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Multistressor effects on marine organisms

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Abstract

Marine ecosystems such as estuaries and coastal zones are vulnerable to chemical contamination arriving from several sources and several other stressors, including variations of temperature, salinity, pH and other abiotic factors. In the last decades, several of these pressures have been increasing at the global scale mainly due to the increase of human population and industrialization, and global climate changes. Thus, there is a high concern regarding their effects on marine ecosystems, estuaries and coastal ecosystems in particular, because of their ecological importance and the valuable services that they provide to the human society. Therefore, more studies are needed to assess the effects of environmental stressors on estuarine and other coastal ecosystems to improve their quality and the management of their resources.

In highly dynamic systems such as estuaries and other coastal areas, biomonitoring studies carried out in natural populations inhabiting the ecosystem under investigation and based on biomarkers are of special interest because they can indicate the biological effects resulting from the simultaneous exposure to several pressures, often called multi-stressors. In the NW Portuguese coast, several biomonitoring programs have been carried out. In the continuation of these programs, the main goal of the present study was to compare the health status of two wild populations of the shrimp *Crangon crangon* (populations from the estuaries of Minho and Douro Rivers), and of mussels (*Mytilus galloprovincialis*) and gastropods (*Monodonta lineata*) collected in São Félix da Marinha and Cabo do Mundo coastal zones, sampling sites in the NW Portuguese coast in late winter/spring2017, using selected biomarkers. These species were selected because: *C. crangon* is sensitive to many multi-stressors (several types of chemical contaminants, abiotic factors variation) andalso is predominant in estuaries. Moreover, *M. galloprovincialis* and *M. lineata* are key species in the inter-tidal zone of the Atlantic rocky shore and they occupy different trophic levels: *M. galloprovincialis* is filter feeder with wide geographical distribution and bioaccumulates a range of chemical contaminants present in seawater, and *M. lineata* species is grazer that feeds mainly on epiphytic algae. The biomarkers were enzymes involved in functions most important for the survival and performance of the animals, and other parameters indicative of biological damage, namely: Cholinesterases (ChEs), Lactate dehydrogenase (LDH), Isocitrate dehydrogenase (IDH), Octopine dehydrogenase (ODH), Glutathione – *S* – Transferase (GST), Catalase (CAT), Lipid peroxidation (LPO) and Carbonyl group oxidation (PO).

Shrimps were collected in the Minho estuary and in the Douro estuary. Mussels and snails were collected in São Félix da Marinha and Cabo do Mundo coastal zones. Minho estuary

and São Félix da Marinha were selected as reference sites whereas Douro estuary and Cabo do Mundo as polluted.

Shrimps collected in Douro estuary presented statistically higher AChE, LPO and lower PO levels than organisms from Minho estuary. The higher levels of AChE and LPO in Douro estuary can be indicatives of higher levels of environmental multistressors such as chemical contaminants and abiotic factors (for example, salinity, temperature, conductivity), among other, than Minho estuary. Chemicals contaminants, such as heavy metals, can induceChEs activity and ROS (Reactive oxygen species) responsible for lipidic damages (LPO).

Regarding coastal zones, mussels collected in Cabo do Mundo presented higher ChEs, CAT activity and lower GST levels comparatively to São Félix do Mundo and snails from Cabo do Mundo presented higher GST and LPO levels and lower CAT activity. Results found in present study relatively to biomarkers determined in both species (*M. galloprovincialis* and *M. lineata*) suggest the need for more biomonitoring studies with these species, in different seasons, complemented with chemical analysis of animal tissues, sediment and water are needed in order to have a more detailed knowledge of the basal levels of the biomarkers and its seasonal variation in each species.

This study increased the knowledge related with the health status of marine ecosystems of NW Portugal and can also help the environmental authorities in making decisions for the protection of these ecosystems.

Resumo

Ecossistemas marinhos tais como estuários e zonas costeiras são vulneráveis a contaminações químicas de diferentes fontes e outros stressores incluindo variações de temperatura, salinidade, pH e outros fatores abióticos. Nas últimas décadas, muitas dessas pressões têm aumentado à escala global, principalmente devido ao aumento da população humana e industrialização, e mudanças climáticas globais. Assim, existe um elevado interesse relativamente aos seus efeitos em ecossistemas marinhos, estuários e zonas costeiras em particular devido à sua importância ecológica e aos valiosos serviços que eles providenciam à sociedade humana. Portanto, mais estudos são necessários para avaliar os efeitos de stressores ambientais em estuários e outros ecossistemas costeiros para melhorar a sua qualidade e a gestão dos seus recursos.

Em sistemas altamente dinâmicos tais como estuários e outras áreas costeiras, estudos de biomonitorização realizados em populações naturais que habitam no sistema em investigação, e baseados em biomarcadores, são de interesse especial porque podem indicar os efeitos biológicos resultantes de exposições simultâneas a muitas pressões, frequentemente chamados multi-stressores. Na costa NO de Portugal, são levados a cabo vários programas de biomonitorização. Na continuação desses programas, o principal objetivo do presente estudo foi comparar o estado de saúde de duas populações selvagens do camarão *Crangon crangon* (populações dos estuários dos rios Minho e Douro), mexilhão (*Mytilus galloprovincialis)* e o gastrópode (*Monodonta lineata*) recolhidos nas zonas costeiras (NO Portugal) de São Félix da Marinha e Cabo do Mundo durante o final do inverno/primavera de 2017. Estas espécies foram selecionadas porque: *C. crangon* é sensível a vários multi-stressores (diferentes tipos de contaminantes químicos, variação de fatores abióticos) e também é predominante em estuários, *M. galloprovinciallis* e *M. lineata* são espécies chave na zona intertidal da costa rochosa atlântica e ocupam diferentes níveis tróficos. *M. galloprovincialis* é filtrador com uma vasta distribuição geográfica e bioacumula uma gama de contaminantes químicos presentes na água, e *M. lineata* alimenta-se principalmente de algas epífitas que raspa das rochas. Os biomarcadores aplicados foram enzimas envolvidas em funções importantes para a sobrevivência e desempenho dos animais nomeadamente: Cholinesterases (ChEs), Lactato desidrogenase (LDH), Isocitrato desidrogenase (IDH), Octopina desidrogenase (ODH), Glutationa S-transferase (GST), Catalase (CAT) e outros parâmetros indicativos de danos biológicos: peroxidação lipídica (LPO) eoxidação de grupos carbonilos das proteínas (PO).

Os camarões foram recolhidos nos estuários dos rios Minho e Douro. Os mexilhões e caracóis foram recolhidos nas zonas costeiras de São Félix da Marinha e Cabo do Mundo.

O estuário do Minho e São Félix da Marinha foram selecionados como locais de referência enquanto que o estuário do Douro e Cabo do Mundo como locais poluídos.

Os camarões recolhidos no estuário de Douro apresentaram níveis significativamente mais elevados de AChE e LPO e mais baixos de PO do que os do estuário de Minho. Níveis elevados de AChE e LPO podem indicar níveis altos de multi-stressores tais como contaminantes químicos e relacionados com fatores abióticos (como por exemplo, temperatura, salinidade, condutividade). Contaminantes químicos tais como metais pesados podem induzir a atividade de ChEs e a presença de ROS (espécies reativas de oxigénio), responsáveis por danos oxidativos nos lípidos (LPO).

Relativamente ás zonas costeiras, os mexilhões recolhidos em Cabo do Mundo apresentaram níveis mais elevados de ChEs e CAT e mais baixos de GST comparativamente a São Félix da Marinha, e os caracóis de Cabo do Mundo apresentaram níveis mais elevados de GST e LPO e mais baixos de CAT do que os de São Félix da Marinha. Estes resultados para ambas espécies sugerem que são necessários mais estudos de biomonitorização com estas espécies, realizados sazonalmente, e complementados com analises químicas dos tecidos animais, sedimentos e águas, de modo a obter-se um conhecimento mais detalhado sobre os biomarcadores ambientais e a sua variação sazonal em cada espécie.

Este estudo pretendeu aumentar o conhecimento relacionado com o estado de saúde dos ecossistemas de NO de Portugal e também ajudar as autoridades ambientais na tomada de decisão para a proteção desses ecossistemas.

Lisf of abbreviations

AChE – Acetylcholinesterase
ALAD – Aminolevulinic acid dehydratase
BHT – Butylated hydroxytoluene
CAT – Catalase
CDNB – 1-chloro-2,4 dinitrobenzene
ChE – Cholinesterase
CM – Cabo do Mundo
CNADS –National Council of Environment and Sustainable Development
DNA – Deoxyribonucleic Acid
DNPH – 2,4-dinitrophenylhydrazin
DTNB – 5,5 – Dithiobis-(2-nitrobenzoic acid)
EROD – Ethoxyresorufin-O-deethylase enzyme
GISD – Global Invasive Species Database
GPx – Glutathione peroxidase
GR – Glutathione reductase
GSH – reduced glutathione
GST – Gluthatione*S*-transferases
IDH – Isocitrate dehydrogenase
LDH – Lactate dehydrogenase
LPO – Lipid peroxidation
NADH - β-nicotinamide adenine dinucleotide reduced form
NW – North West
ODH – Octopine dehydrogenase
PAH – Polycyclicaromatichydrocarbons
PCB – Polychlorinated biphenyls
pH – potential hydrogen
PO- Protein carbonyl oxidation
ROS – Reactive Oxygen Species
SOD – Superoxide dismutase
WAF- Water-Soluble Fraction

Index

List of figures

List of tables

1. Introduction

Marine ecosystems such as estuaries and coastal zones are vulnerable to chemical contamination arriving from several sources and several other stressors (Singh *et al*., 2017), including variations of temperature, salinity, pH and other abiotic factors (Elliott *et al*., 2016; Possingham *et al*., 2017). In the last decades, several of these pressures have been increasing at the global scale mainly due to the increase of human population and industrialization, and global climate changes (Possingham *et al*., 2017). Thus, there is a high concern regarding their effects on marine ecosystems, estuaries and coastal ecosystems in particular, because of their ecological importance and the valuable services that they provide to the human society (Moore *et al*., 2013). Therefore, more studies are needed to assess the effects of environmental stressors on estuarine and other coastal ecosystems to improve their quality and the management of their resources.

One of the methods of environmental monitoring is biomonitoring. In this type of monitoring, one or more selected species is/are used as bioindicators, and biological parameters known as environmental biomarkers can be used to assess the exposure of organisms to environmental stressors, their biological effects, and/or the susceptibility to chemical exposure (Connon *et al*., 2012; Chalghmi *et al*., 2016).Biomonitoring based on biomarkers in wild populations provides information regarding the combined effects resulting from the long-term exposure to the environmental stressors potentially affecting the ecosystem, such as chemical environmental contaminants and abiotic factors (e.g. temperature, pH, salinity) variation (Chalghmi *et al*., 2016; Saleh and Marie, 2016). According to the actual regulations such as the Marine Strategy Framework Directive (MSFD, 2008), integrated monitoring programmes, including biomarkers, and physic and chemical monitoring, should be conducted to assess the quality status of marine waters.

Among marine ecosystems, those of coastal areas, including estuaries, deserve special attention for several reasons. For example, in general, they provide a high diversity of habitats supporting a considerable biodiversity, including populations of species able to survive in these habitats only, and marine species that depend on them for particular phases of their life cycles, such as reproduction, juvenile development, among others (Cairrão *et al*., 2004; Quintaneiro *et al*., 2006; Amorim *et al*., 2016; Whitfield, 2016; EMBOS, 2017) they provide most important services to the human society, including provisioning services (*e.g*. fish and other species for human consumption; water for several purposes; sand for several industries; algae and other species for several industries), regulating services (*e.g.* climate

regulation; detoxification of several pollutants; water quality regulation), cultural services (*e.g.* symbolic, religious, educational), and supporting services (*e.g.* carbon sequestration, energy flux and nutrients cycling) (Kaiser *et al*., 2005; Pollack *et al*., 2013), among several other reasons. Coastal ecosystems are among the most threatened on earth, particularly estuaries and other coastal ecosystems of anthropogenic directly impacted regions (Cairrão *et al*., 2004; Moreira *et al*., 2004; Nicholls and Cazenave, 2010; Bellante *et al*., 2016). Main threats are alterations due to global climate changes, landscape occupation and alterations due to several factors including those resulting from human activities, chemical contamination, bioinvasions, among several others (Moreira *et al*., 2004; Nicholls and Cazenave, 2010; Hong *et al*., 2015).

The Northwest (NW) Portuguese coast and its estuaries are of high ecological and economic value. In addition to the previously mentioned threats, this region is also at risk of suffering the impacts of chemicals release resulting from marine shipping incidents due to its proximity to important maritime traffic routes (CNADS, 2001) and the challenging navigation conditions (Cairrão *et al*., 2004). It has also an important human population density, several industries and economic important activities (e.g. harbours, refinery, fishery ports, tourism, fishery), and several other sources of environmental impact, including the discharges of important hydrographic basins arriving through estuaries (CNADS, 2001; Lima *et al*., 2007). Due to its economic and ecological importance and the existing threats, the NW Portuguese coast and its main estuaries have been investigated from decades regarding several aspects, including in relation to its contamination by chemical contaminants, the effects of pollutants and abiotic factors variation on the health status of wild populations, impacts of biological invasions, among others (Menezes *et al*., 2006; Quintaneiro *et al*., 2006; Cairrão *et al*., 2007; Lima *et al*., 2007; Lima *et al*., 2008; Cairrão *et al*., 2009; Guimarães *et al*., 2009; Tim-Tim *et al*., 2009; Mil-Homens *et al*., 2013; Amorim *et al*., 2016; Gomes *et al*., 2016; Teixeira *et al*., 2016). Among the several wild populations that have been monitoring in this region, the mussel *Mytilus galloprovincialis*, the gastropod *Monodonta lineata* and the shrimp *Crangon crangon* are of special environmental interest mainly because they are key species in the habitats where they occur, they are suitable bioindicators for biomonitoring studies, and the background levels of several biomarkers and their variation along the year in populations of this region are known from previous studies (Quintaneiro *et al*., 2006; Sousa *et al*., 2009; Tim-Tim et al., 2009). Furthermore, these species are of human consumption including in the region, and their exploration for this purpose may be increased thus contributing to food security. *C. crangon* is found in the shallow waters of European seas (Menezes *et al*.,2006) and inhabits in soft bottom estuaries and in coastal lagoons with temperature between 0 to 30°C and salinity between 0 to 35‰(Campos and van der Veer, 2008). It feeds with infaunal

(Oh *et al.*, 2000), epifaunal and demersal organisms (shrimp and fish) during dusk and dawn (Del Norte-Campos and Temming 1994). It has been used as bioindicators for estuaries field studies (Menezes *et al*., 2006; Quintaneiro *et al*., 2006). *M. galloprovincialis* is native of Mediterranean coast and inhabits on temperate rocky shores. It is a filter feeder and feeds by pumping water through enlarged sieve-like gills (Bellante*et al*., 2016). Due to its capacity to accumulate in their tissues a wide range of chemical contaminants has been used in many environmental biomonitoring studies (Moreira *et al*., 2004; Lima *et al*., 2007; Lima *et al*., 2008; Tim-Tim *et al*., 2009). *M. lineata* is widely distributed along the rocky Atlantic intertidal shores, from Scottish and Welsh in the northern to NW Africa in the south (Bode *et al*., 1986) and inhabits from high intertidal to deep sea (Cunha *et al*., 2007). Some examples of biomonitoring studies that were carried out with *M. galloprovincialis*, *M. lineata* and *C. crangon* populations of the NW Portuguese coast, including sampling periods, biomarkers used and other indications are summarized in Table 1. The biomarkers that were used in these studies are enzymes and other biological parameters important for the survival and performance of the animals (Menezes *et al*., 2006; Quintaneiro *et al*., 2006; Lima *et al*., 2007; Lima *et al*., 2008; Tim-Tim *et al*., 2009).

Among the biological parameters that have been used as biomarkers in biomonitoring studies with wild populations, are enzymes and other parameters involved in crucial functions for the survival and performance of organisms (van Gestel and van Brummelen, 1996; Walker *et al*, 2012), such as neurotransmission, cellular energy production, biotransformation and anti-oxidant defences, and parameters indicative of biological damage. Cholinesterases (ChEs) are a family of esterase enzymes widely used in environmental biomonitoring studies. Typical cholinesterases present in vertebrates are divided in acetylcholinesterase (AChE), the enzyme that hydrolyses the neurotransmitter acetylcholine in cholinergic synapses (Payne *et al*., 1996) and that is crucial for cholinergic neurotransmission in the nervous and neuromuscular systems, and pseudocholinesterase (also called butyrylcholinesterase) that as protective function regarding AChE and is important in the detoxification of several xenobiotics (Payne *et al*., 1996). In the nervous system and other tissues of invertebrates AChE can be present (Moreira et al., 2001) and perform a similar activity of the vertebrates' enzyme. However, in the tissues of several species, the ChEs have properties of both AChE and pseudocholinesterase vertebrate enzymes. For example, in cephalothorax of *C. Crangon* predominates AChE (Quintaneiro *et al*., 2006), in haemolymph and gills of *M. galloprovincialis* also predominates AChE but in digestive gland both types are found (AChE and BuChE) (Moreira *et al*., 2001) and in *M. lineata* both types predominate (AChE and BuChE) (Cunha *et al*., 2007). Generally, the inhibition of ChEs activity is considered indicative of exposure (≈ 20% of inhibition) and/or effect (> 20% of inhibition) of anticholinesterase agents (Nunes and

Resende, 2017). Classic anti-cholinesterase agents are organophosphate and carbamate insecticides (Payne *et al*. 1996; Varo´ *et al*, 2002). However, in the last decades, several other environmental contaminants such as some detergents and surfactants, heavy metals, oils and other petrochemical mixtures were found to inhibit ChE activity of wild species at ecological relevant concentrations, and thus a broader use of this enzyme has been made (Martinez-Tabche *et al*. 1996; Guilhermino *et al*., 1998; Schiedek *et al*. 2006). Because pseudocholinesterases are important in the detoxication of several xenobiotics, the activity of these enzymes may be increased in organisms exposed to environmental contamination. For example, ChEs activity decreased for *M. galloprovincialis* but increased for *M. lineata a*nd *N. lapillus* after "Prestige" oil spill. (Tim Tim *et al*., 2009). Another type of enzymes widely used in environmental biomonitoring studies are glutathione-*S*-transferases (GST), involved in the biotransformation of both endogenous substances and xenobiotics by catalysing their conjugation with glutathione (Cunha *et al*., 2005; Hartl *et al*., 2007), facilitating its elimination. These enzymes are also involved in anti-oxidant defences (Nordberg and Arner, 2001). Thus, in biomonitoring studies, GST induction is generally used as an indicative of exposure and effect to chemical agents and other stressors whose biotransformation involves glutathione conjugation and/or to oxidative stress inducers (Nordberg and Arner, 2001; Cunha *et al*., 2005; Hartl *et al*., 2007;). Catalase (CAT), superoxide dismutase (SOD), glutathione reductase (GR) and glutathione peroxidase (GPx) are enzymes involved in anti-oxidant defences that have been also widely used in biomonitoring studies. Because there is a high number of environmental contaminants and other stressors (e.g. temperature) able to induce oxidative stress (Menezes *et al*., 2006). Often, such enzymes are used simultaneously with parameters indicative of oxidative damage such as lipid peroxidation levels (LPO), Carbonyl group oxidation (PO) and DNA oxidation (Chaudiere*,* 1994). Lactate dehydrogenase (LDH) is the enzyme that catalyses the reversible conversion of lactate to pyruvate, an important step in anaerobic cellular energy production (Diamantino *et al*., 2001), whereas isocitrate dehydrogenase (IDH) is an enzyme involved in the aerobic pathway of energy production, being also important for the cellular redox maintenance (Lee *et al*., 2002; Moreira *et al*., 2006). Therefore, the determination of the activity of these enzymes may provide valuable information regarding energy production that is vital for survival and performance. Octopine dehydrogenase (ODH) is an important enzyme for the energetic metabolism of molluscs in anaerobic conditions (Baldwin and Opie, 1978).

In the continuation of the monitoring programmes that have been performed in the NW Portuguese coast, the central objective of the present study was to investigate the health status of *M. galloprovincialis* (shoreline), *M. lineata* (shoreline) and *C. crangon* (Douro and Minho River estuaries) populations of this area.

Table 1: Field studies in NW Portugal costal zones using *C. crangon*, *M. galloprovincialis* and *M. lineata* as bioindicators for spatial and seasonal discrimination of environmental multistressors. The numbers indicate the references: 1 – Quintaneiro *et al*., 2006; 2 - Menezes *et al*., 2006; 3 - Lima *et al*., 2008; 4 – Lima *et al*., 2007; 5 - Tim-Tim *et al*., 2009

Estuary/coast	**Apparent contamination**	**Species**	**Study period**	**Biochemical parameters**	**Observation**	**Methodology**
Minho river	reference	*C. crangon*	Winter 2001 to autumn 2001	low LDH and GST, and high ChE	Low contamination	Organism collection, field biomarker determination at different seasons (1)
Ria de Aveiro	reference			low ChE; High GST in winter and spring; High: LDH in winter, spring and autumn	Run-off from agriculture and chemical stress for LDH	
Douro river	urban and industrial					
Mira channel	agricultural					
Laranjo bay	Heavy metals					
Minho River	Salinity	*C. crangon*		High: AChE at low temperature	non-toxic stressors affect biochemical parameters	Biomarkers levels comparison after exposure to stressors variation (2)
	temperature			High: LDH at high salinity		
	handling stressors			High: AChE, IDH and GST after 30 days		

Continuation of table 1

Estuary/ coast	Apparent contamination	Species	Study period	Biochemical parameters	Observation	Methodology
Carreço	Laboratorial reference	*M. galloprovincialis*	September of 2004	No mutation detected	No mutation detected in mussels from control with WAF –exposed and mussels collected in the field	Ras gene comparison (3)
Leixões harbour	Low petrochemical levels		April of 2005	Detection of single mutation At codon 35 in digestive gland exposed to 12.5%		
Barra	High Petrochemical levels			No mutation detected		
Carreço	Low contamination levels	*M. galloprovincialis*	January of 2005	High values of CAT, SOD, GST, IDH, GPx and LPO were found in mussels from Cabo do Mundo and Leixões harbour. ODH value was high in mussels from Leixões harbour.	Total petroleum hydrocarbons contamination, in decreasing order: Leixões harbour, Viana do Castelo harbour, Vila Chã, Carreço and Cabo do Mundo	Comparison of biomarkers levels in *M. galloprovincialis* and petroleum hydrocarbons contamination levels from different sites (4)
Viana de Castelo	Petrochemical contamination					
Vila Chã	Absence of significant contamination sources					
Cabo do Mundo	Chronically exposed to petrochemicals including PAHs and heavy metals					
Leixões harbour	Petroleum hydrocarbon contamination					

Continuation of table 1

Estuary/coast	Apparent contamination	Species	Study period	Biochemical parameters	Observation	Methodology
Vila Praia de Âncora	low contamination	*M. galloprovincialis* and *M. lineata*	Autumn 2002, and after oil spill, winter 2002, 2003 and summer 2003	*M. galloprovincialis*: ChE levels decreased after oil spill; GST levels increased after oil spill. *M. lineata*: ChE and GST levels increased after oil spill	Spatial and seasonal differences in biomarkers levels were found in both species.	biomarkers comparison levels of mussels before (autumn 2002) and after oil spill(winter 2002/2003, spring and summer 2003)(5)
São Bartolomeu do Mar						
Vila Chã						
Cabo do Mundo	chronically exposed to petroleum-derived hydrocarbons, including PAHs and heavy metals					
Boa Nova						
Homem do Leme	high levels of heavy metals, organic compounds and other chemicals					
Granja	urban, industrial and domestic discharges					

2. Materials and Methods

2.1. Chemicals

The chemicals used in biomarker assays were all of analytical grade and provided by Sigma–Aldrich (USA) or Merck (Germany). The *Bio-rad* reagent used for protein assays was from *Bio-Rad* (Germany). The tests were performed using water test tablets provided by Palintest (United Kingdom).

2.2. Sampling sites brief characterization

The sampling sites were: one sampling site in the estuary of the Douro River (≈41°8'3.45'' N; 8°39'43.91''W) and one sampling site in the estuary of the Minho River (≈ 41°53'27.28'' N; 8°49'81''W) for *C. crangon*, and two sampling sites in the NW coast of Portugal, designed by Cabo do Mundo (≈41°13'62'' N; 8°42'92''W) and São Félix da Marinha (≈41°01'45'' N; 8°38'44''W), for *M. galloprovincialis* and *M. lineate* (Figure 1).

Figure 1: Map of Portugal, showing the location of the sampling sites where animals were collected. *Crangon crangon* specimens were collected in the estuary of Douro River (DE) and in the estuary of Minho River (ME) and *Mytilus galloprovincialis* and *Monodonta lineate* specimens were collected in Cabo do Mundo (CM) and Sao Félix da Marinha (SFM)in the NW Portuguese coast (shoreline).

The Douro River is one of the longest rivers of the Iberian Peninsula (≈ 930 km) with about 98 000 $km^2$of hydrological basin shared between Portugal and Spain (Madureira *et al*., 2010); their banks are densely populated since this river separates two major cities Porto and Vila Nova de Gaia (Madureira *et al*., 2010). This estuary has been polluted by high levels of heavy metals and organic compounds because directly receives urban and industrial discharges, mostly untreated (Mucha *et al*., 2003), although, in last the years, its water quality was considered acceptable in only some sites (Couto *et al*., 2014), due to improvements on collection and treatment of municipal wastes.

The Minho river is also located in Iberian Peninsula with about 300 km long and its hydrological basin has an area of 17 080 km^2 (Sousa *et al*., 2008). This mesotidal estuary is partially mixed; however, during the period of high floods, it tends to evolve towards a salt wedge estuary (Sousa *et al*. 2005). The influence of spring tides extends approximately 40 km upstream, and the tidal freshwater wet lands are located in the upper 30 km (Sousa *et al*., 2008). Its estuary that makes a part of the border between Portugal and Spain and, despite having some localized sources of pollution, has been considered relatively low anthropogenic impacted and was used as reference estuary in previous biomonitoring studies (Cairrão *et al*., 2004; Quintaneiro *et al.*, 2006; Monteiro *et al*., 2007; Guimarães *et al*., 2009).

The sampling site indicated as Cabo do Mundo is localized in the shoreline of the Portuguese NW coast, is a small bay where a stream ends up. It is near to important industrial facilities (and oil refinery that has now a submarine emissary) and a harbour and is chronically exposed to petroleum derived hydrocarbons including polycyclic aromatic hydrocarbons (PAHs) and heavy metals (Salgado and Serra, 2001; Tim -Tim *et al*., 2009).

The sampling site indicated as São Félix da Marinha is localized in the shoreline of the NW Portuguese coast, not far from a village with the same name in the Vila Nova de Gaia municipality. It is an urban shore with sand and rock morphology delimited by dune cord. Several years ago, its suffered the influence of urban and/or industrial contamination, since it was the receiver of an urban stream (Moreira and Guilhermino, 2005), but more recently rehabilitation works were performed, including the implementation of a water-treatment plant, and its water quality improved (Tim-Tim *et al*., 2009), and the beach has now blue flag indicating its good environmental quality.

2.3. Animals collection and transportation to laboratory

A first collection of juvenile shrimps (*C. crangon*) (n=100) was performed in winter 2016 in the Minho estuary, to optimize and get training on sampling and biomarkers determinations. In late winter 2017, the sampling to assess the populations health status was performed. Shrimps were collected in Douro river and Minho river estuaries, *M. galloprovincialis* and *M. lineata* were collected in Cabo do Mundo and São Félix da Marinha (15 animals per sampling site). All animals were collected at low tide, shrimps were collected using a hand-operated net and other species were hand-picked. All animals were transported to the laboratory in appropriated containers with water from the sampling sites in a maximum of two hours. In the laboratory, they were measured using a digital calliper (Electronic Digital calliper). The mean and standard deviation (SD) of the total length (from antenna to uropods) of *C. crangon* specimens was 3.4± 0.4 cm. The mean shell length of *M. galloprovincialis* specimens was 4.2 ± 0.3 cm (anterior-posterior shell length ± standard deviation), whereas for *M. lineata* specimens the mean and SD of the measurement between the base and the top of the shell was 1.75 ± 0.15 cm. After measurements, animals were dissected on ice, and organs and tissues from each animal were isolated on ice, namely: eyes, muscle (in two portions) and digestive gland (in two portions) for *C. crangon*, haemolymph, foot and digestive gland (in two portions) and adductor muscle for *M. galloprovincialis* and gills, foot and digestive gland (in two portions) for *M. lineata*. Each sample was placed into a 2 ml microtube containing the appropriated buffer for each biomarker determination. *C. crangon* eyes were placed in 0.1 M K-phosphate pH = 7.2 for AChE determination, one portion of muscle was placed in tris 0.1 M K-phosphate pH = 7.2 and another in tris K-phosphate pH = 7.8 for LDH and IDH determinations respectively, and for digestive gland, one portion was placed in 0.1 M K-phosphate pH = 6.5 and another in 0.1 M K-phosphate pH = 7.4 for GST and oxidative stress (CAT, LPO and PO) determinations respectively; *M. galloprovincialis* haemolymph was placed in 0.1 M K-phosphate pH = 7.2 for ChEs determination (1:3, v/v), for digestive gland, one portion was placed in tris 0.1 M K-phosphate pH = 7.8 and another in 0.1 M K-phosphate pH = 7.4 for IDH and oxidative stress (CAT, LPO and PO) determinations, gills were placed in0.1 M K-phosphate pH = 6.5 for GST determination and finally adductor muscle in 0.1 M K-phosphate pH = 7.5 for ODH determination; for *M. lineate* two portions of foot were used, one was placed in 0.1 M K-phosphate pH = 7.2 and another in 0.1 M K-phosphate pH = 7.5 for ChEs and ODH determinations respectively, for digestive gland one portion was placed in tris 0.1 M K-phosphate pH = 7.8 and another in 0.1 M K-phosphate pH = 7.4 for IDH and oxidative stress (CAT, LPO and PO) determinations respectively, and gills were placed in 0.1 M K-phosphate pH = 6.5 for GST determination. All samples were then stored at -80°C until further use.

2.4. Abiotic parameters

Simultaneously to the animal sampling performed in the late winter/spring 2017, water salinity, temperature, dissolved oxygen, and conductivity were determined *in situ* using appropriate probes (Multi 340i WTW/tetracom 325 and cellox 325 WTW), and water samples were collected for later determination of concentration of nitrites, nitrates, ammonium, phosphates, and phenol, water hardness and turbidity. All these parameters were determined in the laboratory using a photometer (interface photometer 7500)

2.5. Biomarkers determination in C. crangon

Animals collected in winter 2016 were used in preliminary studies aimed at optimizing the methodologies and get training on biomarkers determinations. Samples of these animals were analysed individually or pooled together (3 animals per pooled sample) to select the number of animals needed for each biomarker in the biomonitoring study. *C. crangon* eye samples, individual or pooled, for ChEs activity determination were homogenized (Ystral D79282 from Power Technology Inc.) in 500 µL or 1000 µL of phosphate buffer (0.1 M, pH = 7.2) for individual and pooled samples respectively. Samples were centrifuged (Eppendorf centrifuge 5810 R) at 3300 g for 3 minutes at 4 °C, the supernatants were collected, and their protein content was determined by the Bradford technique (Bradford,1976), adapted to microplate (Frasco *et al*., 2002) using a calibration curve obtained from concentrations of 0, 0.2, 0.5 and 1.0 $mg.mL^{-1}$(bovin γ-globulin as standard) and the absorbance was read at 600 nm in the microplate reader (BIO-TEK, POWERWAVE 340, USA) at room temperature. The activity of ChEs was determined by the Ellman's method (Ellman *et al.,* 1961), adapted to microplate (Frasco and Guilhermino, 2002), using acetylthiocholine as substrate, and readings were performed at 412 nm (BIO-TEK, POWERWAVE 340, USA).The muscle was separated in two parts, one for LDH activity and the other for IDH activity determination. Muscle samples were homogenised (Ystral D79282 form Power Technology, Inc.) in 1000 µL (pooled samples) or 500 µL (individual samples) in tris-phosphate buffer (0.1 M, pH = 7.2). The recovered supernatant after centrifugation (centrifuge 5810 R) at 15000 g during 15 minutes at 4 °C was used for total protein content and IDH determinations by Ellis and Goldberg method (Ellis and Goldberg, 1971) adapted to microplate (Lima *et al*., 2007), through the measurement of the absorbance of the kinetic reaction product (increase of NADPH production) at 340 nm during 3 minutes (BIO-TEK, POWERWAVE 340, USA). Another portion of muscle of pooled and individuals was used for LDH activity determination. Tissues were homogenised (Ystral D79282 from Power Technology Inc.) after3 cycles of frozen/unfrozen in 1000 µL and 500µL of buffer solution (81.3 mMtris/NaCl-K-phosphate, 203.3 mM NaCl, pH = 7.2) respectively for pooled and individual samples. The supernatant

after centrifugation (Eppendorf centrifuge 5810 R) at 3300 g for 3 minutes at 4°C was used for enzymatic determination. The LDH determination was done by Vassault method (Vassault, *et al*., 1983) adapted to microplate (Diamantino *et al*., 2001) through the measurement of the absorbance of the kinetic reaction (decrease of NADH) at 340 nm during 5 minutes every 20 seconds. The digestive gland tissue for both groups (pooled and individual) was homogenised (Ystral D79282 form Power Technology Inc.) in 1000 µL of phosphate buffer (0.1 M K, pH = 7.4) and the homogenate was separated in two portions. To one portion (200µL) were added 4 µL of BHT 4% solution and was stored at -80°C (SANYO Ultra Low vip Plus Freezer) for LPO determination. Another portion was centrifuged at 10000 g for 20 minutes at 4°C; 30µL of recovered supernatant were used for total protein content determination and the remaining was stored at -80°C for GST, CAT and PO determination. LPO level was determined by Ohkawa and Bird and Draper method (Ohkawa *et al*., 1985; Bird and Draper, 1984**)** through the absorbance read at 535 nm of TBARS formed during the reaction of malondialdehyde and TBA (Jasco V-630 spectrophotometer). GST activity was determined by Habig technique (Habig *et al*.., 1979) adapted to microplate (Frasco and Guilhermino, 2002) through the measurement of the absorbance values of the formation rate of GS-CDNB (during the reaction of GSH and CDNB),read at 340 nm during 5 minutes every 20 seconds. CAT activity was determined by Clairborne method (Clairborne, 1985) through the measurement of the absorbance values of degradation rate of H_2O_2, read at 240 nm during 60 seconds, and PO level was determined by Levine method (Levine *et al*., 1990) through the measurement of the absorbance values read at 370 nm of the hydrozone formed during the reaction of PO and DNPH.

2.6. Biomarkers determination in *Mytilus galloprovincialis* and *Monodonta lineata*

Mussels haemolymph and snails foot were homogenized (Ystral D79282 from Power Technology Inc.) in K-phosphate buffer (0.1 M, pH = 7.2) in 1:3 proportion and in 1000 µL respectively. The ChEs activity were determined as described for *C. crangon* (section 2.5). The digestive gland of both species was separated in two parts, one for IDH and another for CAT, LPO and PO levels determination. These biomarkers were also determined as described *C. crangon* (section 2.5). Gills of both species were homogenized in 500 µL of 0.1 M K-phosphate pH = 6.5 for GST level determination that was determined as described for *C. crangon* (section 2.5). Foot and adductor muscle were homogenized in 500 µL of 0.1M K-phosphate pH = 7.5 for ODH determination according to the Livingstone method (Livingstone *et al*., 1990) through of the readings of the absorbance values of the kinetic reaction of decrease of pyruvate, read at 340 nm during five 3 minutes every 20 seconds.

2.7. Data analyses

Data from the biomonitoring study was analysed in relation to their collection site per parameter and per species. Samples from the Minho estuary were compared with those from the Douro estuary, and samples from São Félix da Marinha were compared with those from Cabo do Mundo. For each abiotic parameter, sites in different estuaries or the 2 sites in the shoreline were compared through the Mann-Whitney U test. For each biomarker, shrimps collected in distinct estuaries, and molluscs collected in São Félix da Marinha and Cabo do Mundo were compared using the Student's *t* test. The SPSS 24.0 package was used and the significance level was0.05.

3. Results

3.1. Preliminary studies with *C. crangon*

The results of the total protein in samples of different organs or tissues of *C. crangon* collected in winter 2016 are shown in the figure 2.The highest protein content was found in the muscle with 12.09 ± 2.49 and 7.29 ± 4.51 mg/ml for pooled and individual samples respectively, whereas the lowest one was found in the eyes (1.31 ± 0.53 and 0.78 ± 0.02 mg/ml for pooled and individual respectively). In all tissues, pooled samples had higher protein content than in individual.

The biomarkers determined are shown in figure 3.Pooled samples had apparently higher mean LDH, GST and CAT levels higher than in individual, whereas the mean levels of AChE, IDH and LPO were apparently lower in pooled samples than in individual. No statistical test was performed for total protein content and biomarkers levels (pooled and individual samples) in preliminary studies with *C. crangon* since only two replicates (n = 2) were used for treatment (pooled or individual).

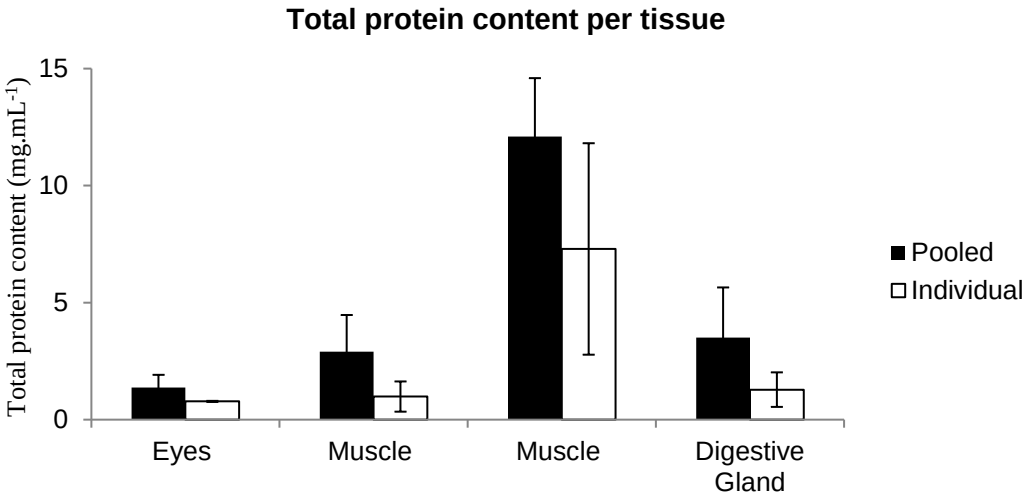

Figure 2: Total protein content (mean ± standard error of the mean) in *Crangon crangon* eyes and other tissues collected in the Minho estuary in winter 2016, of pooled and individual samples n = 2.

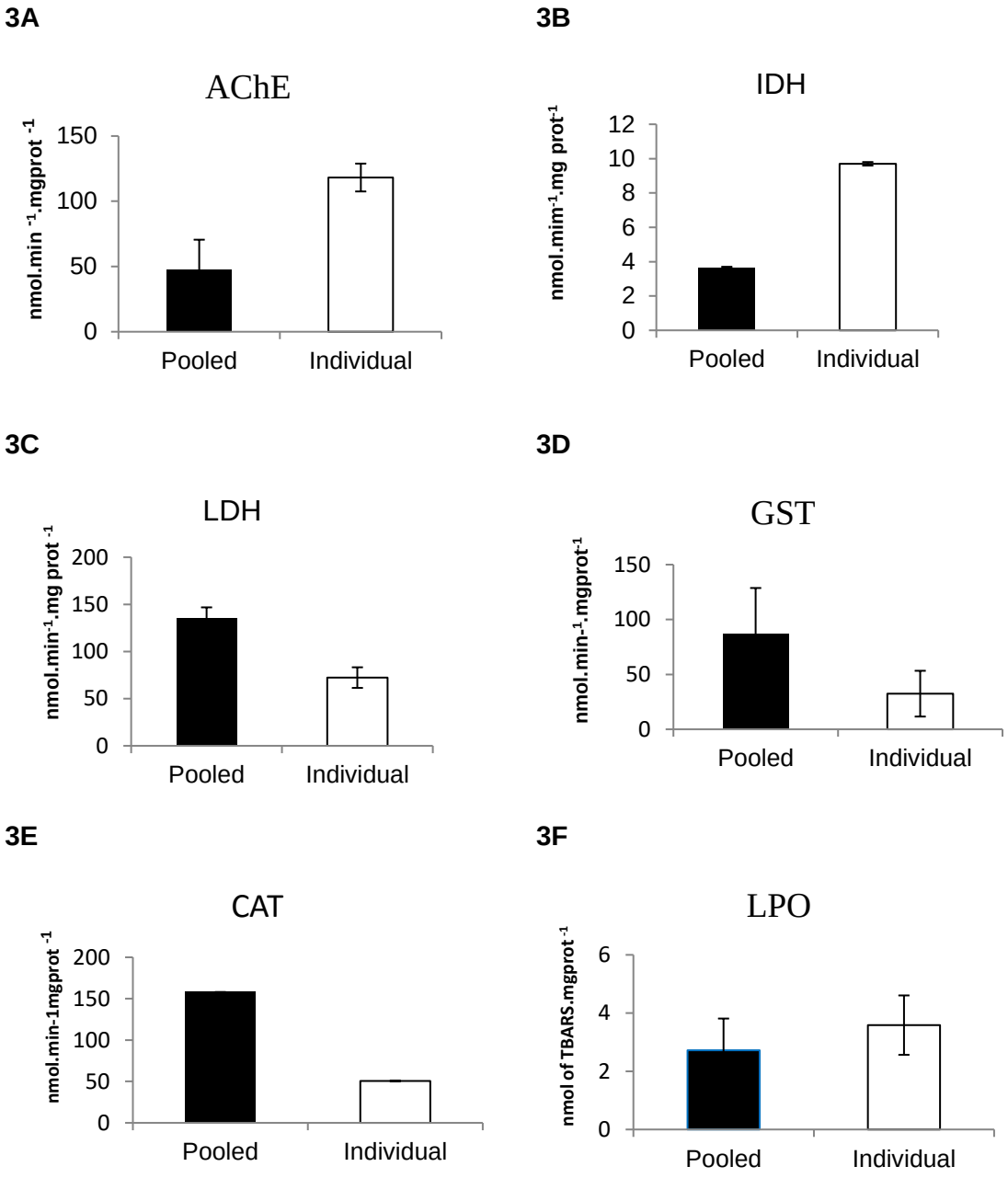

Figure 3: Biomarker levels determined in *C. crangon* collected in Minho estuary in winter2016. Values represent the mean ± standard error of the mean (two replicates) of shrimps in pooled (3 animals) and individual samples. A – Activity of cholinesterase enzymes. B – Activity of isocitrate dehydrogenase enzymes. C – Activity of lactate dehydrogenase enzymes. D – Activity of glutathione *S*-transferases enzymes. E – Activity of catalase enzyme. F – Lipid peroxidation levels.

3.2. Biomonitoring study

3.2.1. Abiotic parameters

The abiotic parameters of the sampling sites are summarized in table 2. Significant differences between the Minho estuary and Douro estuary were found for the following abiotic parameters: salinity ($U = 0.000$, $\rho = 0.046$), temperature ($U = 0.000$, $\rho = 0.046$) and conductivity ($U = 0.000$, $\rho = 0.046$), nitrites ($U = 0.000$, $\rho = 0.046$), nitrates ($U = 0.000$, $\rho = 0.050$), phosphates ($U = 0.000$, $\rho = 0.050$), phenol ($U = 0.000$, $\rho = 0.046$) and turbidity ($U = 0.000$, $\rho = 0.037$) values. No significant differences between the two sites were found for dissolved oxygen, ammonium, iron, and water hardness. The samples of the Douro estuary had significant higher levels of salinity, temperature, conductivity, nitrites, phenol, turbidity and phosphates and lower level of nitrates than Minho estuary samples.

Regarding coastal zones, significant differences for the salinity ($U = 0.000$, $\rho = 0.046$), temperature ($U = 0.000$, $\rho = 0.050$) and conductivity ($U = 0.000$, $\rho = 0.050$) were found. No significant differences between sites were found for dissolved oxygen, nitrites, nitrates, ammonium, phosphates, iron, phenol, hardness and turbidity. The samples of the São Félix da Marinha coastal zone had higher levels of salinity and conductivity and lower level of temperatures than Cabo do Mundo samples.

Table 2: Abiotic parameters determined in the water collected in different sampling sites. The values are the mean of three independent samples with the corresponding standard error of the mean within brackets. * indicates significant differences of abiotic parameters between sites. The values of the Mann-Whitney U-test are also shown ($p \leq 0.05$).

Parameter	Douro estuary	Minho estuary	Statistics	São Félix da Marinha	Cabo do Mundo	Statistics
Salinity ($g.L^{-1}$)	21.90 (±0.17)	10.70 (±1.11)	U = .000; ρ = 0.046*	34.07 (±0.23)	32.13 (±0.4)	U= .000; ρ = 0.046*
Temperature (ºC)	14.47 (±0.12)	13.83 (±0.32)	U= .000; ρ = 0.046*	17.40 (±0.1)	18.07 (±0.38)	U= .000; ρ = 0.050*
Dissolved oxygen ($mg.L^{-1}$)	7.21 (±0.17)	7.41 (±0.28)	U= 2.000; ρ = 0.275	5.08 (±0.13)	5.65 (±1.00)	U= 3.000; ρ = 0.513
Conductivity (mScm)	35.43 (±0.12)	18.51 (±1.59)	U= .000; ρ = 0.046*	52.63 (±0.5)	49.63 (±0.71)	U= .000; ρ = 0.050*
Nitrites($mg.L^{-1}$)	0.03 (±0.01)	0.005 (±0.00)	U= .000; ρ = 0.046*	0.02 (±0.01)	0.03 (±0.02)	U= 4.000; ρ = 0.822
Nitrates ($mg.L^{-1}$)	0.63 (±0.42)	6.27 (±0.61)	U= .000; ρ = 0.050*	3.27 (±0.67)	2.73 (±0.29)	U= 2.000; ρ = 0.268
Ammonium ($mg.L^{-1}$)	0.37 (±0.34)	0.81 (±0.43)	U= 1.000; ρ = 0.127	2.31 (±0.26)	2.00 (±0.49)	U= 3.000; ρ = 0.513
Iron ($mg.L^{-1}$)	0.23 (±0.03)	0.23 (±0.03)	U= 2.000; ρ = 0.197	0.1 (±0.0)	0.20 (±0.09)	U= 1.500; ρ = 0.114
Phenol ($mg.L^{-1}$)	0.07 (±0.01)	0.01 (±0.01)	U= .000; ρ = 0.046*	0.07 (±0.03)	0.15 (±0.4)	U= 3.000; ρ = 0.507
Water Hardness ($mg.L^{-1}$)	263.33 (±110.15)	316.67 (±46.19)	U= 2.500; ρ = 0.369	656.67 (±195.53)	420± (115.33)	U= 1.000; ρ = 0.127
Turbidity (FTU)	6.67 (±3.06)	0.0 (±0.0)	U= .000; ρ = 0.037*	2.00 (±0.46)	2.67 (±2.31)	U= 4.000; ρ = 0.814
Phosphates ($mg.L^{-1}$)	0.27 (±0.05)	0.15 (±0.03)	U= .000; ρ = 0.050*	0.18 (±0.11)	0.32 (±0.32)	U= 4.000; ρ = 0.827

3.2.2. Comparison of *C. crangon* populations from the Douro and Minho estuaries

The results of the biomarkers determined in *C. crangon* are shown in Figure 4. Significant differences between the population of the Minho estuary and the population of the Douro estuary were found for the following biomarkers: ChEs activity [t (26) = 4.179, ρ = 0.000], LPO [t (28) = 2.658, ρ = 0.013] and PO levels [t (20) = - 2.569, ρ = 0.022]. No significant differences between the two populations were found for LDH activity [t (16.269) = 1.205, ρ = 0.246], CAT activity [t (24.928) = - 1.660, ρ = 0.109] and GST activity [t (20.529) = 1.923, ρ = 0.069]. The population of the Douro estuary had significant higher activity of ChEs (Figure 4A), higher LPO levels (Figure 4E), and lower PO levels (Figure 4F) than the Minho estuary population.

3.2.3. Comparison of *M. galloprovincialis* from Cabo do Mundo and São Félix da Marinha

Significant differences in ChEs [t (27.827) = - 3.967, ρ = 0.000], CAT [t (28) = - 3.793, ρ = 0.001] and GST [t (28) = 5.809, ρ = 0.000] activities between mussels collected in Cabo do Mundo and those collected in São Félix da Marinha were found, whereas no significant differences were found for IDH activity [t (25.136) = 0.817, ρ = 0.421],ODH activity [t (26.187) = - 0.107, ρ = 0.915], LPO [t (27.953) = - 0.354, ρ = 0.726] and PO levels [t (16.196) = 1.353, ρ = 0.211], as shown in Figure 5. Mussels collected in Cabo do Mundo had significant higher ChEs activity (Figure 5A) and CAT activity (Figure 5E) significant lower GST activity (Figure 5D) than those collected in São Félix da Marinha.

3.2.4. Comparison of *M. lineata* from Cabo do Mundo and S. Félix da Marinha

The results of the biomarkers determined in *M. lineata* are shown in Figure 6. Significant differences between the population of the Cabo do Mundo and the population of the São Félix da Marinha were found for the following biomarkers: CAT activity [t (28) = 2.211, ρ = 0.035], GST activity [t (28) = - 2.837, ρ = 0.008] and LPO levels [t (28) = - 5.635, ρ = 0.000]. No significant differences between the two populations were found for ChEs activity [t (25.314) = 0.009, ρ = 0.993], IDH activity [t (24.484) = - 0.059, ρ = 0.977], ODH activity [t (19.761) = - 0.808, ρ = 0.426] and PO levels [t (17) = - 1.778, ρ = 0.0.093]. The population of the Douro estuary had significant higher activity of ChEs (Figure 6A), higher LPO levels (Figure 6E), and lower PO levels (Figure 6F) than the Minho estuary population.

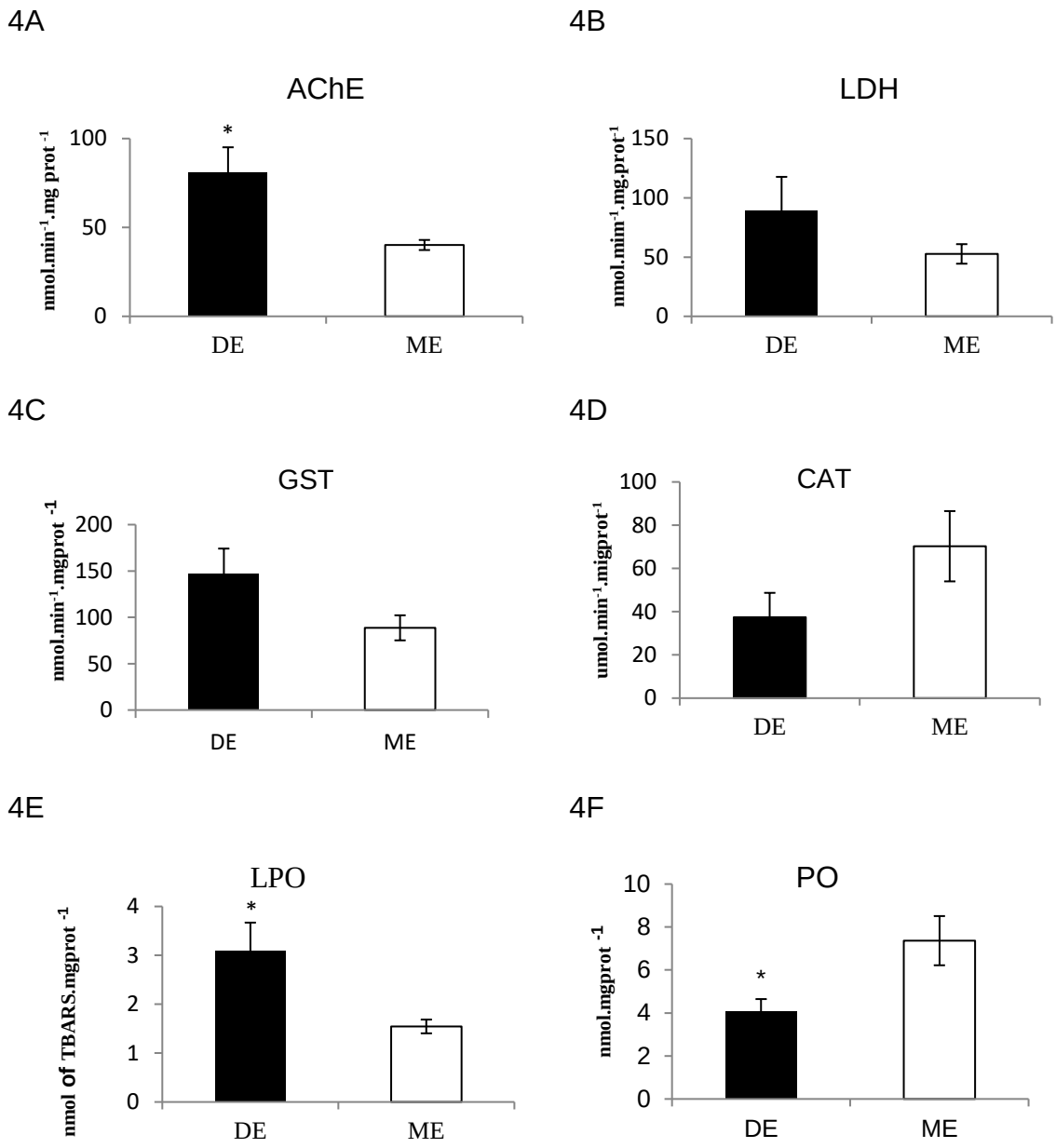

Figure 4: Results of the biomarkers determined in *Crangon crangon* from the populations of the Douro estuary (DE) and of the Minho estuary (ME). The values are the mean of 15 animals with corresponding standard error bars. A – Activity of cholinesterase enzymes. B – Activity of lactate dehydrogenase enzymes. C – Activity of glutathione *S*-transferases enzymes. D – Activity of catalase enzyme. E – Lipid peroxidation levels. F – Carbonyl group oxidation level. * indicates statistically significant differences assessed through the Student's *t* test ($\rho \leq 0.05$).

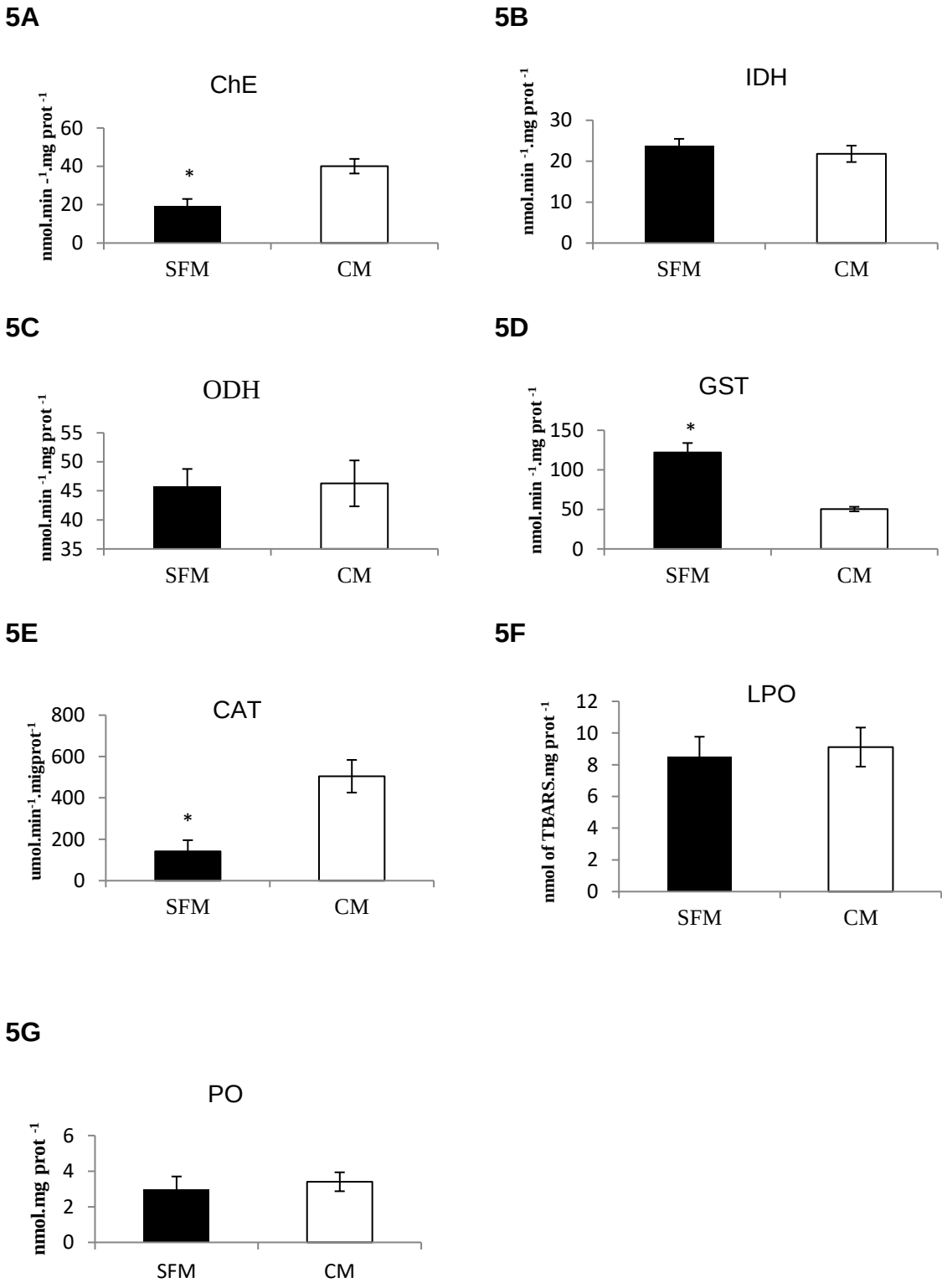

Figure 5: Results of the biomarkers determined in *Mytilus galloprovincialis* from the populations of the Cabo do Mundo (CM) and of the São Félix da Marinha (SFM). The values are the mean of 15 animals with corresponding standard error bars. A – Activity of cholinesterase enzymes. B – Activity of isocitrate dehydrogenase enzymes. C – Activity of octopine dehydrogenase enzymes. D – Activity of glutathione *S*-transferases enzymes. E – Activity of catalase enzymes. F – Lipid peroxidation levels. G – Carbonyl group oxidation level. * indicates statistically significant differences assessed through the Student's *t* test ($\rho \leq 0.05$).

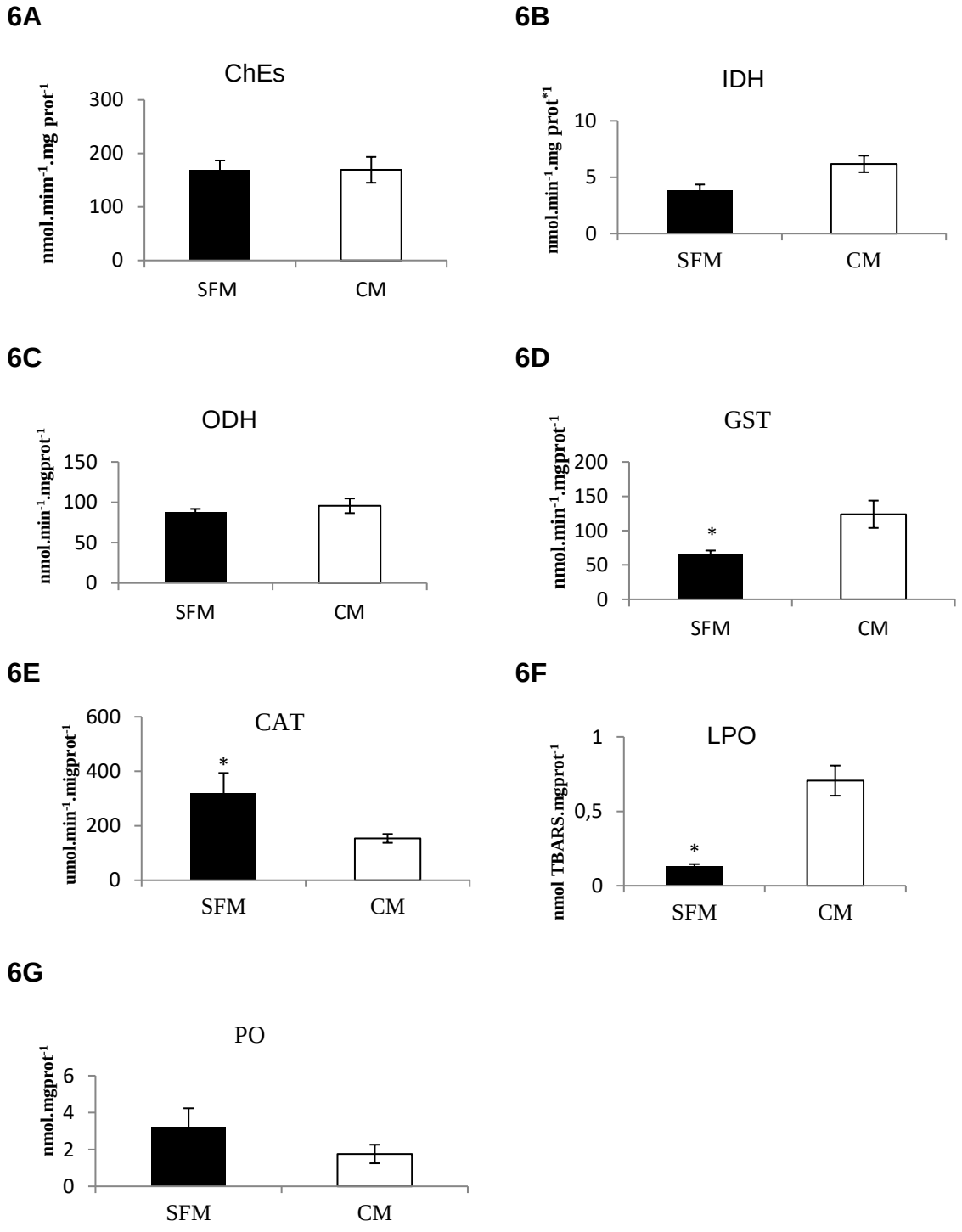

Figure 6: Results of the biomarkers determined in *Monodonta lineata* from the populations of the Cabo do Mundo (CM) and of the São Félix da Marinha (SFM). The values are the mean of 15 animals with corresponding standard error bars. A – Activity of cholinesterase enzymes. B – Activity of isocitrate dehydrogenase enzymes. C – Activity of octopine dehydrogenase enzymes. D – Activity of glutathione *S*-transferases enzymes. E – Activity of catalase enzymes. F – Lipid peroxidation levels. G – Carbonyl group oxidation level. * indicates statistically significant differences assessed through the Student's *t* test ($\rho \leq 0.05$).

4. Discussion

4.2. Preliminary studies

The method of protein determination used in the present study (Bradford, 1976) quantifies the total amount of protein in the samples and not only the protein corresponding to specific enzymes or other individual proteins. Thus, because the activity of the enzymes used as biomarkers are expressed in function of total protein content of the samples, and the determination of protein was based on the relationship between absorbance and protein content of the samples that is not linear at high protein concentrations (Bradford, 1976), high protein content of the samples may introduce errors in the determination of enzymatic activities. In fact, although the protein content of pooled samples was higher than in samples prepared from individual animals (Figure 2), some enzymes had higher activities in individual than in pooled samples (Figure 3). Moreover, the determination of the biomarkers in individual samples allows to take into consideration the individual variation of organisms what is important when studying populations, because individuals have genetic, physiological and other biological differences that should be taken into consideration. Thus, for these reasons, it was decided to carry the biomonitoring study with samples prepared from individual organisms of *C. crangon* populations of the two estuaries.

4.2. Biomonitoring study

4.2.1. Abiotic factors

The significant differences of the abiotic factors found between the Douro estuary and the Minho estuary (Table 2) indicate that, at the time of sampling, the populations of *C. crangon* from distinct estuaries were exposed to different conditions regarding these parameters. A similar conclusion results from the comparison of the abiotic parameters determined in the two sampling sites located in the Portuguese NW coast. Therefore, the differences found in the biomarkers levels may also be due to these abiotic parameters among other factors. Among these parameters, phenol is most important because is directly related to chemical contamination, such as petrochemicals, and the higher level of phenol found in Douro estuary can be an indicative of the presence of petrochemicals or other sources of phenol.

4.2.2. Comparative study of *C. crangon* populations from the Minho and Douro estuaries

The mean (and standard error of the mean) of ChE (Minho estuary: 40 ± 10 nmol/min/mg protein; Douro estuary: 81 ± 14 nmol/min/mg protein), GST (Minho estuary: 86 ± 13 nmol/min/mg protein; Douro estuary: 147 ± 27 nmol/min/mg protein) and LDH (Minho estuary: 53 ± 8 nmol/min/mg protein; Douro estuary: 89 ± 29 nmol/min/mg protein) enzymes determined in the spring present study are in the range of annual mean and variation of corresponding enzymatic activities reported in a biomonitoring study carried out in 2001/2002 that included the populations of *C. crangon* of Minho and Douro estuaries (Quintaneiro *et al*., 2006), despite some methodological differences between the studies, including in some of the tissues used.

The lowest ChEs activity (Figure 4A) recorded in the shrimps from the Minho estuary relative to those of the Douro estuary, suggests higher exposure of the Minho estuary population to anticholinesterase agents than those of the Douro estuary. Such anticholinesterase agents may be organophosphate and carbamate insecticides, metals and components of petrochemical mixtures that have the capability of inhibiting ChEs, detergents, among other possible environmental contaminants (Martinez-Tabche *et al*. 1996; Payne *et al*. 1996; Guilhermino *et al*., 1998; Varó*et al*, 2002; Schiedek *et al*. 2006). Such compounds were found in the two estuaries (Guimarães *et al.*2009). However, in the last years several activities mainly related with tourism (e.g. boat traffic, water-related recreation activities, seasonal increase of habitants and tourism habitations) have been increasing in the Minho estuary area, whereas in the Douro estuary the water quality improvedas are sult of several actions, mainly treatment of urban and industrial effluents. This may explain at least partially, why in the present study a significant higher ChE activity in Douro estuary shrimps than in those from the Minho estuary was found, whereas no significant differences in ChEs activity between the two populations were found by Quintaneiro *et al*. (2006).

The higher LPO levels in shrimps from Douro estuary than those from the Minho estuary (Figure 4F) indicates lipid oxidative damage caused by oxidative stress inducers. Among other possibilities, these may be PAHs and/or PCBs and heavy metals (Cunha *et al*., 2005). The higher PO level (Figure 4G) in Minho estuary than in Douro estuary suggests the higher presence of inducers of oxidative damage in protein in Minho estuary, probably due to increasing tourism activities.

4.2.3. Comparative study of *M. galloprovincialis* populations from São Félix da Marinha and Cabo do Mundo coastal zones

The lowest ChEs activity (Figure 5 A) recorded in the mussels from the São Félix da Marinha relatively to those from Cabo do Mundo, suggests higher exposure of the São Félix da Marinha populations to anticholinesterase agents than those of the Cabo do Mundo. The first group well know of chemicals agents that inhibit cholinesterase enzymes are organophosphate and carbamate insecticides, metals among other possible environmental contaminants (Payne *et al*. 1996; Guilhermino *et al*., 1998; Varó*et al*, 2002). This result is not in agreement with expected if the hypothesis that São Félix da Marinha had suffered environmental rehabilitation after oil spill (Tim-Tim *et al*., 2009). In the other hand, previous study found higher levels of ChEs activity in São Félix da Marinha and the lack of effects on ChE in organisms from Cabo do Mundo even after 'Prestige' oil spill (Tim-Tim *et al*., 2009). The result of the present study can be explained partially by the presence of anti-cholinesterase agents inSão Félix da Marinha and some kind of individual tolerance of organisms from Cabo do Mundo since they are exposed chronically to petrochemical contaminants.

Regarding GST enzyme, the higher level in São Félix da Marinha is an indicative of the presence of GST inducers that may be potentially PCBs and/or PAHs, among other environmental contaminants. This result is not in agreement with a previous study performed by Tim-Tim *et al.* (2009) in the same sampling sites that found an increasing of this enzyme after oil spill.

The higher level of CAT in mussels collected in Cabo do Mundo comparatively to from São Félix da Marinha suggests the presence of ROS inducers that may be, among other, heavy metals, PCBs, PAHs. Such contaminants possibly can be found in this site since it is located close to many industries, including an oil refinery, that are potential sources of these environmental contaminants. (Salgado and Serra, 2001; Tim -Tim *et al*., 2009)

4.2.4. Comparative study of *M. lineata* populations from São Félix da Marinha and Cabo do Mundo coastal zones

Increased GST levels found in organisms from Cabo do Mundo suggests an exposure to GST inducers in this site comparatively to São Félix da Marinha. This result is in agreement with studies performed by Tim-Tim *et al*. (2009) in the same sites and with the same species that confirmed an induction of GST enzyme after oil spill. Higher GST activity may be justified by some oil contamination since Cabo do Mundo is chronically exposed to petrochemicals contaminants (Cairrão *et al*., 2004, Moreira e Guilhermino 2005, Cairrão *et al*., 2007) due to its location near to an oil refinery plant. Lima *et al*. (2007) also found higher levels of GST in

mussels collected in Cabo do Mundo comparatively to other sites with apparently lower contamination levels. The present work suggests that GST enzyme in this species is sensitive to petrochemicals contaminants.

Regarding the biomarkers related with oxidative stress, higher CAT activity in organisms collected in São Félix da Marinha may be an indication of the contamination by ROS comparatively to Cabo do Mundo. The induction of this enzyme can justify the lower LPO level in organisms from São Félix da Marinha since this enzyme is responsible for destruction of ROS maintaining redox balance and avoiding oxidative damage (Chaudiere *et al*., 1984)

5. Final considerations

A more conclusive interpretation of these results would probably be achievable through a long-term biomonitoring programme, as is advisable in risk-assessment studies. This type of approach provides a more detailed knowledge of the basal levels of the biomarkers and its seasonal variation in each species, making it possible to distinguish between pollution–induced effects from those derived from the natural biological cycles and intrinsic characteristics of each species (Moreira and Guilhermino, 2005). Thus, more sampling campaigns and chemical analysis of animal tissues, sediment and water, in different seasons, could improve greatly the significance of our results.

6. References

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