immediately transported to the laboratory. Mussels were sacrificed two hours after collection to ensure equal sampling and transport conditions among sites. From each sampling site, the whole tissue of thirty mussels was isolated for chemical analyses. Moreover, the haemolymph of twenty mussels retrieved from each site, was collected with a 2 mL syringe (0.8 × 40 mm needle; Braun, Melsungen, Germany) from the posterior adductor muscle and diluted (1:2) with ice-cold 100 mM potassium phosphate buffer (pH 7.2) (Merck 5101 and 4873) as described in Moreira et al. (2001) ^[23]. From the same mussels, gills, digestive glands and posterior adductor muscles were immediately isolated and pooled into ten groups for each tissue (one tissue portion of two mussels each) for biomarker determinations. Samples were frozen in liquid nitrogen and stored at -80°C until required for analysis. Finally, the digestive glands of the remaining eight mussels was dissected and stored in RNAlater (Sigma R0901, Steinheim, Germany) at -20°C until further analysis of molecular biology parameters. These additional digestive glands were used due to limitations of the amount of tissue used in the biomarker determinations.

In addition, 108 adult mussels (mean anterior-posterior shell length of 3.5 ± 1.0 cm) were handpicked during low tide in the intertidal zone of S1 (Figure 3.1) to perform a laboratorial exposure to WAF. Mussels collected at S1 were selected to perform the laboratorial exposure since this sampling site exhibited low levels of total petroleum hydrocarbons during a previous survey (see Chapter 1, sections 1.3.2 Chemical analysis).

3.2.4. Laboratory exposure

Adult mussels were acclimatised to laboratory conditions for a period of 48 hours prior to laboratorial exposure to WAF. Mussels were then exposed to different dilutions (0%, 6.25%, 12.5%, 25%, 50% and 100%) of WAF over a period of 21 days. WAF #4 fuel-oil (Galp Energia, SGPS, SA, Portugal) was produced with vacuum-filtered (0.45 µm) and UV-treated seawater according to Singer et al. (2000) ^[24]. WAF was prepared in a 5 L Erlenmeyer flask by stirring 100 g of fuel-oil per litre of seawater for 24 hours, in darkness at 20°C. The WAF mixture was allowed to rest for one hour prior to decantation. Three

mussels were exposed in 1 L glass flasks to 0.8 L of each WAF dilution under controlled laboratorial conditions ($20 \pm 1^\circ\text{C}$; 16:8 L:D cycle). Six replicates of each WAF dilution were preformed. Throughout the exposure period, the media was changed every other day, and mussels were fed with commercial food for marine invertebrates (SERA, Heinsberg, Germany) after each change of the media. Three 100 mL replicates of each WAF dilution were collected in glass flasks at the beginning of the test, as well as after 48 hours of exposure, and frozen at -20°C until further analysis of PAHs concentrations.

At the end of the exposure period, mussels were sacrificed. From each replicate, haemolymph of two mussels was collected from the animal posterior adductor muscle and immediately used for analysis. From the same two mussels, gills, digestive glands and posterior adductor muscles were immediately isolated and pooled into 6 groups for each tissue (one tissue portion of two mussels each) for biomarker determinations. Samples were frozen in liquid nitrogen and stored at -80°C until required for analysis. From the remaining mussel of each replicate, half of the digestive gland was dissected and stored in RNAlater at -20°C until further analysis of molecular biology parameters. These additional digestive glands, as previously explained, were used due to limitations of the amount of tissue used in the biochemical determinations. During the exposure period, mussel mortalities were 38% and 72 % for 50% and 100% WAF, respectively.

3.2.5. Chemical analyses

3.2.5.1. Mussels' tissues

A single analysis of petroleum hydrocarbon was performed in pooled tissues of thirty mussels collected at five sampling sites along the NW coast of Portugal during each sampling period. The analytical procedures for extraction and purification of petroleum hydrocarbons were carried out using the method of CARIPOL/IOCARIBE/UNESCO (1986) ^[25] according to UNEP (1992) ^[26]. Each set of samples was accompanied by a complete blank and a spiked blank which was carried through the entire analytical scheme in identical conditions for all samples. Samples were extracted by homogenisation with a mixture of hexane:methyl chloride (1:1), and an internal standard was added before extraction. The aliphatic and aromatic fractions were purified and separated in three fractions by column chromatography with 10 g each of silica gel/alumina with hexane. The first fraction was eluted with *n*-hexane; the second fraction was eluted with *n*-hexane:methyl chloride (1:1) and the third fraction was eluted only with methyl chloride. The

extracts concentrated containing fraction 1 (aliphatic) and fraction 2 and 3 (aromatics) were rotoevaporated to 1 mL and analysed by gas chromatography. Hydrocarbons were quantified using gas chromatography. Nitrogen was used as carrier gas (flow 1 mL mm⁻¹). The limit of detection for individual aromatic compounds was 0.01 µg g⁻¹ and recovery yields were up to 90%. The AH and UCM was quantified with an *n*-C28 standard. PAHs were identified by comparing their retention times with those from the aromatic analytical standards by Supelco 48743 according to the priority PAHs from method EPA 610.

3.2.5.2. Water-accommodated fraction

Immediately prior to the analysis of PAHs, the samples of WAF were defrosted and vacuum filtered through a glass microfibre filter (0.45 µm) to eliminate any suspension particles that could interfere with the analytical procedure. The analytical procedure started with a solid-phase micro-extraction of the samples using fibres coated with a 100 µm thickness polydimethylsiloxane film. Afterwards, the PAHs determinations were carried out in a gas chromatography (GC, Varian CP-3800) system combined with a split/splitless injector and an ion trap mass spectrometric (MS, Saturn 2200) detection system. The GC-MS determinations were carried out in selected ion monitoring mode. The analytical procedure was validated by adding known concentration of deuterated PAHs to the samples before extraction. The quantification of the PAHs was carried out through response factors obtained from the recovery percentages of standards of deuterated PAHs, with at least one deuterated PAHs per class of aromaticity being used to determine accurate concentrations for all PAHs. Blank solutions were prepared for each sample following its treatment. The value of PAHs quantified for each sample of WAF is the average of two replicates after blank subtraction and are expressed in ng L⁻¹. The methodology herein employed was adapted by Evtugina et al. (2007) ^[27] from the works of King et al (2004) ^[28].

3.2.6. Biomarkers

The following biochemical parameters were selected as biomarkers of antioxidant defence and/or detoxification: SOD, CAT, selenium-dependent glutathione peroxidase (GPx), glutathione reductase (GR), glutathione S-transferases (GST), total glutathione content (tGSx), reduced glutathione (GSH), oxidised glutathione (GSSG), and glutathione

redox status (GSH/GSSG). Levels of lipid peroxides (LPO) were determined as indicators of oxidative cell damage. The activity of NADP⁺-dependent isocitrate dehydrogenase (IDH) was determined as part of the mussels' antioxidant defence system and energetic aerobic metabolism, while octopine dehydrogenase (ODH) was investigated as part of mussels' energetic anaerobic metabolism. Finally, acetylcholinesterase (AChE) activity was quantified to assess mussels' neurotransmission levels. All the biochemical parameters used as biomarkers of antioxidant defence and/or detoxification, as well as oxidative cell damage were determined in mussels' gills and digestive glands. Additionally, IDH was only quantified in mussels' digestive glands because previous studies indicated a very low activity of this enzyme in gill tissue (data not published). The posterior adductor muscle was selected for the quantification of ODH due to the importance of this enzyme on the maintenance of the redox balance of invertebrate muscle tissue during periods of temporary anoxia ^[29]. Finally, AChE was quantified in mussels' haemolymph because this is the tissue in which mussels' AChE presents a higher specific activity when compared with other tissues ^[30].

The activity of SOD was determined according to McCord and Fridovich (1969) ^[31] adapted to microplate. Tissues were homogenised (Ystral homogeniser, Ballrechten-Dottingen, Germany) in 50 mM sodium phosphate buffer (Merck 1.06579 and 1.06345, Darmstadt, Germany) with 1 mM ethylenediaminetetraacetic acid disodium salt dihydrate (Na₂-EDTA, Sigma E4884, Osterode, Germany) (pH 7.8) and centrifuged (Sigma 3K) at 15,000 *g* for 15 min at 4°C. The final concentrations of the assay chemicals, in a final volume of 300 µL, were: 50 mM sodium phosphate buffer with 1 mM Na₂-EDTA (pH 7.8), 0.043 mM xanthine (Sigma X7375), 18.2 µM cytochrome *c* (Sigma C7752) and 0.3 U mL⁻¹ xanthine oxidase (XO, Sigma X1875). The reaction was initiated with the addition of the XO solution, and the reduction of the cytochrome *c* was assessed by the increase of absorbance at 550 nm, using a microplate reader (Bio-Tek®, model Power Wave 340, Winooski, USA). One unit of SOD was defined as the amount of enzyme required to inhibit the rate of reduction of cytochrome *c* by 50%.

The activity of CAT was determined according to Aebi (1984) ^[32]. Tissues were homogenised in 50 mM potassium phosphate buffer (Merck 1.05101 and Merck 1.04873) (pH 7.0) and centrifuged at 15,000 *g* for 15 min at 4°C. The final concentrations of the assay chemicals, in a final volume of 600 µL, were: 50 mM potassium phosphate buffer (pH 7.0) and 10 mM hydrogen peroxide (H₂O₂, Aldrich 21.676, Steinheim, Germany). The reaction was initiated with the addition of the H₂O₂ solution, and its decomposition was assessed by the decrease of absorbance at 240 nm, using a spectrophotometer (Jenway 6405 UV/Vis, Dunmow, England).

The activities GPx and GR were determined according to Flohé and Günzler (1984) ^[33], and Carlberg and Mannervik (1975) ^[34], respectively. The two assays were adapted to microplate. The activity of GST was determined according to Habig et al. (1974) ^[35] adapted to microplate by Frasco et al. (2002) ^[36]. For these three enzymatic assays, tissues were homogenised using 100 mM potassium phosphate buffer with 2 mM Na₂-EDTA (pH 7.5) and centrifuged at 15,000 *g* for 15 min at 4°C. The final concentrations of the chemicals for the GPx assay, in a final volume of 300 µL, were: 100 mM potassium phosphate buffer with 2 mM Na₂-EDTA, 1 mM dithiothreitol (DTT, Sigma D9779) and 1 mM of sodium azide (Sigma S8032) (pH 7.5), 2 mM GSH, 34 U mL⁻¹ GR (Sigma G3664), 0.24 mM β-nicotinamide adenine dinucleotide 2'-phosphate reduced tetrasodium salt (NADPH, Sigma N7505), and 0.6 mM H₂O₂. The reaction was initiated with the addition of the H₂O₂ solution, and the oxidation of NADPH was assessed by the decrease of absorbance at 340 nm, using a microplate reader. The final concentrations of the chemicals for the GR assay, in a final volume of 300 µL, were: 100 mM potassium phosphate buffer with 2 mM Na₂-EDTA (pH 7.5), 0.5 mM GSSG (Sigma G4376) and 0.1 mM NADPH. The reaction was initiated with the addition of the NADPH solution, and the oxidation of NADPH was assessed by the decrease of absorbance at 340 nm, using a microplate reader. The final concentrations of the assay chemicals for the GST assay, in a final volume of 300 µL, were: 100 mM potassium phosphate buffer (pH 6.5), 4 mM GSH and 1 mM 1-chloro-2,4-dinitrobenzene (CDNB, Sigma C6396). The activity of GST was determined by measuring the formation of a thioether by the conjugation of CDNB with GSH. This conjugation is followed by an increase in absorbance at 340 nm, using a microplate reader.

The levels of tGSx and GSSG were determined according to Baker et al. (1990) ^[37]. Tissues were homogenised using 71.5 mM sodium phosphate buffer with 0.63 mM Na₂-EDTA (pH 7.5). Following homogenisation, 5% perchloric acid (Merck 0519) was added to the samples that were centrifuged at 15,000 *g* for 15 min at 4°C. Previous to the enzymatic assay, samples were neutralized with 760 mM potassium hydrogen carbonate (Sigma P4913). The final concentrations of the chemicals for the tGSx quantification, in a final volume of 205 µL, were: 0.15 mM NADPH, 0.85 mM of 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB, Sigma D8130) and 7 U mL⁻¹ GR. A 5% solution of 2-vinylpyridine (Fluka 95040, Steinheim, Germany) was used to conjugate GSH for the GSSG determination. Glutathione equivalents were quantified by monitoring the formation of 5-thio-2-nitrobenzoic acid formed by the conjugation of the SH- group of glutathione and the DTNB at 414 nm, using a microplate reader. Glutathione concentrations were expressed as nmol of GSH equivalents (GSx) per mg of protein (GSx = [GSH] +

2[GSSG]). GSH/GSSG ratio was calculated as number of molecules: $GSH/GSSG = (tGSx - GSSG)/(GSSG/2)$, according to Peña-Llopis et al. (2001) ^[38].

Levels of LPO were measured by the generation of thiobarbituric acid (TBARS)-malondialdehyde (MDA) reactive species, which were referred to MDA equivalents (Ohkawa et al., 1979) ^[39]. Tissues were homogenised using 100 mM potassium phosphate buffer (pH 7.2) and centrifuged at 10,000 *g* for 5 min at 4°C. The reaction mixture contained: 11.4% of homogenate, 4.6% of 10.6 mM sodium dodecyl sulfate (Sigma D2525) with 0.1 mM butylated hydroxytoluene (Aldrich W218405), 40% of 20% acetic acid (Merck 1.00062) (pH 3.5), 40% of 22.2 mM thiobarbituric acid (Sigma T5500), and 4% of nanopure water in a final volume of 700 µL. The reaction mixture was heated in a 95°C water bath for 1 h. Once cold, 175 µL of nanopure water and 875 µL *n*-butanol (Merck 1.01990) and pyridine (Aldrich 270970) (15:1 v/v) were added and thoroughly mixed. Following centrifugation at 10,000 *g* for 5 min, the immiscible organic layer was removed and its absorbance measured at 530 nm, using a microplate reader.

The activity of IDH was determined according to Ellis and Goldberg (1971) ^[40] adapted to microplate. Tissues were homogenised in 50 mM tris(hydroxymethyl)-aminomethane (Tris, Merck 1.08382) buffer (pH 7.8) and centrifuged at 15,000 *g* for 15 min at 4°C. The final concentrations of the assay chemicals, for a final volume of 300 µL, were: 50 mM of Tris buffer (pH 7.8), 0.5 mM β-nicotinamide adenine dinucleotide phosphate (NADP, Sigma N0505), 7 mM DL- isocitric acid (Sigma I1252) and 4 mM manganese chloride tetrahydrate (Merck 1.05927). The reaction was initiated with the addition of the DL-isocitric acid solution, and the reduction of NADP was assessed by the increase of absorbance at 340 nm, using a microplate reader.

The activity of ODH was determined according to Livingston et al. (1990) ^[41] adapted to microplate. Tissues were homogenised in 20 mM Tris buffer (pH 7.5) with 1 mM Na₂-EDTA and 1 mM DTT and centrifuged at 15,000 *g* for 15 min at 4°C. The final concentrations of the assay chemicals, in a final volume of 300 µL, were: 100 mM imidazole hydrochloride (Sigma I3386) buffer (pH 7.0), 0.1 mM β-nicotinamide adenine dinucleotide (NADH, Sigma N8129), 10 mM L-arginine (Aldrich A9,240-6) and 2 mM pyruvic acid sodium salt (Sigma P2256). The reaction was initiated with the addition of the pyruvic acid solution, and the enzyme activity was determined by monitoring the decrease in absorbance due to oxidation of NADH at 340 nm, using a microplate reader.

The activity of AChE was determined according to Ellman et al. (1961) ^[42], adapted to microplate by Guilhermino et al. (1996) ^[43]. The AChE assay was performed directly in mussels' haemolymph diluted (1:2) in ice-cold 100 mM potassium phosphate buffer (pH

7.2), immediately after its collection. The final concentrations of the assay, in a final volume of 300 μ L, were: 100 mM potassium phosphate buffer (pH 7.2), 0.40 mM acetylthiocholine iodide (ATCh, Sigma A5751, Steinheim, Germany) and 0.27 mM DTNB. In this assay the AChE hydrolyses the substrate ATCh in thiocholine and acetate. Following this reaction, the thiocholine reacts with DTND forming a mixed disulphide and the yellow chromophore 5-thio-2-nitrobenzoic acid (TNB). The TNB formation is followed by an increase in absorbance at 412 nm, using a microplate reader. Cholinesterase activity detected in *M. galloprovincialis* haemolymph was previously shown to have properties of true AChE ^[23].

The protein content of the samples was determined by the Bradford method (Bradford, 1976) ^[44], using γ -bovine globulins (Sigma G5009) as standard. All enzymatic assays were preformed at 25°C.

3.2.7. Gene expression

Total RNA extraction was performed with RNeasy reagents according to supplier's instructions (Qiagen Ltd, Crawley, UK), followed by digestion with DNase (Promega GmbH, Madison, USA). The amount of RNA isolated was quantified in a spectrophotometer at 260 nm, and RNA purity was assessed by calculating the ratio between the absorbance at 260 and 280 nm. From each sample, 1 μ g of total RNA was used for the synthesis of the first strand cDNA by RT-PCR using oligo(dT) primers (Invitrogen Ltd., Paisley, UK) in a BioRad iCyclerTM. To identify the putative sequence of the genes of Cu/Zn-SOD and CAT of *M. galloprovincialis*, the synthesised cDNA was used as a template in a PCR preformed with degenerate primers designed based on sequences described by Manduzio et al. (2003) ^[45] in *M. edulis* (Cu/Zn-SOD), and Bilbao et al. (2006) ^[46] in *M. galloprovincialis* (CAT). For both genes, a 50 μ L PCR reaction mixture was prepared with reaction buffer (200 mM Tris-HCl pH 8.4, and 500 mM KCl), 400 μ M of each deoxynucleoside triphosphate, 50 pmol of each degenerate primer, 4 μ L of synthesized cDNA and 1 U Platinum Pfx DNA polymerase (Invitrogen). Following a 2 min denaturation step at 94°C, fragments of each gene were amplified using 35 sequential cycles at 94°C during 30 s for denaturation, 55°C during 30 s for annealing, and 72°C during 45 s for extension, using a BioRad iCyclerTM. A final step of 2 min at 72 °C was performed for a final extension. Following purification of the PCR products, the obtained fragments for each gene were sent for direct sequencing (StabVida, Oeiras, Portugal).

Sequencing results were compared to those in GenBank databases using BLAST, to confirm the nature of the isolated fragments.

Differences in gene expression levels for the genes of Cu/Zn-SOD and CAT isolated from digestive glands of *M. galloprovincialis* were analysed by semi-quantitative PCR. To normalize differences in efficiency during the amplification of the selected genes, 18S rRNA primers were used to amplify a 172 bp fragment as an internal standard (forward – 5'GTGCTCTTGACTGAGTGTCTCG3'; reverse – 5'CGAGGTCCTATTCCATTATTCC3'). For the gene of Cu/Zn-SOD, the specific primers used were: forward – 5'TCTTGAAAGGA GATGGTGCTG3', and reverse – 5'CAATGACACCACAAGCCAGA3', yielding a product of 412 bp. For the gene of CAT, the specific primers used were: forward – 5'GGATTTCATTA CACTTCGACCAG3', and reverse – 5'GGGATCAGTGGAAATTCTCCTT3', yielding a product of 388 bp. The amplification of the selected genes was preformed using a BioRad iCyclerTM in a 50 µL PCR reaction volume containing reaction buffer (200 mM Tris-HCl pH 8.4, and 500 mM KCl), 400 µM of each deoxynucleoside triphosphate, 50 pmol of each primer, 4 µL of synthesized cDNA and 1 U Platinum Pfx DNA polymerase (Invitrogen). Following a 2 min denaturation step at 94°C, fragments of each gene were amplified using 35 sequential cycles at 94°C during 30 s for denaturation, 55°C during 30 s for annealing, and 72°C during 45 s for extension. A final step of 2 min at 72 °C was performed for a final extension. Finally, the PCR products were run in an agarose gel (1.0% agarose, TBE buffer) electrophoresis stained with ethidium bromide.

3.2.8. Data analyses

The results of the biomarkers are presented as means ± standard deviation (SD). Prior to the analysis of variance (ANOVA) of the data, the normality (Kolmogorov–Smirnov normality test) and homogeneity of variance (Hartley, Cochran C, and Bartlett's tests) of data was verified and data transformation applied as required to fulfil ANOVA assumptions^[47]. For parametric data, the comparison of the biomarkers among sampling sites was studied by performing a one way analysis of variance (one-way ANOVA), followed by a Tukey honestly significant difference (HSD) multiple comparison test, whenever applicable^[47]. For non-parametric data, the comparison of the biomarkers among sampling sites was studied by performing a Kruskal-Wallis nonparametric ANOVA followed by a Dunn's test^[47]. Furthermore, a Spearman correlation was preformed to evaluate the degree of relationship between biomarkers and petroleum hydrocarbon levels, as well as biomarkers and physicochemical parameters^[47]. The response of the

biomarkers to petrochemical contamination was also evaluated by multivariate analyses. Triangular similarity matrices were calculated for the biomarkers using the Bray-Curtis similarity coefficient, following a Log (x+1) transformation of the data ^[48]. Using these correlation matrixes, a two dimensional non-metric multidimensional scaling (MDS) was preformed to discriminate the similarities of each sampling site according to biomarker response ^[49]. In addition, a pair-wise comparisons test ANOSIM, which was performed in pre defined sets of sampling sites, confirmed the existence of significant differences between the groups obtained by the MDS analysis ^[49]. A similarity percentages test (SIMPER) was performed to discriminate which biomarkers had more influence on the similarities within groups and dissimilarities among groups obtained by the MDS analysis ^[49]. In addition, principal component analysis (PCA) were preformed to discriminate the similarities of each sampling site as a function of the petroleum hydrocarbon levels measured in mussels' tissue ^[48]. Finally, the biota and/or environment matching (BIOENV) procedure were performed to evaluate which petroleum hydrocarbons better relate with the biomarkers ^[50]. Moreover, for each biomarker assessed after laboratory exposure of mussels to WAF, different treatments were compared using one-way ANOVA, followed by a Dunnett's multiple-comparison test, whenever the respective ANOVA revealed significant differences between 0% WAF and the remaining treatments ^[47]. The normality and homogeneity of variance of data of the biomarkers quantified after laboratorial exposure of mussels to WAF was also verified and data transformation was applied whenever required to fulfil ANOVA assumptions ^[47]. Finally, to evaluate the possible effects of the manipulation of the organisms under laboratorial experimental conditions, differences between biochemical parameters assessed in mussels collected at S1 and in mussels from the 0% WAF after 21 days of exposure were evaluated by Student's *t*-test with independent samples assuming equal variance ^[47].

Statistical analyses of data were performed using Statistica 6.0 (StatSoft, Tulsa, USA), with the exception of the multivariate analyses of the data, which was performed using PRIMER 5 package for Windows (PRIMER-E Ltd., Plymouth, UK).

3.3. RESULTS

3.3.1. Abiotic parameters

Temperature range midpoint values ranged from 13.6°C (S2) to 15.6°C (S4). The highest salinity range midpoint values (35.4 g L⁻¹ at S1 and 32.8 g L⁻¹ at S3) were found in

sites located at open seashore, while the lowest salinity values (29.40 g L^{-1} at S2, 25.40 g L^{-1} at S4, and 31.70 g L^{-1} at S5) were recorded at sites located near the mouth of watercourses. The pH range midpoint values ranged from 8.01 at S2 to 8.34 at S1. Nutrient analysis showed that ammonia concentrations were relatively higher at S4 (3.63 mg L^{-1}) and S2 (1.12 mg L^{-1}) when compared with the values recorded at S1 (0.05 mg L^{-1}) and S3 (0.03 mg L^{-1}). Nitrate values ranged from 1.12 mg L^{-1} at S4 to 0.24 mg L^{-1} at S1. The highest nitrite concentrations were measured in water samples from S4 (0.36 mg L^{-1}), while the lowest values were quantified in water samples from S2 (0.07 mg L^{-1}) and S5 (0.05 mg L^{-1}). Water samples collected at S1 and S3 exhibited no measurable nitrite values. Phosphate concentration values were higher at S4 (1.05 mg L^{-1}), when compared with the remaining sampling sites.

3.3.2. Chemical analyses

3.3.2.1. Mussels' tissues

The results of chemical analyses, determined in single samples of pooled tissues of *M. galloprovincialis* collected at five sampling sites along the NW coast of Portugal, are presented in Table 3.1.

The AH concentrations ranged from $247.13 \text{ } \mu\text{g g}^{-1}$ dry weight (dw) at S5 to $28.19 \text{ } \mu\text{g g}^{-1}$ dw at S2, with the following pattern: $S5 > S1 > S3 > S4 > S2$. The values of petroleum hydrocarbons present in the UCM fraction ranged from $2656.53 \text{ } \mu\text{g g}^{-1}$ dw at S5 to $234.93 \text{ } \mu\text{g g}^{-1}$ dw at S4, with the following pattern: $S5 > S2 > S3 > S1 > S4$. For the total PAHs fraction, the highest value ($642.99 \text{ } \mu\text{g g}^{-1}$ dw) was measured in mussels collected at S2, and the lowest value ($94.81 \text{ } \mu\text{g g}^{-1}$ dw) was measured in mussels collected at S3. The concentration values of the PAHs followed the following pattern: $S2 > S4 > S5 > S1 > S3$. Regarding the results of the 16 priority PAHs, the major contributions for the total PAHs levels present in mussel tissues were given by benzo[a]pyrene (approximately 50% of total PAHs fraction at all sampling sites except S4, which reported for 22%), benzo[k]fluoranthene (approximately 27% of total PAHs fraction at all sampling sites except S4, which reported for 51%), and ideno[1,2,3-cd]pyrene (approximately 18% of total PAHs fraction at all sampling sites). The pattern for total petroleum hydrocarbon found in mussel tissues was: $S5 > S2 > S1 > S3 > S4$.

Table 3.1 Chemical analyses of petroleum hydrocarbons performed in whole tissue of *Mytilus galloprovincialis* collected during April 2005 at five sampling sites (S1-S5) along the NW coast of Portugal.

Petroleum hydrocarbons	Sampling Site				
	S1	S2	S3	S4	S5
AH	94.18	28.19	62.20	33.25	247.14
UCM	686.89	1229.15	788.27	234.93	2656.53
ΣPAHs	189.55	642.99	94.81	228.24	209.61
Acenaphthene	-	-	-	-	0.12
Acenaphthylene	-	0.07	-	0.57	-
Anthracene	0.09	0.21	0.12	0.18	0.28
Benzo[a]anthracene	-	0.13	-	0.10	0.25
Benzo[a]pyrene	101.85	297.87	41.62	64.82	117.84
Benzo[b]fluoranthene	-	0.27	-	0.37	-
Benzo[ghi]perylene	1.71	18.42	1.75	5.06	1.90
Benzo[k]fluoranthene	43.47	185.15	26.57	116.46	57.79
Chrysene	-	0.09	-	0.07	-
Dibenzo[ah]anthracene	-	15.13	0.83	-	-
Fluoranthene	-	0.06	-	-	-
Fluorene	0.11	-	-	0.12	0.30
Indeno[1,2,3-cd]pyrene	27.81	125.42	23.92	40.45	30.95
Naphthalene	14.27	0.03	-	0.04	-
Phenanthrene	0.03	0.14	-	-	0.17
Pyrene	0.20	-	-	-	-

AH – aliphatic hydrocarbons, UCM – unresolved complex mixture, ΣPAHs – polycyclic aromatic hydrocarbons. Data are expressed in $\mu\text{g g}^{-1}$ dry weight.

3.3.2.2. Water-accommodated fraction

The results of chemical analyses of PAHs performed in samples of undiluted WAF collected in the beginning and 48 hours after mussel exposure are presented in Table 3.2. The levels of total PAHs were $5044 \pm 667 \text{ ng L}^{-1}$ at the beginning of the laboratorial exposure of mussels to WAF, and $5644 \pm 498 \text{ ng L}^{-1}$ after 48 hours of exposure. The major contributions for the total PAHs levels present in undiluted WAF were given by naphthalene (43% in the beginning of exposure and 63% 48 hours after exposure), followed by phenanthrene (23% in the beginning of exposure and 9% 48 hours after exposure), fluorene (9% in the beginning and 48 hours after exposure), anthracene (7% in the beginning of exposure and 5% after 48 hours of exposure), chrysene (7% in the beginning of exposure and 4% after 48 hours of exposure), and acenaphthene (6% in the beginning of exposure and 7% after 48 hours of exposure). Finally, 48 hours after

exposure the levels of acenaphtene, acenaphthylene, fluorene, naphthalene and pyrene were higher than in the beginning of the exposure period. All the remaining PAHs presented lower levels after 48 hours of exposure when compared to the beginning of exposure. In control samples there were vestigial quantities of naphthalene, phenanthrene, fluoranthene and chrysene, which might indicate contamination of samples.

Table 3.2 Chemical analyses of polycyclic aromatic hydrocarbons performed in samples of undiluted water-accommodated fraction of #4 fuel-oil collected in the beginning and 48 hours after *Mytilus galloprovincialis* exposure.

Polycyclic aromatic hydrocarbons	Water-accommodated fraction of #4 fuel-oil	
	Beginning of exposure	48 hours after exposure
Σ PAHs	5044 ± 667	5644 ± 498
Acenaphthene	277 ± 60	390 ± 31
Acenaphthylene	82 ± 18	129 ± 16
Anthracene	352 ± 71	278 ± 26
Benzo[a]anthracene	56 ± 0	-
Benzo[a]pyrene	29 ± 5	-
Benzo[b]fluoranthene	50 ± 11	25 ± 22
Benzo[ghi]perylene	55 ± 0	-
Benzo[k]fluoranthene	50 ± 7	30 ± 17
Chrysene	337 ± 78	251 ± 5
Dibenzo[ah]anthracene	46 ± 16	-
Fluoranthene	27 ± 8	20 ± 2
Fluorene	457 ± 109	500 ± 60
Indeno[1,2,3-cd]pyrene	63 ± 0	-
Naphthalene	2164 ± 297	3558 ± 433
Phenanthrene	1134 ± 225	487 ± 103
Pyrene	75 ± 9	77 ± 2

PAHs – polycyclic aromatic hydrocarbons. Data are expressed in ng L⁻¹ of water-accommodated fraction of #4 fuel-oil. Values are presented as mean ± standard deviation (*n* = 6).

3.3.3. Biomarkers

3.3.3.1. Field sampling

The results of the biomarkers are presented in Figure 3.2 to 3.5. One-way ANOVA revealed significant differences among sampling sites for the following oxidative stress

and detoxification parameters determined in *M. galloprovincialis* digestive glands (SOD: $F_{4,45} = 33$, $p \leq 0.001$; CAT: $F_{4,45} = 34$, $p \leq 0.001$; GPx: $F_{4,45} = 29$, $p \leq 0.001$; GR: $F_{4,45} = 14$, $p \leq 0.001$; GST: $F_{4,45} = 33$, $p \leq 0.001$; tGSx: $F_{4,45} = 17$, $p \leq 0.001$; GSH: $F_{4,45} = 5.7$, $p \leq 0.001$; GSSG: $F_{4,45} = 11$, $p \leq 0.001$) and gills (SOD: $F_{4,45} = 3.6$, $p \leq 0.05$; CAT: $F_{4,45} = 4.0$, $p \leq 0.05$; GPx: $F_{4,45} = 22$, $p \leq 0.001$; GR: $F_{4,45} = 23$, $p \leq 0.001$; GST: $F_{4,45} = 5.8$, $p \leq 0.001$; LPO: $F_{4,45} = 6.7$, $p \leq 0.001$; tGSx: $F_{4,45} = 4.3$, $p \leq 0.05$, GSSG: $F_{4,45} = 4.6$, $p \leq 0.05$). One-way ANOVA also revealed that the levels of LPO ($F_{4,45} = 1.4$, $p > 0.05$) and the ratio GSH/GSSG ($F_{4,45} = 0.5$, $p > 0.05$) quantified in mussels' digestive glands, as well as the levels of GSH ($F_{4,45} = 1.9$, $p > 0.05$) and the ratio GSH/GSSG ($F_{4,45} = 1.0$, $p > 0.05$) quantified in mussels' gills did not exhibited significant differences among sampling sites. Results of biomarkers related to energetic metabolism (IDH: $F_{4,45} = 9.9$, $p \leq 0.001$; ODH: $F_{4,45} = 18$, $p \leq 0.001$) and neurotransmission (AChE: non-parametric data $H_{4,45} = 13$, $p \leq 0.05$;) also revealed significant differences among sampling sites.

The values of SOD activity quantified in digestive glands of mussels collected at S4 and S5 were significantly higher than those collected from the remaining sampling sites. Digestive glands of mussels collected at S2 exhibited significantly higher levels of SOD activity that those from mussels collected at S1, but no significant differences were found with those from S3. In gills, the levels of SOD activity were significantly higher in mussels from S5 that in mussels from S1 and S2, but no significant differences were found with those collected at S3 and S4 (Figure 3.2).

The values of CAT activity measured in digestive glands of mussels collected at S4 and S5 were significantly higher than those collected from the remaining sampling sites. Digestive glands of mussels collected at S2 exhibited significantly higher levels of CAT activity than those from S1 and S3. In gills, the significantly higher levels of CAT activity were found in mussels from S2 when compared with mussels from S3-S5, but no significant differences were found with those from S1 (Figure 3.2).

The values of GPx activity quantified in the digestive glands of mussels collected at S2 were significantly higher than in those from the remaining sampling sites. Mussels from S5 exhibited levels of GPx activity quantified in digestive glands significantly higher that those from S1 and S3, but not S4. In gills, the levels of GPx activity were significantly higher in mussels collected at S2, S4 and S5 when compared with those collected at S1 and S3 (Figure 3.2).

The values of GR activity quantified in digestive glands were significantly lower in mussels collected at S2 when compared with mussels collected at the remaining sampling sites. In gills, the levels of GR activity were significantly higher in mussels from S1 and S3

when compared with the remaining sampling sites. Mussels from S5 exhibited levels of GR activity in gills significantly higher than in those from S4, but not from S2 (Figure 3.2).

The levels of GST activities measured in digestive glands of mussels collected at S4 were significantly higher than in mussels from the remaining sampling sites. Mussels from S5 exhibited significantly higher levels of GST activities in digestive glands than mussels from S1 and S2, but no significant differences were found with those from S3.

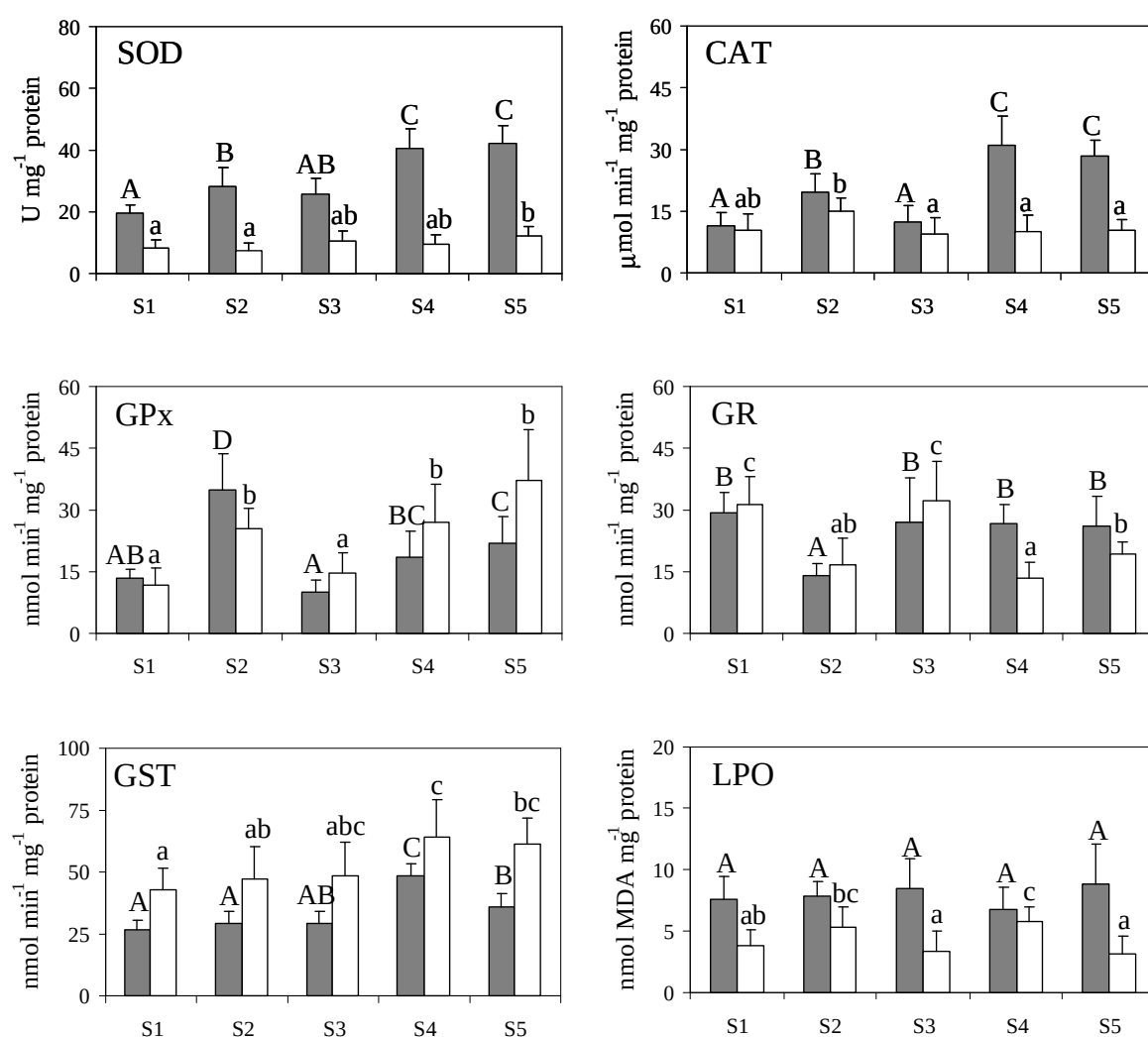


Figure 3.2 Biomarkers analysed in *Mytilus galloprovincialis* collected during April 2005 at five sampling sites (S1-S5) along the NW coast of Portugal. Values are presented as mean $\pm$ standard deviation ($n = 10$) of total superoxide dismutase (SOD), catalase (CAT), selenium-dependent glutathione peroxidase (GPx), glutathione reductase (GR), glutathione S-transferases (GST), lipid peroxides (LPO). Different letters indicate significant differences among sampling sites by Tukey honestly significant difference multiple-comparison test ($p \leq 0.05$) for each biomarker. Capital letters indicate differences in the digestive gland (■) and small letters indicate differences in the gills (□).

In gills, the levels of GST activities were also significantly higher in mussels from S4 when compared with those from S1 and S2. Finally, mussels from S5 exhibited significantly higher levels of GST activities than those from S1, but no significant differences were found with those from S2 and S3 (Figure 3.2).

The levels of LPO quantified in mussels' digestive glands did not exhibit significant differences among the sampling sites. In gills, the levels of LPO quantified in mussels from S4 were significantly higher than in mussels from S1, S3 and S5, but not S2. No significant differences were found in the levels of LPO measured in gills of mussels from S1, S3 and S5 (Figure 3.2).

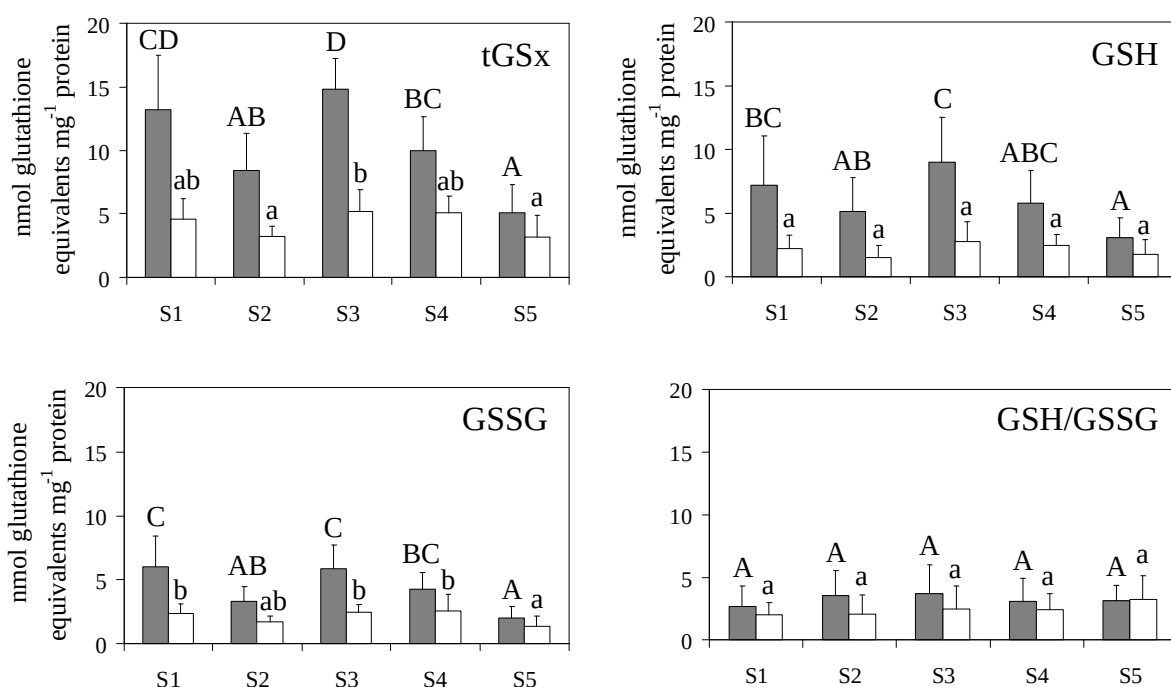


Figure 3.3 Biomarkers analysed in *Mytilus galloprovincialis* collected in April 2005 at five sampling sites (S1-S5) along the NW coast of Portugal. Values are presented as mean $\pm$ standard deviation ($n = 10$) of total glutathione content (tGSx), reduced glutathione (GSH), oxidised glutathione (GSSG), and glutathione redox status (GSH/GSSG). Different letters indicate significant differences among sampling sites by Tukey honestly significant difference multiple-comparison test ($p \leq 0.05$) for each biomarker. Capital letters indicate differences in the digestive gland (■) and small letters indicate differences in gills (□).

In gills, the levels of tGSx quantified in mussels collected at S3 were significantly higher than in mussels collected at S2 and S5, but no significant differences were found with those collected at S1 and S4. No significant differences were found in the levels of tGSx in gills of mussels from S1-S2 and S4-S5 (Figure 3.3).

The levels of GSH quantified in digestive glands of mussels from S3 were significantly higher than in mussels from S2 and S5, but no significant differences were found with those from S1 and S4. Mussels collected at S1 showed significantly higher levels of GSH in digestive glands than mussels collected at S5, but no significant differences were found with those collected at S2 and S4. Finally no significant differences were found in the levels of GSH in digestive glands of mussels from S2 and S4-S5. In gills, no significant differences were found in the levels of GSH among sampling sites (Figure 3.3).

The levels of GSSG measured in digestive glands of mussels from S1 and S3 were significantly higher than in mussels from S2 and S5, but no significant differences were found with those from S4. No significant differences were found between the levels of GSSG in digestive glands of mussels from S2 and S4, as well as mussels from S2 and S5. In gills, the levels of GSSG quantified in mussels from S1, S3 and S4 were significantly higher than in mussels from S5, but no significant differences were found in those from S2 (Figure 3.3). No significant differences were found among sampling sites regarding the GSH/GSSG ratio quantified in mussels' digestive glands and gills (Figure 3.3).

The levels of IDH activity quantified in digestive glands of mussels from S2 were significantly higher than in those from the remaining sites with the exception of S5. Mussels from S5 showed significantly higher levels of IDH activity than those from S4, but no significant differences were found with those from S1 and S3. No significant differences were found in the levels of IDH activity of mussels collected at S1, S3 and S4 (Figure 3.4).

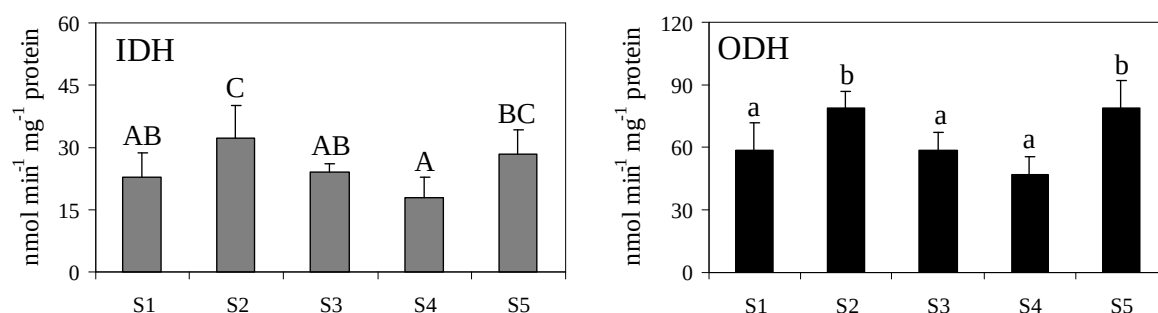


Figure 3.4 Biomarkers analysed in *Mytilus galloprovincialis* collected in April 2005 at five sampling sites (S1-S5) along the NW coast of Portugal. Values are presented as mean $\pm$ standard deviation ($n = 10$) of NADP⁺-dependent isocitrate dehydrogenase (IDH), and octopine dehydrogenase (ODH). Different letters indicate significant differences among sampling sites by Tukey honestly significant difference multiple-comparison test ($p \leq 0.05$) for each biomarker. Capital letters indicate differences in the digestive gland (■) and small letters indicate differences in posterior adductor muscle (■).

The levels of ODH activity determined in posterior adductor muscles of mussels collected at S2 and S5 were significantly higher than in those collected at the remaining sampling sites (Figure 3.4).

The levels of AChE activity quantified in haemolymph of mussels collected at S1 and S2 were significantly higher than in the haemolymph of mussels collected at S5, but no significant differences were found with those collected at S3 and S4. No significant differences were found in the levels of AChE activity of mussels from S3-S5 (Figure 3.5).

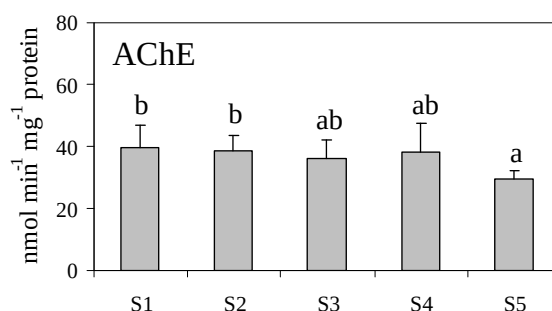


Figure 3.5 Acetylcholinesterase activity analysed in *Mytilus galloprovincialis* collected during April 2005 at five sampling sites (S1-S5) along the NW coast of Portugal. Values are presented as mean $\pm$ standard deviation ($n = 20$) of acetylcholinesterase quantified in mussels' haemolymph. Different letters indicate significant differences among sampling sites by Dunn's test ($p \leq 0.05$).

3.3.3.2. Effects of petroleum hydrocarbons and abiotic parameters on biomarkers

Significant Spearman correlation values ($p \leq 0.01$) were found between some biomarkers and petroleum hydrocarbon levels quantified in mussels' tissue, as well as between some biomarkers and abiotic parameters quantified in water samples from the selected sampling sites (Table 3.3 and 3.4).

The most significant positive correlations ($r > 0.50$) between biomarkers and petroleum hydrocarbons were found between UCM levels and the activities of IDH in digestive glands and ODH in posterior adductor muscle, as well as between PAHs levels and the activities of GPx quantified in mussels' digestive glands. Significant negative correlations were found between the PAHs levels and the activities of GR in gills (Table 3.3).

Table 3.3 Significant Spearman correlation values ($p \leq 0.01$) between petroleum hydrocarbon levels and biomarkers quantified in *Mytilus galloprovincialis* collected during April 2005 at five sampling sites (S1-S5) along the NW coast of Portugal.

Petroleum hydrocarbons	Biomarkers			
	GPx ^a	GR ^b	IDH ^a	ODH ^c
UCM	-	-	0.667	0.756
ΣPAHs	0.786	-0.731	-	-

UCM – unresolved complex mixture, ΣPAHs – total polycyclic aromatic hydrocarbons, GPx – selenium-dependent glutathione peroxidase, GR – glutathione reductase, IDH – NADP⁺-dependent isocitrate dehydrogenase, ODH – octopine dehydrogenase. ^a Digestive glands; ^b Gills; ^c Posterior adductor muscle.

Regarding the correlations between the biomarkers and abiotic parameters quantified in water samples, significant positive correlations were found between salinity and the activities of GR in gills, as well as between the levels of ammonia, nitrates, nitrites, and phosphates, and the activities of CAT in mussels' digestive glands. Significant negative correlations were found between salinity and the activities of CAT in digestive glands, as well as between the levels of ammonia, nitrates, nitrites, and phosphates, and the activities of GR in mussels' gills (Table 3.4).

Table 3.4 Significant Spearman correlation values ($p \leq 0.01$) between abiotic parameters quantified in water samples and biomarkers determined in *Mytilus galloprovincialis* collected during April 2005 at five sampling sites (S1-S5) along the NW coast of Portugal.

Abiotic parameters	Biomarkers	
	CAT ^a	GR ^b
S	-0.707	0.807
NH ₄	0.698	-0.810
NO ₃	0.707	-0.807
NO ₂	0.721	-0.829
PO ₄	0.698	-0.810

CAT – catalase, GR – glutathione reductase, T – temperature, S – salinity, NH₄ – ammonia, NO₃ – nitrate, NO₂ – nitrite, PO₄ – phosphates. ^a Digestive glands; ^b Gills.

3.3.3.3. Integrated data analysis

The response of biomarkers to petrochemical contamination was assessed by performing multivariate and graphical analysis. The results of these analyses are presented in Figure 3.6 and Table 3.5.

The results of the MDS analysis based on the similarity matrix calculated for the biomarkers using the Bray-Curtis similarity coefficient, showed a clear separation of these parameters into three distinct groups: group A, which corresponds to the biomarkers quantified in mussels collected at S1 and S3; group B, which corresponds to the biomarkers quantified in mussels collected at S2; and group C, which corresponds to the biomarkers quantified in mussels collected at S4 and S5 (Figure 3.6). Moreover, the ANOSIM test based on the similarity of the biomarkers revealed significant differences among the three groups ($R = 0.674$; $p < 0.001$).

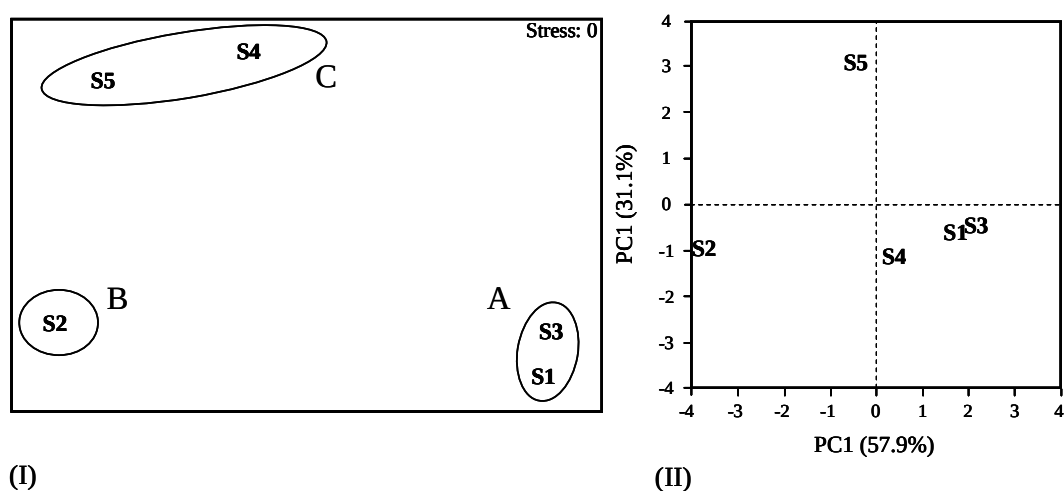


Figure 3.6 Two dimensional non-metric multidimensional scaling (MDS) ordination plot of biomarkers analysed in *Mytilus galloprovincialis* collected during April 2005 at five sampling sites (S1-S5) along the NW coast of Portugal, discriminating the distribution of the sites into three distinct groups (A, B and C) (I). Principal component analysis (PCA) score plot for the five sampling sites as a function of the petroleum hydrocarbon levels measured in mussels' tissue (II). The first two principal components (PC1 and PC2) account for 57.9% and 31.1% of the variance in the data set, respectively.

The results of the SIMPER analysis indicated that the biomarkers that were responsible for the assemblage of sampling sites S1 and S3 in Group A were ODH quantified in mussels' posterior adductor muscles, GST in gills, AChE in haemolymph, as well as GR in both digestive glands and gills, explaining 53% of the similarities within this group (Table 3.5). Likewise, this analysis indicated that the biomarkers that were responsible for the assemblage of sampling sites S4 and S5 in Group C were ODH in posterior adductor muscles, GST in gills, AChE in haemolymph, as well as SOD and GST quantified in mussels' digestive glands, explaining 54% of the similarities within this group (Table 3.5). Moreover, SIMPER analysis indicated that the biomarkers that explained 62% of the dissimilarities between Group A and B were the activities of GPx and GR quantified in mussels' digestive glands and gills, as well as ODH in posterior adductor muscles. About 56% of the dissimilarities between Group A and C were explained by the activities of SOD and CAT quantified in mussels' digestive glands, GPx and GST in gills, as well as ODH in posterior adductor muscles. Finally, 53% of the dissimilarities between Group B and C were explained by the activities of SOD and GPx quantified in mussels' digestive glands, GST in both digestive glands and gills, as well as ODH in posterior adductor muscles (Table 3.5).

The results of the PCA analysis, performed to discriminate the similarities of each sampling site as function of petroleum hydrocarbon levels measured in mussels' tissue indicated that the two principal components accounted for 89% of the overall variability of the data (Figure 3.6). The other components were neglected because they did not provide significant additional explanation to the data. The first principal component, corresponding to the horizontal axis of the plot diagram, accounted for 57.9% of the variability of the data and was clearly associated with the levels of some PAHs, mainly benzo[a]pyrene, benzo[ghi]perylene, and ideno[1,2,3-cd]pyrene. The second principal component, corresponding to the vertical axis of the plot diagram, accounted for 31.1% of the variability of the data and was clearly associated with the levels of UCM, as well as the levels of some PAHs, mainly anthracene and benzo[a]anthracene (Figure 3.6). Finally, the BIOENV analysis indicated that the best correlations between the levels of petroleum hydrocarbons and the biomarkers occurred with the levels of acenaphthylene, anthracene, benzo[a]anthracene, and ideno[1,2,3-cd]pyrene ($r = 0.879$).

Table 3.5 Results of SIMPER analysis indicating which biomarkers contributed most to the overall similarities within each group, and overall dissimilarities between groups of sampling sites.

% Similarity			% Dissimilarity		
Group A	Individual contribution	Cumulative contribution	Group A-B	Individual contribution	Cumulative contribution
ODH ^c	16.05	16.05	GPx ^a	16.92	16.92
GST ^b	11.66	27.71	ODH ^c	14.41	31.33
AChE ^d	9.80	37.51	GR ^b	11.02	42.35
GR ^b	8.52	46.04	GR ^a	10.36	52.71
GR ^a	7.38	53.42	GPx ^b	9.01	61.72
Average similarity of Group A		94.71	Average dissimilarity Group A-B		16.81
Group C			Group A-C		
GST ^b	15.54	15.54	SOD ^a	11.75	11.75
ODH ^c	11.78	27.32	CAT ^a	11.70	23.45
SOD ^a	10.14	37.46	GPx ^b	11.11	34.56
GST ^a	8.97	46.43	GST ^b	10.87	45.43
AChE ^d	7.54	53.88	ODH ^c	10.09	55.52
Average similarity of Group C		87.58	Average dissimilarity Group A-C		19.06
			Group B-C		
			ODH ^c	11.99	11.99
			GST ^b	11.49	23.48
			GPx ^a	10.66	34.14
			SOD ^a	9.53	43.67
			GST ^a	9.48	53.15
			Average dissimilarity Group B-C		15.55

SOD – total superoxide dismutase, CAT – catalase, GPx – selenium-dependent glutathione peroxidase, GR – glutathione reductase, GST – Glutathione S-transferases, ODH – octopine dehydrogenase, AChE - acetylcholinesterase. ^a Digestive glands; ^b gills, ^cposterior adductor muscle, ^dhaemolymph. All values are presented in percentages.

3.3.3.4. Laboratory exposure

During the exposure period, dead organisms were found in 100% and 50% WAF dilutions, with mortalities of 72 % and 38% respectively. As consequence, the response of the selected biomarkers to 100% WAF was not determined due to insufficient sampling material. The results of the biomarkers are presented in Figure 3.7 to 3.10. One-way ANOVA revealed significant differences between organisms exposed to different dilutions of WAF and the control for some antioxidant and/or detoxification parameters determined in *M. galloprovincialis* digestive glands (SOD: $F_{4,25} = 14$, $p \leq 0.001$; CAT: $F_{4,25} = 3.8$, $p \leq 0.05$; GR: $F_{4,25} = 4.5$, $p \leq 0.05$) and gills (SOD: $F_{4,25} = 16$, $p \leq 0.001$; GPx: $F_{4,25} = 2.8$,

$p \leq 0.05$; GR: $F_{4,25} = 4.5$, $p \leq 0.05$; GST: $F_{4,25} = 6.7$, $p \leq 0.001$; LPO: $F_{4,25} = 2.8$, $p \leq 0.05$; tGSx: $F_{4,25} = 6.7$, $p \leq 0.001$). Nevertheless, the Dunnett's multiple-comparison tests did not provide evidence of significant differences between organisms exposed to different dilutions of WAF and the control for the activities of GPx and levels of LPO quantified in mussels' gills. One-way ANOVA also revealed that no significant differences were found between mussels exposed to different dilutions of WAF and the control for the activity levels of GPx ($F_{4,25} = 0.6$, $p > 0.05$) and GST ($F_{4,25} = 0.4$, $p > 0.05$), as well as levels of LPO ($F_{4,25} = 0.3$, $p > 0.05$), tGSx ($F_{4,25} = 1.7$, $p > 0.05$), GSH ($F_{4,25} = 2.5$, $p > 0.05$), GSSG ($F_{4,25} = 0.6$, $p > 0.05$), and GSH/GSSG ratio ($F_{4,25} = 0.1$, $p > 0.05$) quantified in mussels' digestive glands, as well as the activity levels of CAT ($F_{4,25} = 1.6$, $p > 0.05$), and the levels of GSH ($F_{4,25} = 2.5$, $p > 0.05$), GSSG ($F_{4,25} = 2.5$, $p > 0.05$), and GSH/GSSG ratio ($F_{4,25} = 0.4$, $p > 0.05$) quantified in mussels' gills. Moreover, One-way ANOVA also revealed that no significant differences were found between mussels exposed to different dilutions of WAF and the control for biochemical parameters involved in the mussels' energetic metabolism (IDH: $F_{4,25} = 0.6$, $p > 0.05$; ODH: $F_{4,25} = 1.5$, $p > 0.05$) and neurotransmission (AChE: $F_{4,25} = 2.3$, $p > 0.05$).

Compared to the control, a significant induction of SOD activity was found in digestive glands of mussels exposed to all dilutions of WAF, except 6.25%. Induction rates of 64% ($p \leq 0.05$), 75% ($p \leq 0.01$), and 139% ($p \leq 0.01$) in SOD activity were found in digestive glands of mussels exposed to 12.5%, 25% and 50% of WAF respectively. In gills, a significant induction of SOD activity was found in mussels exposed to 25% (233% induction, $p \leq 0.01$) and 50% (331% induction, $p \leq 0.01$) of WAF compared to the control (Figure 3.7).

An induction of 65% ($p \leq 0.05$) in CAT activity levels was found in the digestive glands of mussels exposed to 50 % WAF compared to the control. No significant differences were found in CAT activity levels in gills of mussels exposed to all WAF dilutions compared to the control (Figure 3.7).

No significant differences were found in GPx activity levels in both digestive glands and gills of mussels exposed to all WAF dilutions compared to the control (Figure 3.7).

Regarding the levels of GR activity in digestive glands of mussels at the end of the exposure period, an induction of 44% ($p \leq 0.05$) was found in organisms exposed to 50% WAF compared to the control. Likewise, an induction of 48% ($p \leq 0.05$) was found in the levels of GR activity in gills of mussels exposed to 50% WAF compared to the control (Figure 3.7).

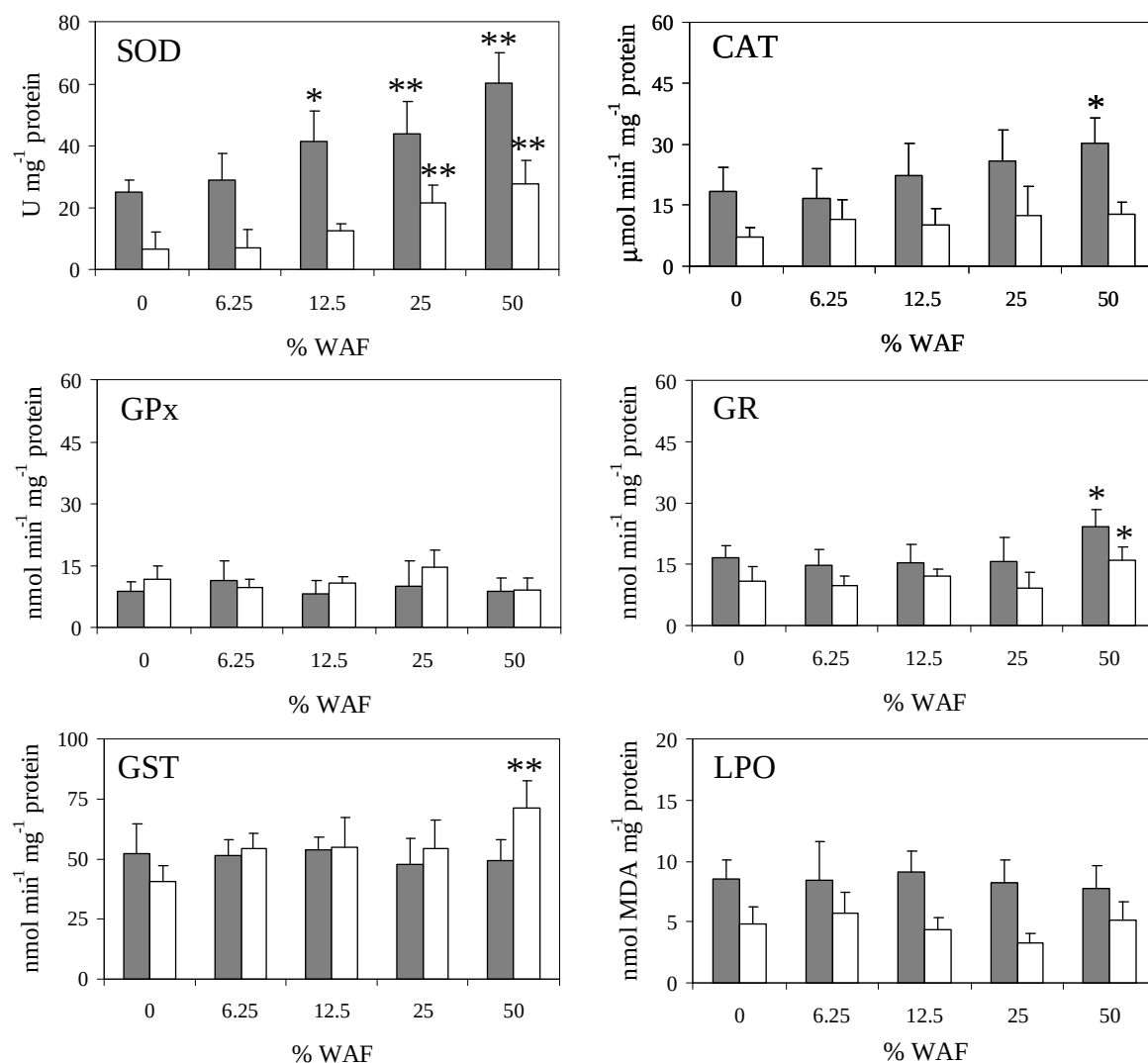


Figure 3.7 Biomarkers analysed in *Mytilus galloprovincialis* following 21 days of exposure to water-accommodated fraction of #4 fuel-oil (WAF) under laboratorial conditions. Values are presented as mean $\pm$ standard deviation ($n = 6$) of total superoxide dismutase (SOD), catalase (CAT), selenium-dependent glutathione peroxidase (GPx), glutathione reductase (GR), glutathione S-transferases (GST), and lipid peroxides (LPO). *($p \leq 0.05$) and **($p \leq 0.01$) indicate significant differences between control and WAF dilutions by Dunnett's multiple-comparison test for each biomarker.

No significant differences were found in GST activity levels in digestive glands of mussels exposed to all WAF dilutions compared to the control. In gills, an induction of 75% ($p \leq 0.01$) was quantified in the levels of GST activity of mussels exposed to 50% WAF compared to the control (Figure 3.7).

No significant differences were found in the levels of LPO quantified in both digestive glands and gills of mussels exposed to all WAF dilutions compared to the control (Figure 3.7).

A significant induction was found in the levels of tGSx in digestive glands of mussels exposed to all WAF dilutions compared to the control. Inductions of 57% ($p \leq 0.01$), 73% ($p \leq 0.01$), 85% ($p \leq 0.01$) and 74% ($p \leq 0.01$) were found in mussels exposed to 6.25%, 12.5%, 25% and 50% of WAF respectively. No significant differences were found in the levels of tGSx quantified in gills of mussels exposed to all WAF dilutions compared to the control. Regarding the remaining parameter involved in the redox status (GSH, GSSG and GSH/GSSG) of mussels, no significant differences were found between the control and all WAF dilutions (Figure 3.8).

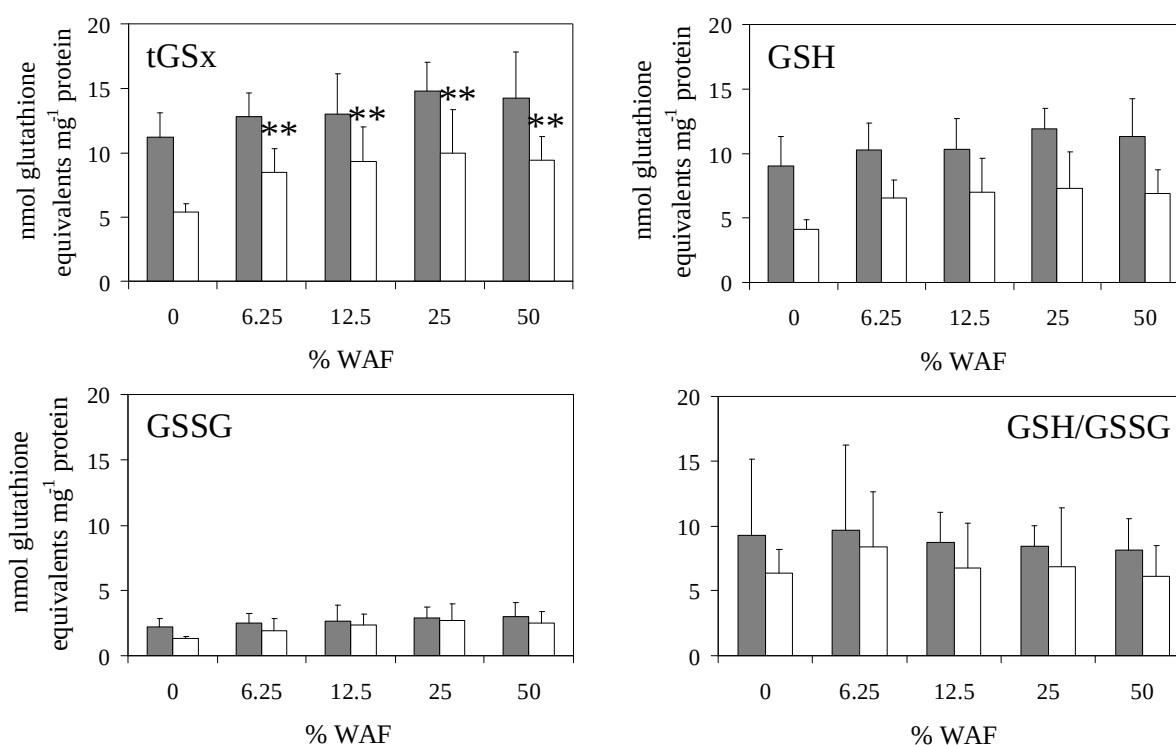


Figure 3.8 Biomarkers analysed in *Mytilus galloprovincialis* following 21 days exposure to water-accommodated fraction of #4 fuel-oil (WAF) under laboratory conditions. Values are presented as mean $\pm$ standard deviation ($n = 6$) of total glutathione content (tGSx), reduced glutathione (GSH), oxidised glutathione (GSSG), and glutathione redox status (GSH/GSSG). *($p \leq 0.05$) and **($p < 0.01$) indicate significant differences between control and WAF dilutions by Dunnett's multiple-comparison test for each biomarker.

Finally, no significant differences were found between control mussels and mussels exposed to all WAF dilutions for parameter involved in energetic metabolism (IDH and ODH), as well as neurotransmission (AChE) (Figure 3.9 and 3.10).

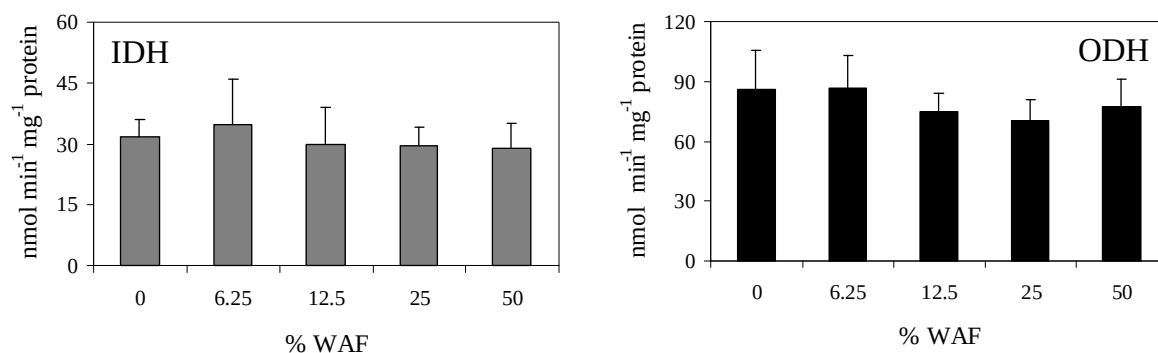


Figure 3.9 Biomarkers analysed in *Mytilus galloprovincialis* following 21 days exposure to water-accommodated fraction of #4 fuel-oil (WAF) under laboratorial conditions. Values are presented as mean $\pm$ standard deviation ($n = 6$) of NADP⁺-dependent isocitrate dehydrogenase (IDH), and octopine dehydrogenase (ODH). $^{*}(p \leq 0.05)$ and $^{**}(p < 0.01)$ indicate significant differences between control and WAF dilutions by Dunnett's multiple-comparison test for each biomarker.

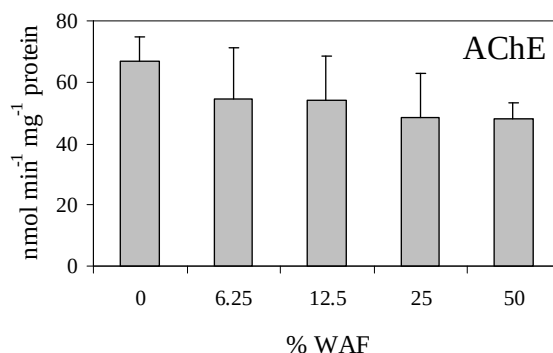


Figure 3.10 Acetylcholinesterase activity analysed in *Mytilus galloprovincialis* following 21 days exposure to water-accommodated fraction of #4 fuel-oil (WAF) under laboratorial conditions. Values are presented as mean $\pm$ standard deviation ($n = 6$). $^{*}(p \leq 0.05)$ and $^{**}(p < 0.01)$ indicate significant differences between control and WAF dilutions by Dunnett's multiple-comparison test.

To evaluate the possible effects of the manipulation of the organisms during laboratorial experimental conditions, differences between biomarkers assessed in mussels collected at S1 and in mussels from the 0% WAF after 21 days of exposure were evaluated. No significant differences were found in the levels of activity of CAT (t -test: $t = -2.0$, $df = 14$, $p > 0.05$), GPx (t -test: $t = -0.1$, $df = 14$, $p > 0.05$) and GST (t -test: $t = -0.5$, $df = 14$, $p > 0.05$) quantified in mussels' gills, as well as in the levels of LPO (t -test: $t = 1.0$, $df = 14$, $p > 0.05$; t -test: $t = 1.4$, $df = 14$, $p > 0.05$) and tGSx (t -test: $t = -1.1$, $df = 14$, $p > 0.05$; t -test: $t = 1.2$, $df = 14$, $p > 0.05$) quantified in digestive glands and gills respectively, and GSH (t -test: $t = 1.0$, $df = 14$, $p > 0.05$) quantified in mussels' digestive glands. Significant higher levels were found on the activity of CAT (t -test: $t = 3.1$, $df = 14$, $p \leq 0.05$), SOD (t -test: $t = 6.3$, $df = 14$, $p < 0.001$), GST (t -test: $t = 6.2$, $df = 14$, $p < 0.001$), and IDH (t -test: $t = 3.3$, $df = 14$, $p \leq 0.05$) quantified in digestive glands, ODH (t -test: $t = 3.4$, $df = 14$, $p \leq 0.05$) quantified in posterior adductor muscles, AChE (t -test: $t = 7.1$, $df = 14$, $p \leq 0.05$) quantified in haemolymph, as well as in the levels of GSH (t -test: $t = 3.7$, $df = 14$, $p \leq 0.05$) quantified in gills, and in the ratio GSH/GSSG (t -test: $t = 3.4$, $df = 14$, $p \leq 0.05$; t -test: $t = 6.3$, $df = 14$, $p < 0.001$) quantified in digestive glands and gills, respectively, of mussels exposed to 0% WAF for 21 days. Significant lower levels were found on the activity of SOD (t -test: $t = -6.3$, $df = 14$, $p < 0.001$) quantified in gills, GPx (t -test: $t = -3.7$, $df = 14$, $p \leq 0.05$) quantified in digestive glands, as well as in GR (t -test: $t = -5.9$, $df = 14$, $p < 0.001$; t -test: $t = -6.8$, $df = 14$, $p < 0.001$) and GSSG (t -test: $t = -3.8$, $df = 14$, $p \leq 0.05$; t -test: $t = -3.0$, $df = 14$, $p \leq 0.05$) quantified in digestive glands and gills, respectively, of mussels exposed to 0% WAF for 21 days.

3.3.4. Gene expression

The genes of Cu/Zn-SOD and CAT and were partially isolated in the marine mussel *M. galloprovincialis* using primers derived from previously published sequences^[45, 46]. Multiple alignments of *M. galloprovincialis* Cu/Zn-SOD and CAT amino acid sequence was performed with ClustalW using known protein sequences from different invertebrate homologues (Figure 3.11 and 3.12).

The putative 412 bp fragment of *M. galloprovincialis* gene of Cu/Zn-SOD herein isolated showed an amino acid sequence 93% identical to other genes of Cu/Zn-SOD of the family **Mytilidae**, as well as 70% identical to the same gene in the oyster *Crassostrea gigas* (Figure 3.11).

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Medulis      MAANIKAVCVLKG DGAVTGTVAFSQQNGDSAVTVTGELTGLAPGEHGFHVHEFGDNTNGC 60
Mgallo       MAANIKAVCVLKG DGAVTGTVAFSQQNGDSAVTVTGELTGLAPGEHGFHVHEFGDNTNGC 60
MgalloPT     -----SQQNGXSAVTVTGELTGLAXGEHGFHVHEFGDNTNGC 37
Cgigas       MSSALKAVCVLKGDSNVTGTVQFSQEAPGTPVTLSGEIKGLTPGQHGFHVHFLFGDNTNGC 60
              **:      :.**:**.**: **: *:***** *****

Medulis      TSAGSHFNPF GKTHGAPGDEERHVGDLGNVLANADGKAEIKITDTKLSLTGPQSIIGRTV 120
Mgallo       TSAGSHFNPF GKTHGAPGDEERHVGDLGNVLANAEGKAEIKITDAKLSLTGPQSIIGRTV 120
MgalloPT     TSAGSHFNPF GKTHGAPGDEERHVGDLGNVLANADGKAEIKITDAKLSLTGPQSIIGRTV 97
Cgigas       TSAGRHFNPFNKEHGVPEDEERHVGDLGNVTAGEDGVAKISITDKMIDLAGPQSIIGRTV 120
              **** ***** * ** * *.***** * . * *.**.* ** :.**:*****

Medulis      VVHADIDDLGKGGGHEL SKTTGNTGGRLACGVIGISKV 158
Mgallo       VVHADIDDLGKGG-HEL SKTTGNAGGRLACGVIGISKV 157
MgalloPT     VVHADIDDLGKGG-HEL SKTTGNAGGRLACXXPXX--- 131
Cgigas       VIHGDVDDLKGGG-HEL SKTTGNAGGRLACGVIGITK- 156
              *.*.*.**:***** *****:*****

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Figure 3.11 Comparison of the deduced Cu/Zn-superoxide dismutase protein sequence of *Mytilus galloprovincialis* (MgalloPT) with selected Cu/Zn-superoxide dismutase protein sequences of invertebrates: *Mytilus edulis* (GeneBank Accession No. CAE46443), a known sequence of *Mytilus galloprovincialis* (CAQ68509), and *Crassostrea gigas* (CAD42722). Asterisks indicate identical amino acids revealed by ClustalW sequence analysis.

Likewise, the putative 388 bp fragment of *M. galloprovincialis* gene of CAT herein isolated showed 90% amino acid sequence identical to other genes of CAT of the family **Mytilidae** (Figure 3.12).

The gene expression of Cu/Zn-SOD and CAT of *M. galloprovincialis* was later analysed. The results showed that there was an increase in the expression of CAT in the digestive glands of mussels collected at S4 and S5, when compared to those collected at S1 and S3 (Figure 3.13). However, no differences in the gene expression of Cu/Zn-SOD in mussels' digestive glands were verified among sampling sites. Regarding the laboratorial exposure of *M. galloprovincialis* to WAF, results also showed an increase in the expression of CAT in the digestive glands of mussels exposed to 50% WAF dilution (Figure 3.13). However, as for field results, no differences were found in the gene expression of Cu/Zn-SOD between mussels exposed to 50% WAF and the control.

Medulis	TPIFFIRDPLMFLPSFIHTQKRNPETHLKDPDMFWDFITLRPETTHQVSFLFSDRGTPDGY	60
Mcalif	TPIFFIRDPLMFLPSFIHTQKRNPETHLKDPDMFWDFITLRPETTHQVSFLFSDRGTPDGF	60
Mgallo	-----RNRETHLKDPDXLWDFITLRPETTHQVSFLFSDRGTPDGY	40
MgalloPT	-----QISLVPSDRGTPDGY	15
	*:~::~ *****:	
Medulis	RRMNGYGSHTFKTVNKDGQAYYCKFHFKTDQGIKCLSAEQADKLSSTDPDYAIRDLYNAI	120
Mcalif	RRMNGYGSHTFKTVNKDGQAYYCKFHFKTDQGIKCLSAEQADKLSSTDPDYAIRDLYNAI	120
Mgallo	RRMNGYGSHTFKTVNKDGQAYYCKFHFKTDQGIKCLSAEQADKLSSTDPDYAIRDLYNAI	100
MgalloPT	RRMNGYGSHTFKTVNKDGQAYYCKFHFKTDQGIKCLSAEQADKLSSTDPDYAIRDLYNAI	75

Medulis	SEGNFPSWSVNVQIMTFEEAENFRYNPFDLTKIWPQGEFPLIPVGRMVLNRNPKNYFAEV	180
Mcalif	SEGNFPSWSLNVQIMTFEEAENFRYNPFDLTKIWPQGEFPLIPVGRMVLNRNPKNYFAEV	180
Mgallo	SEGNFPSWSVNVQIMTFEEAENFRYNPFDLTKIWPQGXFPXIPVGRMVLNRXPKXYFAEV	160
MgalloPT	SEGNFPSWSVNVQIMTFEEXENFRYNPFDLTKIWPQGEFPWXPQXXX-----	122
	*****.***** ***** ** *	
Medulis	EQIAFSPVHMIPGIEASPDKMFQGNRIPRRH	211
Mcalif	EQIAFSPVHMIPGIEASPDKMFQG-----	204
Mgallo	XQ-----	162
MgalloPT	-----	

Figure 3.12 Comparison of the deduced catalase protein sequence of *Mytilus galloprovincialis* (MgalloPT) with selected catalase protein sequences of the **Mytilidae** family: *Mytilus edulis* (GeneBank Accession No. AAT06168), *Mytilus californianus* (AAT06167), and a known sequence of *Mytilus galloprovincialis* (AAV27185). Asterisks indicate identical amino acids revealed by ClustalW sequence analysis.

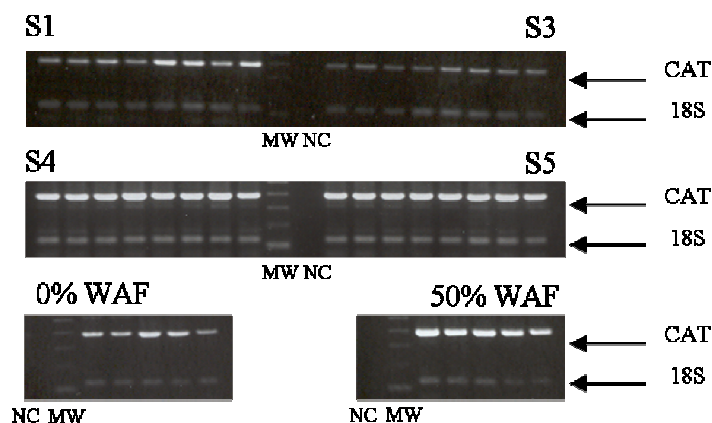


Figure 3.13 Agarose gel stained with ethidium bromide displaying semi-quantitative PCR amplification products of the gene of catalase (388 bp) isolated from *Mytilus galloprovincialis* digestive glands. Gene expression was determined in mussels collected at Carreço (S1), Vila Chã (S3), Cabo do Mundo (S4) and Leixões harbour (S5), as well as in mussels exposed to 0% and 50% water-accommodated fraction of #4 fuel-oil. The 18S rRNA gene (172 bp) was used as housekeeping gene. MW: 100 bp molecular weight ladder; NC: negative control.

3.4. DISCUSSION

A monitoring program was developed by Lima et al. (see Chapter 1 and 2) to evaluate the suitability of a battery of biomarkers quantified in *M. galloprovincialis* to assess the effects of petrochemical contamination along the NW coast of Portugal. The results showed that some of the biochemical parameters implemented as biomarkers were able to discriminate the selected sampling sites according to the levels of petroleum hydrocarbons quantified mussels' tissues. In particular, biomarkers involved in mussels' antioxidant defence system (e.g. SOD and CAT) appeared to be very responsive to this class of contaminants (see Chapter 1 and 2). In the present work, to better understand the toxicity mechanisms induced by petrochemical contaminants in marine mussels, in particular with respect to their antioxidant defence system, we further evaluated the responsiveness of these biomarkers by comparing their discriminative potential in the field with their response following chronic exposure of mussels to WAF under laboratory conditions. Moreover, regarding the results obtained in the field and in the laboratory, and with the aim of developing new tools to assess the effects of petrochemical products at the transcriptional level, the gene expression of two enzymes involved in the *M. galloprovincialis* antioxidant defence system (Cu/Zn-SOD and CAT) was also evaluated.

In the first part of the present study, the results of the chemical analysis performed in mussels' tissues to evaluate the levels of petrochemical contamination along the NW coast of Portugal revealed levels of petroleum hydrocarbons in the same range of the values obtained during the initial survey of January 2005 (see Chapter 1) ^[7]. As reported for January 2005, the levels of PAHs herein presented (642.99 $\mu\text{g g}^{-1}$ dw in S2 to 94.8 $\mu\text{g g}^{-1}$ dw in S3) were higher than those determined in 1998 in *M. galloprovincialis* collected along the region between Vila Chã (S3) and Leixões harbour (S5) (0.60 $\mu\text{g g}^{-1}$ dw to 40.00 $\mu\text{g g}^{-1}$ dw) ^[21], and higher than those obtained at the sampling sites selected for the present work (S1-S5) during a more recent monitoring program performed by Lima et al. (0.32 $\mu\text{g g}^{-1}$ dw to 7.32 $\mu\text{g g}^{-1}$ dw) (unpublished data, see Chapter 2). Even considering the works of Salgado and Serra conducted in 1998 ^[21], and Lima et al. conducted between 2005 and 2006 (see Chapter 1 and 2), the interpretation of the results herein presented may be hampered due to the lack of additional data regarding chemical analysis of petroleum hydrocarbons for the NW coast of Portugal, in particular regarding the levels of UCM.

The highest levels of total petroleum hydrocarbons were found in mussels collected at commercial harbours, with Viana do Castelo harbour (S2) presenting the highest levels of PAHs, and Leixões harbour (S5) presenting the highest levels of AH and UCM. UCM, a fraction of petroleum products that comprises both aromatic and non aromatic compounds can be used as an indicator of the degradation of petrogenic products by weathering processes ^[51, 52, 53, 54]. High ratios of unresolved to resolved petroleum hydrocarbons (UCM/total petroleum hydrocarbons), such as those found in Leixões harbour (S5) may reflect long-term contamination by petrogenic products that can enter the docks either by sporadic fuel spills from fishing vessels or from maintenance activities in oil terminals. Unexpectedly, high ratios of unresolved to resolved petroleum hydrocarbons were also found in Vila Chã (S3), a site classified as having low levels of petrochemical contamination ^[7]. However, these results are not related with high levels of UCM but with low levels of AH and PAHs. Furthermore, low ratios of unresolved to resolved petroleum hydrocarbons, mainly due to high levels of PAHs, were found in Viana do Castelo harbour (S2) and near an oil refinery (S4), which indicated possible recent discharges of petrochemical products into these areas. The toxicity of UCM has not been extensively studied. However, it is known that the oxidation of non-aromatic hydrocarbons, as well as some aromatic hydrocarbons present in this petroleum fraction, can enhance toxicity mechanisms in aquatic organisms ^[52, 55]. Interestingly, UCM was the petroleum fraction that related better with the response of biomarkers in the monitoring program implemented by Lima et al. between 2005 and 2006 (unpublished data, see Chapter 2). Finally considering the levels of PAHs, one of the petroleum fractions known to be highly toxicity to aquatic organisms, we can classify the sampling sites selected for the present work as having high (S2 - Viana do Castelo harbour), moderate (S4 – Cabo do Mundo; S5 – Leixões harbour), and low (S1 – Carreço; S3 – Vila Chã) levels of petrochemical contamination. These ranking is in agreement with the results found during January 2005 by Lima et al. (2007) ^[7]. With the aim of assessing the potential effects induced by these levels of petrochemical hydrocarbons in *M. galloprovincialis*, several biochemical parameters involved in key physiological processes of mussels (antioxidant defences, detoxification, energetic metabolism and neurotransmission) were applied as biomarkers.

It has been reported that in aquatic organisms the production of ROS can be enhanced in the presence of organic and inorganic contaminants, which can lead to cellular damage through protein oxidation, lipid peroxidation and DNA damage ^[13, 15, 56]. In the absence of stressors there is a balance between the generation of ROS during normal metabolism and their detoxification by enzymatic and non-enzymatic antioxidant defence mechanisms. In the event of an imbalance causing an increase in the production of ROS,

the activity of antioxidant enzymes such as SOD, CAT and GPx is enhanced to eliminate the excess ROS and prevent cellular damage ^[13, 15, 56]. In particular, to eliminate ROS such as organic and inorganic peroxides, the enzyme GPx oxidises GSH to its disulfide form (GSSG). However, the GSH/GSSG ratio needs to remain high in order to maintain the redox homeostasis of the cell ^[57]. Consequently, when the organism is under oxidative stress, cellular levels of GSH are maintained by the enzyme GR which converts GSSG back into GSH. The regeneration of GSH from GSSG occurs at the expense of NADPH, which is posteriorly restored by the pentose phosphate pathway or by NADP⁺-dependent IDH ^[57, 58]. In addition, it has also been reported that molluscs' GST, a family of multi-functional enzymes involved in Phase II of biotransformation processes, also has an important role in their antioxidant defence system. Besides the conjugation of electrophilic xenobiotics with GSH, it has been reported that mussels' GST enzymes also present a distinct GSH peroxidase activity particularly in gills ^[59].

The results obtained in the field showed that mussels collected near an oil refinery (S4) and at Leixões harbour (S5) presented significantly higher levels of SOD and CAT activity quantified in digestive glands than those collected from the remaining sampling sites. These results are in agreement with those obtained at the same sampling sites during other surveys performed by Lima et al. (see Chapter 1 and 2). A study conducted by Livingstone et al. (1992), which aimed to compare the levels SOD and CAT activity between vertebrates and invertebrates, revealed that invertebrates presented higher levels of SOD and CAT activity than vertebrates, reinforcing the importance of these two enzymes in the antioxidant defence system of organisms such as *M. galloprovincialis* ^[60]. In aquatic organisms the enzymes SOD and CAT are the first lines of antioxidant defences, and appear to be highly responsive to increasing levels of contaminant stimulated ROS production ^[15]. In fact, significant correlations between PAHs levels and the activities of these two antioxidant enzymes have been found in mussels collected in the Mediterranean Sea and Gulf of Cádiz ^[61, 62, 63]. However, in the present work, no significant correlations were found between the levels of SOD and CAT activity and petroleum hydrocarbon levels as reported in other surveys conducted in the same sampling sites (see Chapter 1 and 2). This might indicate the presence other classes of contaminants (e.g. metals, PCBs) at S4 and S5, which are able to induce the production of superoxide anions (O₂⁻), as well as hydrogen peroxide (H₂O₂) ^[15].

Significantly higher levels of GST activity were also detected in mussels collected near an oil refinery (S4) than in those collected at the remaining sites. As previously discussed, S4 was the site selected for this work that presented the highest levels of PAHs. These results are in agreement with previous field studies conducted by our

research group that have demonstrated a similar relationship between levels of petrochemical contamination and GST activity in mussels ^[7, 18]. Moreover, Gowland et al. (2002) found that high molecular weight PAHs with five and six rings seem to have a greater role in the induction of GST activity in mussels than low molecular weight PAHs with two to four rings ^[64]. In fact, the major contributions for the levels of total PAHs quantified in mussel tissues during this study were given by benzo[a]pyrene, benzo[k]fluoranthene (both with five rings), and ideno[1,2,3-cd]pyrene (with six rings). However, no significant correlations were found between the activity of these multi-functional enzymes involved in mussels' detoxification processes and the fractions of petroleum hydrocarbons quantified in the present study. Finally, higher levels of GST activity were detected in mussels' gills than in digestive glands. These results are the opposite of what was verified for the enzyme CAT, which presented higher activity levels in mussels' digestive glands. This situation, which was also verified in other surveys performed by Lima et al. (see Chapter 1 and 2), may suggest that an increase in the levels of GST activity, in particular its peroxidase activity, might act as a cellular compensation mechanism when CAT activity is low, in order to protect against ROS induced damage ^[59].

As previously described, the detoxification of contaminants, through the action of GST, and the detoxification of ROS, through the action of some antioxidant enzymatic defences may lead to depletion of GSH, which needs to be quickly replaced to maintain the cellular redox balance ^[58]. In the present work, while significantly high levels of GPx activity were quantified in both digestive glands and gills of mussels collected in Viana do Castelo harbour (S2), significantly low levels of GPx activity were quantified in mussels collected from sites located in open seashore (S1 and S3). Contrary to these results, significantly low levels of GR activity were found in both digestive glands and gills of mussels collected at S2, while significantly high levels of GR activity were quantified in mussels collected at S1 and S3. A similar situation has been reported by Lima et al. in the survey conducted during January 2005 ^[7]. These results are in agreement with the levels of GSH found during the present study. Mussels collected at S2, which presented high levels of GPx activity and low levels of GR activity, also presented significantly lower levels of GSH in digestive glands when compared with those collected from S1 and S3. However, unexpectedly low levels of GSSG were also found in mussels from S2, and high levels of GSSG were found in mussels collected from S1 and S3. These results might explain why no significant differences were found in the cellular redox status (GSH/GSSG) of mussels among sampling sites. As discussed in previous chapters (see discussions of Chapter 1 and 2), for a full understanding of the cellular redox status of mussels, the

activity of enzymes involved in GSH synthesis (γ -glutamylcystein synthetase and GSH synthetase) and GSH cellular transport (γ -glutamyl transpeptidase), should be considered ^[58]. In the present work, the concentrations of PAHs quantified in mussels' tissues was significantly correlated with levels of GPx activity quantified in mussels' digestive glands and GR quantified in mussels' gills. However, while GPx activity in digestive glands did not present significant correlations with abiotic parameters, indicating its suitability as biomarker of petrochemical contamination, GR activity in gills seems to be highly influenced by salinity, as well as by the levels of ammonia, nitrates, nitrites and phosphates.

The levels of LPO were quantified in the present work as an indicator of oxidative damage. The results showed that no significant differences were found in the levels of LPO measured in digestive glands of mussels among sampling sites, indicating that the levels of antioxidant defences active in this organ were enough to fight contaminant stimulated ROS production. However, in gills, which are more exposed to environmental stressors and contaminants, significantly high levels of LPO were quantified in mussels collected at S4 and S2, which were the sampling sites that presented the highest levels of PAHs. Still, no significant correlations were found between LPO and petroleum hydrocarbons quantified in the present work.

In addition, IDH, which apparently is a regulator of cellular antioxidant defences, mainly through the regeneration of NADPH oxidised by GR during the reduction of GSSG to GSH, was also assessed as a possible biomarker for petrochemical contamination ^[65, 66]. The results obtained for this enzyme during the present work indicated that significantly high levels of IDH were measured in mussels collected from Viana do Castelo harbour (S2) and Leixões harbour (S5), presenting a good correlation with the levels of UCM quantified in mussels tissues. Moreover, since no significant correlations were found with abiotic parameters, we suggest that IDH might be a suitable biomarker for petrochemical contamination. Similar results were found for the levels of ODH activity. ODH, which is a pyruvate oxidoreductase enzyme with a function similar to lactate dehydrogenase in vertebrates, is involved in the anaerobic metabolism of several invertebrates by regenerating NAD^+ during anaerobic glycolysis ^[67]. In the present work, significantly high levels of ODH were found in mussels collected from both commercial harbours (S2 and S5). It has been reported that under stressful conditions mussels reduce cellular respiration as an attempt to conserve energy. Under these circumstances, the rate of cellular oxygen uptake may be insufficient and anaerobic metabolism needs to be enhanced to cope with this respiratory deficit and to supply extra ATP ^[68]. Moreover, a significant positive correlation was found between the UCM fraction and the activity levels

of ODH, which apparently were not influenced by abiotic parameters. Similar results were reported by Lima et al. (unpublished data, see Chapter 2) following a long-term monitoring program conducted in the same region of the NW coast of Portugal, who suggested this enzyme as a possible biomarker for petrochemical contamination.

Finally, the enzyme AChE, which breaks down of the neurotransmitter acetylcholine during the transmission of nerve impulses across cholinergic synapses, has been widely used as an indicator of neurotoxicity in marine invertebrates ^[69]. In the present work, significantly lower levels of AChE activity were found in mussels collected at Leixões harbour (S5) when compared with the organisms collected at the remaining sampling sites. However, no significant correlations were found between the activity levels of this enzyme and the petroleum hydrocarbon fractions quantified in mussels' tissues. Its inhibition has been widely used as a specific biomarker for organophosphate and carbamate pesticides, but significant inhibitions in AChE activity have also been reported in *M. galloprovincialis* exposed to petrochemical contamination by our research group ^[18].

The results of the MDS analysis indicated that the response of the biomarkers selected for this study was able to discriminate the sampling sites into three groups as reported for previous surveys (see Chapter 2). This separation seems to be in agreement with the classification made according to the levels of PAHs: S2 (highly contaminated), S4 and S5 (moderately contaminated) and S1 and S3 (low contamination). SIMPER analysis indicated that the biomarker responsible for the assemblage of S1 and S3 into one group and S4 and S5 into another group were AChE, ODH and GST in gills, which is in agreement with the results found during the biomonitoring program conducted by Lima et al. for the same region of Portugal (unpublished data, see Chapter 2). Moreover, the biomarkers that distinguish S2 from S1 and S3 were GPx and GR quantified in digestive glands and gills, as well as ODH. Ultimately, the biomarkers that distinguish S4 and S5 from S1 and S3 were SOD and CAT in digestive glands, and ODH. Moreover, the PCA analysis indicated that the petroleum hydrocarbons herein quantified explained 89% of the separation of the sampling sites into three distinct groups. However, by comparing the plots of the MDS and PCA analysis we can conclude that site S4 might be under the influence of other classes of contaminants since its location does not match both plots. Finally, the BIOENV analysis indicated that a good correlation was found between the levels of PAHs quantified in mussels' tissues and the overall response of the selected biomarkers.

The second part of this study intended to assess the specific response of the selected biomarker following exposure of *M. galloprovincialis* to petroleum products under controlled laboratorial conditions. At the end of the 21 days of laboratorial exposure

mussels' mortality rates were 38% and 72% for 50% and 100% WAF dilutions respectively. These values showed that the majority of WAF dilutions prepared during this laboratorial exposure was sublethal for adult specimens of *M. galloprovincialis*. Moreover, the results of the chemical analysis performed on samples of undiluted WAF indicated that the total PAHs to which mussels were exposed were ecologically relevant, in particular to the sampling sites selected for this work (approximately 5000 ng L⁻¹ at the beginning of the WAF exposure). Values in the same order of magnitude as those herein presented have been previously found by Salgado and Serra (2001) in water samples collected along the NW coast of Portugal ^[21]. Only water samples collected from sites located closest to the oil refinery exhibited higher concentrations of total PAHs ^[21].

Following the 21 days of mussels' exposure to WAF, the biomarkers that showed to be more responsive to the concentrations of total PAHs were related with the organisms' antioxidant defence system and detoxification processes. Biomarker results showed that mussels exposed to WAF exhibited significant inductions in the activity levels of SOD, CAT and GR in digestive glands, as well as SOD, GR and GST in gills. Furthermore, significantly higher levels of tGSx were also measured in gills of mussels following WAF exposure. In the present work a significant induction of CAT activity was quantified in digestive glands of mussels exposed to 50% WAF, while a significant induction of GST activity was quantified in the gills of the same organisms. These results are in agreement with the field results obtained in the present work, as well as with other surveys performed by Lima et al. (see Chapter 1 and 2), which suggest that an increase in the levels of GST activity, in particular its peroxidase activity, might act as a cellular compensation mechanism when CAT activity is low as it happens in mussels' gills ^[60].

Acute laboratorial bioassays lasting 96 hours have been previously performed by our research group with *M. galloprovincialis* (unpublished data), *Paracentrotus lividus* ^[70], and *Pomatoschistus microps* ^[71] exposed to WAF, as well as *M. galloprovincialis* exposed to fuel-oil collected following the "Coral Bulker" oil spill ^[18]. The results herein presented are in agreement with the results obtained during these previous studies, which showed GST quantified in mussels and fish gills as being one of the most responsive biomarkers to WAF exposure. The results of these studies revealed significant inductions of GST activity levels, however, results obtained with *P. lividus* showed significant inhibition of GST activity suggesting a different action mechanism ^[18, 70, 71]. Despite these results, which indicated that GST could be a suitable biomarker to assess the effects of petrochemical contamination, no significant correlations were found with this parameter and the levels of petroleum hydrocarbons quantified in the tissues of mussels collected during the field study here presented. Nevertheless, when looking at the integrated

analysis of the results for this field study, as well as the results of the long-term monitoring program performed by Lima et al during 2005 and 2006 for the same region of the NW coast of Portugal (unpublished data, see Chapter 2), GST showed to be one of the biomarkers responsible for the assemblage of the sampling sites into distinct groups according to the MDS analysis. Zhang et al. (2004) reported for *Carassius auratus* that GST showed an induction in activity following 15 days of exposure to diesel oil, however, following 25 days GST activity levels returned to values similar to the control ^[72]. A similar pattern might explain why there was no correlation with the levels of petroleum hydrocarbons and the GST levels quantified in mussels collected along the NW coast of Portugal; however only a longer exposure to WAF under laboratorial conditions or manipulative field work (e.g. 3 months exposure) could reveal if a similar pattern occurs for the GST of *M. galloprovincialis*.

From the studies performed by our research group in which aquatic organisms were exposed to WAF only that performed by Vieira et al. (2008) tested the suitability of antioxidant parameters as possible biomarkers for petrochemical contamination ^[71]. The results of this study showed that *P. microps* exposed to WAF only exhibited a significant induction of CAT activity quantified in the fish liver ^[71]. Contrary to our results, no significant inductions of SOD and GR activity were verified by Vieira et al. (2008) in *P. microps* following WAF exposure ^[71]. Earlier studies have reported that adverse effects induced by sublethal concentrations of contaminants can sometimes be difficult to observe in aquatic organisms following short periods of exposure, however, such effects can be induced following chronic exposure ^[73]. Moreover, as previously discussed in this Chapter, SOD activity levels seem to be higher in invertebrates than vertebrates, showing their importance as first line of antioxidant defences in invertebrates ^[60].

The field results previously presented indicated that the enzymatic activities of GPx, GR, IDH and ODH were correlated with the levels of petroleum hydrocarbons quantified in mussels' tissues. However, surprisingly only GR activity was induced in *M. galloprovincialis* following WAF exposure. No significant differences were found between the activity levels of GPx, IDH and ODH quantified in mussels exposed to WAF when compared to control organisms. These results are also contrary to those reported by Reid and MacFarlane (2003) ^[74], which indicated GPx as a suitable biomarker for field studies conducted with the gastropod *Austrocochlea porcata* since it exhibited a dose-dependent induction to WAF of a crude oil. To better understand the response of these enzymes to chronic petrochemical contamination, as previously suggested, future laboratorial works should be performed exposing mussels to WAF for longer periods (e.g. 3 months).

In addition, it is important to mention that significant differences were found between the activity levels of some enzymes quantified in mussels collected from S1 and control mussels following the 21 days of laboratorial exposure. In particular, extremely low levels of GPx and GR activities were detected in both digestive glands and gills of mussels exposed to WAF. Future works involving exposure of mussels to WAF under laboratorial conditions should be conducted using a semi-continuous water flow system, in order to minimise stress induced by the manipulation of organisms and to better control the exposure conditions.

In the third part of this study we assessed the putative effects of petrochemical contamination in the gene expression of Cu/Zn-SOD and CAT. The aim of this work was to assess the suitability of these parameters to be applied as biomarkers in field studies using *M. galloprovincialis* as a bioindicator. Fragments of the genes of Cu/Zn-SOD and CAT isolated from digestive glands of *M. galloprovincialis* showed respectively 93% and 90% of homology with known genes of *Mytilus* spp. and *Crassostrea gigas*. No differences in gene expression of Cu/Zn-SOD were found among mussels collected along the NW coast of Portugal, as well as between control mussels and mussels exposed to WAF in laboratorial conditions, which contradicts the biochemical results previously discussed. A possible explanation for these distinct results might be related with the fact that total SOD activity was assessed in biochemical assays, while gene expression was only investigated in one isoform. Besides Cu/Zn-SOD, other isoforms of this enzyme are known (e.g. Fe-SOD and Mn-SOD) ^[45]. The selection of Cu/Zn-SOD for this study was based on the works of Manduzio et al. (2003) ^[45], which reported that the main isoform of SOD detected in mussels' digestive glands and gills was Cu/Zn-SOD, while Mn-SOD only had a weak participation in the total SOD activity ^[45]. Regardless these findings we suggest that in future work the gene expression of other isoforms of SOD should be investigated. Moreover, in the present study we used semi-quantitative PCR to study changes in gene expression. However, to increase the accuracy of future results we also suggest the use of quantitative real-time PCR.

Finally, the gene expression of CAT was induced in mussels collected near an oil refinery (S4) and in Leixões harbour when compared with those collected from sites classified as having lower levels of petrochemical contamination (S1 and S3). Likewise, the gene expression of CAT was also induced in mussels exposed to 50% WAF in comparison with control mussels, indicating a good dose-response to petrochemical exposure. To our knowledge, works comprising the study of CAT gene in mussels of the genus *Mytilus* have focused mainly on the characterization of the gene and not so much on the effects of contaminants ^[46]. Only the works developed by Dondero and co-workers

in which the mussels' gene expression profile was evaluated in organisms exposed to a crude oil mixture, and in organisms caged along a copper pollution gradient, indicated the gene of CAT showed changes in expression following microarray analysis ^[75, 75]. These results indicate the potential of CAT gene expression to be used as a biomarker of contamination in field studies. However, further studies need to be conducted to assess the response of this gene to other types of contaminants, as well as to abiotic parameters.

3.5. CONCLUSIONS

In the present study results of field work indicated that the selected battery of biomarkers allowed the discrimination of the sampling sites according to levels of petrochemical contamination, indicating its suitability to be applied in monitoring programs. These results also indicated that the separation of the sites located near an oil refinery and commercial harbours (S4 and S5) from those with less contaminated (S1 and S3) was mainly due to the activity levels of SOD and CAT. Moreover, these antioxidant enzymes also showed a dose-dependent response following mussels' exposure to WAF. Activity levels of SOD and CAT measured in mussels' digestive glands exhibited an induction of 138% and 65% respectively for the 50% WAF dilution. In light of these results, the response of the genes of Cu/Zn-SOD and CAT on mussels' digestive glands was investigated. Results showed that the gene expression of CAT corresponded well with its enzymatic activity in mussels chronically exposed to petrochemical products, showing its role as a major defence against oxidative stress induced by this class of contaminants. However, further work needs to be developed to confirm its suitability as a biomarker for petrochemical contamination.

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CHAPTER 4

Ras gene in marine mussels: a molecular level response to petrochemical exposure

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ABSTRACT

Mussels are susceptible to numerous toxicants and are often employed as bioindicators. This study investigated the status of the *ras* proto-oncogene in *Mytilus galloprovincialis* following petrochemical exposure. A *M. galloprovincialis* homologue of the vertebrate *ras* gene was isolated, showing conserved sequence in regions of functional importance and a high incidence of polymorphic variation. Mutational damage was investigated in mussels chronically exposed to the water-accommodated fraction of #4 fuel-oil (WAF), and in mussels collected along the NW coast of Portugal in sites with different levels of petrochemical contamination. A *ras* gene point mutation was identified in the codon 35 of one individual exposed to 12.5% WAF. No mutations were detected in mussels from the WAF control or environmental samples. This represents the first report of a *ras* gene mutation, experimentally-induced by petrochemical exposure, in an invertebrate species.

Keywords: *ras* gene, *Mytilus galloprovincialis*, mutation, biomarker, genotoxicity, petroleum hydrocarbon

4.1. INTRODUCTION

Following the development of urban and industrial centres, polycyclic aromatic hydrocarbons (PAHs), which are important components of petrochemical products, have become an increasingly widespread class of environmental contaminants. Their presence in the environment is of great concern since they have been identified as genotoxic and carcinogenic ^[1]. Some PAHs can interact directly with DNA, whereas others require metabolic biotransformation prior to induction of genetic damage ^[2]. Such differences in activity and carcinogenicity are related to the chemical structure of each specific PAH, namely the number of rings, the fusion site, the extent of condensation or the site and degree of methylation ^[3].

It is well established that non-polar xenobiotics with low reactivity can undergo metabolic transformation via the detoxification system, resulting in highly reactive electrophilic forms that can, in turn, interact with nucleophilic DNA. The function of this detoxification system is to protect the cell against xenobiotics by metabolizing such non-polar chemicals to water-soluble forms that may be easily excreted by the organism ^[2]. The cytochrome P450 mixed function oxidase system metabolizes selected PAHs to phenols and dihydrodiols, which, in turn, can be conjugated with glutathione by glutathione S-transferases to more soluble compounds that can be easily excreted. However, during this process, electrophilic epoxides and quinones may be also formed. These may react with DNA, forming DNA adducts, or causing oxidative DNA damage through the production of reactive oxygen species (ROS) ^[1, 2, 4]. DNA adducts, increased quinone and ROS production have been detected in invertebrates ^[4, 5, 6, 7], fish ^[8, 9], and humans ^[10] exposed to high levels of hydrocarbons. Furthermore, if left un-repaired, DNA lesions may result in mutations, and if a mutation occurs in a proto-oncogene or tumour suppressor gene a pre-neoplastic lesion may occur ^[11].

Recent studies concerning the development of carcinogenesis in mammals and fish reported mutant forms of the *ras* gene in a large proportion and wide variety of tumours, being the most common mutations reported at codons 12, 13 and 61 ^[1, 12]. The *ras* gene encodes a GTP/GDP binding protein that is responsible for the transduction of mitogenic signals from the cellular membrane to the nucleus ^[12]. When this gene suffers a mutation in one of the codons mentioned above, the encoded protein presents inhibition of GTPase activity. This enzymatic inhibition occurs because codons 12 and 13 encode for amino acids that form the binding pocket for the GTP, and codon 61 encodes for amino acids involved in the hydrolysis of GTP to GDP ^[13]. Consequently, mutations occurring in these hot-spots will affect both activator site and activator function of the encoded protein,

inhibiting GTPase activity ^[12]. So, it is often the case that activated *ras* genes are involved in aberrant cell proliferation, altered cell checkpoint control and cell differentiation ^[1].

A number of *Mytilus* sp. cancer genes, including the *ras* gene, have recently been characterized ^[14, 15], however, the role of *ras* in the carcinogenesis process and its' mutational activation by PAHs has yet to be studied in invertebrate species. The aim of this study was to investigate the effect of petrochemical contamination exposure on the *ras* gene in the marine invertebrate *Mytilus galloprovincialis*.

4.2. MATERIAL & METHODS

4.2.1. Sample collection

In September 2004, a single adult *M. galloprovincialis* mussel was handpicked during low tide in the intertidal zone at sampling site S3 (Figure 4.1) and used for the isolation of the normal *ras* gene sequence of this species. During April 2005, mussels were sampled from two additional sites along the NW coast of Portugal (Figure 4.1). Sampling site S1 has lower levels of hydrocarbon contamination compared with sampling site S2 ^[16]. After sampling, mussels were transported to the laboratory in thermally insulated boxes and immediately sacrificed. A portion of gonad and digestive tissues were dissected from each mussel and stored in RNAlater (Sigma, Germany) at -20°C until further analysis.

4.2.2. Experimental exposure

Adult mussels were collected from S1, a relatively clean sampling site (Figure 4.1), and acclimatised to laboratory conditions for a period of 48 hours. Mussels were then exposed to different dilutions (0%, 6.25%, 12.5%, 25%, 50% and 100%) of water-accommodated fraction of #4 fuel-oil (WAF) over a period of 21 days. WAF of fuel oil #4 (Galp Energia, SGPS, SA, Portugal) was produced with vacuum-filtered (0.45 µm) and UV-treated seawater according to Singer et al. (2000) ^[17]. WAF was prepared in a 5 L Erlenmeyer flask by mixing 100 g of fuel oil per litre of seawater for 24 hours, in darkness at 20°C. The WAF mixture was allowed to rest for one hour prior to decanting. Three mussels were exposed in 1 L glass flasks to 0.8 L of each WAF dilution under controlled conditions (20 ± 1°C; 16:8 L:D cycle). Six replicates of each WAF dilution were preformed. Throughout the exposure period, the media was changed every other day, and mussels

were fed with commercial food for marine invertebrates (SERA, Germany) after each change of the media. During the exposure period, mussel mortalities were 38% and 72 % for 50% and 100% WAF, respectively. At the end of the exposure period, mussels were sacrificed. A portion of gonad and digestive tissues were dissected from each mussel and stored in RNAlater at -20°C until further analysis.

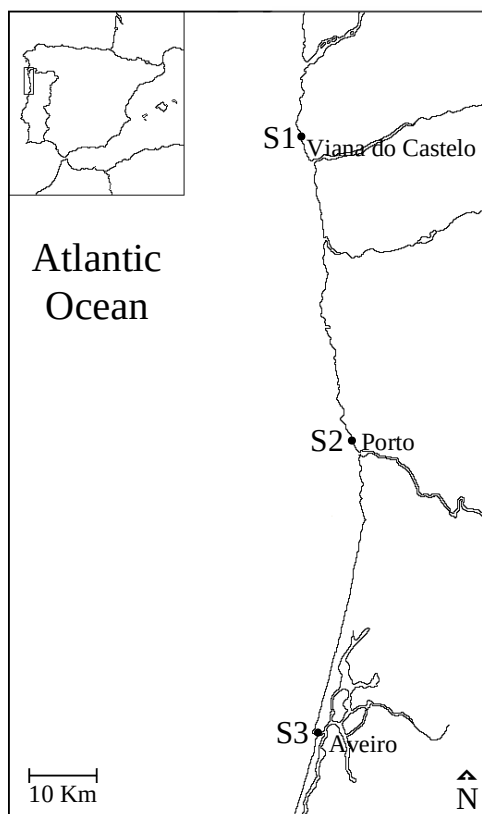


Figure 4.1 Map of the North-western coast of Portugal, showing the location of sampling sites. S1: Carreço (41°44'33"N; 08°52'43"W), S2: Leixões harbour (41°10'58"N; 08°41'56"W), S3: Barra (40°37'36"N; 08°44'47"W). Sampling site S1 has relatively low levels of hydrocarbon contamination compared with S2, which is considered highly contaminated by petrochemical products.

4.2.3. Isolation of total RNA and RT-PCR

RNA extractions were carried out with RNeasy reagents (Qiagen Ltd, U.K.). First strand cDNA was obtained using 1 µg of total RNA and oligo d(T) primers (Invitrogen Ltd.,

UK). The obtained cDNA was used as template to amplify exon 1 and part of exon 2 of the *ras* gene of *M. galloprovincialis*. A 50 µL PCR reaction was performed in reaction buffer (200 mM Tris-HCl pH 8.4, and 500 mM KCl), 400 µM of each deoxynucleoside triphosphate, 50 pmol of each primer (forward 5'ATGACGGAATACAAGCT3'; reverse 5'ATGAGAACGGGAGAAGGA3'), 4 µL of synthesized cDNA and 1 U Platinum Taq DNA polymerase (Invitrogen Ltd., U.K.). After a 2 min denaturation step at 94°C, the 231 bp *ras* fragment was amplified in a BioRad iCycler™ using 35 sequential cycles at 94°C for 30 s, 58°C for 30 s, 72°C for 30 s, followed by a final 2 min extension at 72°C. The sequence obtained from the cloned *ras* fragment subsequently served as a starting point for 3' RACE primer design.

4.2.4. RACE isolation of 3' end *ras* cDNA

The mRNA was purified from one control gonad total RNA (1 µg) using SMART™ RACE cDNA amplification reagents and protocol (BD Biosciences Clontech, U.K.). The 3' end of the *ras* gene of *M. galloprovincialis* was obtained using a gene specific primer: 5'GGAGCTGGTGGCGTAGGCAAAAGTGC3'. Amplification was performed in 50 µL reactions using a BioRad iCycler™ for 5 cycles at 94°C for 5 s and 72°C for 3 min, 5 cycles at 94°C for 5 s, 70°C for 10 s and 72°C for 3 min, followed by 25 cycles at 94°C for 5 s, 68°C for 10 s, and 72°C for 3 min. The RACE products obtained were analysed on an agarose gel, excised and purified using a Qiaquick spin column (Qiagen Inc., U.K.). Purified cDNA was ligated into a TA cloning vector (Invitrogen Ltd., U.K.). Recombinant plasmids were transformed and selected using kanamycin LB plates. Plasmid DNA was purified for DNA sequence analysis using commercial sequencing (MWG Biotech, Germany) to verify the identity of the product.

4.2.5. *Ras* gene mutation analysis

cDNA from WAF-exposed and environmental samples were used as template to amplify exons 1 and 2 of the *ras* proto-oncogene of *M. galloprovincialis*. For each reaction, 4 µL of template cDNA was used in 50 µL reaction mixture containing 200 µM of each deoxynucleoside triphosphate, 50 pmol of primers (forward – 5'ATGACGGAATACAAGCT 3'; reverse – 5'TACCAAGACCATTGGCTC3'), and 1 U Platinum Pfx DNA polymerase (Invitrogen Ltd., U.K.) in reaction buffer (200mM Tris-HCl (pH 8.4) and 500mM KCl). After a 2 min denaturation step at 94°C, 38 cycles of denatur ation at 94°C for 30 s, annealing at

58°C for 30 s, and extension at 72°C for 30 s were conducted using a BioRad iCycler™. A final extension step at 72°C for 2 min was performed after the last cycle. The 342 bp *ras* cDNA fragment amplified was directly sequenced (MWG Biotech, Germany) in both directions in order to identify and characterise any mutations present.

4.2.6. *Ras* gene expression analysis

Expression levels of *ras* gene were analysed by semi-quantitative RT-PCR. The concentration of isolated RNA was measured by UV-spectroscopy at 260 nm and 1 µg of total RNA from each sample was used for the reverse transcription reaction. In order to normalize differences in efficiency during amplification, 18S rRNA primers were used to amplify a 172 bp fragment as an internal standard (forward – 5'GTGCTCTTGACTGAGTG TCTCG3'; reverse – 5'CGAGGTCCTATTCCATTATTCC3'). The *ras* specific primers used were: forward – 5'ATGACGGAATACAAGCT3'; reverse – 5'TACCAAGACCATTGGCTC3', yielding a product of 342 bp. Amplifications were performed with a BioRad iCycler™ in 50 µL reaction volumes 200 µM of each deoxynucleoside triphosphate, 50 pmol of primers, and 1 U Platinum Pfx DNA polymerase in reaction buffer (200mM Tris-HCl (pH 8.4) and 500mM KCl) (Invitrogen Ltd., UK). A 2 min denaturation step at 94°C was followed by 38 sequential cycles at 94°C for 30 s, 58°C for 30 s and 72°C for 30 s, followed by a final 2 min extension at 72°C, were conducted using a BioRad iCycler™. A volume of 15 µL of each PCR product was taken for agarose gel electrophoresis (1.0% agarose, TBE buffer).

4.2.6. Chemical analysis of whole tissues

A single analysis of petroleum hydrocarbon was performed in pooled tissues of thirty mussels collected in two sampling sites (S1–S2) along the NW coast of Portugal (Figure 4.1). The analytical procedures for extraction and purification of petroleum hydrocarbons were carried out using the method of CARIPOL/IOCARIBE/UNESCO (1986) ^[18] according to UNEP (1992) ^[19]. Each set of samples was accompanied by a complete blank and a spiked blank which was carried through the entire analytical scheme in identical conditions for all samples. Samples were extracted by homogenisation with a mixture of hexane:methyl chloride (1:1), an internal standard was added before extraction. The aliphatic and aromatic fractions were purified and separated in three fractions by column chromatography with 10 g each of silica gel/alumina with hexane. The first fraction

was eluted with *n*-hexane; the second fraction was eluted with *n*-hexane: methyl chloride (1:1) and the third fraction was eluted only with methyl chloride. The extracts concentrated containing fraction 1 (aliphatic) and fractions 2 and 3 (aromatics) were rotoevaporated to 1 mL and analysed by gas chromatography. Hydrocarbons were quantified using gas chromatography. Nitrogen was used as carrier gas (flow 1 mL mm⁻¹). The limit of detection for individual aromatic compounds was 0.01 µg g⁻¹ and recovery yields were up to 90%. The aliphatic hydrocarbons (AH) and unresolved complex mixture (UCM) was quantified with an *n*-C28 standard. PAHs were identified by comparing their retention times with those from the aromatic analytical standards by Supelco 48743 according to the priority PAHs from method EPA 610.

4.3. RESULTS

4.3.1. Isolation of the normal *ras* gene of *Mytilus galloprovincialis*

Primers derived from previously published sequences for the exons 1 and 2 of the *M. edulis* *ras* gene ^[14] were used to amplify the *ras* gene from *M. galloprovincialis* gonad cDNA. RT-PCR produced one band of expected size (231 bp), which was isolated and sequenced. This fragment was used to design a gene-specific primer to generate a putative sequence of 516 bp with a 3'RACE reaction. The *M. galloprovincialis* *ras* cDNA isolated by 3'RACE reaction contained a complete open reading frame (GenBank Accession No. **DQ305041**), and the predicted amino acid sequence revealed a 195 amino acid protein. Multiple alignment of the *ras* deduced amino acid sequence of *M. galloprovincialis* was performed with ClustalW using *ras* proteins from different invertebrate and vertebrate homologues. The analysis revealed a strong homology between the *M. galloprovincialis* *ras* protein and the *Ki-ras* proteins (Figure 4.2). Furthermore, several *ras* gene sequence variations were identified in gonad cDNA, comprising polymorphic substitutions occurring predominantly at the third base position within each codon (Figure 4.3). This polymorphic variation, which did not result in a change of the encoded amino acid sequence, was found between codon 12 and codon 26. The remainder of the coding region was identical.

Amplification of exons 1 and 2 of *ras* gene cDNAs synthesised with RNA isolated from digestive gland of control *M. galloprovincialis* samples produced fragments of 342 bp, and were directly sent for sequencing. Several *ras* gene sequence variations, similar to those found in gonad cDNA, were identified (Figure 4.3).

		12	13			35	
		↓	↓			↓	
M.gallo	MTEYKLVVVGAGG	GVGKS	SALT	TIQLIQNH	FVEEYD	PR	IEDSYRRQVVIDGETCLLDILDTAG 60
M.edulis	MTEYKLVVVGAGG	GVGKS	SALT	TIQLIQNH	FVEEYD	PT	IEDSYRKQVVIDGETCLLDILDTAG 60
S.mansoni	MTEYKLVVVGAGG	GVGKS	SALT	TIQLIQNH	FVEEYD	PT	IEDSYRKQMVIDGEICLLDILDTAG 60
O.mykiss-K	MTEYKLVVVGAGG	GVGKS	SALT	TIQLIQNH	FVDEYD	PT	IEDSYRKQVVIDGETCLLDILDTAG 60
H.sapiens-K	MTEYKLVVVGAGG	GVGKS	SALT	TIQLIQNH	FVDEYD	PT	IEDSYRKQVVIDGETCLLDILDTAG 60
H.sapiens-H	MTEYKLVVVGAGG	GVGKS	SALT	TIQLIQNH	FVDEYD	PT	IEDSYRKQVVIDGETCLLDILDTAG 60
H.sapiens-N	MTEYKLVVVGAGG	GVGKS	SALT	TIQLIQNH	FVDEYD	PT	IEDSYRKQVVIDGETCLLDILDTAG 60

	61						
	↓						
M.gallo	QEEYSAMRDQYMRT	GEGFL	CVFAV	NN	TKSFED	INQYRE	QIKRVKDADEVPMVLVGNKVDL 120
M.edulis	QEEYSAMRDQYMRT	GEGFL	CVFAV	NN	TKSFED	INQYRE	QIKRVKDADEVPMVLVGNKVDL 120
S.mansoni	QEEYSAMRDQYMRT	GEGFL	CVFAV	NN	SKSYDD	INQYRE	QIKRVKDADEVPMVLVGNKVDL 120
O.mykiss-K	QEEYSAMRDQYMRT	GEGFL	CVFAI	NN	TKSFED	IHHYRE	QIKRVKDESDVPMVLVGNKCDL 120
H.sapiens-K	QEEYSAMRDQYMRT	GEGFL	CVFAI	NN	TKSFED	IHHYRE	QIKRVKDESDVPMVLVGNKCDL 120
H.sapiens-H	QEEYSAMRDQYMRT	GEGFL	CVFAI	NN	TKSFED	IHQYRE	QIKRVKSDDVPMVLVGNKCDL 120
H.sapiens-N	QEEYSAMRDQYMRT	GEGFL	CVFAI	NN	SKSFAD	INLYRE	QIKRVKSDDVPMVLVGNKCDL 120
	***** ** ** ** ***** ***** **						
M.gallo	PTRTVDAKQARP	VADSYN	IPYVET	SAKTRQ	GVDDA	FYTLV	REIRKYKERKGPKKKKKKKK 180
M.edulis	PTRTVGAKQARP	VADSYN	IPYVET	SAKTRQ	GVDDA	FYTLV	REIRKYKERKGPKKGKKKPR 180
S.mansoni	TNRSVCTEEAKS	LAHSYN	IPYVET	SAKTRQ	GVDDA	FHKL	VREIRKSKEKKGKDRKKRKRK 180
O.mykiss-K	PSRTVDTKQAQ	DLARTY	GIPFIET	SAKTRQ	GVDDA	FYTLV	REIRKHK-EKMSK----- 172
H.sapiens-K	PSRTVDTKQAQ	DLARSY	GIPFIET	SAKTRQ	GVDDA	FYTLV	REIRKHK-EKMSKDGGKKKKK 179
H.sapiens-H	AARTVESRQAQ	DLARSY	GIPYIET	SAKTRQ	GVDDA	FYTLV	REIRQHKLRLNPPDESGPG 180
H.sapiens-N	PTRTVDTKQA	HELAKSY	GIPFIET	SAKTRQ	GVDDA	FYTLV	REIRQYRMKKLNSSDDGTQG 180
	* * * * * ***** ** ***** *						
M.gallo	KYSALYHCLPY	SESY					195
M.edulis	-----CLLI	----					184
S.mansoni	-----CCIQ	----					184
O.mykiss-K	-----						
H.sapiens-K	KSK--TKCVIM	----					188
H.sapiens-H	CMS--CKCVLS	----					189
H.sapiens-N	CMG--LPCVVM	----					189

Figure 4.2 Comparison of the deduced *ras* protein sequence of *Mytilus galloprovincialis* (GenBank Accession No. [DQ305041](#)) with selected *ras* protein sequences of invertebrates and vertebrates: *Mytilus edulis* (AAT81171); *Schistosoma mansoni* (AAB09439); *Oncorhynchus mykiss c-Ki-ras-1* (A54321); *Homo sapiens Ki-ras-2* (AAB59444), *N-ras* (AAM12633), *H-ras-1* (AAB02605). Asterisks indicate areas showing homology. Arrows indicate mutational hot spots (codons 12, 13, and 61); arrows and dark highlighting indicate site of mutation at codon 35 in the *ras* gene of *M. galloprovincialis* exposed to 12.5% of water-accommodated fraction of #4 fuel-oil. Light highlighting indicates polymorphic variation.

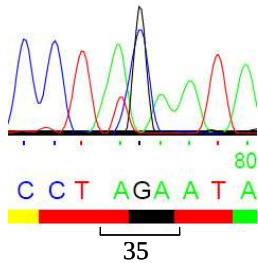
GGT¹², GGC^{(GGT)¹³}, GTA^{(GTT)¹⁴}, GGC^{(GGT)¹⁵}, AAA¹⁶, AGT¹⁷, GCA^{(GCC)¹⁸},
TTA^{(TTG, CTA, CTG)¹⁹}, ACC²⁰, ATC^{(ATA)²¹}, CAA²², CTT²³, ATA^{(ATT)²⁴}, CAG^{(CAA)²⁵}, AAT²⁶

Figure 4.3 Nucleotide sequence of normal *Mytilus galloprovincialis* *ras* gene from nucleotides 12 to 26, with parenthesis showing polymorphic variations.

4.3.2. *Ras* gene mutation analysis

Mutations in the *ras* gene of *M. galloprovincialis* were screened by direct sequencing of cDNA isolated from digestive glands of mussels environmentally contaminated by PAHs and mussels exposed to different dilutions of WAF. No mutations were detected in mussels from the control of the WAF- exposure or in mussels collected in the field. A single mutation was detected at codon 35 in a *M. galloprovincialis* digestive gland sample of an individual exposed to 12.5% of WAF (Table 4.1).

Table 4.1 Summary of mutational alterations observed in the *ras* gene of *Mytilus galloprovincialis*.

Sample	Position	Mutation	Putative consequence	Electrophoreogram
MYTD3DG5	codon 35	C → G	Thr → Arg	
		A → T	Thr → Ser	
		AC → TG	Thr → STOP	

C – cytosine, G – guanine, A – adenine, T – thymine, Thr – threonine, Arg – arginine, Ser – serine.

4.3.3. *Ras* gene expression analysis

The expression of *ras* gene of *M. galloprovincialis* decreased in PAH-contaminated samples and 100% WAF-exposed samples compared with reference samples (Figure 4.4). *Ras* gene expression was higher in digestive gland samples compared with gonad samples from the same individuals (Figure 4.4).

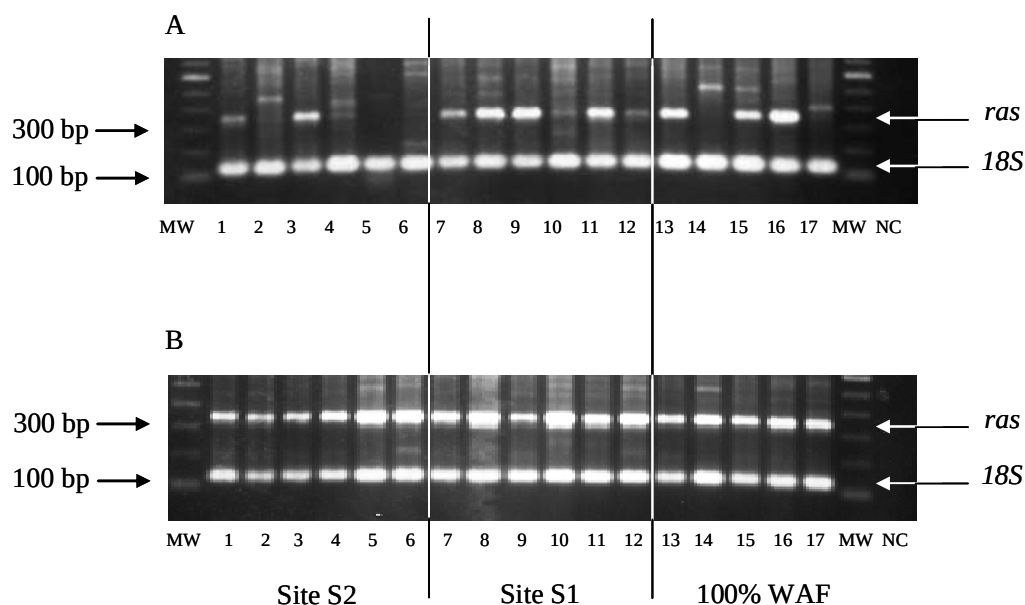


Figure 4.4 Agarose gel stained with ethidium bromide displaying semi-quantitative PCR amplification products of *ras* gene (342 bp) and *18S rRNA* gene (172 bp) from *Mytilus galloprovincialis*. MW: 100 bp molecular weight ladder; NC: negative control; 1-6: mussels from the contaminated site S2; 7-12: mussels from reference site S1; 13-17: mussels exposed to 100% WAF. A: Gonad; B: Digestive gland.

4.3.4. Chemical analysis of whole tissues

High tissue levels of hydrocarbons; 210 $\mu\text{g g}^{-1}$ dry weight (dw) of total PAHs, 2657 $\mu\text{g g}^{-1}$ dw of UCM, and 247 $\mu\text{g g}^{-1}$ dw of AH were observed in *M. galloprovincialis* sampled from the Portuguese coast at site S2.

4.4. DISCUSSION

Herein, we report the cDNA sequence of the *ras* gene in *M. galloprovincialis*. The predicted amino acid sequence of *M. galloprovincialis ras* gene displays conserved structural domains suggesting that the functional role may also be conserved (Figure 4.2).

Direct sequencing of exons 1 and 2 of *ras* gene (342 bp) of different gonad and digestive gland samples, isolated from control *M. galloprovincialis*, revealed several nucleotide variations within this fragment (Figure 4.3), none of which led to an alteration in

the predicted amino acid sequence. One of the codons presenting polymorphic variation was codon 13, a *ras* gene mutational hotspot. *Ras* polymorphism has previously been reported in a small number of fish species, namely *Oryzias latipes* ^[20], *Oncorhynchus gorbuscha* ^[21], and *Anguilla anguilla* ^[22]. However, the level of polymorphic variation observed in the *M. galloprovincialis* *ras* gene is significantly higher and may indicate the presence of a second *ras* gene. It is important to note that a similar pattern of polymorphic variation at codons 13-15, 18-19, 21, 24-25 was found in two samples of *M. trossulus* presenting haemic neoplasia ^[15]. Despite the significant degree of genome instability indicated by the polymorphic variation found in *M. galloprovincialis*, no neoplastic lesion was found by histological analyses of the same samples, as has been reported in *M. trossulus* with haemic neoplasia (unpublished data).

M. galloprovincialis, like many others species of aquatic organisms that inhabit estuaries and coastal zones, are exposed to urban and industrial pollutants such as PAHs. Mussels are often selected as a sentinel organism to monitor aquatic pollution for several reasons: they are sessile, filter feeding, distributed worldwide and also cultivated for human food consumption ^[23]. Mussels, and related bivalve species, also appear to be susceptible to neoplastic damage and as such could provide both: an opportunity for assessing the levels of genotoxicity, and a means to determine the aetiology of observed genetic damage in the aquatic environment. The range of neoplasms observed include: haemocytic in *Mytilus* sp. ^[24, 25] and *Mya arenaria* ^[26]; gonadal in several marine bivalve species ^[27, 28, 29]; digestive in *Macoma balthica* experimentally exposed to contaminated sediments ^[30]; gill in *M. balthica* ^[31]; as well as kidney and heart in *Crassostrea virginica* following laboratory and field controlled exposure to contaminated sediments ^[32, 33].

In cases of environmentally-induced neoplasia, the identification of the causative agents and their role in the molecular aetiology has yet to be achieved. Here, we have attempted to develop *ras* gene status as an early warning biomarker of petrochemical contamination. The current methods for assessing genetic damage in aquatic invertebrates are restricted to micronucleus frequency ^[34], Fast Micromethod[®] ^[35], Comet assay ^[36], and flow cytometry ^[37]. Such methods detect gross DNA alteration providing a correlation of DNA damage with contaminant exposure but do not provide actual cause-and-effect or mechanistic detail.

Ras gene mutations in fish have been related with PAH exposure ^[12] and are thought to be an early event in the carcinogenesis process ^[38]. The *ras* gene in invertebrates has previously been isolated in *M. edulis* and *M. trossulus* ^[14, 15], as well as in a number of other invertebrates, however no information regarding its' mutational activation is currently available.

Herein, the presence of a *ras* gene mutation in an invertebrate species has been reported for the first time at codon 35, in an individual exposed to 12.5% of WAF. Residue 35 corresponds to the effector region of the *ras* protein and may potentially affect protein function ^[39].

The dilution and duration of the WAF-exposure has previously been found to induce enzymatic changes in vertebrates ^[40] and invertebrates ^[16], namely enhancement of antioxidant defences. This work involved a relatively short term single exposure, failing to reflect the multi-step development of cancer which is normally a long and gradual process. Moreover, no neoplastic lesions were found by histological analysis (unpublished data). Tumours have previously been observed in *M. edulis* after a short 36 day continual exposure to PAHs loaded-dredge spoils ^[33]. Studies using 3 week-old medaka fish (*O. latipes*) also indicate that a single exposure over a 6 month grow out period is sufficient to induce *ras* gene mutations ^[20]. The organisms used in this study were, however, adults and arguably at a less susceptible life stage for acquiring genetic damage and neoplastic lesions. Constant or sporadic WAF-exposure over an extended period of 6 months or longer, is a more likely strategy to induce neoplastic lesions in future work using *M. galloprovincialis* in order to study the correlation between the development of mutations with phenotypic changes.

Sampling site S2 is located inside Leixões harbour, at the mouth of the Leça River, which comprises the largest seaport infrastructure in the North of Portugal. Due to intense vessel traffic and to oil terminal activity, the harbour is constantly subjected to petroleum hydrocarbon contamination ^[41, 42, 43]. Studies have previously reported that similar levels of PAHs can induce genotoxic damage as measured by micronuclei frequency ^[7] yet no *ras* gene mutations were found in these environmentally exposed mussels' samples.

A separate mechanism of *ras*-implicated carcinogenesis involves overexpression of the gene. RT-PCR semi-quantitative analysis revealed differences in the *ras* gene expression levels between gonad and digestive gland of *M. galloprovincialis*, as well as differences related to exposure history (Figure 4.4). *Ras* gene expression was induced in digestive gland samples compared with gonad samples from the same individuals. Differences in tissue levels of gene expression may relate to either natural turnover rates (gonads may be at a resting stage) or to relative exposure (digestive gland would be in direct contact with the contaminants in contrast to the gonad tissue). Tissue differences in *ras* gene expression levels would therefore need to be taken into account in developing a biomarker of hydrocarbon exposure based on this gene's expression levels. In terms of exposure history, the expression of *ras* gene was slightly downregulated in PAH-

contaminated samples and in 100% WAF-exposed samples compared with reference samples. However, upregulation of *ras* gene expression is involved in PAHs-induced neoplastic development ^[44]. Consequently, the relationship between *ras* gene expression levels, PAHs exposure history and the neoplastic development process may not be as simple as thought for vertebrate species.

4.5. CONCLUSIONS

In summary, a single *M. galloprovincialis* *ras* gene mutation, though no induction in *ras* gene expression levels, was observed in an individual exposed to WAF under laboratory conditions. No mutations were detected in mussels sampled at a site of high PAH contamination. This is the first report of a *ras* gene mutation in any invertebrate species. A high incidence of (exon 1) *ras* gene polymorphic variation in *M. galloprovincialis* was also observed and may indicate the presence of a second *ras* gene in these species.

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PART IV

GENERAL CONCLUSIONS

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FINAL REMARKS

As proposed, the present dissertation contributed for the assessment of the ecotoxicological effects of petrochemical products on natural populations of *Mytilus galloprovincialis* inhabiting rocky shores along the NW coast of Portugal.

The study conducted in Chapter 1 intended to evaluate the suitability of a monitoring program designed to assess the effects of petrochemical contamination, by using a battery of biomarkers to detect the presence of petrochemical products along the NW coast of Portugal. Results showed significant correlations between some of the biomarkers and petroleum hydrocarbons, which allowed the discrimination of the sampling sites according to the levels of petrochemical contamination. However, significant correlations were also found between some of the biomarkers and abiotic parameters quantified in water samples. Further studies need to be undertaken to address the effects of such parameters on the selected biomarkers, in particular ammonia, nitrites, nitrates and phosphates. This approach constitutes a research strategy that has been recommended by Sheehan and Power (1999), as well as Bodin and co-workers (2004), since it is important to separate the effects which are due to chemical contamination from those which are due to natural fluctuations of both abiotic parameters and the mussels' annual physiological cycle ^[1, 2]. Since the selected monitoring approach appeared to be able to discriminate different levels of contamination along the NW coast of Portugal, a long-term monitoring program was planned to investigate the spatial and temporal trends of petrochemical contamination in the NW coast of Portugal during twelve months.

In Chapter 2, it was recognised that the multivariate and graphical analyses implemented was a valuable tool for the interpretation of complex sets of chemical and biomarker data obtained during long-term monitoring programs, as previously reported by Astley et al. (1999) for data obtained in the Tees Estuary ^[3]. These analyses illustrated that some of the selected biomarkers were able to discriminate the selected sampling sites according to the levels of contamination. The activity of octopine dehydrogenase (ODH), which is involved in the mussels' anaerobic metabolism; acetylcholinesterase, which is involved in the mussels' neurotransmission processes; glutathione S-transferases, which are involved in the mussels' detoxification processes; as well as some

oxidative stress parameters such as superoxide dismutase (SOD), catalase (CAT), lipid peroxidation, and levels of reduced and oxidised glutathione, were shown to have the greatest influence upon sampling site discrimination. Moreover, these multivariate and graphical analyses also illustrated that biomarkers quantified in mussels sampled from sites which were potentially less impacted exhibited significant differences in their response throughout the year, while those quantified in mussels from sites which were potentially more impacted did not demonstrate seasonal fluctuations. This suggests that the effects of high levels of contamination may overlap those of abiotic factors. In particular, Anderson and Lee (2006) ^[4] stated that for a biomarker to be used to monitor petrochemical contamination, its response needs to be exclusively linked to petroleum exposure and not be strongly influenced by internal and external confounding factors. Herein, we observed that the activity of ODH presented a significant positive correlation with the levels of unresolved complex mixture and apparently was not influenced by seasonality indicating its suitability as biomarker. However, to better understand the application of this biochemical parameter as a biomarker for petrochemical contamination, further studies need to be performed to investigate its responsiveness to other contaminants, such as PCBs or metals.

In Chapter 3, to better understand the toxicity mechanisms induced by petrochemical contaminants in marine mussels, in particular with respect to their antioxidant defence system, the responsiveness of a battery of biomarkers was evaluated by comparing their discriminative potential in the field with their response following chronic exposure of mussels to fuel-oil under laboratory conditions. Regarding the biochemical results obtained during this study, which showed that the enzymes SOD and CAT were the most responsive biomarkers, the gene expression of these antioxidant enzymes of *M. galloprovincialis* was also evaluated. Results showed that CAT gene expression corresponded well with its enzymatic activity in mussels chronically exposed to petrochemical products, showing its role as a major defence against oxidative stress induced by this class of contaminants. No significant changes were verified in the gene expression of mussels' Cu/Zn-SOD. As such, the investigation of the effects of chronic exposure of petrochemical products on the gene expression of other isoforms of this enzyme (e.g. Mn-SOD) should be done.

In Chapter 4, a novel biomarker that could have a specific response to petrochemical products in mussels was investigated. Results showed that a single *M. galloprovincialis* *ras* gene mutation, though no induction in *ras* gene expression levels, was observed in a mussel exposed to WAF under laboratory conditions. No mutations were detected in mussels sampled at a site with high PAH contamination. This is the first

report of a *ras* gene mutation in any invertebrate species. Moreover, a high incidence of (exon 1) *ras* gene polymorphic variation in *M. galloprovincialis* was also observed and may indicate the presence of a second *ras* gene in these species. Further work needs to be conducted to assess the applicability of mutational damage of mussels' *ras* gene as a specific biomarker for petrochemical contamination, as well as to find a relationship between mutations and possible phenotypic changes, such as the development of neoplasia.

In conclusion, the monitoring strategy implemented to assess the spatial and temporal trends of petrochemical contamination along the NW coast of Portugal was appropriate since it was possible to discriminate the levels of petroleum hydrocarbon contamination present in each sampling site according to biomarker responses quantified in *M. galloprovincialis*. This strategy is therefore recommended for future work. Moreover, regarding the development of new tools to assess the effects of petrochemical contamination at the transcriptional levels in *M. galloprovincialis*, results showed that an increase in the gene expression of CAT, as well as the development of mutational damage in the *ras* gene of mussels chronically exposed to petrochemical products seem to have potential to be used as biomarkers.

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