Universidade de Aveiro Departamento de Biologia
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METODOLOGIAS PARA UM DESENVOLVIMENTO SUSTENTADO DE ECOSSISTEMAS ESTUARINOS

METHODOLOGIES FOR A SUSTAINABLE DEVELOPMENT OF ESTUARINE ECOSYSTEMS

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Doutor em Biologia, realizada sob a orientação científica da Doutora Lúcia Guilhermino, Professora Catedrática do Instituto de Ciências Biomédicas de Abel Salazar da Universidade do Porto e do Doutor Fernando Morgado, Professor Auxiliar com Agregação do Departamento de Biologia da Universidade de Aveiro.

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To my parents, my grandmother and my sister

To Luísa

"A ciência não é apenas compatível com a espiritualidade;
ela é uma profunda fonte de espiritualidade."

Carl Sagan (1934 - 1996)

O Júri

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palavras-chave

Ria de Aveiro, hidrocarbonetos aromáticos policíclicos, metais, *Pomatoschistus microps*, biomarcadores, performance natatória, bioacumulação, índices de condição, estatística multivariada.

resumo

As zonas costeiras, estuarinas e lagunares são consideradas áreas muito produtivas e dotadas de grande biodiversidade sendo, por isso, consideradas de elevado valor ecológico e económico. No entanto, nas últimas décadas tem vindo a verificar-se um aumento da contaminação destes ecossistemas como resultado de diversas actividades antrópicas. As abordagens actualmente disponíveis para avaliação do impacto da poluição em ecossistemas estuarinos e lagunares apresentam diversos tipos de lacunas, pelo que é importante desenvolver metodologias mais eficazes com organismos autóctones. Neste contexto, o objectivo central desta dissertação consistiu em desenvolver e validar métodos ecologicamente relevantes para avaliação da contaminação estuarina e dos seus efeitos, utilizando o góbio-comum (*Pomatoschistus microps*), quer como organismo-teste quer como espécie sentinela, devido à importante função que desempenha nas cadeias tróficas de diversos estuários da costa Portuguesa. A Ria de Aveiro foi seleccionada como área de estudo principalmente pelo facto de possuir zonas com diferentes tipos de contaminação predominante e de haver conhecimento científico de base abundante e de elevada qualidade sobre este ecosistema.

Na primeira fase do estudo, foram investigados os efeitos agudos de dois hidrocarbonetos aromáticos policíclicos (HAPs) (benzo[a]pireno e antraceno), de um *fuel*-óleo e de dois metais (cobre e mercúrio) em *P. microps*, utilizando ensaios laboratoriais baseados em biomarcadores e em parâmetros comportamentais, os quais foram avaliados utilizando um dispositivo expressamente desenvolvido para o efeito, designado por *speed performance device* (SPEDE). Como biomarcadores foram utilizados parâmetros envolvidos em funções fisiológicas determinantes para a sobrevivência e desempenho dos animais (neurotransmissão, obtenção de energia, destoxificação e defesas anti-oxidantes), nomeadamente a actividade das enzimas acetilcolinesterase, lactato desidrogenase, CYP1A1, glutationa S-transferases, glutationa reductase, glutationa peroxidase, superóxido dismutase, catalase, tendo ainda sido determinados os níveis de peroxidação lipídica como indicador de danos oxidativos. De forma global, os resultados indicaram que os agentes e a mistura testados têm a capacidade de interferir com a função neurológica, de alterar as vias utilizadas para obtenção de energia celular, induzir as defesas antioxidantes e, no caso do cobre e do mercúrio, de causarem peroxidação lipídica. Foram ainda obtidas relações concentração-resposta a nível dos parâmetros comportamentais testados, nomeadamente a capacidade de nadar contra a corrente e a distância percorrida a nadar contra o fluxo de água, sugerindo que os agentes testados podem, por exemplo, diminuir a capacidade de fuga aos predadores, as probabilidades de captura de presas e o sucesso reprodutivo.

Na segunda fase, tendo sido já adaptadas técnicas para determinação de vários biomarcadores em *P. microps* e estudada a sua resposta a dois grupos

resumo

de poluentes particularmente relevantes em ecossistemas estuarinos e lagunares (metais e HAPs), foi efectuado um estudo de monitorização utilizando *P. microps* como bioindicador e que incluiu diversos parâmetros ecológicos e ecotoxicológicos, nomedamente: 20 parâmetros indicativos da qualidade da água e do sedimento, concentração de 9 metais em sedimentos e no corpo de *P. microps*, 8 biomarcadores e 2 índices de condição na espécie seleccionada. A amostragem foi efectuada em quatro locais da Ria de Aveiro, um considerado como referência (*Barra*) e três com diferentes tipos predominantes de contaminação (*Vagueira*, *Porto de Aveiro e Cais do Bico*), sazonalmente, durante um ano. Os resultados obtidos permitiram uma caracterização ecotoxicológica dos locais, incluindo informação sobre a qualidade da água, concentrações de contaminantes ambientais prioritários nos sedimentos e nos tecidos de *P. microps*, capacidade desta espécie para bioacumular metais, efeitos exercidos pelas complexas misturas de poluentes presentes em cada uma das zonas de amostragem nesta espécie e possíveis consequências para a população. A análise multivariada permitiu analisar de forma integrada todos os resultados, proporcionando informação que não poderia ser obtida analisando os dados de forma compartimentalizada. Em conclusão, os resultados obtidos no âmbito desta dissertação indicam que *P. microps* possui características adequadas para ser utilizado como organismo-teste em ensaios laboratoriais (e.g. abundância, fácil manutenção, permite a determinação de diferentes tipos de critérios de efeito utilizando um número relativamente reduzido de animais, entre outras) e como organismo sentinela em estudos de monitorização da poluição e da qualidade ambiental, estando portanto de acordo com estudos de menor dimensão previamente efectuados. O trabalho desenvolvido permitiu ainda adaptar a *P. microps* diversas técnicas bioquímicas vulgarmente utilizadas como biomarcadores em Ecotoxicologia e validá-las quer no laboratório quer em cenários reais; desenvolver um novo bioensaio, utilizando um dispositivo de teste especialmente concebido para peixes epibentónicos baseado na *performance* natatória de uma espécie autóctone e em biomarcadores; relacionar os efeitos a nível bioquímico com parâmetros comportamentais que ao serem afectados podem reduzir de forma drástica e diversificada (e.g. aumento da mortalidade, diminuição do sucesso reprodutivo, redução do crescimento) a contribuição individual para a população. Finalmente, foi validada uma abordagem multidisciplinar, combinando metodologias ecológicas, ecotoxicológicas e químicas que, quando considerada de forma integrada utilizando análises de estatística multivariada, fornece informação científica da maior relevância susceptível de ser utilizada como suporte a medidas de conservação e gestão em estuários e sistemas lagunares.

keywords

Aveiro lagoon, polycyclic aromatic hydrocarbons, metals, *Pomatoschistus microps,* biomarkers, swimming performance, bioaccumulation, condition indexes, multivariate statistics.

abstract

Coastal, estuarine and lagoon ecosystems have been considered of high ecological and economic value due to their considerable productivity and biodiversity. However, in the last decades they have been increasingly contaminated as a result of several anthropogenic activities. Since the currently available approaches present several types of limitations, it is important to develop more effective methodologies with autochthonous organisms. In this context, the central objective of this dissertation was to develop and validate ecologically relevant methodologies for the assessment of estuarine contamination and its effects, using the common goby (*Pomatoschistus microps*) both as test-organism and sentinel species, due to the important role that it plays in food webs of several Portuguese estuaries. The Aveiro lagoon was selected as study area mainly because sites with different types of predominant contamination may be found and a considerable amount of scientific information is available.

In the first phase of the study, the acute effects of two polycyclic aromatic hydrocarbons (PAHs) (benzo[a]pyrene and anthracene), a fuel-oil and two metals, copper and mercury, on *P. microps* were assessed, using laboratory bioassays based on biomarkers and behaviour parameters which were evaluated using a device expressly developed for the purpose thereafter designed as *speed performance device* (SPEDE). Parameters involved in physiological functions crucial for the survival and performance of animals (neurotransmission, energetic metabolism, detoxification and anti-oxidant defences) were used as biomarkers, namely: acetylcholinesterase, lactate dehydrogenase, CYP1A1, glutathione S-transferases glutathione reductase, glutathione peroxidase, superoxide dismutase and catalase. Lipid peroxidation, an indicator of oxidative damage, was also determined. The overall results indicated that the tested agents and the mixture have the capability to interfere with the neurological function of *P. microps*, change the cellular pathways of energy production and induce antioxidant defences. Mercury and copper were also found to cause lipid peroxidation. Furthermore, concentration-response relationships were obtained for behaviour parameters, namely the ability of swimming against water-flow and covered distance when swimming against water-flow, suggesting that exposure of fish to tested chemicals may reduce, for example, their ability to escape from predators, their prey-capture rates and their reproductive success.

In the second phase of the study, after adaptation of biomarkers' techniques to

abstract

P. microps and their validation with two groups of pollutants particularly relevant in estuarine and lagoon ecosystems (metals and PAHs), a monitoring study was performed using *P. microps* as bioindicator, including several ecological and ecotoxicological parameters, namely: 20 parameters indicative of water and sediment quality, concentrations of 9 metals in sediments and *P. microps* tissues, 8 biomarkers and 2 condition indexes in the selected species. Sampling was conducted in four sites of the Aveiro lagoon, a reference (*Barra*) and three contaminated sites with different types of predominant contamination (*Vagueira*, *Aveiro Harbour* and *Cais do Bico*), seasonally during a year. The obtained results allowed the ecotoxicological characterization of sampling areas, including information on water quality, concentrations of metals in sediments and in *P. microps* body, metals bioaccumulation by this species, effects resulting from exposure to different complex mixtures of pollutants present in distinct sampling areas and possible consequences for *P. microps* population. Multivariate analysis allowed the integration of all the relevant results, providing important information which could not be obtained by fragmented data analysis. In conclusion, the results of the present thesis indicate that *P. microps* has suitable characteristics (*e.g.* abundance, easy-maintenance in laboratory, size allowing the determination of different types of effect criteria using a relatively small number of animals, among others) to be used as both test-organism in laboratory tests and sentinel species in monitoring studies, therefore in good agreement with smaller studies previously carried out. This work also allowed the adaptation of several biochemical techniques commonly used as biomarkers in Ecotoxicology to *P. microps* and their validation both in laboratorial conditions and real scenarios; the development of a new bioassay, using a test device specially designed for epibenthic fish, based on swimming performance of a indigenous species and biomarkers; to relate biochemical effects with behavioural endpoints directly related to the individual contribution (e.g. mortality, reproduction, growth) to the evolution of the population. Finally, a multidisciplinary approach combining ecological, ecotoxicological and chemical methodologies was validated. The integration of data from such approach through multivariate analysis provided important information that may be used as scientific support for conservation and management of estuarine and lagoon systems.

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AUTHOR'S DECLARATION

The author declares that the experiments carried out and described within this thesis respect national and international safety regulations and ethical principles for animal welfare.

PUBLICATIONS

The following papers resulted from the experimental work done in the scope of this thesis:

Vieira L.R., Sousa A., Frasco M.F., Lima I., Morgado F., Guilhermino L. 2008. Acute effects of benzo[a]pyrene, anthracene and a fuel oil on biomarkers of the common goby *Pomatoschistus microps* (Teleostei, Gobiidae). *Science of The Total Environment*, **395**: 87-100.

Vieira L.R., Gravato C., Soares A.M.V.M., Morgado F., Guilhermino L. Acute effects of copper and mercury on the estuarine fish *Pomatoschistus microps*: linking biomarkers to behaviour (*accepted for publication in Chemosphere*).

Vieira, L.R., Nogueira A.J.A., Soares A.M.V.M., Morgado F., Guilhermino L. Biomonitoring study in a shallow lagoon using *Pomatoschistus microps* as bioindicator: multivariate approach integrating ecological and ecotoxicological parameters (*to be submitted to Environmental Science and Pollution Research*).

Chapter 1. General Introduction

1.1. INTRODUCTION

In the last decades, the contamination of estuaries and lagoons has been considerably increasing worldwide as a result of anthropogenic activities. These ecosystems are recognized as an important component of continental coasts in terms of their biological importance and utilization by humans (Cooper *et al.*, 1994, Marques *et al.*, 2004), being crucial to the life history and development of many species (Chapman and Wang, 2001). Estuaries and lagoons are interface ecosystems that couple continental and marine environments, receiving bio-geochemical active inputs from land, rivers and coastal seas (Lopes *et al.*, 2005). The importance of estuarine systems and their association to coastal waters have been enhanced by several authors. In fact, they are nursery areas for several species, including fish, and therefore they have a determinant role in supporting the offshore stocks of economically valuable species (Gillanders *et al.*, 2003; Able, 2005). In fact, estuaries are particularly used by juveniles of many fish species because of the potential advantages they provide for growth and survival of young fish, namely high prey availability, refuge from predators and good environmental conditions for a rapid growth (Lenanton and Potter, 1987; Beck *et al.,* 2001). However, with the increase of human population and the industrialization of human societies, these areas have been increasingly impacted with negative effects on the biota. Pollution, which may affects both the biotic and abiotic components of the ecosystems, is one of the main treads to these ecosystems.

In several estuaries and other coastal areas around the world, petrochemical products are one of the main types of environmental contaminants. They may enter into these ecosystems as a result of harbour activities, petrochemical industry, shipping transport and other anthropogenic activities, as well as from natural sources. In the last decades, fuel oil spills such as the recent accident with the tanker *Prestige* in the Galician coast, have highlighted the ecological and socio-economic problems inherent to this class of contaminants (Vieira *et al.*, 2008). Among petrochemical products, fuel-oils are of special concern mainly because their widespread use and toxicity. They are complex mixtures that contain polycyclic aromatic hydrocarbons (PAHs), metals and other compounds (Albaigés and Bayona, 2003). PAHs are known to be determinant for the toxicity elicited by these environmental contaminants to aquatic organisms (Anderson, 1977; Connell and Miller, 1981; Spies, 1987). This class of contaminants have been found to induce adverse effects

on fish growth (Hannah *et al.*, 1982; Ostrander *et al.*, 1990), reproduction (Thomas, 1988, White *et al.*, 1999; Monteverdi, 2000) and survival (Collier and Varanasi, 1991; Hawkins *et al.*, 1991). Furthermore, after biotransformation, these compounds may originate reactive products that bind to DNA causing mutations or other alterations on the genetic material (Hall and Glower, 1990; Marvin *et al.*, 1995; Woodhead *et al.*, 1999), further leading to tumours. Despite the considerable amount of studies that has been performed on the toxicity of PAHs to marine organisms, gaps of knowledge still exist especially on ecological relevant autochthonous fish from the South Europe that are not used for human consumption.

Metals are common contaminants of estuaries and coastal areas. Besides petrochemical products, other sources are industrial and urban effluents and mining activities. They are persistent in the environment, are bioaccumulated by several species and organic forms of some of them (e.g. methylmercury) are biomagnified in food webs. Non-essential heavy metals are usually potent toxicants and their bioaccumulation in tissues may lead to intoxication, decreased fertility, cellular and tissue damage, cell death and dysfunction of a variety of organs (Oliveira-Ribeiro *et al.*, 2000). Essential metals such as copper, magnesium and zinc have normal physiological regulatory functions (Hogstrand and Haux, 2001), but may also be accumulated by organisms reaching toxic levels (Rietzler *et al.*, 2001). As for PAHs, despite the considerable amount of research done on the effects of metals on living organisms, more information is still needed particularly in relation to their effects on non-commercial estuarine species from the South Europe.

The contamination of estuaries and coastal areas by petrochemical products and metals and its effects on wild organisms have been assessed through monitoring programmes, some of them including sub-individual endpoints known as environmental biomarkers (Abreu *et al*., 2000; Viguri *et al*., 2002; Buet *et al*., 2006; Martinez-Gomez *et al*., 2006; Ferreira *et al.*, 2008; Guilherme *et al*., 2008; Guimarães *et al.*, 2009). Since they are measured at a low biological organization level, biomarkers detect early responses to pollution exposure before higher levels of biological organization (e.g. population) become affected. Therefore, they allow the adoption of protective measures before the situation becomes difficult to revert. However, since both biotic and abiotic factors may influence the response of several biomarkers, it is important to have baseline values for key species of the ecosystem to be used as reference. In addition and despite the intensive work that

has been done in the last decades, it still is necessary to standardize and validate protocols for measuring biomarkers in autochthonous species, especially in key species of South Europe ecosystems. Furthermore, integrating data from biomarkers and other biological endpoints with abiotic changes is a priority, since chemicals may induce toxic effects directly on the organisms and/or decrease the quality of environment as life-support with negative effects on the biota.

Among animals inhabiting estuaries and coastal lagoons, fish are of great interest since different species may occupy distinct ecological niches, they are sensitive to several environmental contaminants and some species have economic importance. Consequently, several fish have been used as sentinel species in estuarine and other coastal monitoring programs (Solé *et al.*, 2006; Webb *et al.*, 2005; Arruda *et al.*, 1993; Cabral *et al.*, 2007; Rodrigues *et al*., 2006). One of this species is the common goby, *Pomatoschistus microps* Krøyer (1838) (Figure 1.1.) that is one of the most abundant fish species in estuaries, lagoons and shores of Europe (Salgado *et al.*, 2004; Arruda *et al.*, 1993).

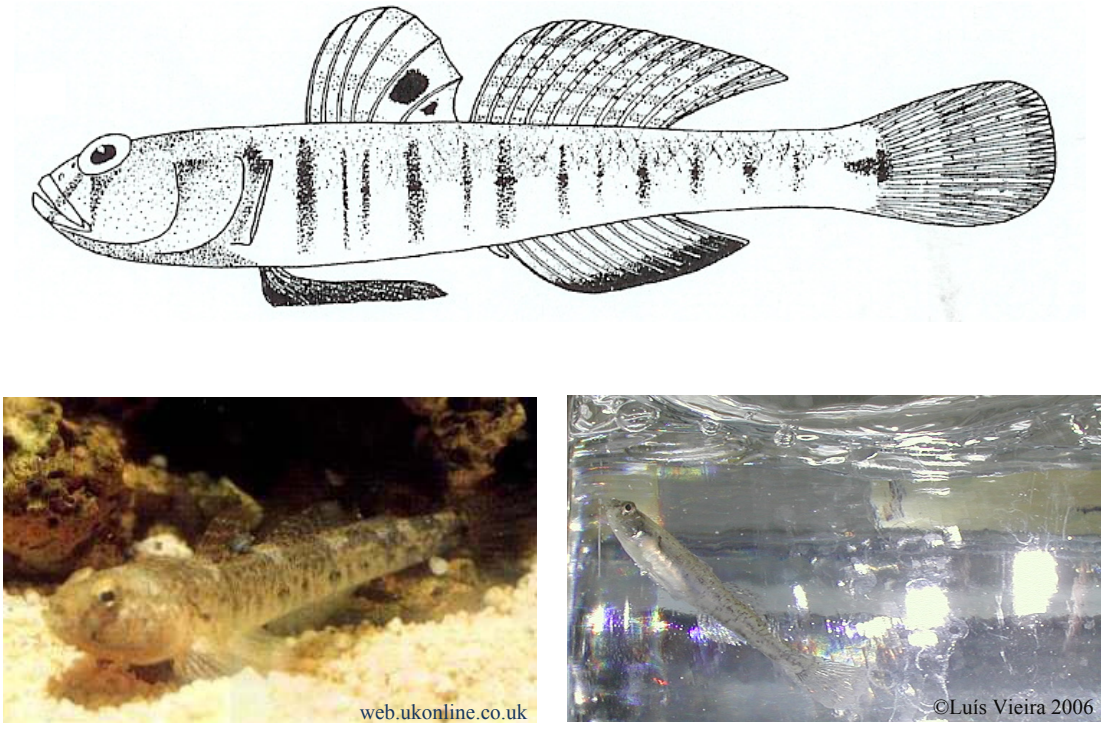

Figure 1.1. – The common goby, *Pomatoschistus microps* (Krøyer, 1838) (figure adapted from Miller *et al.*, 1986).

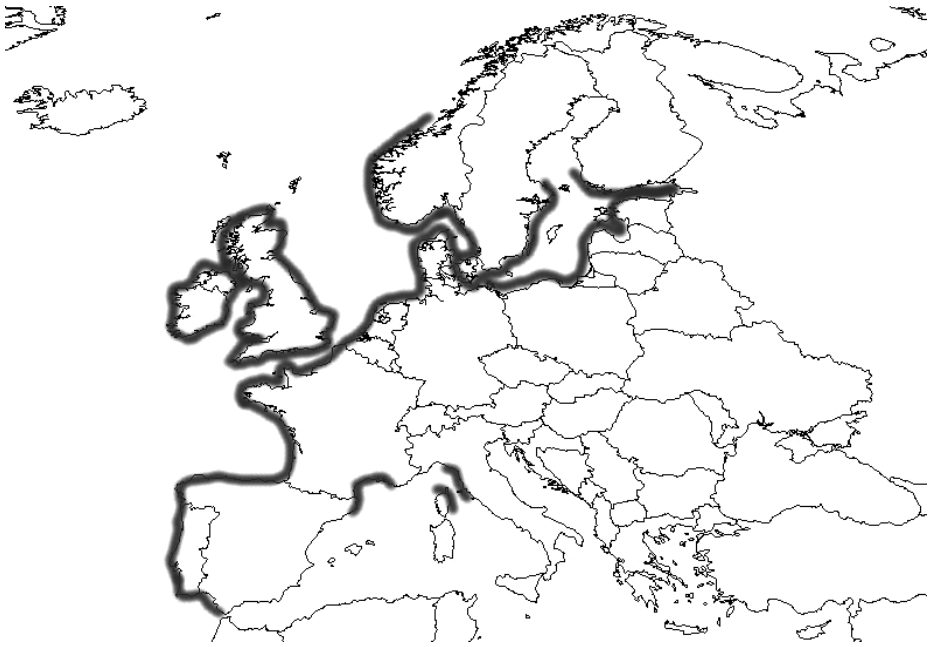

Figure 1.2. – Geographic distribution of *Pomatoschistus microps* (figure adapted from Miller *et al*., 1986).

Its geographic distribution ranges from the coast of Norway to the Gulf of Lion (Miller *et al.*, 1986) (Figure 1.2.) and its great adaptability endows it the potential capacity to successfully occupy different biotopes (Bouchereau and Guelorget, 1998).

P. microps is an epibenthic euryhaline small fish (11-64 mm long) living in semi-enclosed lagoon-like environments (Pampoulie, 2001) where it has an important function as intermediary predator, feeding on plankton, macro- and meiofauna and being prey of several larger fishes and birds (Doornbos and Twisk, 1987; Miller *et al.*, 1986; Arruda *et al.*, 1993). Adults feed at the surface of the sediment on amphipods, isopods, chironomid larvae and polychaetes, while the juveniles' diet consists largely of interstitial copepods (Ehrenberg *et al.*, 2005; Zloch *et al.*, 2005). It is able to spend its entire life cycle within an estuary (Healey, 1971).

In the NW coast of Portugal, the common goby is an abundant species that can be collected all over the year in areas with different types and levels of environmental contamination. In addition to its ecological relevance, it is easy to maintain in the laboratory, it is sensitive to several chemicals that occur as environmental contaminants in estuaries and other coastal ecosystems, and it was successfully used both as test organism and bioindicator in previous studies (Monteiro *et al.*, 2006, 2007).

1.2. GENERAL AND SPECIFIC OBJECTIVES OF THE THESIS

The central objective of the present study was to develop and validate ecologically relevant methodologies to assess the effects of pollution on estuarine and other coastal ecosystems using autochthonous fish as test organisms and sentinel species. To attain this central objective, in a first phase of the study, protocols for measuring several biomarkers in *P. microps* tissues were adapted and validated, a new device and protocols for measuring behavioural parameters in this species were developed and validated and the effects of common pollutants of estuarine areas on the common goby were investigated in laboratorial conditions. Then, in the second phase of the study, an approach integrating ecological and ecotoxicology parameters and multivariate statistics was validated in the Aveiro lagoon taking advantage of the existence of sites with different types of main pollution.

Therefore, the specific objectives of the present study were:

(i) To investigate the effects of two different PAHs and a complex petrochemical mixture on the common goby, *Pomatoschistus microps*, using selected biomarkers as effect criteria.

(ii) To investigate possible links between biomarkers and swimming performance in the estuarine fish *Pomatoschistus microps* acutely exposed to metals (copper and mercury).

(iii) To validate an integrated approach, including ecological and ecotoxicological parameters and to evaluate the effects of pollution on estuarine fish in real scenarios, using the common goby *Pomatoschistus microps* (Krøyer, 1838) as bioindicator and the Aveiro lagoon (NW coast of Portugal) as case study area.

1.3. OUTLINE OF THE THESIS

The general thesis framework with specific aims of each section is presented in Figure 1.3.

It is structured in five chapters:

Chapter 1. General introduction

Introduces the work and explains the structure of the thesis.

Chapter 2. Acute effects of benzo[a]pyrene, anthracene and a fuel oil on biomarkers of the common goby *Pomatoschistus microps* (Teleostei, Gobiidae)

In this chapter, the protocols for measuring several biomarkers were adapted to *P. microps* and they were used as endpoints to evaluate the effects of two PAHs (benzo[a]pyrene and anthracene) and a fuel-oil on this species.

Chapter 3. Acute effects of copper and mercury on the estuarine fish *Pomatoschistus microps*: linking biomarkers to behaviour

Biomarkers have been considered by some authors has having low ecological relevance because they are sub-individual parameters. Therefore, a top issue in Ecotoxicology is to establish relationships between biomarkers and parameters with higher ecological relevance. In the present chapter, the information provided by biomarkers was related to the information provided by behaviour endpoints using a device developed and validated specifically for this purpose: the Swimming Performance Device (SPEDE) that was designed to measure swimming resistance to water-flow and covered distance while swimming against water-flow, with epibenthic fish. Here, the effects of metals (Hg and Cu) on *P. microps* were investigated.

Chapter 4. Biomonitoring study in a shallow lagoon using *Pomatoschistus microps* as bioindicator: multivariate approach integrating ecological and ecotoxicological parameters

Since laboratorial bioassays are not enough to assess the effects of pollution in complex ecosystems such as estuaries and lagoons where complex mixtures of contaminants are present, field studies are necessary. Among these, monitoring programmes are of high importance especially when including parameters measured in wild populations of autochthonous species. Therefore, in this chapter, an integrated approach, including ecological and ecotoxicological parameters was used to evaluate the effects of pollution on estuarine fish in real scenarios, using the common goby as bioindicator and the Aveiro lagoon (NW coast of Portugal) as case study area. The selected approach included fourteen water quality variables, sediment characteristics, the concentrations of nine metals in sediments and in the fish body, fish condition indexes, eight biomarkers and multivariate statistics (Redundancy and Principal Response Curves analysis) to integrate the information provided by different parameters.

Chapter 5. Concluding Remarks

This section makes some final remarks based on the conclusions of different studies carried out.

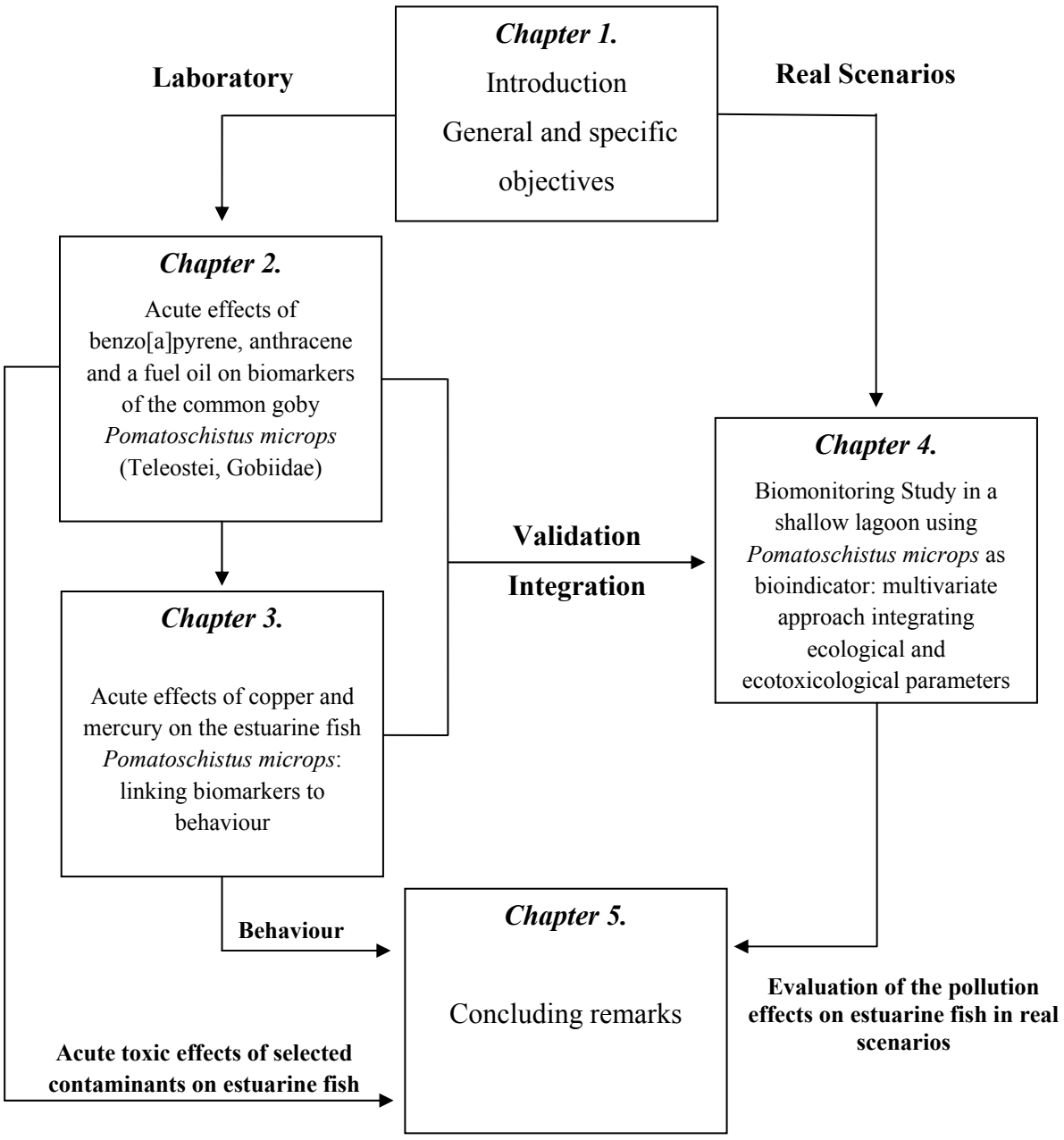

Figure 1.3. - Framework of the thesis with specific aims.

1.4. REFERENCES

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Chapter 2. Acute effects of benzo[a]pyrene, anthracene and a fuel oil on biomarkers of the common goby *Pomatoschistus microps* (Teleostei, Gobiidae)

Acute effects of benzo[a]pyrene, anthracene and a fuel oil on biomarkers of the common goby *Pomatoschistus microps* (Teleostei, Gobiidae).

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2.1. ABSTRACT

The objective of this study was to investigate the effects of two different PAHs and a complex petrochemical mixture on the common goby, *Pomatoschistus microps*, using selected biomarkers as effect criteria. Benzo[a]pyrene (BaP) and anthracene were used as reference substances, while the water accommodated fraction of #4 fuel-oil (#4 WAF) was used as an example of a petrochemical mixture. *P. microps* was used since it is both a suitable bioindicator and a good test organism. Groups of fish were exposed to different concentrations of each of the test substances for 96 h and the activities of several enzymes commonly used as biomarkers were determined at the end of the bioassays. All the substances inhibited *P. microps* acetylcholinesterase (AChE) indicating that they have at least one mechanism of neurotoxicity in common: the disruption of cholinergic transmission by inhibition of AChE. An induction of lactate dehydrogenase (LDH) activity was found in fish exposed to BaP or to anthracene, suggesting an increase of the anaerobic pathway of energy production. On the contrary, inhibition of LDH was found in fish exposed to #4 WAF, suggesting a distinct effect of the mixture. An induction of *P. microps* glutathione S-transferase (GST) activity was found in fish exposed to BaP or to #4 WAF, while an inhibition was observed after exposure to anthracene. These results suggest that GST is involved in the detoxification of BaP and #4 WAF, but not of anthracene. All the substances increased catalase activity and isolated PAHs also increased superoxide dismutase, glutathione reductase and glutathione peroxidase activities, while #4 WAF did not cause significant alterations on these enzymes. These results suggest that all the substances may induce oxidative stress on *P. microps*, with BaP and anthracene apparently having more oxidative stress potential than #4 WAF.

Keywords: *Pomatoschistus microps*, acetylcholinesterase, lactate dehydrogenase, glutathione S-transferases, anti-oxidant enzymes, benzo[a]pyrene, anthracene, fuel oil

2.2. INTRODUCTION

Coastal and estuarine areas are productive ecosystems with a high biodiversity, and, thus, they are considered of great ecologic and economic value. Petrochemical products may enter into aquatic ecosystems as a result of harbour activities, petrochemical industry, shipping transport and other anthropogenic activities, as well as from natural sources. In the last decades, fuel oil spills such as the recent accident with the tanker *Prestige* in the Galician coast, have highlighted the ecological and social-economic problems inherent to this class of contaminants.

The NW coast of Portugal belongs to the so-called "risk" area of the Iberian coast regarding shipping accidents due to adverse sea conditions in some periods of the year, maritime currents and characteristics of the coast that make it particularly dangerous for navigation (Lima *et al.*, 2007). Therefore, it is very important to recognize in advance the effects of fuel oils, polycyclic aromatic hydrocarbons (PAHs) and other components of petrochemical products on native organisms, considered suitable for use in the assessment of the impact of potential accidents. Basic knowledge about the potential adverse effects on wild species is also crucial to mitigate effects and to help in population recovery if necessary.

Among petrochemical products, fuel-oils are of special concern because they are widespread in aquatic ecosystems and have been found to have a high toxicity to aquatic organisms. They are complex mixtures that contain PAHs, metals and other compounds (Albaigés and Bayona, 2003). PAHs are known to be determinant for the toxicity elicited by these environmental contaminants to aquatic organisms (Anderson, 1977; Connell and Miller, 1981; Spies, 1987).

Petrochemical products and/or PAHs have been found to induce adverse effects on fish growth (Hannah *et al.*, 1982; Ostrander *et al.*, 1990), reproduction (Thomas, 1988; White *et al.*, 1999; Monteverdi, 2000) and survival (Collier and Varanasi, 1991; Hawkins *et al.*, 1991). Furthermore, after biotransformation, these compounds may originate

reactive products that bind to DNA and may cause mutations or other alterations on the genetic material (Hall and Glower, 1990; Marvin *et al.*, 1995; Woodhead *et al.*, 1999). For example, in fish, the PAH benzo[a]pyrene (BaP) was found to cause mutations in the oncogene *ras* (Rotchell *et al.*, 2001), while the PAH anthracene was found to alter gene expression in the mummichog (*Fundulus heteroclitus*) (Peterson and Bain, 2004). In this species and in the steelhead trout (*Salmo gairdneri*), carcinogenic effects induced by BaP exposure were also found (Black *et al.*, 1988).

In fish, BaP and PAHs in general, are subject to biotransformation in a first step by enzymes of the P450 system. An induction of cytochrome P4501A (CYP1A) has been found in several species exposed to these xenobiotics, including in the Arctic charr (*Salvelinus alpinus*) (Wolkers *et al.*, 1996), in the common carp (*Cyprinus carpio*) (Van der Weiden *et al.*, 1993), in the European eel (*Anguilla anguilla*) (Lemaire-Gony and Lemaire, 1992) and in the turbot (*Scophthalmus maximus*) (Peters *et al.*, 1997). In this first step of the biotransformation of these compounds, several metabolites are formed, some of which are subject to further transformation by conjugation with endogenous substances. A possible pathway is the conjugation with glutathione, a reaction catalysed by glutathione S-transferases (GST), a family of enzymes that is also involved in the prevention of lipid peroxidation (LPO). Glutathione conjugation seems to be an important pathway of detoxification of BaP, at least in some species, since an induction of GST activity has been found in fish exposed to this xenobiotic, including in the Japanese sea bass (*Lateolabrax japonicus*) (Jifa *et al.*, 2006) and in the sea bass (*Dicentrarchus labrax*) (Gravato and Guilhermino, 2009). However, inhibition of GST activity after exposure to BaP has also been found, for example in the rockfish *Sebastiscus marmoratus* (Wang *et al.*, 2006). Therefore, the role of this enzyme on PAHs detoxification in fish deserves further research.

PAHs have been also found to induce oxidative stress and to cause lipid peroxidation (LPO) in several fish species (Orbea *et al.*, 2002; Reid and MacFarlane, 2003; Jifa *et al.*, 2006; Gravato and Guilhermino, 2009). However, distinct and even contradictory effects of PAHs and fuel oils on anti-oxidant enzymes have been reported. For example, catalase (CAT) activity was found to be increased in the sea bass (*D. labrax*) exposed to BaP (Gravato and Guilhermino, 2009) but no changes were found in the same species exposed to 3-methylcholanthrene (3MC) (Lemaire *et al.*, 1996), suggesting that distinct substances may have different effects on this enzyme. In addition, an opposite

answer of anti-oxidant enzymes over time has been also reported. For example, during the first days of exposure of the goldfish (*Carassius auratus*) to the water-soluble fraction of a diesel oil, an increase of superoxide dismutase (SOD) activity was observed, while in the next days a gradual decrease was recorded (Zhang *et al.*, 2004), indicating that the time of exposure may also induce different answers from anti-oxidant enzymes. Furthermore, a sort of bell-shaped pattern for these enzymatic activities in response to the increase of the concentration of PAHs has been reported for several fish, including the sea bass (*D. labrax*) and the rockfish (*S. marmoratus*): the activity increases until a certain concentration and then progressively decreases despite the increase of the exposure concentration (Wang *et al.*, 2006; Gravato and Guilhermino, 2009). Therefore, since anti-oxidant enzymes of fish have been used as biomarkers in areas polluted with petrochemical products, it is convenient to clarify their pattern of answer to petrochemical products and their components.

Another enzyme that has been used as an environmental biomarker is lactate dehydrogenase (LDH) which is a key enzyme in the anaerobic pathway of energy production, being particularly important for muscular physiology in conditions of chemical stress when high levels of energy may be required in a short period of time (De Coen *et al.*, 2001). Also in the case of fish LDH, contradictory answers to PAHS and fuel oils exposure can be found in the literature. For example, Tintos *et al.* (2008) observed no significant effects of BaP on rainbow trout (*Oncorhynchus mykiss*) LDH activity, while an increase of LDH activity was found in the crimson-spotted rainbowfish (*Melanotaenia fluvialis*) exposed to the WAF of a dispersed crude oil (Pollino and Holdway, 2003) and inhibition of this enzymatic activity was found in the Atlantic salmon (*Salmo salar*) exposed to the WAF of "Bass Strait" crude oil (Gagnon and Holdway, 1999). Thus, the effects of PAHs on LDH also require more investigation.

Another important aspect regarding the toxicity of PAHs and fuel-oils is the potential that some of these compounds and mixtures seem to have to inhibit the activity of acetylcholinesterase (AChE) and, thus, to disrupt cholinergic neurotransmission. This is an effect that may have severe repercussions in functions determinant for the survival and performance of the organism, such as feeding, predator avoidance, swimming and survival to toxicant exposure. In fact, several recent studies performed with invertebrates and fish reported inhibition of this enzyme after exposure to fuel-oil and/or to PAHs (Moreira *et al.*,

2004; Zapata-Pérez *et al.*, 2004; Barsiene *et al.*, 2006). However, no effects on AChE in fish exposed to PAHs have been also reported (Jifa *et al.*, 2006). Therefore, this is also a subject that needs further research since this enzyme has been used in biomonitoring studies in estuarine and coastal ecosystems contaminated with petrochemical products (Bucalossi *et al.* 2006; Lehtonen and Schiedek, 2006; Monteiro *et al.*, 2007).

The common goby, *Pomatoschistus microps* Krøyer (1838), is among the most abundant fish species in estuaries, lagoons and shores of Europe (Arruda *et al.*, 1993; Salgado *et al.*, 2004), with a geographic distribution ranging from the coast of Norway to the Gulf of Lion (Miller *et al.*, 1986). It has an important function in estuarine ecosystems, since it is an intermediary predator in food webs connecting macro- and meiofauna with larger predator fish (Miller *et al.*, 1986; Arruda *et al.*, 1993). In the NW coast of Portugal, it has been found in both reference and contaminated estuaries, including in those impacted by petrochemical products. It is both a suitable test organism and a good bioindicator (Monteiro *et al.*, 2005, 2006a). In addition, it was validated for use as a sentinel species in the European project EROCIPS (INTERREG III B "Atlantic Area", code 168 – EROCIPS) aimed to develop and validate methods for the integrated assessment of the impact of oil spills and other accidents resulting from shipping activities in coastal and estuarine ecosystems. Due to these favourable characteristics, it was used as test organism in the present study.

The mechanisms of toxicity and detoxification of fuel oils and of PAHs in fish are not fully understood and contradictory effects on enzymes commonly used as biomarkers have been reported. Therefore, the central objective of the present study was to investigate the effects of two different PAHs and a complex petrochemical mixture on *P. microps*, using selected biomarkers as effect criteria. This will allow going inside the mechanisms of toxicity of these compounds in fish, also giving a contribution to understand the different results that have been published in the literature about the effects of these pollutants on enzymes commonly used as biomarkers, such as AChE, GST, LDH and anti-oxidant enzymes namely CAT, SOD, glutathione peroxidase (GPx) and glutathione reductase (GR).

Benzo[a]pyrene (BaP) and anthracene were used as reference substances, since a considerable amount of literature about their effects on aquatic organisms exist. In addition, they are included in the list of priority pollutants of the US Environmental

Protection Agency (US EPA) due to their toxicological features (EPA, 1995), they are common contaminants of estuaries, coastal areas and other aquatic ecosystems and they have been also found in tissues of fish (Baumard *et al.,* 1998). The #4 fuel-oil (Anglo-Saxon terminology) was used as an example of a widely used fuel oil that frequently is released in the environment during anthropogenic activities, such as marine traffic. A fuel oil was tested because this type of petrochemical mixtures may induce toxic effects considerably different from their individual components due to toxicological interactions.

2.3. MATERIAL AND METHODS

2.3.1. Chemicals

Benzo[a]pyrene (CAS no. 50-32-8) and anthracene (CAS no. 120-12-7) were purchased from Sigma–Aldrich Chemical (Steinheim, Germany), and were used with 97% and >99% purity, respectively. The #4 fuel oil (Anglo-saxon terminology) was kindly provided by Dr. Jorge Ribeiro from "PETROGAL" (Galp Energia, SGPS, SA, Portugal). The chemicals for enzymatic analysis were acquired from Sigma–Aldrich Chemical (Steinheim, Germany), except acetone (Merck, Darmstadt, Germany) and the Bradford reagent (Bio-Rad, Munich, Germany).

2.3.2. Sampling of P. microps

P. microps juveniles (2.5 – 3 cm long) were captured during Spring in a low-impacted site in the Minho river estuary (41º 53' 26.8''N, 8º 49' 29.2''W) (NW of Portugal) (Figure 2.1.). River Minho estuary was chosen for fish sampling due to its characteristics of low urban industrial and agricultural contamination (Ferreira *et al.*, 2003) and because it has been used as a reference estuary in previous studies with this species (Monteiro *et al.*, 2005, 2006a).

Fish were collected using a hand operated net at low tide. During the collection period, water salinity changed from 6 to 8 and the water temperature from 18.3 to 19.5 ºC. Selected specimens were immediately transported to the laboratory in 30L containers with aeration. In the laboratory, fish were submitted to an acclimation period of two weeks in artificial medium which was prepared by dissolving aquarium salt (SERA® Premium – Sea

Salt – D52518 Heinsberg, Germany) in distilled water until reaching a salinity of 6; after stirring in a vortex with a magnetic stirrer for about 20 minutes, the salinity was again measured and corrected if necessary. The medium was changed every other day. Fish were kept in 60 L glass aquaria with internal filters and an aeration system, in a photoperiod (16h L: 8h D) and temperature controlled room (20±1^{o}C) and fed with commercial food (TetraMin®).

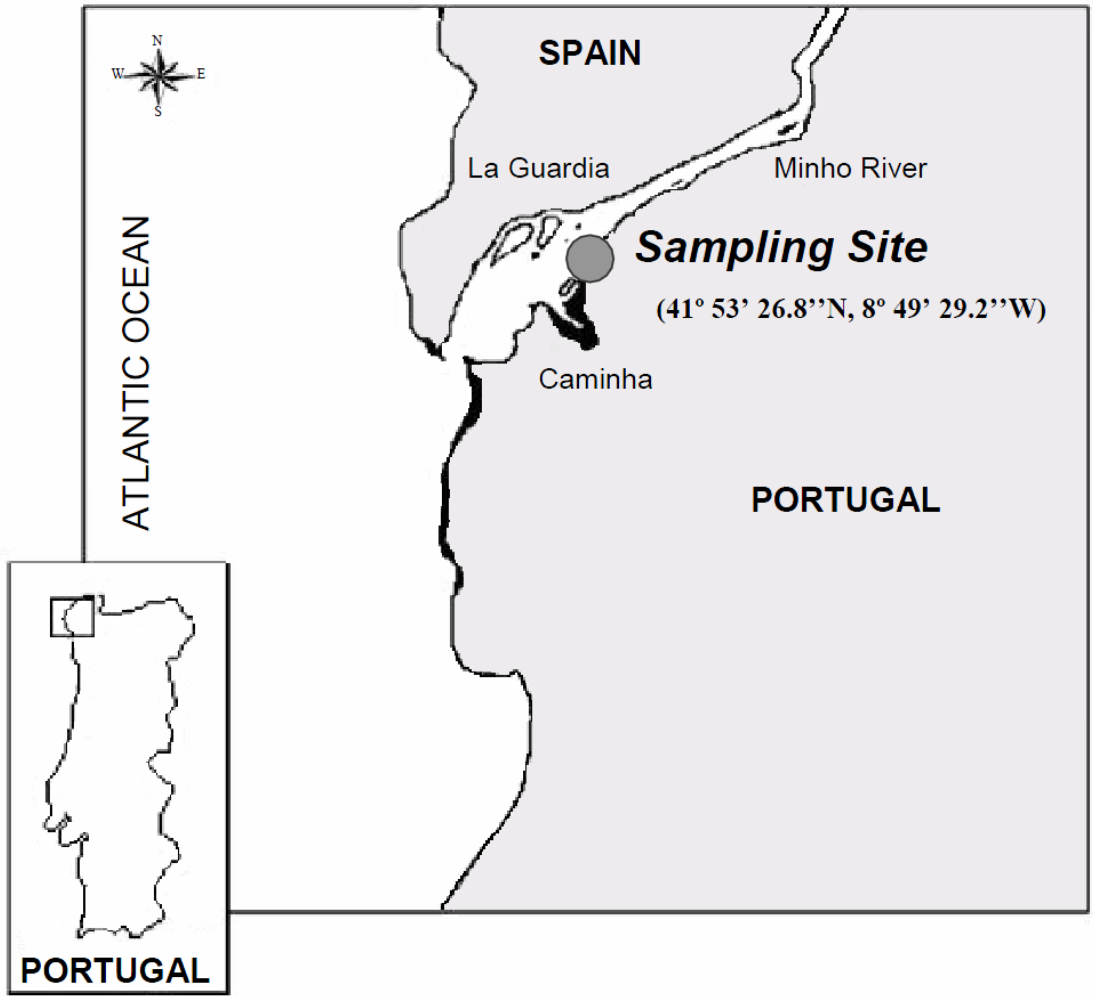

Figure 2.1. – Map of the Minho river estuary (NW Portugal) showing the location of the sampling site (41° 53′ 26.8″N, 8° 49′ 29.2″W).

2.3.3. Laboratorial toxicity tests

The experimental design generally followed recommendations of OECD guidelines (OECD, 1993), with the modifications indicated below.

Stock solutions of BaP (10 mg/L) and anthracene (5 mg/L) were prepared in 50% (v/v) of acetone/ultra-pure water. Test solutions of each chemical (1, 2, 4, 8 and 16 µg/L for BaP; and 0.25, 0.5, 1, 2 and 4 µg/L for anthracene) were prepared by dilution of the respective stock solution in the artificial medium (salinity 6). These concentrations were selected based on the results of previous LC_{50} assays. Although the ranges tested included concentrations equal for both PAHs (1, 2 and 4 µg/L), they differed in the lower and upper concentrations due to the differences of acute toxicity of BaP and anthracene to *P. microps*. The petrochemical mixture tested was the #4 fuel oil. Its WAF was prepared by stirring 100 g of fuel-oil per litre of medium (salinity 6), in a vortex with a magnetic stirrer for 24 hours in the dark, at 20 ºC, as described by Singer *et al.* (2000). This stock solution was diluted with artificial medium to obtain the tested sublethal concentrations: 7.5, 15 and 30 % (v/v). In each bioassay the control (artificial medium) and a second control with acetone in the maximum concentration used in test solutions (0.8 mL of acetone per litre) were included in the experimental design.

For each toxicity test, twenty-seven fish were used per treatment. They were individually exposed for 96 hours to 500 mL of each test solution in 1 L glass recipients. During the tests, photoperiod, temperature and aeration conditions were similar to those used in the acclimation period, and no food was provided. Medium temperature, salinity, conductivity, pH, O_2 concentrations were monitored every 24 hours.

2.3.4. Biological material

Following the exposure period, fish were sacrificed by decapitation. All tissues were isolated, homogenized (Ystral homogenizer, Ballrechten-Dottingen, Germany) in appropriate buffers, and centrifuged (SIGMA 3K 30) at 4 ºC. One head, one dorsal muscle and two gills were used for AChE (phosphate buffer 0.1 M, pH 7.2), LDH (Tris-NaCl buffer 0.1 M, pH 7.2), and GST (phosphate buffer 0.1 M, pH 6.5) determinations, respectively. A pool of three livers was used for determination of CAT (phosphate buffer 50 mM, pH 7.0), SOD (phosphate buffer 50 mM, pH 7.8, with 1 mM Na_2EDTA), GPx and GR (phosphate buffer 0.1 M, pH 7.5). Following homogenization, samples were

centrifuged for 3 min at 3300g for AChE and LDH, 30 min at 9000g for GST, and 15 min at 15000g for SOD, CAT, GR and GPx determinations. Finally, supernatants were recovered and kept at -80 ºC until being used for enzymatic determinations.

2.3.5. Enzymatic activities

In a previous study, it was found that the soluble fraction of *P. microps* head homogenates contain mainly acetylcholinesterase (AChE) (Monteiro *et al.*, 2005). Therefore, AChE activity was determined according to Ellman's method (Ellman *et al.*, 1961) adapted to microplate (Guilhermino *et al.*, 1996), using 0.500 mL of fish head homogenate. *P. microps* LDH was determined by the method of Vassault (1983) adapted to microplate (Diamantino *et al.*, 2001). GST was assessed according to Habig *et al.* (1974), with some modifications of the original protocol (Frasco and Guilhermino, 2002). The activities of GR (Carlberg and Mannervik, 1975), GPx (Flohé and Günzler, 1984) and SOD (McCord and Fridovich, 1969) were also adapted to microplate (Lima *et al.*, 2007). All these enzymatic activities were measured in a microplate reader BIO-TEK, model POWERWAVE 340. CAT activity was measured according to the method of Aebi (1984) in a spectrophotometer JENWAY, model 6405 UV/VIS. Protein content was determined by the Bradford method (Bradford, 1976) adapted to microplate. Enzymatic activities were determined at 25 ºC and expressed as activity per mg of protein. One unit (U) of SOD activity was defined as the amount of enzyme required to inhibit the rate of reduction of cytochrome *c* by 50%. For CAT activity, U was defined as 1 μmol/min and for the remaining enzymes as 1 nmol/min.

2.3.6. Statistical Analyses

Statistical analyses were performed using STATISTICA 6.0© software package. For each enzyme, different treatments were compared using one-way analysis of variance (ANOVA). Dunnett's test was applied if significant differences among different treatments were detected by ANOVA (Zar, 1996). For each biomarker, the normality of data from the different treatments was tested (Kolmogorov-Smirnov normality test) and the homogeneity of variance was verified (Barlett's test) (Zar, 1996).

2.4. RESULTS

In the bioassays with BaP and anthracene and for all the enzymatic determinations performed, no significant differences were found between fish of the control and acetone control groups (Figures 2.2. and 2.3.).

2.4.1. Effects of benzo[a]pyrene

BaP caused a significant inhibition of AChE activity ($F_{(6, 56)} = 10.404$, $p \leq 0.05$; LOEC = 2 µg/L), with more than 30% of inhibition at the concentrations equal or higher than 4 µg/L (Figure 2.2.A). Fish exposed to BaP showed an increase of LDH activity ($F_{(6, 56)} = 91.222$, $p \leq 0.05$; LOEC = 1 µg/L), with 83% of increase at the highest concentrations tested (Figure 2.2.B). They also showed an induction of GST activity ($F_{(6, 14)} = 9.028$, $p \leq 0.05$; LOEC = 4 µg/L) with 17% of increase at 4 µg/L and 23% of increase at 8 µg/L (Figure 2.2.C). The activity of all the enzymes involved in the antioxidant defences was significantly increased in fish exposed to BaP (CAT: $F_{(6, 14)} = 3.329$, $p \leq 0.05$; SOD: $F_{(6, 14)} = 5.338$, $p \leq 0.05$; GR: $F_{(6, 14)} = 11.230$, $p \leq 0.05$; GPx: $F_{(6, 14)} = 39.005$, $p \leq 0.05$), with LOECs of 4 µg/L for CAT and GPx and 16 µg/L for SOD and GR (Figures 2.2.D, E, F, G).

2.4.2. Effects of anthracene

Significant differences between the control and anthracene exposed fish were found for all the enzymes: AChE: $F_{(6, 46)} = 4.128$, $p \leq 0,05$; LDH: $F_{(6, 41)} = 48,223$; GST: $F_{(6, 14)} = 49.682$, $p \leq 0,05$; CAT: $F_{(6, 14)} = 5.876$, $p \leq 0,05$; SOD: $F_{(6, 14)} = 12.777$, $p \leq 0,05$; GR: $F_{(6, 12)} = 5.991$, $p \leq 0,05$ and GPx: $F_{(6, 12)} = 6.434$, $p \leq 0,05$). A significant decrease of AChE activity was found at the highest tested concentration (LOEC = 4 µg/L), corresponding to 52% of inhibition (Figure 2.3.A). LDH activity was induced by concentrations equal or highest than 0.25 µg/L, with 99% of induction of the highest concentration tested (Figure 2.3.B). GST activity was inhibited by concentrations equal or highest than 0.5 µg/L, with 42% of inhibition of the highest tested concentration (Figure 2.3.C).

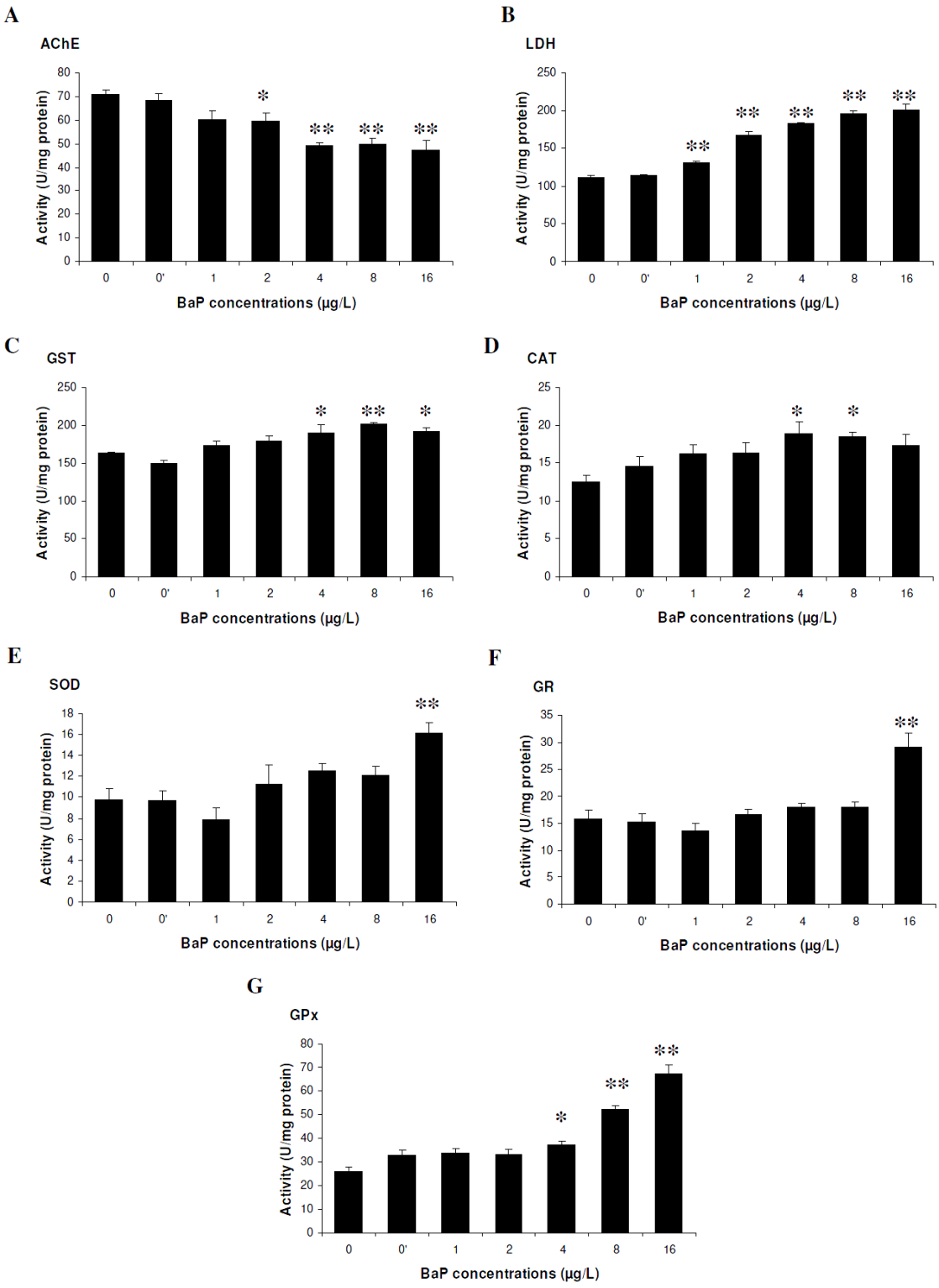

Figure 2.2. – Effects of benzo[a]pyrene on (A) AChE, (B) LDH, (C) GST, (D) CAT, (E) SOD, (F) GR and (G) GPx activities of *P. microps*. Values indicate the means ± S.E.M. (n=27). 0 – Control; 0´- Solvent control; * - Significantly different from the control group ($p \leq 0.05$ Dunnett Test); ** - Significantly different from the control group ($p \leq 0.01$ Dunnett Test). U/mg protein = 1 µmol/min for CAT activity, the amount of enzyme required to inhibit the rate of reduction of cytochrome *c* by 50% for SOD activity and 1 nmol/min for the other enzymes.

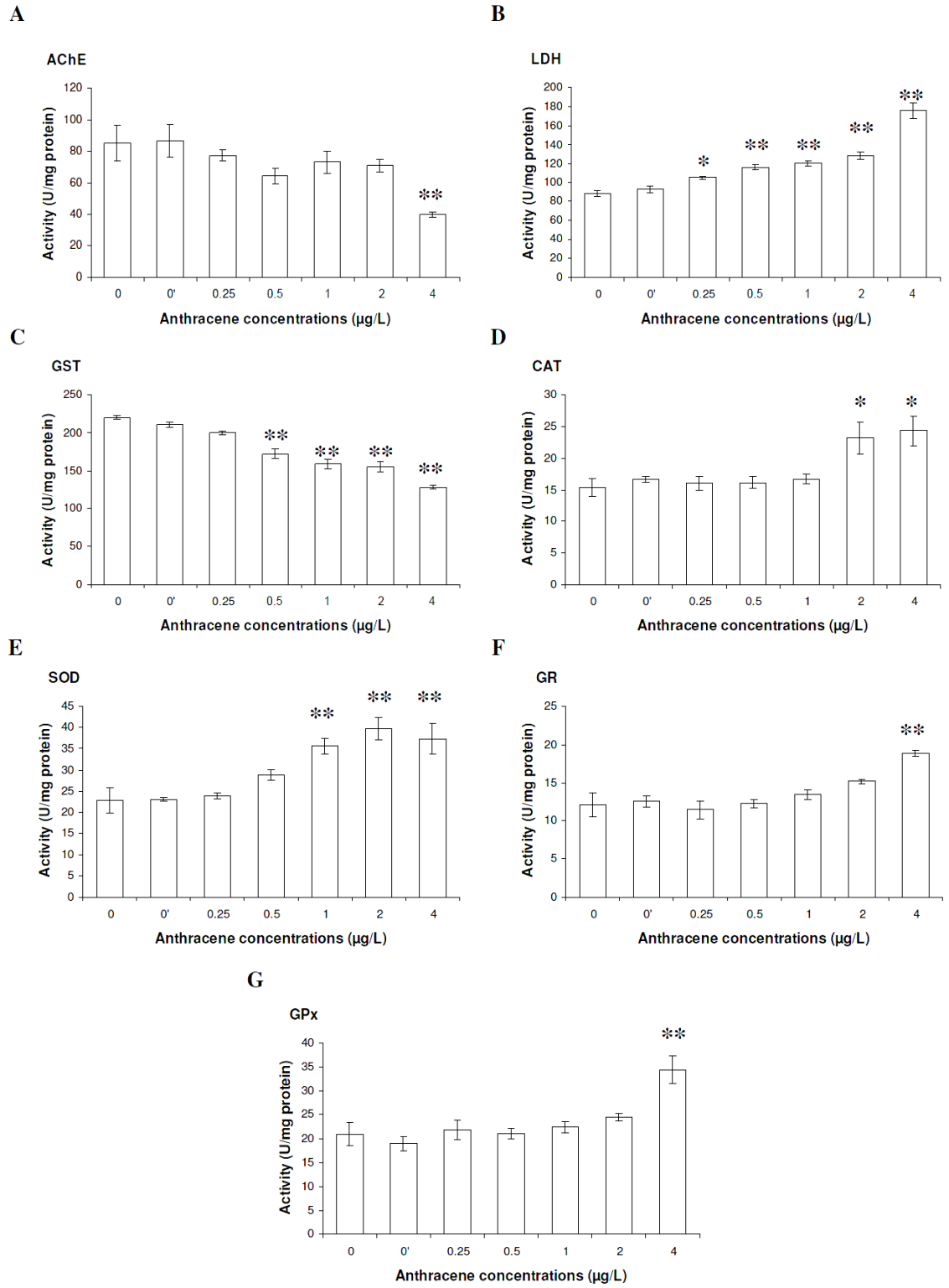

Figure 2.3. – Effects of anthracene on (A) AChE, (B) LDH, (C) GST, (D) CAT, (E) SOD, (F) GR and (G) GPx activities of *P. microps*. Values indicate the means ± S.E.M. (n=27). 0 – Control; 0´- Solvent control; * - Significantly different from the control group ($p \leq 0.05$ Dunnett Test); ** - Significantly different from the control group ($p \leq 0.01$ Dunnett Test).U/mg protein = 1 µmol/min for CAT activity, the amount of enzyme required to inhibit the rate of reduction of cytochrome *c* by 50% for SOD activity and 1 nmol/min for the other enzymes.

The activity of all the enzymes involved in the antioxidant defences was induced in exposed fish with LOECs of 2 µg/L for CAT (Figure 2.3.D), 1 µg/L for SOD (Figure 2.3.E) and 4 µg/L for GR and GPx (Figures 2.3.F, G). Percentages of induction at the highest tested concentration were 58% for CAT, 63% for SOD, 55% for GR and 64 % for GPx.

2.4.3. Effects of #4 WAF

Significant effects on *P. microps* AChE ($F_{(3, 29)} = 27.700$, $p \leq 0.05$), LDH ($F_{(3, 28)} = 7.491$, $p \leq 0.05$), GST ($F_{(3, 8)} = 30.937$, $p \leq 0.05$) and CAT ($F_{(3, 8)} = 21.477$, $p \leq 0.05$) were found in fish exposed to #4 WAF (Figure 2.4.).

AChE activity was significantly inhibited, with a LOEC of 7.5% of #4 WAF and an inhibition of 46% at the highest concentration tested (30%) (Figure 2.4.A). LDH activity was significantly decreased in fish exposed to concentrations equal or highest than 7.5% of #4 WAF, with 39% of inhibition at the highest concentration tested relatively to the control group (Figure 2.4.B). The activity of both GST and CAT was increased following exposure to #4 WAF with LOECs of 15% #4 WAF (Figures 2.4.C, D) and percentages of induction relatively to the control group of 43% and 305%, respectively. No significant differences were found in SOD, GR and GPx activities following exposure to #4 WAF (Figures 2.4.E, F, G).

2.5. DISCUSSION

In the present study, the acute toxicity of two well-known PAHs (BaP and anthracene) and of the #4 WAF to *P. microps* was investigated using enzymatic biomarkers as effect criteria. Enzymatic activities in non-exposed fish, *i.e.*, determined using the control groups of the three bioassays performed, are similar to those reported in previous studies with fish (Table 2.1.).

The concentrations of BaP (1 to 16µg/L) and anthracene (0.25 to 4 µg/L) tested in the present study can be considered ecologically relevant since they have been found in sediments, water column and organisms from estuaries polluted with petrochemical products.

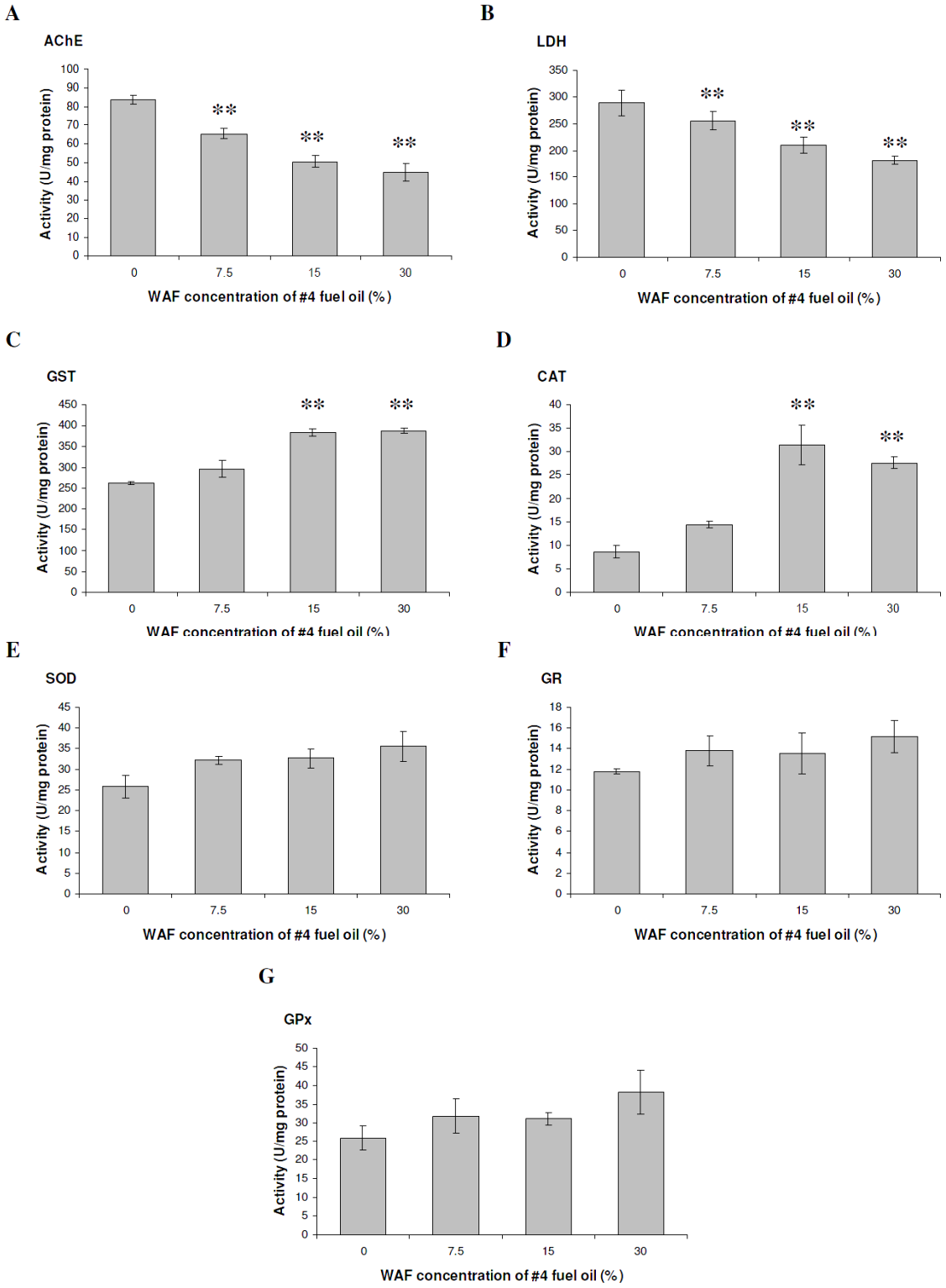

Figure 2.4. – Effects of fuel elutriate on (A) AChE, (B) LDH, (C) GST, (D) CAT, (E) SOD, (F) GR and (G) GPx activities of *P. microps*. Values indicate the means ± S.E.M. (n=27). 0 – Control; 0´- Solvent control; * - Significantly different from the control group ($p \leq 0.05$ Dunnett Test); ** - Significantly different from the control group ($p \leq 0.01$ Dunnett Test). U/mg protein = 1 µmol/min for CAT activity, the amount of enzyme required to inhibit the rate of reduction of cytochrome c by 50% for SOD activity and 1 nmol/min for the other enzymes.

For example, BaP concentrations of 667 µg/g of organic content (OC) and anthracene concentrations of 32 µg/g of OC were found in sediments of the Santander Bay, Spain (Viguri *et al.*, 2002), while BaP concentrations ranging from 12.2 to 96.8 µg/L and anthracene concentrations ranging from 3.55 to 24.4 µg/L were found in pore water of the Jiulong River Estuary and in Western Xiamen Sea, China (Maskaoui *et al*., 2002). In this study, the concentrations of these two PAHs in surface water ranged from 0.56 to 3.32 In addition, mean concentrations of anthracene between 23.9 (min: 1.42; max: 337) ng/g dry weight (d.w.) and 227 (min: 33.7; max: 1878) ng/g d.w., and mean concentrations of BaP between 0.36 (min: not detected; max: 374) ng/g d.w. and 25.2 (min: 0.12; max: 1727) ng/g d.w. were found in European eel (*A. anguilla*) juveniles collected in the Rhône delta, Southern France (Buet *et al.*, 2006). Furthermore, in a recent study performed by our team, anthracene concentrations ranging from 0.1 to 2 ng/g d.w., BaP concentrations ranging from 0.5 to 8.2 ng/g d.w. and total concentrations of 16 PAHs between 3.7 and 104.8 ng/g d.w. were found in sediments of four estuaries of the NW coast of Portugal (unpublished results from our group obtained in the scope of the EROCIPS project, www.erocips.org).

In the last decades, AChE activity of different species has been found to be inhibited by environmental contaminants other than organophosphate and carbamate insecticides, such as metals, detergents and surfactants, used engine oil and complex mixtures of pollutants (NRC, 1985; Gill *et al.*, 1990; Payne *et al.*, 1996; Guilhermino *et al.*, 1998, 2000). Regarding the effects of PAHs on this enzyme and on cholinesterases (ChE) in general, contradictory effects may be found in the literature with some studies reporting inhibition and others indicating no-effects. For example, Zapata-Pérez *et al.* (2004) observed an AChE inhibition in Nile tilapia (*Oreochromis niloticus)* following BaP exposure, while Jifa *et al.* (2006) reported no inhibition of AChE in the Japanese sea bass (*L. japonicus*) after exposure to BaP and Solé *et al.* (2008) found no AChE inhibition in juvenile sole (*Solea senegalensis*) exposed to the WAF of the ‘‘*Prestige*’’ fuel oil. Several factors may account for these apparently contradictory effects, mainly the species and the compounds tested. In the present study, for both PAHs and for the #4 WAF, inhibition of *P. microps* AChE was found, indicating at least one mechanism of neurotoxicity in common among them: the capability of disrupting cholinergic neurotransmission through the inhibition of AChE.

Table 2.1. – Acetylcholinesterase (AChE), lactate dehydrogenase (LDH), glutathione S-transferases (GST), catalase (CAT), superoxide dismutase (SOD), glutathione reductase (GR) and glutathione peroxidase (GPx) activities determined in non-exposed fish of several species.

	Enzymatic activities (U/mg protein)* in non-exposed fish							
	AChE	LDH	GST	CAT	SOD	GR	GPx	**Ref.**
Anguilla anguilla	-------	-------	350-400 (gills)	-------	-------	-------	100-150 (gills)	Ahmad *et al.*, 2006
Brycon cephalus	-------	-------	88.2 (liver)	-------	12.6 (liver)	-------	29.5 (liver)	Monteiro *et al.,* 2006b
Carassius auratus	-------	-------	20 (liver)	15-20 (liver)	8-10 (liver)	-------	-------	Liu, *et al.*,2006
Carassius auratus	-------	-------	-------	14.73 (liver)	-------	-------	-------	Zhang *et al.*, 2004
Gambusia holbrooki	40-60 (head)	-------	-------	35-40 (liver)	-------	-------	-------	Nunes *et al.*, 2004
Gambusia yucatana	45 (head)	-------	-------	-------	-------	-------	-------	Rendón-von Osten *et al.*, 2005
Lateolabrax japonicus	-------	-------	20 (liver)	60 (liver)	10-20 (liver)	-------	40-60 (liver)	Jifa *et al.*, 2006
Oncorhyncus mykiss	-------	-------	203 (liver)	-------	-------	18.1 (liver)	8.2 (liver)	Vigano *et al.*, 1995
Oreochromis mossambicus	-------	77 (brain)	-------	-------	-------	-------	-------	Rao, 2006
Poecilia reticulata	145 (muscle)	-------	-------	-------	-------	-------	-------	Garcia *et al.*, 2000
Pomatoschistus microps	80 (head)	146 (muscle)	215 (gills)	12.3 (liver)	19.1 (liver)	13.4 (liver)	22.8 (liver)	**Present study**
Pomatoschistus microps	80-90 (head)	120 (muscle)	118 (gills)	-------	-------	-------	-------	Monteiro *et al.*, 2006a
Serranus cabrilla	85.8 (brain)	-------	-------	-------	-------	-------	-------	Sturm *et al.,* 1999

* For all enzymes U = nmol.min^{-1} with the exception of CAT for which U = µmol.min^{-1} and SOD (U = the amount of enzyme required to inhibit the rate of reduction of cytochrome *c* by 50%).

Anthracene seems to be a more potent inhibitor of this enzyme than BaP since, at 4 µg/l, it caused 52% of inhibition while BaP only caused 30% of AChE inhibition.

Recent studies have shown that some petrochemical mixtures (*e.g.* fuel oils) may also decrease the AChE activity of some aquatic species. For example, Barsiene *et al.* (2006) described a decreased AChE activity in the flounder (*Platichthys flesus*) following an oil spill in the Baltic Sea. Therefore, the inhibition of *P. microps* head AChE by # 4 WAF found in the present work is in good agreement with these studies. Since several metals (*e.g.* zinc, mercury, copper, lead) potentially present in fuel oils and other petrochemical mixtures have the potential to inhibit the ChE activity of several species (Gill *et al.*, 1990; Garcia *et al.*, 2000; Frasco *et al.*, 2005), they can also contribute to the anti-cholinesterase effect of these products. However, some authors found no inhibition of fish AChE after exposure to WAF of fuel oils (Solé *et al.*, 2008). This may be due to differential composition of the mixtures, mainly their content in AChE inhibitors, and also to different methods of preparing the WAF that may lead to different concentrations of AChE inhibitors in test media.

The inhibition of AChE by BaP, anthracene and the #4 WAF found in the present study ranged from 23 to 52%. These inhibitions are above the 20% inhibition that has been considered as indicative of exposure to anti-ChE agents (Ludke *et al.*, 1975). As a whole, these findings suggest that AChE activity of fish may be used as an environmental biomarker in ecosystems contaminated by petrochemical products, therefore, in good agreement with the opinion of Buet *et al.* (2006).

In the present study, BaP exposure resulted in 66% of LDH activity increase at 4 µg/L and of 84% at 16 µg/L, while anthracene increased LDH activity by 99% at 4 µg/L. These results indicate that anthracene and BaP have a similar effect on LDH activity, but anthracene seems to have a more pronounced effect on LDH activity than BaP. These findings also suggest that animals are getting additional energy from the anaerobic pathway in an attempt to support the processes (*e.g.* detoxification mechanisms) needed to face chemical exposure. These results are in good agreement with findings from the literature reporting an increase of LDH in fish after exposure to PAHs. For example, Oikari and Jimenez (1992) observed higher LDH activity in plasma of sunfish hybrids (*Lepomis macrochirus* x *L. cyanellus*) after administration of BaP.

Considering now the results of the bioassay with # 4 WAF, a reduction of 39% in LDH activity at 30% of WAF was found. This potential interference of the mixture with the energetic balance may have negative consequences in the performance of the organisms both at short and long term. The reduction of LDH activity after exposure to #4 WAF is in good agreement with the findings of Gagnon and Holdway (1999), who also found a significant inhibition of this enzyme in the Atlantic salmon (*S. solar*) exposed for 12 days to the WAF of "Bass Strait" crude oil. However, induction of LDH by WAF was described in the Australian crimson-spotted rainbowfish (*M. fluviatilis*) exposed for 3 days to the WAF of a dispersed crude oil (Pollino and Holdway, 2003). Therefore, the response of LDH to fuel-oils seems to depend of mixture tested and, thus, of its composition.

In the case of *P. microps*, a similar effect (increase) of both PAHs on LDH activity was found, while the opposite effect was observed for the fuel-oil. This difference may be due to the presence of metals in the fuel oil some of which have been found to inhibit the LDH activity of fish (Castro *et al.*, 2004; Osman *et al.*, 2007). Since different components of fuel oils may have opposite effects of LDH activity of fish, care should be taken when using this enzyme as a biomarker in sites contaminated with petrochemical products because the overall result may be "no effect" and this may lead to erroneous conclusions.

GSTs are important enzymes in the detoxification processes since they catalyse the conjugation of both endogenous substances and xenobiotics with glutathione (GSH). GSH plays an important role in the detoxification of electrophilic substances and prevention of cellular oxidative stress (Hasspieler *et al.*, 1994). They can also bind, store and/or transport a number of compounds that are not conjugated with GSH (Parkinson, 2001). In the present study, BaP caused an induction of GST activity, while anthracene caused its inhibition. These results indicate that GST and/or the mechanisms controlling its production or GSH availability respond differently to distinct PAHs. The induction of GST by BaP suggests that in *P. microps,* GSH conjugation is involved in BaP removal, therefore in good agreement with the general detoxification pathway described for this compound (Di Giulio *et al.*, 1995). Gowland *et al.* (2002) found that high molecular weight PAHs with 5- and 6-rings have a more pronounced role than low molecular weight compounds with 2- to 4-rings in inducing GST activity. This may be related to their different affinities for the aryl hydrocarbon receptor (Ah-R) since Ah-R ligands enhance induction of phase II enzymes such as GST in fish (Goksøyr and Husøy, 1998; Taysse *et*

al., 1998). BaP is a well known Ah-R ligand (Billard *et al.*, 2006) with high affinity to this receptor, while anthracene has a considerable lower affinity to this receptor (Barron *et al.*, 2004; Incardona *et al.*, 2006). Other possible explanations are (i) that anthracene directly binds to GST causing its inhibition and (ii) since anthracene considerably increases the activity of GPx requiring GSH, the levels of this molecule available may be not enough to assure the function of GST and, thus, the enzyme is inhibited due to the lack of GSH that is being used in the process dealing with oxidative stress. However, it should be work noted that BaP also increased the activity of GPx and GST inhibition was not observed. Thus, this finding makes the last hypotheses weaker than the others.

P. microps GST was induced after exposure to #4 WAF. This result is in good agreement with the increased activities found in the goldfish (*C. auratus*) exposed to water soluble fractions of diesel oil (Zhang *et al.*, 2004). Moreover, in a field study, Martínez-Gómez *et al.* (2006) found elevated GST activities in the four-spot megrim (*Lepidorhombus boscii*) from the most impacted area after the *Prestige* oil spill.

In the present study, different PAHs had opposite effects on GST activity. In real scenarios, usually several PAHs are present in ecosystems contaminated by petrochemical products. Therefore, since different PAHs may have opposite effects on GST activity of fish, care should be taken when using this enzyme as a biomarker in sites contaminated with several PAHs because the overall result may lead to erroneous conclusions. Furthermore, it is interesting to note that the induction caused by #4 WAF was almost the double that the induction caused by BaP alone indicating that the mixture is more efficient in inducing GST activity, suggesting synergism among at least part of its components. Therefore, our findings about GST activity seem to support the questions raised by Billiard *et al.* (2006) from their experiments with Ah-R ligands and cytochrome P4501A1 inhibitors about the efficacy of using additive models of toxicity for PAHs in risk assessments for these compounds.

In the present study, both BaP and anthracene were found to significantly induce all the anti-oxidant enzymes tested, namely SOD, CAT, GR and GPx, which are crucial in the detoxification of oxyradicals to non-reactive molecules (Van der Oost *et al.*, 2003). These results suggest that both PAHs induce the production of $O_2^{\bullet-}$ which is converted to hydrogen peroxide (H_2O_2) by action of SOD and then that H_2O_2 is converted into water by action of CAT and/or GPx. The pathway involving GPx also detoxify lipid peroxides

(Winston and Di Giulio, 1991) and requires GR to catalyse the transformation of the oxidized disulfide form of glutathione (GSSG) to the reduced form (GSH), by making use of the oxidation of NADPH to $NADP^+$, which is further recycled mainly by the pentose phosphate pathway. Since in the case of fish exposed to individual PAHs all the anti-oxidant enzymes measured were found to be induced, it is likely to conclude that a considerable amount of $O_2^{\bullet-}$ is produced originating abundant H_2O_2 that needs to be detoxified by both CAT and GPx pathways. Considering now the results obtained in fish exposed to the #4 WAF, only CAT was found to be significantly induced at the concentrations tested. This suggests a low production of $O_2^{\bullet-}$ (for each no increase of SOD activity is needed) leading to a low amount of H_2O_2 in the cell for which only the pathway of CAT is enough to remove it. However, these results are quite surprising since GPx has been considered the main antioxidant enzyme for the removal of H_2O_2 in animal cells because CAT has a considerable lower affinity for H_2O_2 (Izawa *et al.*, 1996). From these results, one can conclude that all the tested substances (BaP, anthracene and #4 WAF) have the capability of inducing oxidative stress on *P. microps* if the anti-oxidative stress defences of the cell are overtaken. However, these findings also suggest that both PAHs are more potent in inducing oxidative stress than #4 WAF, probably because the mixture also contain substances that might have no oxidative-stress effects and/or that have opposite effects on SOD, GR and GPx resulting in the overall balance of no effects on the activities of these enzymes. However, from these results, is evident the existence of differences in the mechanisms used by *P. microps* to deal with oxidants generated by the exposure to isolated PAHs and those resulting from the exposure to the mixture. Unfortunately, our experimental design does not allow going further into this question.

The induction of GPx, SOD and CAT by both PAHs found in the present study is in good agreement with the findings of Jifa *et al.* (2006) in the Japanese sea bass (*L. japonicus*) exposed to BaP. The increase of CAT activity by #4 WAF is in agreement with the results obtained by Sturve *et al.* (2006), who also found a significant increase of CAT activity in the Atlantic cod (*Gadus morhua*) exposed to North Sea oil. However, no effects on anti-oxidant enzymes, including CAT, were observed in soles (*S. senegalensis*) exposed to the WAF of the ‘‘*Prestige*’’ fuel-oil (#6 fuel-oil) (Solé *et al.,* 2008). The differences between our findings and those that have been reported by other authors dealing with fuel-oils may be due to several factors, including differences in the composition of the mixtures,

differences in the mechanisms of toxicity and detoxification among distinct fish species and/or differences in the preparation of WAF that may lead to important differences in the compounds present in test media and/or in their concentrations. In any case, the evidences from our study support the opinion that has been expressed by several authors that to avoid misinterpretations all the anti-oxidant enzymes should be measured and that their results should be analysed as a whole.

2.6. CONCLUSIONS

In the present study, *P. microps* AChE activity was significantly inhibited by BaP, anthracene and #4 WAF. These results indicate that the three substances have at least one mechanism of neurotoxicity in common: the disruption of cholinergic neurotransmission through the inhibition of AChE activity. Furthermore, since AChE inhibitions between 30 and 52% were found at ecological relevant concentrations, they indicate the suitability of *P. microps* AChE for use as an environmental biomarker in ecosystems polluted by petrochemical products. A significant induction of LDH activity was found in fish exposed to BaP and to anthracene, suggesting an increase of the anaerobic pathway of energy production. On the contrary, inhibition of LDH was found in fish exposed to the fuel oil, suggesting a distinct effect of the mixture. A significant induction of *P. microps* GST was found in fish exposure to BaP and #4 WAF, while an inhibition was observed in fish exposed to anthracene. These results may be due, at least in part, to different affinities for the Ah-R receptor from which GST induction is dependent and suggest that GST is involved in the detoxification of BaP and #4 WAF, but not of anthracene. They also indicate that care should be taken when using this enzyme as a biomarker in ecosystems contaminated with different PAHs because they might have opposite effects on GST activity. As a whole, the results from anti-oxidant enzymes indicate that all the tested substances may induce oxidative stress on *P. microps* and suggest that isolated PAHs may be stronger inducers of anti-oxidant enzymes than complex mixtures such as fuel oils due to opposite effects (direct and/or indirect) that some of the components of the mixtures may have on these enzymes.

2.7. ACKNOWLEDGEMENTS

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Chapter 3. *Acute effects of copper and mercury on the estuarine fish Pomatoschistus microps: linking biomarkers to behaviour*

Acute effects of copper and mercury on the estuarine fish *Pomatoschistus microps*: linking biomarkers to behaviour

(*Manuscript accepted for publication in Chemosphere*)

3.1. ABSTRACT

The main objective of the present study was to investigate possible links between biomarkers and swimming performance in the estuarine fish *Pomatoschistus microps* acutely exposed to metals (copper and mercury). In independent bioassays, *P. microps* juveniles were individually exposed for 96 h to sub-lethal concentrations of copper or mercury. At the end of the assays, swimming performance was measured using a device specially developed for epibenthic fish (SPEDE). Furthermore, the following biomarkers were measured: lipid peroxidation (LPO) and the activity of the enzymes acetylcholinesterase (AChE), lactate dehydrogenase (LDH), glutathione S-transferases (GST), 7-ethoxyresorufin-*O*-deethylase (EROD), superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GR) and glutathione peroxidase (GPx). LC_{50}s of copper and mercury (ionic concentrations) were 568 µg/L and 62 µg/L, respectively. Significant and concentration-dependent effects of both metals on swimming resistance and covered distance against water flow were found at concentrations equal or higher than 50 µg/L for copper, and 3 µg/L for mercury (ionic concentrations). In addition, significant alterations of both metals on the biomarkers were found: inhibition of AChE and EROD activities, induction of LDH, GST and anti-oxidant enzymes, and increased LPO levels, with LOEC values ranging from 25 to 200 µg/L for copper and from 3 to 25 µg/L for mercury (ionic concentrations). Furthermore, for both metals significant and high correlations were found between behaviour parameters and some biomarkers. Finally, multivariate data analysis showed that swimming resistance and covered distance against water flow seem to be highly associated to AChE activity, which suggests the relevance of measuring the effects on the activity of this enzyme. In addition, negative and significant correlations between LPO and swimming performance endpoints were found, suggesting that lipid peroxidation caused by mercury and copper may interfere with fish performance.

Keywords: *Pomatoschistus microps*, metals, swimming performance, biomarkers

3.2. INTRODUCTION

Metals are an important group of estuarine pollutants. They are known to be able to disturb the integrity of biochemical and physiological mechanisms in aquatic organisms, including estuarine fish. Among metals, copper and mercury are of special concern since they are considerably toxic to aquatic animals at ecologically relevant concentrations (Mzimela *et al.*, 2002; Zhang *et al.*, 2005). Copper is a trace element that plays a fundamental role in the biochemistry of organisms, including aquatic ones that can take it up directly from water (Grosell *et al.*, 2003). However, at high concentrations it can become toxic (Lam *et al.*, 1998; Alquezar *et al.*, 2008). Mercury is considered one of the most dangerous metals in the aquatic environment (Goyer *et al.*, 1995), mainly because organic forms can be biomagnified in trophic chains representing an increased risk for top predators (Waring *et al.*, 1996; MacDougal *et al.*, 1996), including humans consuming contaminated fish.

Biomarkers have been widely used in estuarine and coastal ecosystems to assess the exposure and/or effects of pollution on native populations of fish. Due to their importance in the tolerance capability of organisms to pollution exposure, lipid peroxidation (LPO) levels and the activity of the enzymes cholinesterases (ChE), glutathione S-transferases (GST), ethoxyresorufin O-deethylase (EROD), lactate dehydrogenase (LDH), glutathione reductase (GR), catalase (CAT) and glutathione peroxidase (GPx) are among the most used biomarkers in marine ecosystems (for detailed descriptions of the role of these enzymes see for example Guilhermino *et al.*, 1998*;* García *et al.*, 2000; Lima *et al.*, 2007; Gravato *et al.*, 2008; Vieira *et al.*, 2008). The effects of copper and mercury on biomarkers of fish have been studied in both field and laboratorial conditions and contradictory effects have been reported in the literature (Nemcsok *et al.*, 1984; Suresh *et al.*, 1992; Radhakrishnaiah *et al.*, 1993; Pedrajas *et al*., 1995; Sastry *et al.*, 1997; Roméo *et al.*, 2000; Dautremepuits *et al.*, 2002; Antognelli *et al.*, 2003; Elia *et al.*, 2003; Romani *et al.*, 2003; Ahmad *et al.*, 2005; Sanchez *et al.*, 2005; Atli *et al.*, 2006; Liu *et al.*, 2006; Vutukuru *et al.*, 2006; Varo *et al.*, 2007) apparently related with species differences and exposure conditions, among other factors. Therefore, more research on this subject is still needed.

Despite the high value of biomarkers as early warning tools, the significance of some environmental studies based on biomarkers has been questioned mainly due to the fact that alterations induced at sub-individual level do not necessary have negative reflexes at higher levels of biological organization. Without questioning the general veracity of the argument, it is our opinion that the problem is the lack of knowledge on relationships between biomarkers and parameters considered "ecological relevant" and, thus, that more research is needed on this matter to take full advantage of these powerful tools. Therefore, to contribute for this question, the central objective of this study was to investigate possible relationships between biomarkers (AChE, LDH, EROD, GST, GPx, GR, CAT and LPO) and swimming performance in marine fish acutely exposed to copper or mercury.

The above mentioned biomarkers were selected because they play a decisive role in functions determinant for the survival and performance of fish under chemical stress, while swimming performance was selected because its impairment may decrease the capability of fish to escape from predators, to capture preys and to reproduce, in any case decreasing the contribution of the animals for the population. The common goby, *Pomatoschistus microps* Krøyer (1838), was selected as test organism since it is an abundant fish species in estuaries, lagoons and shores of Europe (Arruda *et al.*, 1993; Salgado *et al.*, 2004), has an important function in estuarine ecosystems as an intermediary predator in food webs connecting macro- and meiofauna with larger predator fish (Miller *et al.*, 1986; Arruda *et al.*, 1993) and has been found to be a suitable test organism and an adequate bioindicator in previous studies (Monteiro *et al.*, 2005, 2006; Vieira *et al.*, 2008). Swimming performance was assessed using a new device, the Swimming Performance Device (SPEDE), specially designed to measure swimming resistance to water-flow (swimming resistance) and covered distance while swimming against water-flow (covered distance) in epibenthic fish.

3.3. MATERIAL AND METHODS

3.3.1. Chemicals

Copper sulphate ($CuSO_4$) and mercury chloride ($HgCl_2$) were purchased from MERK (Germany) and Sigma–Aldrich Chemical (Steinheim, Germany), respectively. The chemicals for enzymatic analysis were acquired from Sigma–Aldrich Chemical (Steinheim, Germany).

3.3.2. Fish sampling and maintenance in the laboratory

P. microps juveniles (2.5 – 3 cm long) were captured in a low-impacted site in the Minho River estuary (41º 53' 26.8''N, 8º 49' 29.2''W) (NW of Portugal) (Figure 3.1.), during low tide using a hand operated net. This estuary was chosen due to its characteristics of low urban, industrial and agricultural contamination (Ferreira *et al.*, 2003) and because it has been used as a reference estuary in previous studies with this species (Monteiro *et al.*, 2005, 2006). During the collection period, water salinity varies from 5 to 8 and the water temperature between 17.5 to 18.5 ºC. After being collected, specimens were immediately transported alive to the laboratory in 30L containers with aeration. In the laboratory, fish were submitted to an acclimation period of two weeks in artificial medium which was prepared by dissolving aquarium salt (SERA® Premium – Sea Salt – D52518 Heinsberg, Germany) in distilled water until reaching a salinity of 6 g/L; after stirring in a vortex with a magnetic stirrer for about 20 minutes, the salinity was again measured and corrected if necessary. The medium was changed every other day. Fish were kept in 60 L glass aquaria with internal filters and an aeration system, in a photoperiod (16h L: 8h D) and temperature controlled room (20±1^{o}C), being feed with commercial fish food (TetraMin®).

3.3.3. Bioassays

Ninety-six hours acute bioassays were performed following, in general, OECD guidelines for fish acute bioassays (guideline OECD 203, 92/69/EC, method C1) (OECD, 1993), but exposing fish individually and using the artificial medium above described as test medium. Test chemicals were copper sulphate and mercury chloride. Stock solutions of both chemicals were prepared in ultra-pure water at ionic concentrations: 10 mg/L and 5 mg/L for Cu^{2+} and Hg^{2+}, respectively. In independent bioassays, test solutions were prepared by dilution of stock solutions in artificial medium (prepared as indicated in 3.3.2.) to obtain the following final ionic concentrations: 25, 50, 100, 200, 400, 800 and 1600 µg/L for copper; and 3.125, 6.25, 12.5, 25, 50, 100 and 200 µg/L for mercury. Fish (2.0 – 2.7 cm long) were individually exposed in 1L polyethylene-terephthalate test chambers for 96 hours.

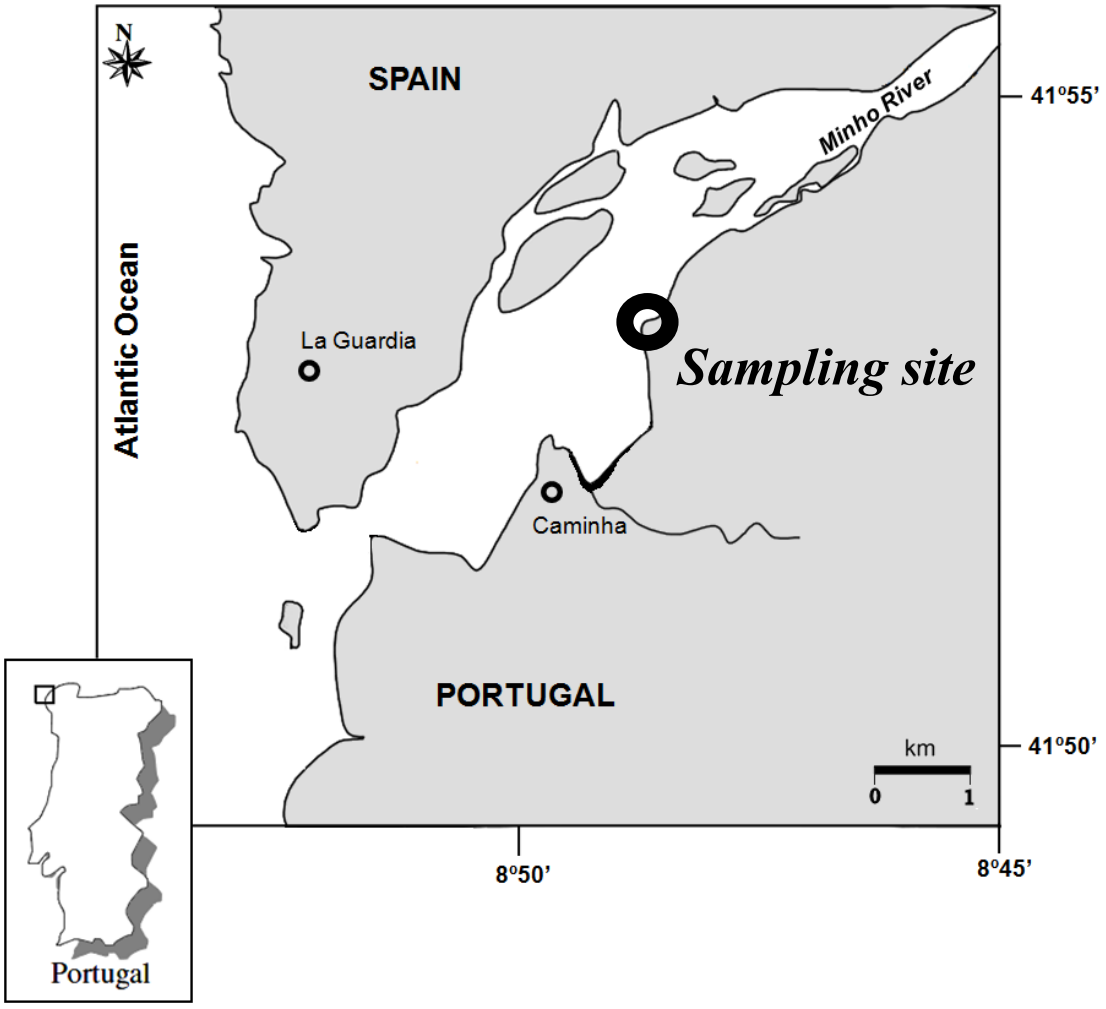

Figure 3.1. – Map of the Minho river estuary (NW Portugal), showing the location of the sampling site (41º 53' 26.8''N, 8º 49' 29.2''W).

During the tests, photoperiod, temperature and aeration conditions were similar to those used in the acclimation period and no food was provided. Water temperature, conductivity, salinity, pH, dissolved oxygen (DO) and fish mortality were monitored every 24 hours. Twenty-seven fish were used per treatment. Following the exposure period (96 hours), the concentrations causing LC_{50} values were calculated for both metals, based on the mortality recorded during the exposure period.

At the end of the bioassays, the swimming performance and several biomarkers were determined as described below.

3.3.3.1. Swimming performance

The swimming performance of fish was assessed using a new device built for studies with epibenthic fish: the Swimming Performance Device (SPEDE) (Figure 3.2.). SPEDE was based on a device previously developed and validated with the seabass (*Dicentrarchus labrax*) by this research group (Gravato and Guilhermino, 2009). SPEDE (Figure 3.2.) is a closed system composed by: two taps (1A and 1B) to maintain water flow at 2L/min; a plastic tube (main tube) 1.2m long, 1.3cm height and 1.9cm width (2); a tilted tube (3) connecting the main tube to a net basket (4) which is filled with water at 50% of its total volume; a water recipient (5) containing the net basket, an electric water pump (6) and devices for measuring temperature, conductivity and salinity (7A), pH (7B) and DO (7C); and connection tubes (8). As shown in the 3D view of the main tube (Figure 3.2., down), it has an open section with 80 cm long with a scale (mm) and it is full of water (artificial water, salinity 6 g/L) during the test. The main tube has an inclination of 5º, water flows from the taps (1A and 1B) to the tilted tube (3).

Fish swimming performance was evaluated by two endpoints: (i) the time born by the fish until being dragged by the water flow (swimming resistance) and (ii) the distance covered when swimming against the water flow (covered distance). For this, fish were introduced at the middle of the open part of the main tube (position 2B), they were left to get a stable position, and then the time (seconds) while they were able to resist to the water flow (i.e. until being dragged in the direction of 2A) was counted and considered as the swimming resistance, while the distance covered when swimming against the water flow (i.e. the distance covered while resisting to the water flow), measured (mm) in the main tube scale in the direction 2A → 2C, was considered as the covered distance. The swimming test was performed once per fish. After being dragged by the water flow through the tilted tube (3), fish fall into the net basket (4) and were put back into their original test chambers where they stand for two hours before being used for biomarkers analysis.

3.3.3.2. Biomarkers determination

Fish were sacrificed by decapitation and the following tissues were isolated: head, dorsal muscle, gills and liver. Three heads were used to prepare one sample for AChE determination, three dorsal muscle pieces were used to prepare 1 sample for LDH analysis and three pairs of gills were used to prepare 1 sample for GST analysis. Three livers were used to prepare 1 sample for LPO, EROD, CAT, SOD, GR and GPx analysis. For the LPO assay, livers were weighed using a Kern 770 balance.

Tissues were homogenised (Ystral homogenizer, Ballrechten-Dottingen, Germany) in different buffers according to the biomarker to be measured: head in phosphate buffer (0.1 M, pH 7.2), muscle in Tris-NaCl buffer (0.1 M, pH 7.2) and gills in phosphate buffer (0.1 M, pH 6.5). Following homogenisation, head and muscle samples were centrifuged for 3 min at 3300g, while gill samples were centrifuged for 30 min at 9000g. All supernatants were recovered and kept at -80 ºC until further analysis. Livers were homogenised in 1:10 (w/v) of phosphate buffer (0.05 M, pH 7.0, with 0.1% Triton X-100). For LPO assay, 200 µL were put in an eppendorf with 4 µL of butylated hydroxytoluene, for each sample, and stored at -80 ºC, while the remanding liver homogenate was centrifuged for 15 min at 15000g; the supernatant was collected and divided in aliquots for EROD and anti-oxidant enzymes analyses and stored at -80 ºC.

AChE activity was determined according to the Ellman's method (Ellman *et al.*, 1961) adapted to microplates (Guilhermino *et al.*, 1996), using a BIO-TEK POWERWAVE 340 microplate reader. In a previous study, it was found that the soluble fraction of *P. microps* head homogenates contains mainly AChE (Monteiro *et al.*, 2005). Frasco *et al.* (2005) reported that some metals, including copper and mercury, may react with the products of the Ellman's technique and suggested the use of the *o*-nitrophenyl acetate assay to assess the effects of metals on AChE in some conditions. In this context and to compare the results, both techniques were used in this work. *P. microps* LDH was determined by the method of Vassault (1983), adapted to microplate (Diamantino *et al.*, 2001). EROD was quantified by the methodology described by Burke and Mayer (1974) with some modifications.

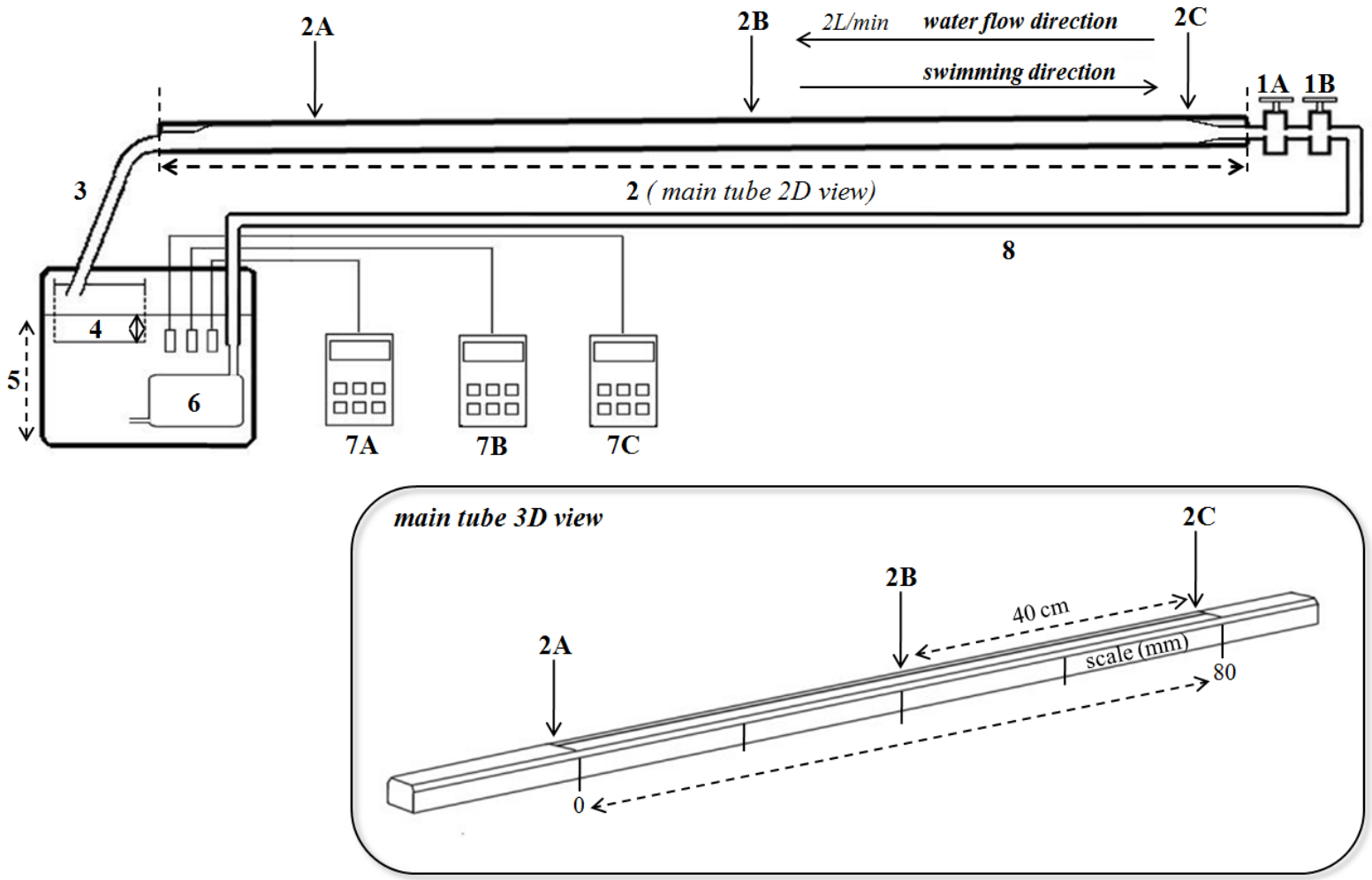

Figure 3.2. – Swimming Performance Device (SPEDE). It is a closed system consisting of two taps (1A and 1B); a 1.2m plastic tube (main tube) (2); a tilted tube (3) connecting the main tube to a net basket (4); a water recipient (5); an electric water pump (6); devices for measuring temperature, conductivity and salinity (7A), pH (7B) and DO (7C) and connection tubes (8). The main tube has an inclination of 5º. A 3D view of the main tube is represented below the main scheme and indicates an open section with 80 cm long with a scale (mm) where swimming performance endpoints are measured, from 2A to 2C, as well as the position where fish are introduced, at the middle of the open part of the main tube (2B).

LPO levels were measured by quantification of thiobarbituric acid reactive substances (TBARS) and expressed as nmol TBARS/g tissue (Ohkawa *et al.*, 1979). GST was assessed according to Habig *et al.* (1974), with some modifications of the original protocol (Frasco and Guilhermino, 2002). The activities of GR (Carlberg and Mannervik, 1975), GPx (Flohé and Günzler, 1984) and SOD (McCord and Fridovich, 1969) were also measured in microplates (Lima *et al.*, 2007). CAT activity was measured according to the method of Aebi (1984) in a JENWAY, model 6405 UV/VIS, spectrophotometer. Enzymatic activities were determined at 25ºC and expressed as activity per mg of protein (second protein determination performed after the enzymatic analysis, as indicated below). One unit (U) of SOD activity was defined as the amount of enzyme required to inhibit the rate of reduction of cytochrome *c* by 50%. For CAT, one U was defined as 1 μmol/min; for EROD activity as 1 pmol/min and for the remaining enzymes as 1 nmol/min.

Prior to enzymatic analysis, sample protein was normalised to 0.3 mg/ml in AChE and GST samples and to 0.9 mg/ml in LDH, EROD, CAT, SOD, GR and GPx samples (Vieira *et al.*, 2008). After enzymatic analysis, the amount of protein in each sample was determined again and this value was used to express enzymatic activities. All the protein determinations were done by the Bradford method (Bradford, 1976) adapted to microplate.

3.3.4. Statistical Analyses

For each enzyme, swimming resistance time and swimming covered distance results, different treatments were compared using one-way analysis of variance (ANOVA), followed by the Dunnett's comparison test whenever applicable (Zar, 1996). Data were previously tested for distribution normality (Kolmogrov-Smirnov normality test) and homogeneity of variance (Barlett's test) (Zar, 1996). The t-test of Student was employed to compare the two techniques used to quantify AChE activity (Zar, 1996). Pearson correlation coefficient was used to measure the correlation between each biomarker and swimming resistance time, and between each biomarker and swimming covered distance. In addition, data were also analysed through principal component analysis (PCA) (Zar, 1996). In all statistical analysis, differences were considered statistically significant when $p \leq 0.05$. Statistical analyses were performed using STATISTICA 6.0© and SPSS 14.0© software packages, with the exception of PCA that was performed using the software CANOCO 4.52©.

3.4. RESULTS

All the mentioned concentrations are ionic concentrations.

3.4.1. Lethal effects of copper and mercury on P. microps

Considering the copper bioassay, the lowest concentration causing 100% of fish mortality was 1600 µg/L at 96 h, while the highest concentration causing no fish mortality was 25 µg/L at 96 h. After 96 h of exposure, no mortality was observed up to 25 µg/L, while exposure of fish to 50, 100, 200, 400, 800 and 1600 µg/L resulted in 4%, 19%, 4%, 44%, 70% and 100% of mortality, respectively. LC_{50} values calculated for 24, 48, 72 and 96 h of exposure are shown in Table 3.1.

Regarding the mercury bioassay, the lowest concentration causing 100% of fish mortality was 200 µg/L at 72 h, while the highest concentration causing no fish mortality was 6.3 µg/L at 96 h. After 96 h of exposure, no mortality was observed in the control and at 6.25 µg/L, while exposure of fish to 3.1, 12.5, 25, 50, 100 and 200 µg/L resulted in 4%, 26%, 33%, 44%, 74% and 100% of mortality, respectively. Mercury LC_{50} values calculated for 24, 48, 72 and 96 h of exposure are shown in Table 3.1.

3.4.2. Effects of copper and mercury on behaviour

In the copper bioassay, fish showed a lethargic and erratic swimming behaviour starting at 48h when exposed to 800 µg/L and 1600µg/L, and at 96 h when exposed to 400µg/L. Significant differences in both post-exposure swimming resistance ($F_{(5,\ 66)}$ = 138.9, ≤ 0.05) and covered distance ($F_{(5,\ 66)} = 54.0$, $p \leq 0.05$) were found (Figure 3.3.).
No Observed Effect Concentration (NOEC), Lowest Observed Effect Concentration (LOEC) and the 50% Effective Concentrations (EC_{50}) for behavioural parameters are indicated in Table 3.2. The most sensitive parameter was covered distance with an EC_{50} of 12.3 µg/L (95% Confidence Limits (CL): 0.5 - 24.8).

Table 3.1. – Copper and mercury ionic concentrations causing 10% (LC_{10}) and 50% (LC_{50}) of mortality on *P. microps* after 24, 48, 72 and 96 hours of exposure with corresponding 95% confidence limits (95% CL).

	24	48	72	96
Cu^{2+} (µg/L)				
LC_{10}	**746.7**	**382.8**	**281.96**	**161.5**
(95%CL)	(491.4 - 973.5)	(-71.21 - 669.8)	(142.7 - 394.9)	(-89.31 - 305.03)
LC_{50}	**1735**	**1337**	**842.3**	**568.1**
(95%CL)	(1430 - 2297)	(1005 - 2077)	(721.1 - 1003)	(419.8 - 858.1)
Hg^{2+} (µg/L)				
LC_{10}	**44.94**	**8.201**	**9.295**	**6.346**
(95%CL)	(-432.3 - 130.3)	(-106.3 - 51.63)	(-34.01 - 30.14)	(-36.01 - 25.66)
LC_{50}	**228.6**	**130.3**	**71.03**	**61.89**
(95%CL)	(138.5 - 2240)	(85.77 - 256.6)	(50.02 - 115.8)	(42.92 - 101.6)

Regarding the mercury bioassay, *P. microps* showed lethargic and erratic swimming behaviour after 24, 48 and 72 h when exposed to 200µg/L, 100µg/L and 50 µg/L, respectively. Significant differences in both post-exposure swimming resistance ($F_{(5, 66)} = 114.4$, $p \leq 0.05$) and covered distance ($F_{(5, 66)} = 21.2$, $p \leq 0.05$) were found (Figure 3.4.). NOEC, LOEC and EC_{50} values for mercury exposure are indicated in Table 3.2. The most sensitive parameter was the covered distance, with an EC_{50} of 1.2 (95% CL: 0.522 - 2.028) µg/L.

3.4.3. Effects of copper and mercury on biomarkers

Copper caused a significant inhibition of *P. microps* AChE activity as indicated by the two different techniques used to measure enzymatic activity (Figure 3.5.A): the Ellman technique (Figure 3.5.A (a)) ($F_{(5, 42)} = 100.4$, $p \leq 0.05$; LOEC = 25µg/L) and the method using *o*-nitrophenyl acetate as substrate (Figure 3.5.A (b)) ($F_{(5, 42)} = 118.4$, $p \leq 0.05$; LOEC = 25µg/L). The *o*-nitrophenyl method was apparently more sensitive causing 79% of inhibition at 400 µg/L. In this bioassay, significant differences among different treatments were also found for LDH activity ($F_{(5, 42)} = 44.8$, $p \leq 0.05$; LOEC = 25µg/L) (Figure 3.5.B).

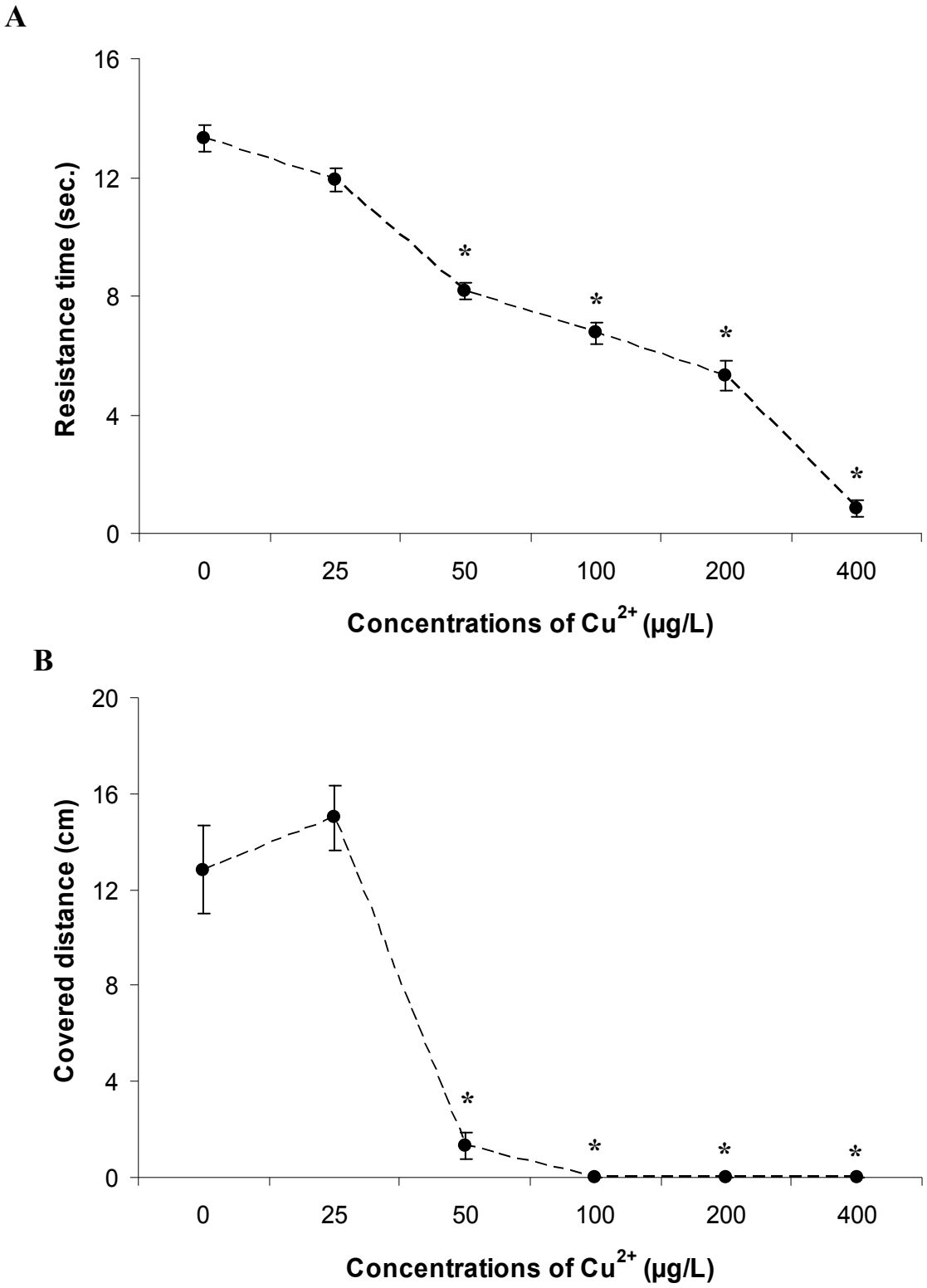

Figure 3.3. – Effects of copper on *P. microps* swimming resistance against water-flow (swimming resistance, A) and covered distance while swimming against water flow (covered distance, B). The values are the mean with corresponding ± S.E.M. 0 – Control; * Significantly different from the control group ($p \leq 0.05$ Dunnett Test). Swimming resistance decreases of 39%, 49%, 60% and 93% in relation to controls were observed at 50µg/L, 100µg/L, 200µg/L and 400µg/L, respectively.

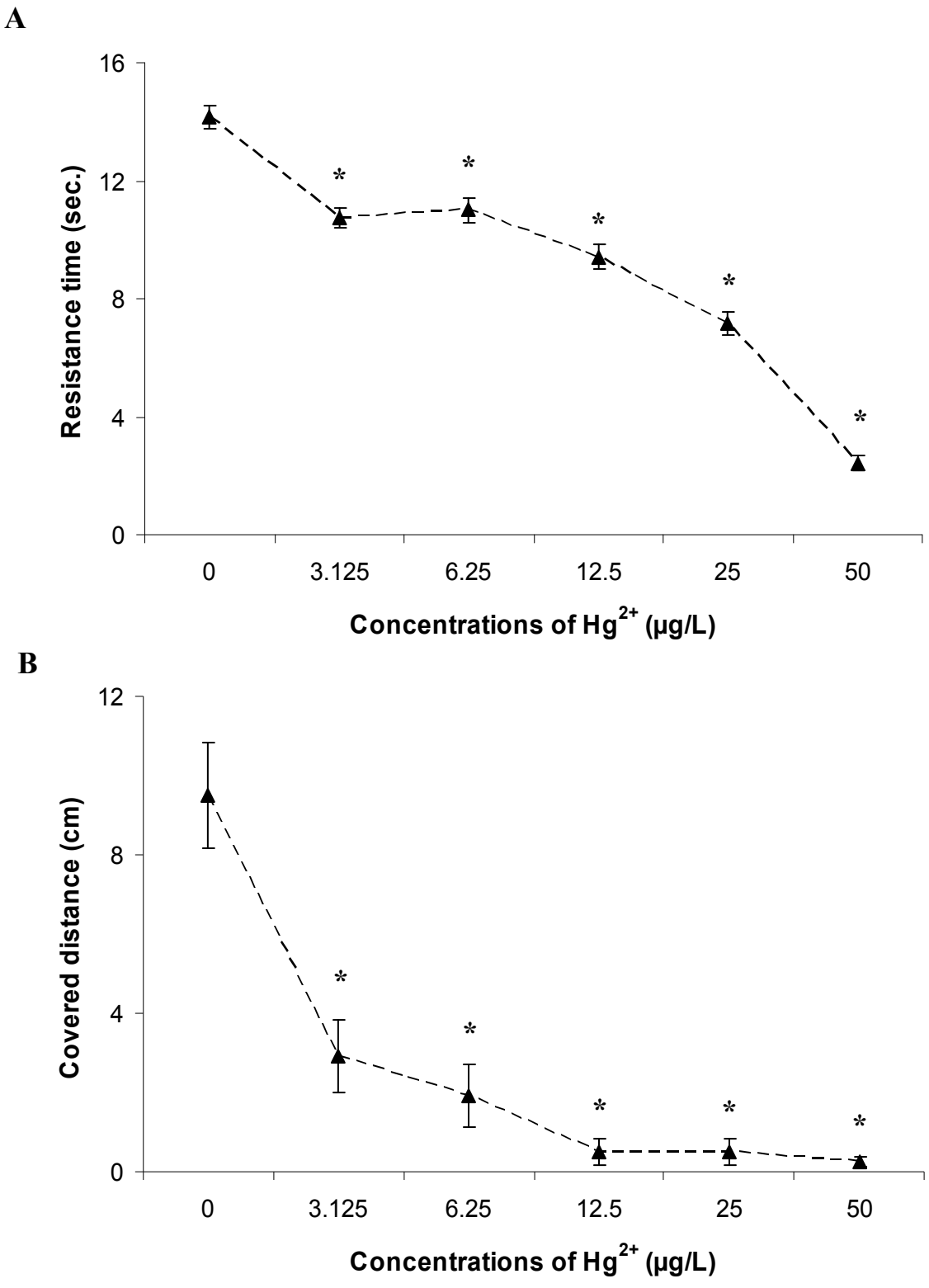

Figure 3.4. – Effects of mercury on *P. microps* swimming resistance against water-flow (swimming resistance, A) and covered distance while swimming against water flow (covered distance, B). The values are the mean with corresponding ± S.E.M. 0 – Control; * Significantly different from the control group ($p \leq 0.05$ Dunnett Test). Swimming resistance decreases 24%, 22%, 34%, 49% and 82% of reduction at 3.125µg/L, 6.25 µg/L, 12.5 µg/L, 25 µg/L and 50µg/L, respectively.

Table 3.2. – Ecotoxicological parameters for copper and mercury obtained in bioassays with *P. microps*: No Observed Effect Concentration (NOEC), Lowest Observed Effect Concentration (LOEC) and 50% Effective Concentrations (EC_{50}) for behavioural and biomarkers responses determined after 96 hours of exposure. For both metals, the values are ionic concentrations. For EC_{50}s, 95% confidence limits are indicated within brackets.

	Cu^{2+} (µg/L)			Hg^{2+} (µg/L)		
	NOEC	LOEC	EC_{50}	NOEC	LOEC	EC_{50}
Parameter						
Swimming resistance	**25**	**50**	**97.58** (49.81 - 201.4)	**<3.125**	**3.125**	**18.07** (6.764 - 251.9)
Covered distance	**25**	**50**	**12.33** (0.517 - 24.82)	**<3.125**	**3.125**	**1.244** (0.522 - 2.028)
AChE (a)	**<25**	**25**	**385.9** (284.4 - 608.1)	**<3.125**	**3.125**	**93.57** (54.12 - 264.5)
AChE (b)	**<25**	**25**	**119.2** (99.82 - 143.6)	**<3.125**	**3.125**	**27.45** (19.37 - 46.82)
EROD	**<25**	**25**	**385.1** (266.2 - 698.3)	**<3.125**	**3.125**	**1.148** (0.001 - 3.962)

The response of *P. microps* LDH to copper showed a distinct behaviour according the exposure concentrations: reduction of enzymatic activity at low concentrations (21% at 25 µg/L and 16% at 50µg/L), no significant alterations at 100 µg/L and a significant increase at 200 (23%) and 400 (47%) µg/L. A concentration-dependent decrease of EROD activity was found in copper exposed fish ($F_{(5, 42)} = 71.1$, $p \leq 0.05$, LOEC 25 µg/L) with 54% of EROD inhibition relatively to the control group at 400 µg/L (Figure 3.5.C). Significant changes of GST activity were also observed ($F_{(5, 42)} = 129.7$, $p \leq 0.05$; LOEC = 200 µg/L) with 99% of induction at 400 µg/L (Figure 3.5.D). A significant increase of all the anti-oxidant enzymes activity was also found (CAT: $F_{(5, 42)} = 143.2$, $p \leq 0.05$; SOD: $F_{(5, 42)} = 533.7$, $p \leq 0.05$; GR: $F_{(5, 42)} = 139.7$, $p \leq 0.05$; GPx: $F_{(5, 42)} = 185.6$, $p \leq 0.05$) (Figure 3.5.E, F, G, H) with LOECs ranging from 25 to 50 µg/L. Exposure to copper at concentrations equal or higher than 25 activity µg/L caused lipid peroxidation ($F_{(5, 42)} = 303.4$, $p \leq 0.05$) with 233% of increase at 100 µg/L (Figure 3.5.I). Among all the endpoints tested, the most sensitive was AChE (using the *o*-nitrophenyl acetate method), with an EC_{50} of 119.2 (99.82 - 143.6) µg/L (Table 3.2.).

Exposure to mercury caused a significant inhibition of *P. microps* AChE activity in *P. microps*, as indicated by the two different techniques used to measure enzymatic activity (Figure 3.6.A): the Ellman technique (Figure 3.6.A (a)) ($F_{(5, 42)} = 64.1$, $p \leq 0.05$; LOEC = 3.1 µg/L) and the method using *o*-nitrophenyl acetate as substrate (Figure 3.6.A (b))($F_{(5, 42)} = 167.5$, $p \leq 0.05$; LOEC = 3.1 µg/L). As observed for copper exposure, the *o*-nitrophenyl method was apparently more sensitive causing 54% of inhibition at 50µg/L.

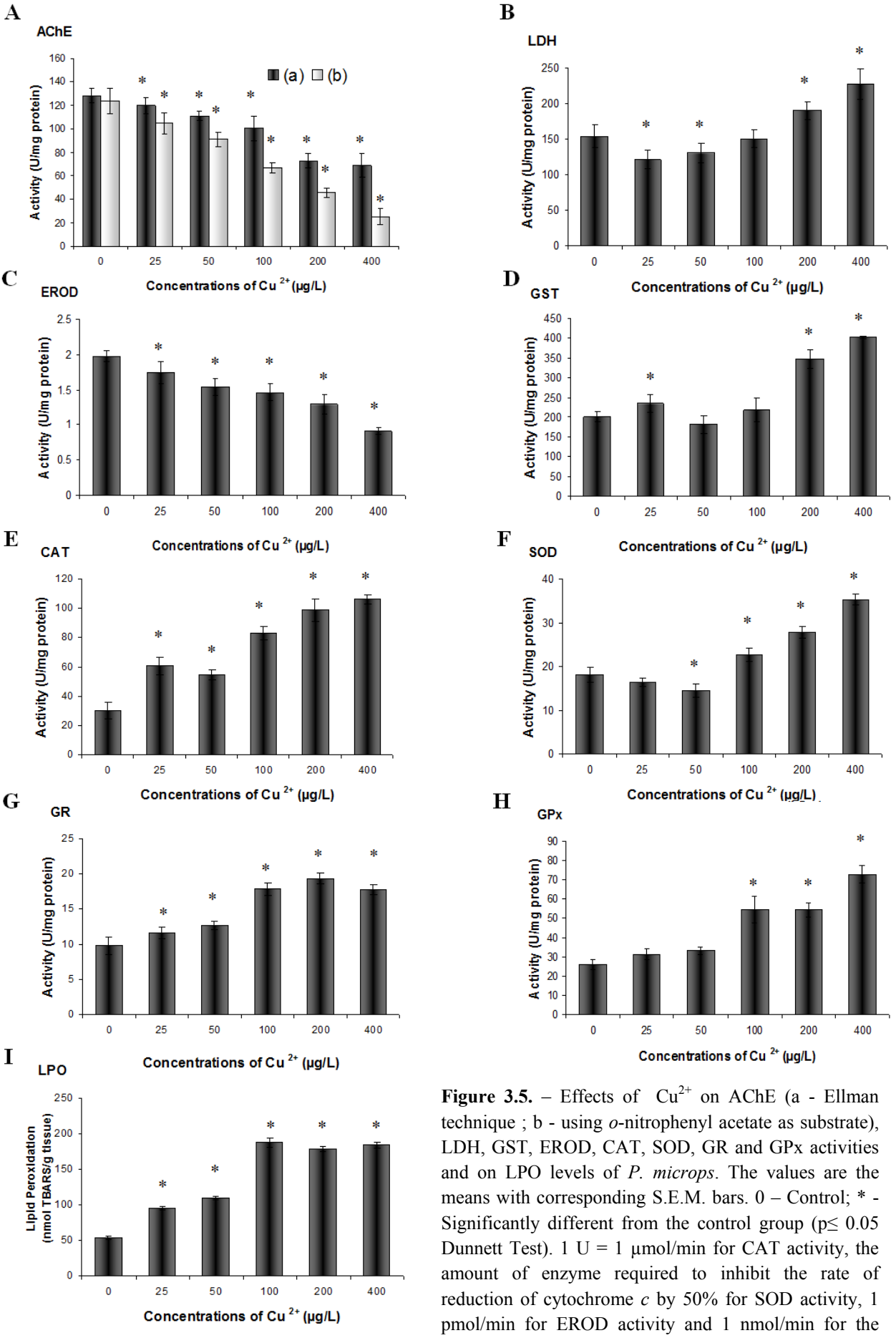

Figure 3.5. – Effects of Cu^{2+} on AChE (a - Ellman technique ; b - using *o*-nitrophenyl acetate as substrate), LDH, GST, EROD, CAT, SOD, GR and GPx activities and on LPO levels of *P. microps*. The values are the means with corresponding S.E.M. bars. 0 – Control; * - Significantly different from the control group ($p \leq 0.05$ Dunnett Test). 1 U = 1 µmol/min for CAT activity, the amount of enzyme required to inhibit the rate of reduction of cytochrome *c* by 50% for SOD activity, 1 pmol/min for EROD activity and 1 nmol/min for the other enzymes.

LDH activity was significantly induced ($F_{(5, 42)}$ = 137.7, $p \leq 0.05$; LOEC = 25µg/L) in fish exposed to concentrations 3.1 µg/L, 25 µg/L and 50 µg/L, 43% and 13% of induction at the highest concentrations tested relatively to the control group (Figure 3.6.B). Mercury caused a significant decrease of EROD activity ($F_{(5, 42)}$ = 167.7, $p \leq 0.05$, LOEC 3.1µg/L) with 73% and 68% of EROD inhibition relatively to the control group at 12.5 µg/L and 50 µg/L, respectively (Figure 3.6.C). A significant induction of GST activity was observed ($F_{(5, 42)}$ = 98.1, $p \leq 0.05$; LOEC = 3.1 µg/L), showing an almost bell-shaped pattern with a maximum of induction of 47% at 25 µg/L and a further decrease (25%) the highest concentration tested (Figure 3.6.D). The activity of all the anti-oxidant enzymes was induced in exposed fish (CAT: $F_{(5, 42)}$ = 375.5, $p \leq 0.05$; SOD: $F_{(5, 42)}$ = 32.9, $p \leq 0.05$; GR: $F_{(5, 42)}$ = 211.9, $p \leq 0.05$; GPx: $F_{(5, 42)}$ = 354.121, $p \leq 0.05$) (Figure 3.6.E, F, G, H), with LOECs of 3.125 µg/L. Exposure to mercury caused lipid peroxidation at all the concentrations tested ($F_{(5, 42)}$ = 243.8, $p \leq 0.05$), with 255% of increase relatively to the control group at 50 µg/L (Figure 3.6.I). Among all the endpoints tested, the most sensitive parameter was EROD activity with an EC_{50} value of 1.2 (CL: 0.001 - 3.962) µg/L (Table 3.2.).

3.4.4. Linking biomarkers to behaviour

In fish exposed to metals, significant correlations were found between several biomarkers and behavioural endpoints (Table 3.3.). For both metals, positive and significant correlations between AChE and swimming resistance were found. In addition, for mercury, positive and significant correlations were also found for covered distance. For both metals, significant correlations between the two behavioural endpoints and LPO were found and, in general, also with anti-oxidant enzymes. In addition, in the case of copper, a positive and significant correlation between EROD and swimming resistance was also observed, while under mercury exposure, the correlation was significant with covered distance (Table 3.3.).

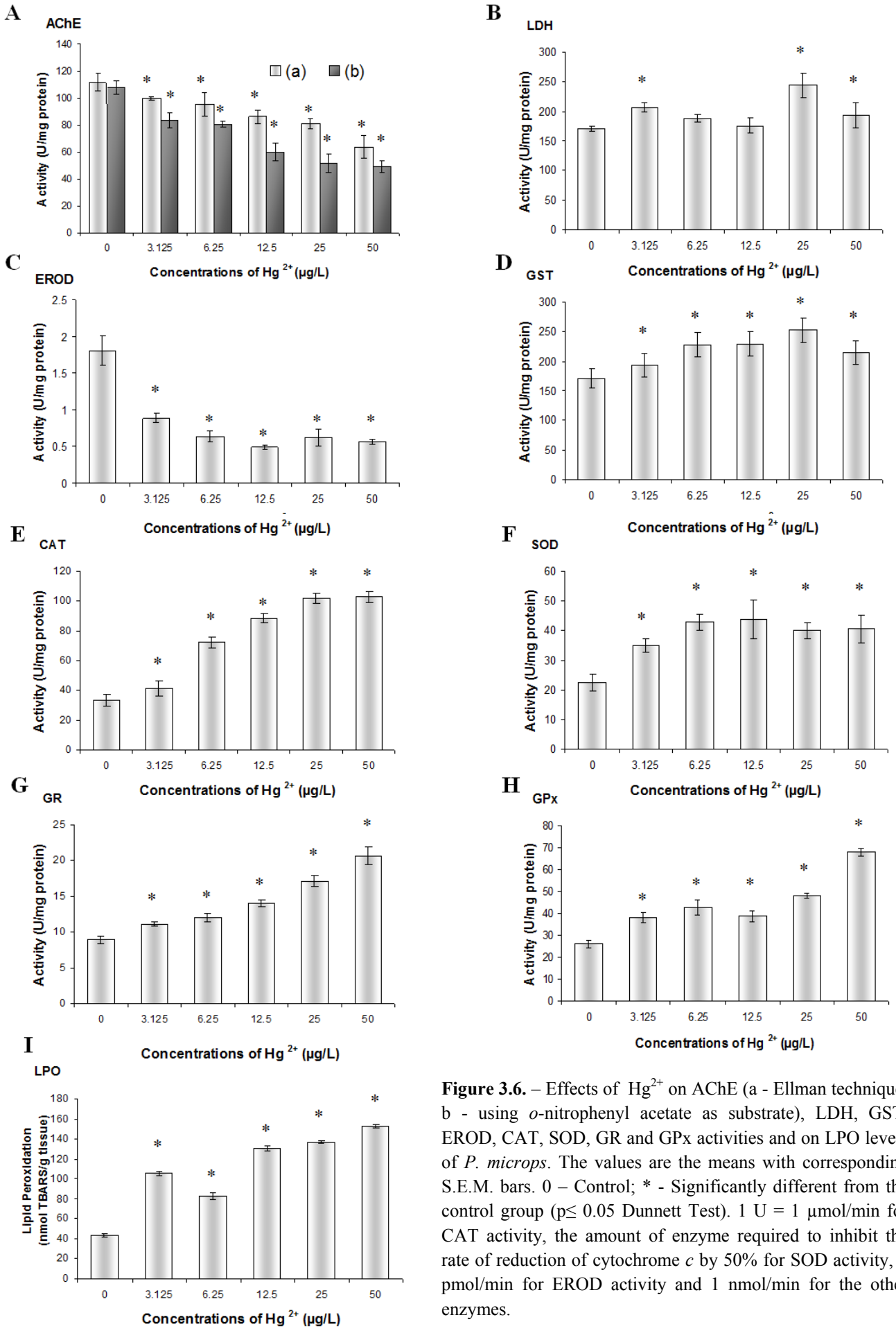

Figure 3.6. – Effects of Hg^{2+} on AChE (a - Ellman technique; b - using *o*-nitrophenyl acetate as substrate), LDH, GST, EROD, CAT, SOD, GR and GPx activities and on LPO levels of *P. microps*. The values are the means with corresponding S.E.M. bars. 0 – Control; * - Significantly different from the control group ($p \leq 0.05$ Dunnett Test). 1 U = 1 µmol/min for CAT activity, the amount of enzyme required to inhibit the rate of reduction of cytochrome *c* by 50% for SOD activity, 1 pmol/min for EROD activity and 1 nmol/min for the other enzymes.

A PCA was used to simultaneously analyze swimming performance and biomarkers response in fish exposed to copper and mercury (Figures 3.7 and 3.8).

In the case of copper (Figure 3.7.), the two axes explained 97.8% of total variation; the first axis (horizontal) explained 86.4% of total variation, while the second axis (vertical) explained 11.4% of total variation. The first axis clearly separates two groups of parameters: the first including AChE, EROD, swimming resistance and covered distance and, the second, including LDH, GST, anti-oxidant enzymes and LPO.

In the case of mercury exposure (Figure 3.8.), the two displayed axis explained 90.2% of the total variation: the first axis (horizontal) explained 78.9% of total variation, while the second axis (vertical) explained 11.3%. As for copper, the first axis clearly separates AChE, EROD and behavioural parameters from LDH, GST, LPO and anti-oxidant enzymes.

Table 3.3. - Pearson correlation coefficients (*p ≤ 0.05) for the correlations between biomarkers (AChE ((a)-Ellman assay; (b) – using *o*-nitrophenyl substract), LDH, EROD, GST, CAT, SOD, GR, GPx and LPO) and the behavioural parameters quantified in *P. microps* after exposure to copper and mercury.

	Pearson correlation coefficient			
	Copper exposure		***Mercury Exposure***	
Biomarker	Swimming resistance	Covered distance	Swimming resistance	Covered distance
AChE *(a)*	0.941*	0.770	0.984*	0.812*
AChE *(b)*	0.977*	0.805	0.890*	0.916*
LDH	-0.813*	-0.544	-0.387	-0.457
EROD	0.991*	0.782	0.676	0.990*
GST	-0.796	-0.429	-0.518	-0.835*
CAT	-0.911*	-0.719	-0.835*	-0.838*
SOD	-0.860*	-0.562	-0.587	-0.952*
GR	-0.851*	-0.829*	-0.985*	-0.749
GPx	-0.954*	-0.743	-0.972*	-0.724
LPO	-0.879*	-0.836*	-0.896*	-0.899*
Swimming resistance		0.843*		0.751
Covered distance	0.843*		0.751	

3.5. DISCUSSION

In the present study, ionic concentrations of copper ranging from 25 to 400 µg/L and of mercury ranging from 3.1 to 50 µg/L were tested. These concentrations are ecologically relevant since they compare with those that have been found in sediments, water and organisms from several estuaries, including some were *P. microps* naturally occurs. For example, concentrations of copper between 9 and 232 mg/Kg dry weight (d.w.) were found in sediments of the Esmoriz-Paramos coastal lagoon in Portugal (Fernandes *et al.*, 2007), copper concentrations ranging from 0.6 to 25 µg/L in water, from 17.4 to 2672 µg/g (d.w.) in sediments and from 2.9 to 39.5 µg/g (d.w) in the yellow perch (*Perca flavescens*) were found in lakes of Sudbury (Ontario, Canada) (Pyle *et al.*, 2005), while copper concentrations of 2.5 ±1 µg/g (d.w.) were determined in *P. microps* from the Seine estuary (France) (Miramand *et al.*, 1998). Morillo *et al.* (2005) observed water concentrations of copper ranging from 9.0±3.2 µg/L to 479±300 µg/L in the Huelva estuary, Spain. In Portugal, Hg concentrations ranging from 0.001 to 0.598 µg/g (d.w.) were found in sediments from the "Ria Formosa" lagoon (Bebianno, 1995), concentrations from 2.3 to 343 µg/g (d.w.) and of 2.0 µg/g were found in sediments and in *Dicentrarchus labrax* liver from the "Aveiro" lagoon (Pereira *et al.*, 1998; Abreu *et al.*, 2000), while water concentrations in a particular site of the Aveiro lagoon ranged from 0.1667 to 1.9255 µg/L in surface waters and from 1.1205 – 3.3948 µg/L in bottom waters (Guilherme *et al.*, 2008). Geffen *et al.* (1998) reported mercury concentrations between 29.8 and 43.93 µg/g (wet weight w.w.) in *Pomatoschistus minutus* from the Isle of Man, U.K., while Sellanes *et al.* (2002) reported concentrations of Hg in tissues of several species of fish from Cabo Frio (Rio de Janeiro, Brazil) ranging from 0.019 to 0.117 ppm.

3.5.1. Lethal effects

LC_{50} values indicated that mercury is more toxic to *P. microps* than copper, with a difference of about nine fold. LC_{50}s obtained in the present study (568.1 µg/L for copper, 61.9 µg/L for mercury) compare with corresponding values that have been published in the literature for other species of fish.

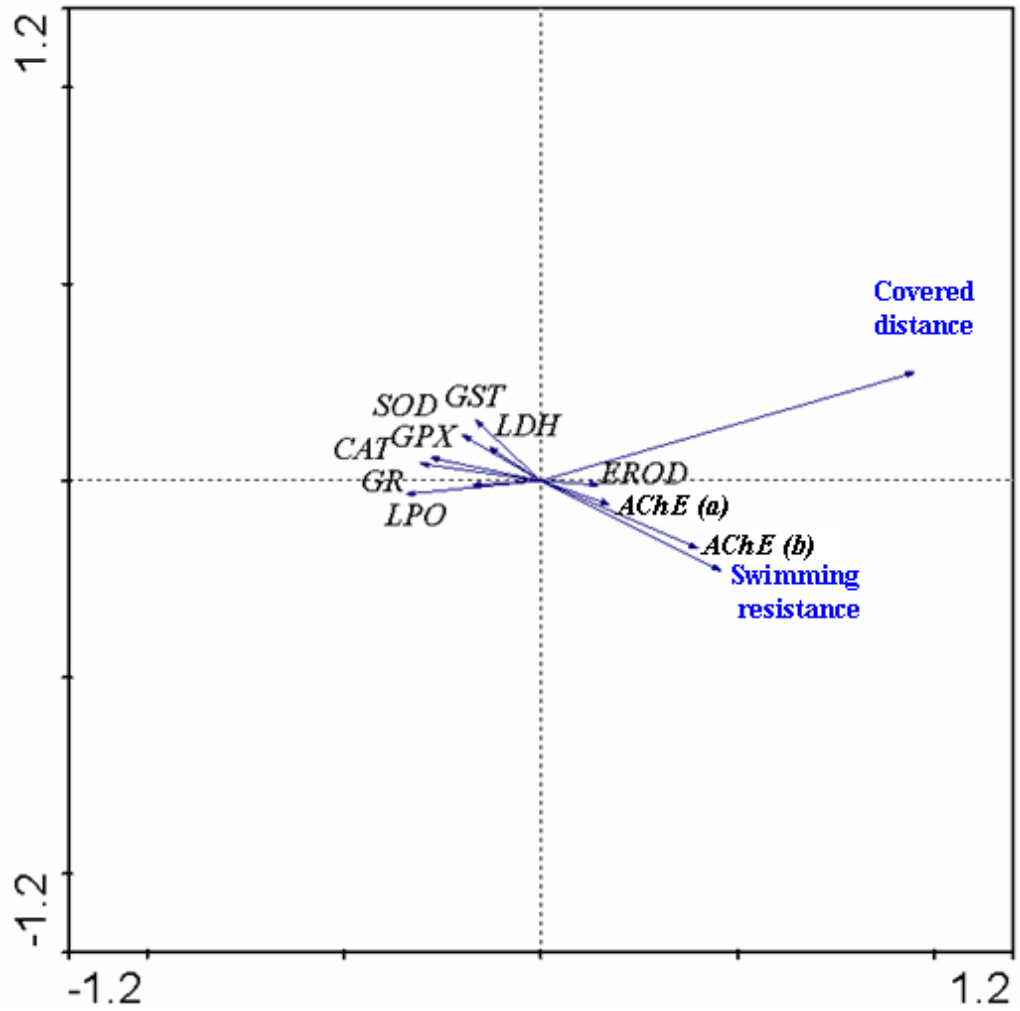

Figure 3.7. – PCA diagram showing the biomarkers assayed and their relation with swimming resistance and covered distance in *P. microps* exposed to copper. AChE (a) - Ellman technique and AChE (b) - using *o*-nitrophenyl acetate as substrate. The first axis (horizontal) displays 86.4% of total variation and the second axis (vertical), 11.4%. Total variation explained: 97.8%.

For example, copper 96h LC_{50} values of 1.4 mg/l (5.5 mg/l as $CuSO_4$) for the Flying barb (*Esomus danricus*), 0.246 mg/L for *Varicorhinus barbatus* and 1140 µg/L for the sheepshead (*Archosargus probatocephalus*) were reported (Steele, 1983; Shyong and Chen, 2000; Vutukuru *et al.*, 2006). For mercury, 96h LC_{50} values of 75 µg/L for the catfish (*Sarothrodon mossambicus*), 33 µg/L for the rainbow trout (*Salmo gairdneri*), 110 µg/L for the banded killifish (*Fundulus diaphanous*) and 90 µg/L for the striped bass (*Roccus saxatilis*) were found (Rehwoldt *et al.*, 1972; Hale, 1977; Das *et al.*, 1980).

3.5.2. Behavioural effects

Behaviour links physiological functions with ecological processes (Scott and Sloman, 2004) and therefore it has been considered of relevance when studying the effects of pollution (Atchinson *et al*., 1987), particularly in fish where several ecological relevant behavioural endpoints are easy observed and quantified in a controlled setting (Scott and Sloman, 2004). *P. microps* is a migratory epibenthic intermediary predator fish frequently inhabiting areas with a considerable hydrodynamism. It moves regularly to avoid low salinities, low temperatures and other adverse conditions. Its capability of swimming, including against water flow, is crucial to its survival.

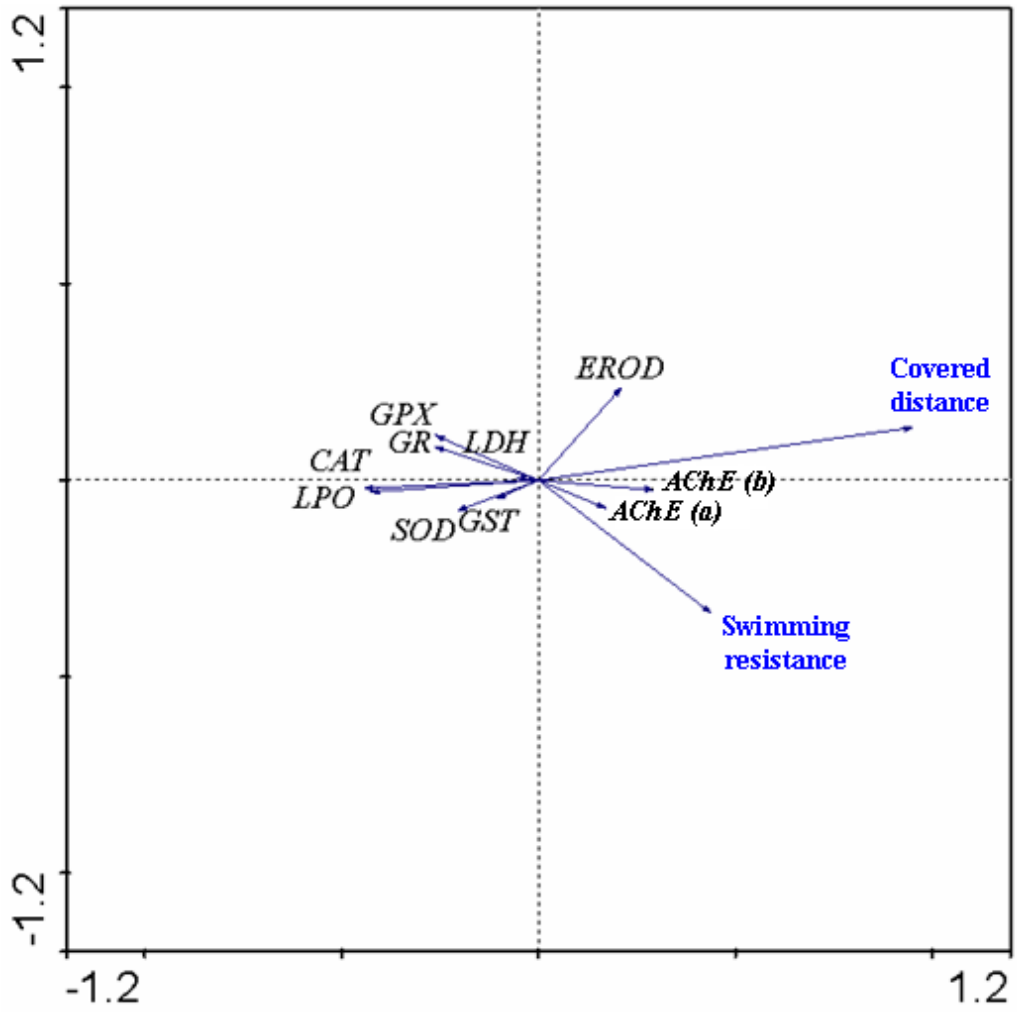

Figure 3.8. – PCA diagram showing the biomarkers assayed and their relation with swimming resistance and covered distance in fish exposed to mercury. AChE (a) - Ellman technique and AChE (b) - using *o*-nitrophenyl acetate as substrate. The first axis (horizontal) displays 78.9% of total variation, the second axis (vertical), 11.3%. The total variance explained by the two axes is 90.2%.

SPEDE was based on these principles and allowed the easy quantification of the individual performance when swimming against water flow, through the swimming time until being drag way, and the distance covered when swimming against the water flow. The results show a clear concentration-dependent loss of swimming resistance in fish exposed to metals, as well as a considerable reduction of the covered distance, with mercury having effects at lower concentrations than copper. The effects in behavioural endpoints were well below LC_{50} values, indicating that these endpoints are far more sensitive than mortality, and compare to some of the biomarkers used. Therefore, SPEDE seems to be a valuable device to assess the effects of metals on *P. microps* behaviour, allowing the quantitative measurement of ecological relevant behavioural endpoints. Further studies are needed to test its efficacy towards other groups of important environmental contaminants.

In the present study, alterations of *P. microps* behaviour were found in fish exposed to ionic concentrations equal or higher than 50 µg/L of copper and 3.1 µg/L of mercury, therefore in good accordance with results that have been reported for other fish: from 2.5 to 10 mg/L of copper sulphate for the flying barb, *Esomus danricus* (Vutukuru *et al.*, 2006) and 10 mg/L of copper (as $CuCl_2$) for the brown trout, *Salmo trutta* (Beaumont *et al.*, 2000). In fish, most studies on mercury induced behavioural changes are restricted to waterborne exposure, mostly reporting structural damage to the olfactory organs and disturbed sensory behaviour (Hara *et al.*, 1976; Baatrup and Doving, 1990; Baatrup, 1991; Ribeiro *et al.*, 1995).

3.5.3. Effects on biomarkers

It is well known that some metals can alter the activity of several enzymes by binding to their functional groups or by displacing the metal associated with the enzyme (Viarengo, 1985). In good agreement, laboratorial and field studies published in the last decades indicate effects of some metals, including copper and mercury, on several enzymatic biomarkers. In some cases (e.g. AChE, GST, EROD activities), apparently contradictory effects have been reported (i.e. inhibition, induction) even when considering only *in vivo* studies. Several factors may contribute to the differences found including the species studied, the properties of the enzymes present in the analysed tissue, the type of study (field or laboratorial), experimental conditions (e.g. temperature, test medium), time and intensity of the exposure, chemical form of the test compound, among others.

In several aquatic animals, AChE and other ChEs have been found to be inhibited by copper and mercury, both *in vivo* and *in vitro* conditions (Gill *et al.*, 1990; Bocquené *et al.*, 1995; Suresh *et al.*, 1992; Garcia *et al.*, 2000; Elumalai *et al.*, 2007; Roméo *et al.*, 2006). However, no significant effects on AChE (Frasco *et al.*, 2008) and increases of AChE activity have been also reported (Dethloff *et al.*, 1999; Romani *et al.*, 2003). In the case of inorganic mercury, the type of cholinesterase inhibition seems to be dependent of the presence and sensitivity of a free sulfhydryl group in the enzyme and reversible inhibition, enzyme denaturation and protein aggregation may occur (Frasco *et al.*, 2007). Since metals can interfere with the Ellman's technique (Ellman *et al.*, 1961) and *o*-nitrophenyl acetate is degraded also by non-specific esterases (Frasco *et al.*, 2005), both techniques were used in the present study to assess the effects of copper and mercury on *P. microps* AChE. For both metals, a significant and concentration-dependent inhibition of AChE activity was found, mercury being a more efficient inhibitor than copper. These results indicate that toxicants are causing neurotoxic effects through the inhibition of AChE and, thus, that fish neurologic and neuromuscular functions are impaired at least at the highest concentrations tested. The technique using substrate *o*-nitrophenyl acetate gave a more pronounced AChE inhibition than the Ellman's technique. This may be due to the presence of esterases other than ChE in head homogenates contributing to the degradation of the substrate that it is not specific for ChE. For this reason and as indicated by Frasco *et al.* (2005), the technique using *o*-nitrophenyl acetate should only be used when working with purified extracts of ChE, with pure enzymes or after a previous characterization of the enzymes present in the tissue used as enzymatic source. The comparison of the two methods using *P. microps* head homogenates performed in the present work also indicates that the technique used to measure AChE activity may have influence on the results.

Changes in LDH activity have been used as an indicative of alterations in the pathways of cellular energy production induced by toxicants (De Coen *et al.*, 2001; Monteiro *et al.*, 2006; Vieira *et al.*, 2008). In the present study, both copper and mercury significantly induced *P. microps* LDH activity at the highest concentrations tested, suggesting an increase of the use of the anaerobic pathway of energy production in fish under copper or mercury stress. These results are in good agreement with the enhanced LDH activity found in rosy barb (*Puntius conchonius*) muscle exposed to mercuric

chloride (Gill *et al.*, 1990) and disagree with the inhibition of LDH found in bony fish (*Sparus auratus*) muscle exposed to 0.5 ppm of copper (Antognelli *et al.*, 2003).

EROD activity of *P. microps* exposed to metals showed a significant inhibition (68% for mercury and 54% for copper) at the highest concentrations tested, indicating a decrease in the capability of phase I biotransformation in the presence of these metals. This may have serious implications for fish even if they are not exposed to foreign chemicals other than metals since some toxic endogenous substances need the intervention of this enzyme to be eliminated. The inhibition of *P. microps* found agrees with the reductions on EROD activity previously found in aquatic animals exposed to metals (Roméo *et al.*, 1994; Guilherme *et al.*, 2008), including to copper (Oliveira *et al.*, 2004; Sanchez *et al.*, 2005) and mercury (Viarengo *et al.*, 1997). EROD inhibition by metals may be due to its ability to react with sulfhydryl groups of the enzyme, resulting in protein conformational changes and thereby preventing their normal function (Oliveira *et al.*, 2004). *P. microps* EROD activity was found to be more inhibited by mercury than by copper, in good agreement with results from the literature (Viarengo and Nott, 1993; Viarengo *et al.*, 1997; Canesi *et al.*, 1999; Sen and Semiz 2007).

In the present study, both copper and mercury significantly increased gill GST activity. At least two different hypotheses, not mutually exclusive, may be raised to explain these results: (i) since GST is a cofactor for glutathione peroxidase, the increase of this enzyme to face oxidative stress needs more co-factor and, thus, the levels of GST are also enhanced, and (ii) since GST determinations were performed in gills that constitute a first barrier against the entrance of toxicants in fish body and GSTs have the capability of bind/store or transport substances, the increase of GST levels observed may correspond to a first attempt to overcome metal stress by producing a high amount of enzyme that will be then available to bind metals, decreasing their local concentration and, therefore, their uptake by the organism. Also for this enzyme, inhibition, no effects and induction have been reported after exposure to copper (Sanchez *et al.*, 2005; Liu *et al.*, 2006). However, no direct comparisons with our study are possible since these studies were performed in liver, while our GST determinations where performed in gills and GSTs present in the two tissues may not behaviour in a similar way. It is interesting to note the bell-shaped pattern of GST response to mercury (Figure 3.6.). This GST pattern showing a decrease of enzymatic activity at high concentrations may be due to glutathione (GSH) depletion at the

highest concentrations as suggested previously (Elumalai *et al.*, 2007). A bell-shaped GST inhibition by mercury was also reported by Elia *et al.* (2003) in the catfish (*Ictalurus melas*).

Significant inductions of all anti-oxidant enzymes activities were observed in copper and mercury exposed fish, indicating that both metals are inducing oxidative stress on fish, with effects already induced as indicated by the significant increase of lipid peroxidation levels at all the concentrations of metals tested. In general, these results are in agreement with previous findings published by other authors (Pedrajas *et al.*, 1995; Berntssen *et al.*, 2003; Elia *et al.*, 2003) and confirm the oxidative stress potential of copper and mercury to *P. microps*, as found for other species (Bano and Hasan, 1989; Salonen *et al.*, 1995; Roméo *et al.*, 2000; Florence *et al.*, 2002; Berntssen *et al.*, 2003).

3.5.4. Linking biomarkers to behaviour

The integrated analysis of all the parameters showed, both for copper and mercury stress, an association between AChE and the swimming performance of fish, in good agreement with the significant and positive correlations found between the activity of this enzyme and both behavioural endpoints. These results suggest that AChE inhibition is involved in the reduced swimming performance of fish probably due to the impairment of neuromuscular function and/or to central nervous effects. EROD also appears associated with this group of parameters, possibly because the tested metals inhibit both enzymes through the same mechanism. The association between anti-oxidant enzymes, LPO and LDH suggests that the anaerobic pathway of energy production is enhanced to face oxidative stress. GST is also associated with oxidative stress parameters suggesting that increased gill GST activity are required to cope oxidative stress, probably because this enzyme is a cofactor for glutathione peroxidase. The overall results also suggest that the presence of Reactive Oxygen Species (ROS) and lipid damage are negatively related with swimming performance of fish, EROD and AChE activities.

3.6. CONCLUSIONS

The new device especially designed to quantify two behavioural endpoints in epibenthic fish (SPEDE) was proven to be efficacious against metals, allowing the quantification of behavioural alterations at ecological relevant concentrations. Both copper and mercury inhibited *P. microps* AChE, increased the use of the anaerobic pathway of energy production, caused oxidative stress and lipid peroxidation. Significative and positive correlations were found between the biomarkers AChE and EROD and the behavioural parameters, while negative correlations were found between all the other biomarkers and behavioural parameters. The integrated analysis of data associated AChE, EROD and swimming performance of fish by opposition to anti-oxidant enzymes, LDH, GST and LPO. Therefore, at least in the case of metals, biomarkers such as AChE and LPO are ecological relevant parameters.

3.7. ACKNOWLEDGEMENTS

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Chapter 4. Biomonitoring study in a shallow lagoon using Pomatoschistus microps as bioindicator: multivariate approach integrating ecological and ecotoxicological parameters

Biomonitoring study in a shallow lagoon using *Pomatoschistus microps* as bioindicator: multivariate approach integrating ecological and ecotoxicological parameters

(*to be submitted to Environmental Science and Pollution Research*)

4.1. ABSTRACT

The central objective of this study was to validate an integrated approach, including ecological and ecotoxicological parameters, to evaluate the effects of pollution on estuarine fish in real scenarios, using the common goby *Pomatoschistus microps* (Krøyer, 1838) as bioindicator and the Aveiro lagoon (NW coast of Portugal) as case study area. The methodology included fourteen water quality variables, sediment characteristics, the concentrations of nine metals in sediments and in the fish body, fish condition indexes, eight biomarkers and multivariate statistics (Redundancy and Principal Response Curves analysis) to integrate the information provided by different parameters. The study was conducted over one year with seasonal sampling (winter, spring, summer and autumn) at four sampling sites with different contamination histories. The integrated approach indicated significant differences between the reference site and the remaining ones, both in biological and environmental descriptors. Bioaccumulation factors (BAFs) suggested that fish bioaccumulated some metals, especially Zn. Overall, the results indicated that several biomarkers and the concentrations of Al and Pb in fish body were suitable discriminatory parameters, separating the contaminated sites from the reference one. On the contrary, the Fulton Condition Index was not a good discriminatory parameter. Furthermore, PRC analysis provided useful information regarding the discriminating power of parameters inside both biological and environmental descriptors groups. The selected integration approach through multivariate analysis provided important information that may be used as scientific support for conservation and management of estuarine and lagoon systems.

Keywords: *Pomatoschistus microps*, estuaries, metal pollution, ecological and ecotoxicological parameters, biomarkers, multivariate analysis.

4.2. INTRODUCTION

Coastal transition ecosystems, such as estuaries and coastal lagoons, are among the most productive and valuable aquatic ecosystems on earth. They are recognized worldwide as an important component of continental coasts in terms of their biological importance and human utilization (Marques *et al.*, 2004), being crucial to the life history and development of many species (Chapman and Wang, 2001). A considerable part of these areas around the world have been increasingly contaminated by chemicals resulting from anthropogenic activities. Some of these chemicals have been found at concentrations high enough to cause adverse effects on the biota (Van der Oost *et al.*, 2003). They may also alter water and sediments characteristics decreasing their overall quality to support the community of organisms living in their dependency. Therefore, monitoring programs have been carried out to assess the type and levels of environmental contaminants in sediments and water (e.g. Bebianno, 1995; Ajmone-Marsan *et al.*, 2008; Vicente-Martorell *et al.*, 2009), and the bioaccumulation of pollutants by selected species, especially those for human consumption (Chi *et al.*, 2007). However, chemical monitoring is restricted to the quantification of a limited number of substances present in the environment and/or organisms without providing relevant information on their biological significance (Livingstone *et al.*, 1991). In addition, often in estuaries and coastal areas, chemicals are present as complex mixtures and, thus, toxicological interactions are likely to occur. Therefore, chemical analyses *per si* are not sufficient to describe the adverse effects of these complex mixtures (Ozmen *et al.*, 2006). Furthermore, in these ecosystems, considerable variations of physico-chemical parameters use to occur and several species are already living in their tolerance limits relatively to some of these factors. Thus, the interactions between natural stressors (e.g. temperature, salinity, pH and light) and pollutants may be an additional tread at least to some species. Therefore, considering global changing scenarios, it is urgent to develop cost-effective methods to assess the impact of pollution on sensitive and important ecosystems such as estuaries and lagoons. Particular attention should be given to field approaches assessing both direct toxic effects on organisms and alterations on the abiotic component of the ecosystem.

The contamination of estuarine and coastal waters by metals derived from anthropogenic activities has long been a concern (Van der Oost *et al.*, 2003). They are

common contaminants of these ecosystems, they accumulate in sediments and in organisms and they are toxic at ecological relevant concentrations. Despite the essential role of some metals, several of those commonly found in estuaries and lagoons are able to disrupt several physiological functions of organisms (Frasco *et al.*, 2005, 2007) with potential effects at higher levels of biological organization. Furthermore, since some species occupying low trophic levels are able to accumulate metals (Cairrão *et al.*, 2007), predators of these species may be exposed to toxic doses even if biomagnification does not occur.

Among animals inhabiting coastal lagoons, fish are of great interest since distinct species may occupy different ecological niches, they are sensitive to several environmental contaminants and some species have economic importance. Therefore, several fish have been used as sentinel species in monitoring programs carried out in estuaries, lagoons and coastal areas (Arruda *et al.*, 1993, Cabral *et al.*, 2007; Rodrigues *et al.*, 2006; Solé *et al.*, 2006, Webb, 2005). One of this species is the common goby, *Pomatoschistus microps* Krøyer (1838) (Monteiro *et al.*, 2007), which is an abundant fish in estuaries, lagoons and shores of Europe (Arruda *et al.*, 1993; Salgado *et al.*, 2004) where it plays an important ecological function as intermediary predator connecting macro- and meiofauna with larger predator fish (Miller *et al.*, 1986; Arruda *et al.*, 1993).

In the last years, several monitoring programs with fish included environmental biomarkers to assess biological adverse effects of chemicals present in water, sediments and/or fish body (Lopes *et al.*, 2001; Monteiro *et al.*, 2007; Guimarães *et al.*, 2009). These parameters allow the early diagnosis of effects resulting from stress exposure long before they become evident at the population level where adverse effects may be difficult to revert. Enzymes involved in physiological processes determinant for the survival and performance of organisms are among the most used biomarkers in biomonitoring studies. Some examples are: acetylcholinesterase (AChE) which plays a determinant role in cholinergic neurotransmission; lactate dehydrogenase (LDH) which is a key enzyme in the anaerobic pathway of energy production; glutathione S-transferases (GST) that are involved in detoxification and in lipid peroxidation prevention; catalase (CAT), superoxide dismutase (SOD), glutathione reductase (GR) and glutathione peroxidase (GPx) which are part of the anti-oxidant defences, and lipid peroxidation (LPO) that has been used as a marker of oxidative damage (Solé *et al.*, 2006; Monteiro *et al.*, 2007; Ferreira *et al.*, 2008;

Falfushynska *et al.*, 2009; Guimarães *et al.*, 2009). These biomarkers used simultaneously with health condition indexes, such as the Fulton condition factor (FCF) and the hepatosomatic index (HSI), determined in representative samples and in relation to a reference population, use to give a good indication of the general health *status* of the population in relation to chemical exposure and its effects.

The central objective of this study was to validate an integrated approach, including ecological and ecotoxicological parameters, to evaluate the effects of pollution on estuarine fish in real scenarios, using the common goby as bioindicator and the Aveiro lagoon (NW Portugal) as case-study area. The approach included twenty physico-chemical parameters indicative of water quality and sediment characteristics, the concentrations of nine metals in sediments and in the fish body, eight biomarkers determined in different tissues of fish, two health condition indexes and multivariate analysis to integrate the information provided by different parameters.

4.3. MATERIAL AND METHODS

4.3.1. Chemicals

The chemicals for enzymatic analysis were purchased from Sigma–Aldrich® Chemical (Steinheim, Germany), except the Bradford reagent (Bio-Rad®, Munich, Germany). The chemicals used for water analysis were from Palintest® (Palintest LTD – England).

4.3.2. Short description of the study area

The Aveiro lagoon is located in the NW coast of Portugal (Figure 4.1). It is approximately 45 km long and 10 km wide, covering a minimum area of approximately 66 km^2 at low spring tide, and reaching a maximum of 83km^2 at a high spring tide (Dias *et al.*, 2001). This lagoon is supplied with freshwater by two main rivers: the Antuã river (5 m^3 s^{-1} average flow) and the Vouga river (50 m^3 s^{-1}) (Dias *et al.*, 1999). It is a very irregular and complex coastal lagoon, composed by long and narrow channels, with a high longitudinal development organized by successive ramifications from the mouth, as an arborescent network system (Morgado *et al.*, 2003; Leandro *et al.*, 2007). This lagoon is

composed of a wide range of biotopes (e.g. wetlands, salt marshes and mudflats) used as nursery areas for many valuable species belonging to different animal groups, such as bivalves, crustaceans, fish and birds (Lopes *et al.*, 2007).

4.3.3. Sampling sites

The study was performed during one-year (2005-2006), with seasonal sampling (winter, spring, summer and autumn). In the lagoon, four sites were selected reflecting different degrees of anthropogenic contamination by metals and other chemicals. A global position system (GPS) (Garmin GPSMAP 60CSX) was used to determine the coordinates of the sampling sites (Figure 4.1.), which are briefly described below:

Barra (40º37'50.91''N, 8º44'38.96''W) is located near to the artificial connection of lagoon to the sea. This sampling site was chosen as reference site due to its low levels of environmental contamination (Cerqueira and Pio, 1999; Quintaneiro *et al.*, 2006) and because it has been used as a reference site in previous studies with *P. microps* (Monteiro *et al.*, 2005, 2006) and other species (Quintaneiro *et al.*, 2006; Guilherme *et al.*, 2008).

Vagueira (40º34'24.32''N, 8º45'20.60W) is located in the Mira channel of the Aveiro lagoon which receives agriculture and animal farming run-off and, therefore, it is likely to be contaminated by fertilizers and pesticides used in crop fields and additives used for animal growth.

Harbour (40º39'19.56''N, 8º42'13.00''W) is located in the Aveiro harbour. It is contaminated by petrochemical products and their components, including metals (Hall *et al.*, 1987; Pacheco and Santos, 2001).

Cais do Bico (*C. Bico*) (40º43'46.96''N 8º39'00.13''W) is located near an area known as Laranjo bay which is described as polluted by metals (Monterroso *et al.*, 2003a, 2007).

4.3.4. Water and sediment analysis

Water analyses were performed in samples collected monthly during one year, from winter 2005/06 to the autumn 2006, including at the time of fish sampling. Temperature (ºC), salinity (‰), conductivity (µS/m) and pH were measured *in situ* at the four sampling sites during low tide with a multi-parameter probe (Hydrolab DS5X– Hach Environmental).

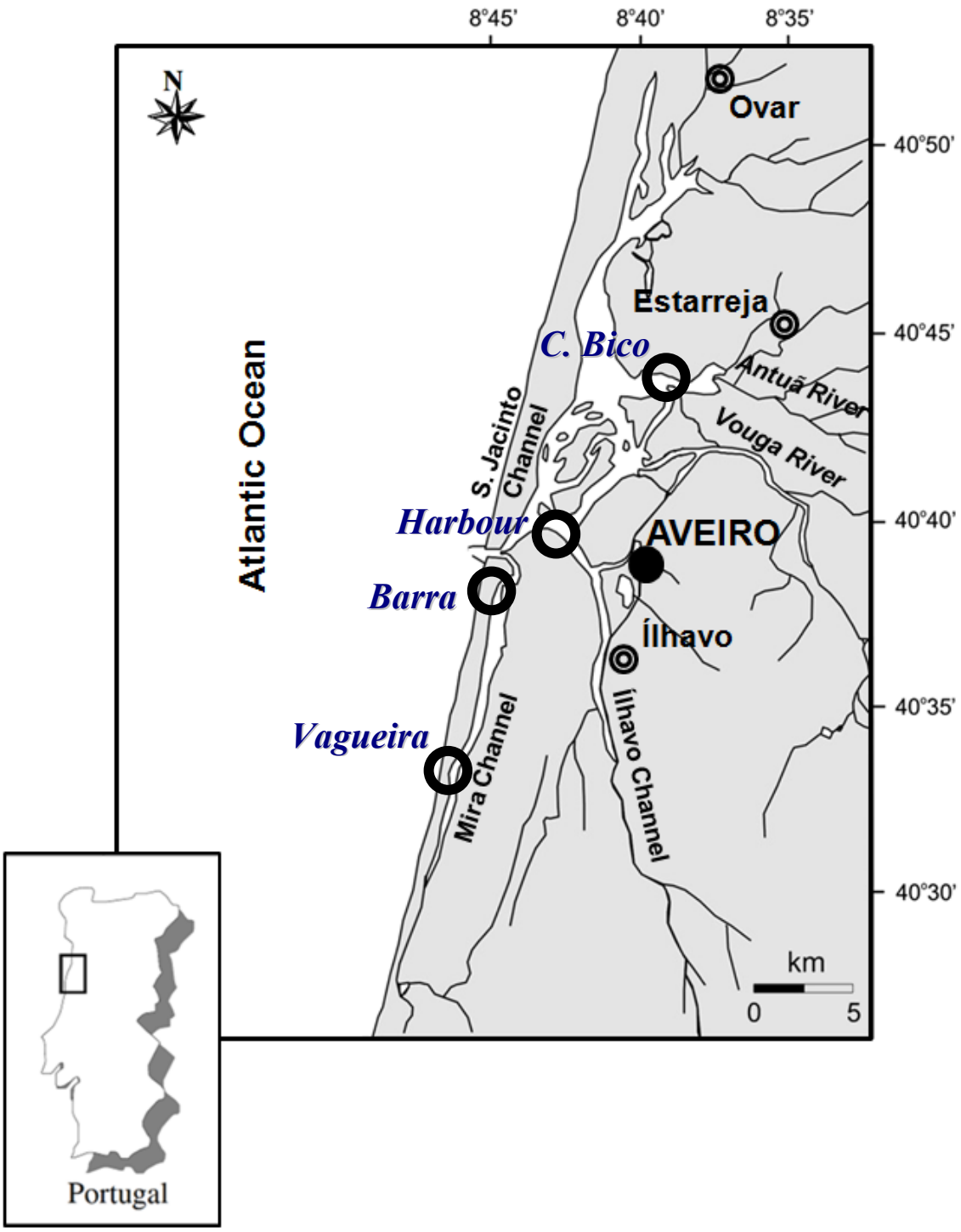


Figure 4.1. – The Aveiro lagoon indicating the location of the selected sampling sites, main channels and Rivers: *Barra* (40º37'50.91''N, 8º44'38.96''W), *Vagueira* (40º34'24.32''N, 8º45'20.60W), *Harbour* (40º39'19.56''N, 8º42'13.00''W) and *C. Bico* (40º43'46.96''N 8º39'00.13''W).

Dissolved oxygen (DO) (%) was measured *in situ* using a portable meter (LDO HQ10 – Hach Environmental). At each sampling site, subsurface water samples were also collected to 0.5 L polyethylene-terephthalate bottles and stored at -20°C for analysis. Levels of nitrates, nitrites, ammonia, phosphates, silica, phenol, iron, hardness and turbidity were measured using commercial photometer kits (Photometer 7000, Palintest, Kingsway, England). In addition, sediment samples for particle size determination (granulometry), organic matter content, chlorophyll *a*, *b* and *c,* and phaeopigments assays were collected. For each parameter, 10 replicates from the first 1cm of sediment were collected per site, with a 100 mL syringe. GR, OM, chlorophylls and phaeopigments were measured seasonally from winter 2005/2006 to the autumn 2006. Samples collected for granulometry and organic matter determinations were stored in plastic bags, commonly used for keeping human food, previously washed with nitric acid and covered with aluminium foil. Once in the laboratory, samples were stored at -20ºC until analysis. Granulometry determination involved a splitting process of the sediment into a sand fraction (particles greater than 63 µm) and a silt-clay fraction (SCF) (particles less than 63 µm), following the methodology described by Holme and McIntyre (1971). The organic matter content was estimated after combustion of previously dried samples, following the methodology indicated by Strickland & Parsons (1972). For chlorophylls and phaeopigments determinations, each sediment replicate was added to a 50mL polyethylene centrifuge tube with 30mL of 90% acetone and 0.2mL of magnesium carbonate (1g/100mL). At each sampling site, the centrifuge tubes were mixed thoroughly and, in the lab, they were kept in the fridge for 24h until the assay. Chlorophyll *a*, *b* and *c* and phaeopigments were estimated spectrophotometrically following the methodology described in Strickland and Parsons (1972), with small adaptations.

4.3.5. Fish sampling

P. microps juveniles (2.5 – 3 cm long) were captured seasonally (winter 2005/06 to autumn 2006) at the four sampling sites using a hand operated net at low tide. Approximately 105 animals were collected at each site for biomarkers and 360 individuals for determination of the concentrations of metals. After being collected, animals were immediately transported alive to the laboratory in refrigerated and aerated boxes.

4.3.6. Morphometric parameters and condition indexes

In the laboratory, fish were weighted, measured and sacrificed by decapitation. Then, the eviscerated body weight was also determined. All the weight determinations were done using an analytical balance (KERN 770). Liver was isolated on ice and individually weighted using the same analytical balance (KERN 770).

Two condition indexes were determined: the Fulton Condition Factor (FCF) and the hepatosomatic index (HIS) as:

$$FCF = 100 \times \frac{W}{L^3}$$

Where W is the total fish weight (g) and L is the total body length (cm) (Pyle *et al.*, 2005).

$$HIS = \frac{l}{b} \times 100$$

where l is the weight of liver (g) and b is the total body weight (g) (Lloret *et al.*, 2002)

4.3.7. Biomarkers analysis

From each fish, head, gills, dorsal muscle and liver were isolated on ice. The heads of 15 fish were put in 4.5 ml of phosphate buffer (0.1 M, pH 7.2) and used to prepare one sample for AChE determinations; pieces of dorsal muscle from 15 different fish were put in 4.5 ml of Tris-NaCl buffer (0.1 M, pH 7.2) and used to prepare one sample for LDH determinations; 15 pairs of gills were put in 4.5 ml of phosphate buffer (0.1 M, pH 6.5) and used to prepare one sample for GST analysis and 15 livers were put in 1:10 (w/v) of phosphate buffer (0.05 M, pH 7.0, with 0.1% Triton X-100) and used to prepare samples for LPO and anti-oxidant enzymes. Seven replicate pooled samples were prepared per site and per biomarker, in a total of 105 fish per site.

Samples were homogenised on ice using an Ystral homogenizer (Ballrechten-Dottingen, Germany) and centrifuged (SIGMA 3K 30 centrifuge) as follows: for 3 min at 4 ºC and 3300g for AChE and LDH samples; for 30 min at 4 ºC 9000g for GST

determinations. After homogenization of AChE, LDH and GST samples, the supernatant of each homogenate was collected and stored at -80ºC until further analysis. In the case of homogenised liver, 200 µL were collected from each sample and then stored at -80ºC after addition of 4 µL of butylated hydroxytoluene (BHT). The remanding homogenised liver of each pooled sample was centrifuged (SIGMA 3K 30 centrifuge) for 15 min at 15000g (4 ºC). Each of the final supernatants was divided in aliquots for CAT, SOD, GR and GPx assays and stored at -80ºC.

Prior to enzymatic analysis, the protein of each sample was normalised to 0.3 mg ml^{-1} in the case of samples for AChE and GST determinations, and to 0.9 mg ml^{-1} in the case of samples for LDH, CAT, SOD, GR and GPx determinations (Vieira *et al.*, 2008). The protein content was determined by the Bradford method (Bradford, 1976) adapted to microplate as indicated in Guilhermino *et al.* (1996).

AChE activity was determined according to the Ellman's method (Ellman *et al.*, 1961) adapted to microplate (Guilhermino *et al.*, 1996), using acetylthiocholine as substrate and a microplate reader BIO-TEK, model POWERWAVE 340. In a previous study, it was found that the soluble fraction of *P. microps* head homogenates contains mainly acetylcholinesterase (AChE) (Monteiro *et al.*, 2005). LDH was determined by the method of Vassault (1983), adapted to microplate (Diamantino *et al.*, 2001), using a microplate reader BIO-TEK, model POWERWAVE 340. GST was assessed according to Habig *et al.* (1974), with the modifications of the original protocol indicated in Frasco and Guilhermino (2002), in a microplate reader BIO-TEK, model POWERWAVE 340. The activities of GR, GPx and SOD were determined according to the methods of Carlberg and Mannervik (1975), Flohé and Günzler (1984) and McCord and Fridovich (1969), respectively, adapted to microplate (Lima *et al.*, 2007). A microplate reader BIO-TEK, model POWERWAVE 340 was used. CAT activity was measured according to the method of Aebi (1984) in a spectrophotometer JENWAY, model 6405 UV/VIS. LPO levels were determined by the quantification of thiobarbituric acid reactive substances (TBARS) as in Ohkawa *et al.* (1979) and expressed as nmol of TBARS per g of tissue, using a spectrophotometer JENWAY, model 6405 UV/VIS. At the end of enzymatic and LPO analysis, the amount of protein in each sample was again determined and this value was used for expressing enzymatic activity.

All the enzymatic activities were determined at 25 ºC and expressed as activity per mg of protein. One unit (U) of SOD activity was defined as the amount of enzyme required to inhibit the rate of reduction of cytochrome *c* by 50%. For CAT activity, one U was defined as 1 µmol/min and for all the remaining enzymes one U was equivalent to 1 nmol/min.

4.3.8. Chemical Analysis

The following metals were measured in sediments and in the fish body: Al, Pb, Cd, Cr, Co, Cu, Ni, Zn and Hg.

Sediment samples for chemical analysis were collected in the field and transported to the laboratory as previously indicated (point 2.4). In the laboratory, the sediments were dried in a drying oven (Raypa®) at 40ºC, they were put in previously nitric acid washed plastic bags, covered with aluminium foil and sent for Terracon Laboratorium, (Jüterbog, Germany) where the determination of heavy metals content was done. Here, 2 g of homogenised sediment was weighed into a 250 ml reaction glass vessel (accuracy 0.1 mg); 5 ml of nitric acid (68%, trace select quality) and 15 ml of hydrochloric acid (37%, trace select quality) were added ("aqua regia" acid digestion). The reaction vessel with cooler and vapour trap was heated at 180°C for 2 hours and the acidic solution was filled to 50 ml in a volumetric flask with 5% of nitric acid solution. After filtration, the metals were measured by ICP-OES. An aliquot of the sediment sample was used to determine the dry matter (mT) at 105°C. Metals were determined with ICP-OES (Model ARL 3410) after calibration with external standards for each element. Mercury (Hg) was determined with Hydrid-AAS (Model UNICAM 939) after calibration with an external standard.

Fish collected in the field for chemical analysis were weighted in an analytical balance, KERN 770). Then, groups of 120 individuals (1 replicate) were put in 15 mL Falcon tubes, previously washed with nitric acid, protected from light with aluminium foil, and kept at -20°C until analysis. Three replicates were prepared per site and season. Samples were lyophilised (freeze drying) for 4 days in a EZ-DRY lyophilizer (model nº EZ55OQ – FTS Systems, USA) and packed in Falcon tubes previously washed with nitric acid, protected from light with aluminium foil. In the laboratory of chemical analysis, 0.5 to 0.8 g of the fish sample were weighed (accuracy 0.1 mg), put into a 150 ml glass reaction vessel and 5 ml of sulphuric acid (98%, trace select quality) were added. After

reaction overnight ("wet washing" with sulphuric acid to destroy the organic part of the sample), 1 ml of nitric acid and 1 ml of hydrogen peroxide were added. The vessel with a vapour trap was heated in a microwave digestion apparatus according to a temperature program up to 200°C for 45 minutes. If the solution was not clear after the first digestion, further 1 ml of nitric acid and 1 ml of hydrogen peroxide were added and a new microwave digestion was started. These steps were repeated until a clear and colourless solution was obtained ("total acidic microwave digestion"). The digestion was carried out with the original air-dried (max. 40°C) fish sample. The acidic solution was filled to 50 ml in a volumetric flask with 5% nitric acid solution. After filtration, the metals were measured by ICP-OES (Model ARL 3410) after calibration with external standards for each element. Mercury (Hg) was determined with Hydrid-AAS (Model UNICAM 939) after calibration with an external standard.

4.3.9. Bioaccumulation factors

The bioaccumulation factor (BAF) was calculated for each metal according to the following formula (Barron, 1995):

$$BAF = \frac{Cfish}{Csed}$$

Where, *Cfish* is concentration (mg/kg) of metal in the fish tissue and *Csed* is the concentration (mg/kg) of the same metal in the sediment. For each metal and site, the C*sed* was the mean of the concentrations individually determined for the three samples of sediments collected at that site; the BAF was calculated for each fish as the ratio between the concentration of metal in that fish and the mean of sediment samples concentration.

4.3.10. Statistical analysis of data

Variables were tested for normality (Anderson-Darling normality test) and homogeneity of variance (Levine's test) (Zar, 1996). Strong departures from normality and homoscedasticity were corrected with log(x), log (x+1), arcsin[sqrt(x)] or sqrt(x) transformations, as appropriate. The values of each environmental variable, each metal determination, each condition index and each biomarker were compared over time by

Repeated Measures Analysis of Variance (RM-ANOVA) using sampling site as factor. When statistically significant differences among sites were found, post hoc comparisons were done against "*Barra*" using the Dunnet test (Zar, 1996). Season was selected as a random factor; therefore no multiple comparisons were performed for this factor. The null hypothesis of no differences between reference and contaminated sites was tested, except in the case of metals in sediments where the null hypothesis was that contaminant levels in the sediment of the contaminated sites were less or equal to the levels of the reference site.

In addition, the ordination technique of redundancy analysis (RDA), an ordination method of direct gradient analysis (ter Braak and Prentice, 1988) was done to assess the relationship between biological and environmental data. The biomarkers, condition indexes and metals analysed in fish were selected as species data (later on referred as biological descriptors), water parameters and chemical analysis of metals in sediments were selected as environmental data (later on referred to as environmental descriptors) and environmental parameters in sediment were selected as covariables data. Monte Carlo permutations were used to assess statistical significance of the canonical axes. Moreover, the least significant environmental variables, after Monte Carlo permutations under reduced model, were automatically excluded from the graphical representation.

The biological and environmental data were also analysed by Principal Response Curves (PRC). PRC is based on RDA, the constrained form of Principal Component Analysis (Van den Brink and ter Braak, 1999). The analysis resulted in diagrams showing the seasons on the x-axis and the first Principal Component of parameters on sites, on the y-axis. This yield a diagram showing the deviations in time of the most contaminated sites in relation to the reference (*Barra*). The weights of analysed parameters, biological descriptors (biomarkers, condition indexes and metals analysed in fish) and environmental descriptors (metals analysed in sediment and environmental parameters measured in water) are presented in separate diagrams indicating the affinity of the parameters with responses displayed in the first diagram.

Statistical analyses were performed using the software Minitab 14.0® (Minitab Inc, USA), with the exception of RDA and PRC analysis that were done using the software CANOCO 4.520© for Windows (Biometris, The Netherlands) (ter Braak and Smilauer, 1998).

4.4. RESULTS

4.4.1. Characterization and comparison of sampling sites

The mean, maximum and minimum values of the parameters measured in water and sediments are described in the Annex 4.1. The results from RM-ANOVA for environmental variables measured both in water and sediment are shown in Table 4.1. Significant differences among seasons and sites were found for all the tested parameters in both water and sediments (Table 4.1.). *Vagueira* showed significant differences relatively to the reference site (*Barra*) in the following parameters: temperature, conductivity, salinity, pH, nitrates, nitrites, ammonium, phosphates, silica, phenol, hardness, turbidity, sediment chlorophyll *a* and *c*, and sediment organic matter. *Harbour* showed significant differences relatively to *Barra* in water nitrates, nitrites, ammonium, phosphates, silica, iron, hardness and turbidity, and sediment chlorophyll *c*, phaeopigments and silt clay fraction. *C. Bico* showed significant differences relatively to *Barra* in water conductivity, salinity, nitrates, phosphates, silica, iron, hardness and turbidity, and sediment chlorophyll *a* and *b*, and organic matter. It should be work noted that all the contaminated sites had higher values of nitrates and phosphates than *Barra*, while *Vagueira* and *Harbour* also had higher concentrations of nitrites and ammonia than *Barra*.

The mean concentrations of the nine metals determined in the sediments collected seasonally at the four sampling sites are shown in Annex 4.2. Cd was only found in one season (winter) and in one site (*Vagueira*) and thus it was not included in the statistical analysis. Significant differences among sites and seasons were found for all the metals, with the exception of Pb variation among seasons and Cu among sites (Table 4.2.). Relatively to the reference site, *Vagueira* showed significantly higher concentrations of Al, Cr, Co and Ni. *C. Bico* showed statistically significant higher concentrations of Al, Pb, Co, Zn and Hg relatively to *Barra*, while no statistically significant differences between *Harbour* and *Barra* were found for any of the metals determined in the sediments.

4.4.2. Bioconcentration of metals

The concentrations of the nine metals determined in *P. microps* collected at the four sampling sites in different seasons are shown in the Annex 4.3. and the results of statistical analysis are indicated in Table 4.3.

Table 4.1. – Results from Repeated Measures Analysis of Variance (RM-ANOVA), for environmental variables measured both in water and sediment, using sampling site as factor. The Dunnet Comparisons results represents the post hoc comparisons with the reference site (*Barra*), performed using the Dunnet Simultaneous Test. Season was selected as a random factor; therefore no multiple comparisons were performed. Temp – temperature, Cond – conductivity, Sal – salinity, DO - dissolved oxygen, NO_3 – nitrates, NO_2 – nitrites, NH_4 – ammonia, PO_4 – phosphates, SiO_2 – silica, C_6H_5OH – phenol, Fe-w – iron (in water), Hard – hardness, Turb – turbidity, Chl *a* – Chlorophyll *a*, Chl *b* – Chlorophyll *b*, Chl *c* – Chlorophyll *c*, Phaeo – phaeopigments, SCF – Silt Clay Fraction and OM– organic matter.

Water	Parameter	Factor	Statistics	Dunnet Comparisons (*)
	Temp	site season	$F_{(3,41)}$= 7.44 (p<0.001) $F_{(3,41)}$= 539.58 (p<0.001)	***Barra*** *Harbour C. Bico Vagueira*
	Cond	site season	$F_{(3,41)}$= 7.52 (p<0.001) $F_{(3,41)}$= 35.39 (p<0.001)	*C. Bico Vagueira Harbour* ***Barra***
	Sal	site season	$F_{(3,41)}$= 12.37 (p<0.001) $F_{(3,41)}$= 120.17 (p<0.001)	*C. Bico Vagueira Harbour* ***Barra***
	pH	site season	$F_{(3,41)}$= 4.24 (p<0.05) $F_{(3,41)}$= 14.49 (p<0.001)	*Vagueira C. Bico* ***Barra*** *Harbour*
	DO	site season	$F_{(3,41)}$= 3.99 (p<0.05) $F_{(3,41)}$= 49.60 (p<0.001)	*Harbour Vagueira* ***Barra*** *C. Bico*
	NO_3	site season	$F_{(3,41)}$= 18.74 (p<0.001) $F_{(3,41)}$= 31.66 (p<0.001)	***Barra*** *Harbour Vagueira C. Bico*
	NO_2	site season	$F_{(3,41)}$= 3.45 (p<0.05) $F_{(3,41)}$= 6.10 (p<0.05)	***Barra*** *C. Bico Harbour Vagueira*
	NH_4	site season	$F_{(3,41)}$= 11.26 (p<0.001) $F_{(3,41)}$= 15.62 (p<0.001)	***Barra*** *C. Bico Harbour Vagueira*
	PO_4	site season	$F_{(3,41)}$= 4.92 (p<0.05) $F_{(3,41)}$= 8.20 (p<0.001)	***Barra*** *Harbour C. Bico Vagueira*
	SiO_2	site season	$F_{(3,41)}$= 11.51 (p<0.001) $F_{(3,41)}$= 64.23 (p<0.001)	***Barra*** *C. Bico Vagueira Harbour*
	C_6H_5OH	site season	$F_{(3,41)}$= 5.35 (p<0.05) $F_{(3,41)}$= 14.34 (p<0.001)	***Barra*** *C. Bico Harbour Vagueira*
	Fe-w	site season	$F_{(3,41)}$= 8.46 (p<0.001) $F_{(3,41)}$= 21.44 (p<0.001)	***Barra*** *Vagueira Harbour C. Bico*
	Hard	site season	$F_{(3,41)}$= 22.13 (p<0.001) $F_{(3,41)}$= 38.45 (p<0.001)	*C. Bico Vagueira Harbour* ***Barra***
	Turb	site season	$F_{(3,41)}$= 11.53 (p<0.001) $F_{(3,41)}$= 18.16 (p<0.001)	***Barra*** *Vagueira Harbour C. Bico*
Sediment				
	Chl *a*	site season	$F_{(3,153)}$= 27.91 (p<0.001) $F_{(3,153)}$= 22.24 (p<0.001)	*C. Bico* ***Barra*** *Harbour Vagueira*
	Chl *b*	site season	$F_{(3,153)}$= 11.97 (p<0.001) $F_{(3,153)}$= 28.28 (p<0.001)	*C. Bico Harbour* ***Barra*** *Vagueira*
	Chl *c*	site season	$F_{(3,153)}$= 12.10 (p<0.001) $F_{(3,153)}$= 17.00 (p<0.001)	*C. Bico* ***Barra*** *Harbour Vagueira*
	Phaeo	site season	$F_{(3,153)}$= 17.88 (p<0.001) $F_{(3,153)}$= 38.98 (p<0.001)	*Harbour C. Bico* ***Barra*** *Vagueira*
	SCF	site season	$F_{(3,153)}$= 12.59 (p<0.001) $F_{(3,153)}$= 14.59 (p<0.001)	*Harbour Vagueira C. Bico* ***Barra***
	OM	site season	$F_{(3,153)}$= 30.92 (p<0.001) $F_{(3,153)}$= 4.66 (p<0.05)	***Barra*** *Harbour Vagueira C. Bico*

(*) Sites statistically not different from the control are united by an underscore.

Table 4.2. – Results from RM-ANOVA statistical analysis, for the nine metals measured in sediment, using sampling site as factor. The Dunnet Comparisons results represents the post hoc comparisons with the reference site (shown in bold), performed using the Dunnet Simultaneous Test. Season was selected as a random factor; therefore no multiple comparisons were performed. NS = not significant at $p<0.05$.

Parameter (*)	Factor	Statistics	Dunnet Comparisons (**)
Al	site season	$F_{(3,41)}$= 16.73 (p<0.001) $F_{(3,41)}$= 4.05 (p<0.05)	*Harbour* ***Barra*** *C. Bico Vagueira*
Pb	site season	$F_{(3,41)}$= 7.97 (p<0.001) $F_{(3,41)}$= 0.30 (NS)	***Barra*** *Harbour Vagueira C. Bico*
Cr	site season	$F_{(3,41)}$= 10.05 (p<0.001) $F_{(3,41)}$= 6.89 (p<0.05)	*Harbour* ***Barra*** *C. Bico Vagueira*
Co	site season	$F_{(3,41)}$= 13.60 (p<0.001) $F_{(3,41)}$= 7.40 (p<0.001)	*Harbour* ***Barra*** *C. Bico Vagueira*
Cu	site season	$F_{(3,41)}$= 2.43 (NS) $F_{(3,41)}$= 6.83 (p<0.05)	*Harbour* ***Barra*** *Vagueira C. Bico*
Ni	site season	$F_{(3,41)}$= 5.76 (p<0.05) $F_{(3,41)}$= 6.09 (p<0.05)	*Harbour* ***Barra*** *C. Bico Vagueira*
Zn	site season	$F_{(3,41)}$= 17.31 (p<0.001) $F_{(3,41)}$= 4.94 (p<0.05)	*Harbour* ***Barra*** *Vagueira C. Bico*
Hg	site season	$F_{(3,41)}$= 11.70 (p<0.001) $F_{(3,41)}$= 28.51 (p<0.001)	***Barra*** *Vagueira Harbour C. Bico*

(*) Cd had only one record, in sediment, during the year (winter), therefore was not included in ANOVA analysis.

(**) Sites united by an underscore correspond to the situation where the null hypothesis (H_o: $\mu_{contaminated} \leq \mu_{reference}$) was not rejected.

Statistically significant differences among sites were found for Al, Cd, Zn and Hg. Relatively to fish from the reference site, animals from *C. Bico* showed significantly higher levels of Zn and Hg, fish from *Vagueira* showed significantly lower concentrations of Al, while those collected at *Harbour* had significantly lower concentrations of Al and higher levels of Zn (Table 4.3. and Annex 4.3.). The BAFs calculated for each metal are displayed in Table 4.4. and the results of RM-ANOVA analysis are indicated in Table 4.5. Since Cd concentrations in sediments were detected only once, it was not possible to calculate the BAFs for this metal. BAFs higher than 1 were found for Cu, Zn Hg at all the sampling sites, for Cr at *Barra* and *Harbour*, for Co at *Harbour*, for Ni at *Barra*, *Vagueira* and *C. Bico*. The highest BAFs were found for Zn reaching 20.96. Significant differences in BAFs among sites and seasons were found for all the metals, except for Pb and Hg among sites, and Cu among sites and seasons. Statistically significant lower BAFs relatively to the reference site were found for Al and Ni in fish from *Vagueira*, and higher BAFs relatively to *Barra* were found for Co and Zn in fish from the *Harbour*.

Table 4.3. – Results from RM-ANOVA statistical analysis, for the nine metals measured in *P. microps* tissues, using sampling site as factor. The Dunnet Comparisons results represents the post hoc comparisons with the reference site (shown in bold), performed using the Dunnet Simultaneous Test. Season was selected as a random factor; therefore no multiple comparisons were performed. NS = not significant at $p<0.05$.

Parameter	Factor	Statistics	Dunnet Comparisons (*)
Al	site	$F_{(3,41)}= 4.45$ ($p<0.05$)	*Harbour Vagueira C. Bico* ***Barra***
	season	$F_{(3,41)}= 34.50$ ($p<0.001$)	
Pb	site	$F_{(3,41)}= 0.46$ (NS)	*Harbour* ***Barra*** *C. Bico Vagueira*
	season	$F_{(3,41)}= 3.58$ ($p<0.05$)	
Cd	site	$F_{(3,41)}= 4.88$ ($p<0.05$)	*C. Bico Harbour* ***Barra*** *Vagueira*
	season	$F_{(3,41)}= 11.37$ ($p<0.001$)	
Cr	site	$F_{(3,41)}= 0.12$ (NS)	*C. Bico* ***Barra*** *Vagueira Harbour*
	season	$F_{(3,41)}= 74.60$ ($p<0.001$)	
Co	site	$F_{(3,41)}= 0.11$ (NS)	*C. Bico Vagueira Harbour* ***Barra***
	season	$F_{(3,41)}= 30.51$ ($p<0.001$)	
Cu	site	$F_{(3,41)}= 0.11$ (NS)	***Barra*** *Harbour C. Bico Vagueira*
	season	$F_{(3,41)}= 2.69$ (NS)	
Ni	site	$F_{(3,41)}= 2.00$ (NS)	*Harbour Vagueira* ***Barra*** *C. Bico*
	season	$F_{(3,41)}= 0.63$ (NS)	
Zn	site	$F_{(3,41)}= 8.40$ ($p<0.001$)	***Barra*** *Vagueira C. Bico Harbour*
	season	$F_{(3,41)}= 39.18$ ($p<0.001$)	
Hg	site	$F_{(3,41)}= 7.85$ ($p<0.001$)	*Harbour Vagueira* ***Barra*** *C. Bico*
	season	$F_{(3,41)}= 21.04$ ($p<0.001$)	

(*) Sites statistically not different from the control are united by an underscore.

4.4.3. Condition indexes and biomarkers in fish

The variation of *P. microps* condition indexes and biomarkers captured at different seasons and sampling sites is shown in Table 4.6. and the results of statistical analysis are shown in Table 4.7. Significant differences in FCF and HSI were found among sampling sites and seasons (Table 4.7.). Statistically significant higher HSI were found in fish collected at all the contaminated sites relatively to those from the reference site. Despite significant differences in FCF among fish from different sites were also found, the Dunnet Simultaneous Test was not able to discriminate the different groups of fish (Table 4.7.).

Statistically significant differences among sites and seasons were found for all the tested biomarkers (Table 4.7.). For all the biomarkers, the contaminated sites were significantly different from the reference site. Relatively to the fish collected at *Barra*, those from *Vagueira*, *Harbour* and *C. Bico* showed significantly inhibition of AChE (24%, 25%, 27%, respectively; % of inhibition calculated from annual means) and LDH activities (Table 4.6.). Increased LPO levels and activities of GST and anti-oxidant enzymes were found in fish from *Vagueira*, *Harbour* and *C. Bico* relatively to those from *Barra* (Table 4.6.).

Table 4.4. – Annual bioaccumulation Factor (BAF) values for *Barra*, *Vagueira*, *Harbour* and *C. Bico*, for each metal, based in sediment and fish data. Values represent the mean ± S.E.M. The Cd concentrations were not included in BAF results.

BAF	*Barra*	*Vagueira*	*Harbour*	*C. Bico*
Al	**0.196** ± 0.056	**0.045** ± 0.013	**0.140** ± 0.041	**0.113** ± 0.024
Pb	**0.776** ± 0.224	**0.709** ± 0.204	**0.562** ± 0.162	**0.484** ± 0.139
Cr	**1.085** ± 0.313	**0.484** ± 0.135	**1.844** ± 0.535	**0.180** ± 0.052
Co	**0.493** ± 0.142	**0.766** ± 0.221	**3.664** ± 1.058	**0.375** ± 1.08
Cu	**1.622** ± 0.468	**3.530** ± 1.019	**4.068** ± 1.174	**2.956** ± 0.853
Ni	**3.067** ± 0.885	**1.086** ± 0.313	**0.619** ± 0.179	**1.386** ± 0.401
Zn	**8.001** ± 2.309	**6.761** ± 1.860	**20.96** ± 6.052	**6.069** ± 1.240
Hg	**2.678** ± 0.802	**3.417** ± 0.986	**2.436** ± 0.703	**2.925** ± 0.547

Table 4.5. – Results from RM-ANOVA statistical analysis, for each metal BAF, using sampling site as factor. The Dunnet Comparisons results indicate the post hoc comparisons with the reference site (control - shown in bold), performed using the Dunnet Simultaneous Test. Season was selected as a random factor; therefore no multiple comparisons were performed. NS = not significant at $p<0.05$.

Parameter (*)	Factor	Statistics	Dunnet Comparisons (**)
Al	site season	$F_{(3,41)}$= 3.20 ($p<0.05$) $F_{(3,41)}$= 8.71 ($p<0.001$)	*Vagueira C. Bico Harbour* ***Barra***
Pb	site season	$F_{(3,41)}$= 0.21 (NS) $F_{(3,41)}$= 3.38 ($p<0.05$)	*C. Bico Harbour Vagueira* ***Barra***
Cr	site season	$F_{(3,41)}$= 7.25 ($p<0.05$) $F_{(3,41)}$= 4.67 ($p<0.05$)	*C. Bico Vagueira* ***Barra*** *Harbour*
Co	site season	$F_{(3,41)}$= 11.33 ($p<0.001$) $F_{(3,41)}$= 13.26 ($p<0.001$)	*C. Bico* ***Barra*** *Vagueira Harbour*
Cu	site season	$F_{(3,41)}$= 2.07 (NS) $F_{(3,41)}$= 1.80 (NS)	***Barra*** *C. Bico Vagueira Harbour*
Ni	site season	$F_{(3,41)}$= 3.96 ($p<0.05$) $F_{(3,41)}$= 7.50 ($p<0.001$)	*Harbour Vagueira C. Bico* ***Barra***
Zn	site season	$F_{(3,41)}$= 18.07 ($p<0.001$) $F_{(3,41)}$= 5.90 ($p<0.05$)	*C. Bico Vagueira* ***Barra*** *Harbour*
Hg	site season	$F_{(3,41)}$= 0.72 (NS) $F_{(3,41)}$= 3.14 ($p<0.05$)	*Harbour* ***Barra*** *C. Bico Vagueira*

(*) The Cd concentrations were not included in ANOVA analysis.

(**) Sites statistically not different from the control are united by an underscore.

Table 4.6. – Local and seasonal variation of condition indexes and biomarkers measured in *P. microps*, collected at the four sampling sites located in the Aveiro lagoon. FCF - Fulton Condition Factor and HIS - Hepatosomatic Index. The mean enzymatic activity, LPO and condition values, per year, for each location are shown in the grey column. Values indicate the mean ± S.E.M. U/mg protein = 1 µmol/min for CAT activity, the amount of enzyme required to inhibit the rate of reduction of cytochrome *c* by 50% for SOD activity and 1 nmol/min for the other enzymes. AChE - acetylcholinesterase, LDH - lactate dehydrogenase, GST - glutathione S-transferases, CAT – catalase, SOD - superoxide dismutase, GR - glutathione reductase, GPx - glutathione peroxidase and LPO - lipid peroxidation.

	Barra					***Vagueira***					***Harbour***					***C. Bico***				
Condition Indexes	**WINTER**	**SPRING**	**SUMMER**	**AUTUMN**	**Annual mean**	**WINTER**	**SPRING**	**SUMMER**	**AUTUMN**	**Annual mean**	**WINTER**	**SPRING**	**SUMMER**	**AUTUMN**	**Annual mean**	**WINTER**	**SPRING**	**SUMMER**	**AUTUMN**	**Annual mean**
FCF	**1.032**	**0.994**	**0.783**	**0.803**	**0.904**	**1.003**	**0.875**	**0.768**	**0.704**	**0.838**	**1.279**	**1.034**	**0.773**	**0.725**	**0.953**	**0.973**	**0.832**	**0.704**	**0.805**	**0.829**
	± 0.045	± 0.021	± 0.025	± 0.052	± 0.027	± 0.071	± 0.012	± 0.033	± 0.076	± 0.033	± 0.049	± 0.032	± 0.042	± 0.042	± 0.047	± 0.027	± 0.026	± 0.033	± 0.072	± 0.027
HSI	**4.371**	**4.401**	**4.461**	**3.638**	**4.217**	**5.917**	**5.943**	**5.671**	**4.582**	**5.529**	**5.942**	**5.020**	**5.475**	**5.071**	**5.378**	**7.192**	**5.473**	**5.207**	**5.488**	**5.841**
	± 0.373	± 0.235	± 0.175	± 0.181	± 0.136	± 0.202	± 0.216	± 0.255	± 0.361	± 0.165	± 0.252	± 0.251	± 0.201	± 0.151	± 0.125	± 0.355	± 0.066	± 0.154	± 0.231	± 0.185
Biomarkers																				
AChE (U/mg protein)	**39.97**	**31.12**	**44.67**	**27.82**	**35.90**	**27.65**	**30.72**	**35.95**	**14.95**	**27.32**	**29.82**	**26.99**	**34.51**	**15.88**	**26.81**	**31.61**	**27.01**	**31.81**	**14.05**	**26.12**
	± 1.235	± 1.355	± 2.028	± 1.105	± 1.471	± 2.608	± 1.641	± 0.972	± 0.763	± 1.681	± 2.045	± 1.298	± 1.277	± 0.758	± 1.479	± 1.598	± 0.884	± 1.105	± 0.762	± 1.489
LDH (U/mg protein)	**252.4**	**171.4**	**244.4**	**205.1**	**218.3**	**219.1**	**142.7**	**235.7**	**104.7**	**175.6**	**189.2**	**131.9**	**161.2**	**145.9**	**157.1**	**173.6**	**154.8**	**186.7**	**121.6**	**159.2**
	± 5.741	± 6.584	± 6.482	± 4.325	± 6.832	± 4.662	± 1.763	± 4.009	± 3.276	± 10.50	± 4.225	± 3.365	± 6.426	± 4.741	± 4.679	± 4.446	± 7.799	± 5.821	± 3.774	± 5.415
GST (U/mg protein)	**59.82**	**59.07**	**48.11**	**35.57**	**50.65**	**90.11**	**65.24**	**54.75**	**48.74**	**64.71**	**108.8**	**99.22**	**69.36**	**53.85**	**82.82**	**118.8**	**86.35**	**52.73**	**59.32**	**79.32**
	± 0.798	± 3.156	± 1.068	± 1.626	± 2.097	± 3.572	± 2.636	± 2.273	± 2.757	± 3.325	± 2.605	± 2.058	± 1.477	± 2.091	± 4.373	± 2.076	± 3.913	± 2.609	± 2.718	± 5.203
CAT (U/mg protein)	**6.292**	**3.175**	**4.743**	**6.679**	**5.223**	**9.322**	**8.302**	**7.044**	**13.35**	**9.506**	**9.362**	**8.816**	**8.482**	**11.33**	**9.499**	**11.35**	**7.303**	**6.881**	**10.13**	**8.919**
	± 0.321	± 0.253	± 0.453	± 0.247	± 0.308	± 0.445	± 0.468	± 0.571	± 0.202	± 0.501	± 0.218	± 0.471	± 0.386	± 0.256	± 0.268	± 0.321	± 0.437	± 0.524	± 0.375	± 0.413
SOD (U/mg protein)	**0.494**	**1.167**	**1.075**	**0.658**	**0.849**	**0.992**	**3.364**	**2.052**	**2.144**	**2.138**	**1.216**	**2.346**	**1.526**	**1.251**	**1.585**	**1.323**	**2.529**	**1.338**	**0.911**	**1.526**
	± 0.019	± 0.134	± 0.025	± 0.017	± 0.631	± 0.021	± 0.156	± 0.059	± 0.075	± 0.167	± 0.055	± 0.121	± 0.077	± 0.048	± 0.095	± 0.036	± 0.041	± 0.024	± 0.027	± 0.117
GR (U/mg protein)	**2.853**	**2.261**	**5.686**	**4.752**	**3.888**	**6.627**	**3.893**	**9.578**	**11.19**	**7.824**	**7.269**	**4.148**	**8.580**	**10.16**	**7.542**	**9.679**	**5.216**	**7.277**	**9.967**	**8.035**
	± 0.129	± 0.047	± 0.311	± 0.309	± 0.288	± 0.332	± 0.054	± 0.511	± 0.456	± 0.567	± 0.351	± 0.045	± 0.252	± 0.214	± 0.440	± 0.301	± 0.062	± 0.411	± 0.246	± 0.395
GPx (U/mg protein)	**7.017**	**5.351**	**14.56**	**5.957**	**8.222**	**14.08**	**14.09**	**32.16**	**19.17**	**19.88**	**17.61**	**16.82**	**17.87**	**15.45**	**16.94**	**16.02**	**10.44**	**30.41**	**17.35**	**18.56**
	± 0.362	± 0.287	± 0.837	± 0.362	± 0.753	± 0.157	± 0.372	± 0.846	± 1.250	± 1.469	± 0.329	± 0.515	± 0.484	± 0.572	± 0.291	± 0.586	± 0.376	± 0.737	± 0.403	± 1.431
LPO (nmol TBARS/g tissue)	**88.46**	**90.51**	**47.25**	**40.38**	**66.65**	**185.9**	**176.6**	**65.64**	**135.1**	**140.9**	**122.8**	**126.1**	**62.36**	**91.21**	**100.6**	**171.5**	**158.7**	**79.67**	**124.4**	**133.6**
	± 1.513	± 3.101	± 1.822	± 2.012	± 4.541	± 2.248	± 2.197	± 2.116	± 1.663	± 9.183	± 2.567	± 1.098	± 1.445	± 1.917	± 5.068	± 2.179	± 2.815	± 3.246	± 2.044	± 6.957

Table 4.7. – RM-ANOVA statistical analysis results for measured condition indexes and biomarkers in *P. microps*, using sampling site as factor. The Dunnet Comparisons results indicate the post hoc comparisons with the reference site (shown in bold), performed using the Dunnet Simultaneous Test. Season was selected as a random factor; therefore no multiple comparisons were performed. For each biomarker and condition index, the full names are shown in Table 4.6.

	Parameter	Factor	Statistics	Dunnet Comparisons (*)
Condition Indexes				
	FCF	site season	$F_{(3,105)}$= 5.66 (p<0.05) $F_{(3,105)}$= 37.99 (p<0.001)	*C. Bico Vagueira* ***Barra*** *Harbour*
	HSI	site season	$F_{(3,105)}$= 27.56 (p<0.001) $F_{(3,105)}$= 12.39 (p<0.001)	***Barra*** *Harbour Vagueira C. Bico*
Biomarkers (**)				
	AChE	site season	$F_{(3,105)}$= 33.60 (p<0.001) $F_{(3,105)}$= 99.29 (p<0.001)	*C. Bico Harbour Vagueira* ***Barra***
	LDH	site season	$F_{(3,105)}$= 43.66 (p<0.001) $F_{(3,105)}$= 66.38 (p<0.001)	*Harbour C. Bico Vagueira* ***Barra***
	GST	site season	$F_{(3,105)}$= 56.88 (p<0.001) $F_{(3,105)}$= 110.32 (p<0.001)	***Barra*** *Vagueira C. Bico Harbour*
	CAT	site season	$F_{(3,105)}$= 66.29 (p<0.001) $F_{(3,105)}$= 47.72 (p<0.001)	***Barra*** *C. Bico Harbour Vagueira*
	SOD	site season	$F_{(3,105)}$= 97.17 (p<0.001) $F_{(3,105)}$= 88.44 (p<0.001)	***Barra*** *C. Bico Harbour Vagueira*
	GR	site season	$F_{(3,105)}$= 93.52 (p<0.001) $F_{(3,105)}$= 107.31 (p<0.001)	***Barra*** *Harbour Vagueira C. Bico*
	GPx	site season	$F_{(3,105)}$= 129.20 (p<0.001) $F_{(3,105)}$= 71.27 (p<0.001)	***Barra*** *Harbour C. Bico Vagueira*
	LPO	site season	$F_{(3,105)}$= 167.78 (p<0.001) $F_{(3,105)}$= 198.36 (p<0.001)	***Barra*** *Harbour C. Bico Vagueira*

(*) Sites statistically not different from the control are united by an underscore.

(**) In post hoc comparisons all sites were different from control.

4.4.4. Integrated data analysis

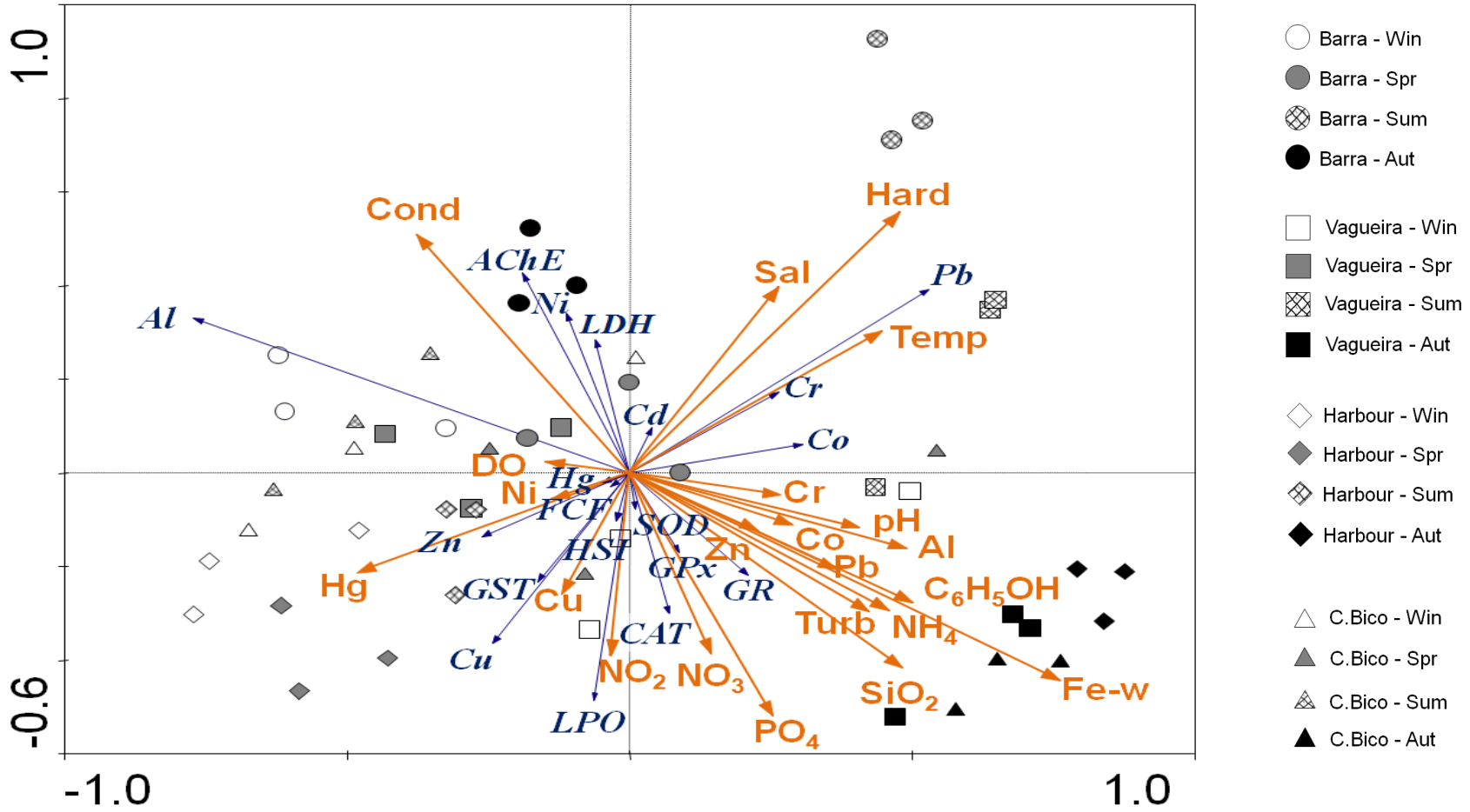
The results of RDA are shown as a triplot ordination diagram (Figure 4.2.). The first two axes of the RDA analysis accounted for 62.6% (53.1% for biological descriptors and additional 9.5% for the interaction between biological and environmental data) of the overall canonical variability of the data. The first RDA axis (horizontal) accounted for 43.6% of the total canonical variability and was strongly associated with a seasonality gradient. Considering biological descriptors, this axis was particularly associated with Al (negative part) and Pb (positive part) concentrations in fish, while regarding environmental descriptors it was associated with Hg (negative part) and with temperature, iron, silica, turbidity, ammonia, phenol, pH, Al, Pb, Co, Cr, and Zn (positive part). The second constrained axis (vertical) accounted for additional 9% of the canonical variability and was

influenced by contamination since the reference site (*Barra*) appears separated from the contaminated sites, especially in autumn and summer. The positive part of the axis is particularly associated with AChE, LDH and Ni (in fish) and conductivity, salinity and hardness (in water). The negative part of axis is mainly associated with LPO, HSI, antioxidant enzymes and Cu concentrations in fish, with nitrites, nitrates and phosphates in water and Cu in sediments.

The PRC diagrams displayed in Figures 4.3. and 4.4. showed seasonal differences between *Barra* and the contaminated sites, considering the environmental and biological descriptors, respectively. Figure 4.3. shows the PRC for the first and second axis (PRC1 and PRC2), resulting from the analysis of environmental descriptors (water parameters and metals in the sediment). Seasonal changes, sampling sites and replication accounted for 32.5%, 62.5% and 5% of the variance, respectively. The PRC1 (Figure 4.3.A) accounted for 48.6% of total variance. Differences between contaminated sites and *Barra* were found to be mainly associated with Al, Zn and Cr concentrations in sediments (Figure 4.3.A). PRC2 analysis (Figure 4.3.B) accounted for 22.9% of total variance. For this second axis, the differences observed between contaminated sites and reference were found to be mainly associated with turbidity. Figure 4.4. describes the PRC for the first and second axis (PRC1 and PRC2) resulting from the analysis of biological descriptors of *P. microps* (biomarkers, condition indexes and metals). Seasonal changes, sampling sites and replication accounted for 47.8%, 34.9% and 17.3% of the variance, respectively. The PRC1 and PRC2 accounted for 37.8% and 22.3% of total variance, respectively.

Considering PRC1 analysis (Figure 4.4.A), the descriptors mainly responsible for deviations of *Vagueira*, *Harbour* and *C. Bico* relatively to the reference site were GPx, Pb GR and CAT. It is interesting to note that in summer all the sites are more close *Barra* relatively to biological descriptors. For the PRC2 analysis (Figure 4.4.B) the differences observed between contaminated sites and *Barra* were found to be mainly associated with Ni and Pb levels in *P. microps* body.

Figure 4.2. – Redundancy analysis (RDA) ordination diagram with biological and environmental data: the biomarkers, condition indexes and metals analysed in fish were selected as biological descriptors (blue), while water parameters and chemical analysis of metals in sediments were selected as environmental descriptors (orange). Environmental parameters analysed in sediment were selected as covariables data. The sampling sites are indicated as: circles – *Barra*; squares – *Vagueira*; rhombus – *Harbour* and triangles – *C. Bico*). For each sampling site is, also, indicated the season: Win – winter; Spr – spring; Sum – summer and Aut – autumn. First axis is horizontal, second axis is vertical. Temp – temperature, Cond – conductivity, Sal – salinity, Turb – turbidity, Hard – hardness, DO - dissolved oxygen, NO_3 – nitrates, NO_2 – nitrites, NH_4 – ammonia, PO_4 – phosphates, C_6H_5OH – phenol, SiO_2 – silica, Fe-w – iron (in water).



Vieira L.R. (2009)

Figure 4.3. – First Principal Response Curves (PRC) resulting from the analysis of the environmental descriptors (water parameters and metals analysed in sediment), for first axis (A) and second axis (B). The lines represent the course of each sampling site levels in time. The descriptors weight (b_k) can be interpreted as the affinity of each described parameter with the Principal Response Curves.

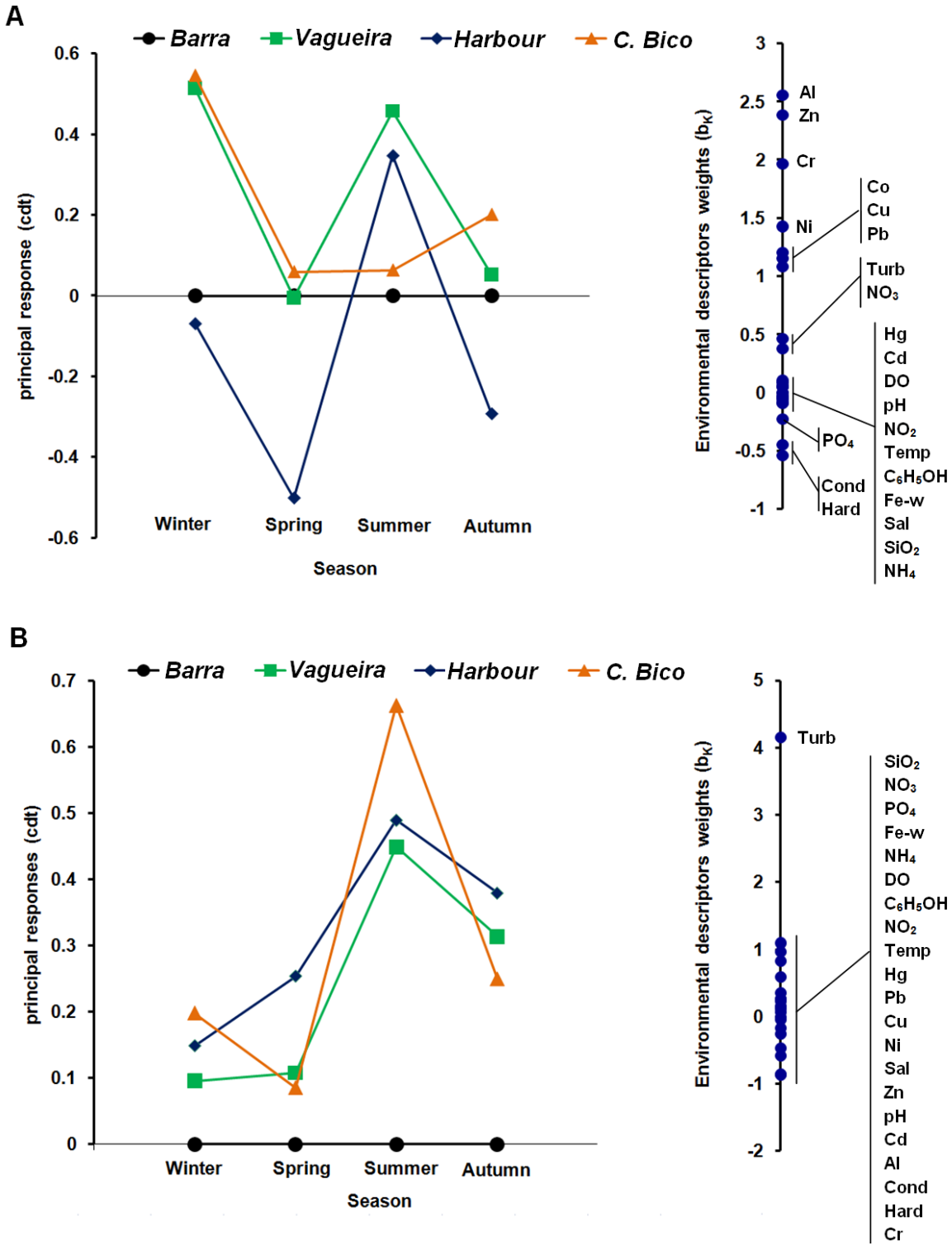
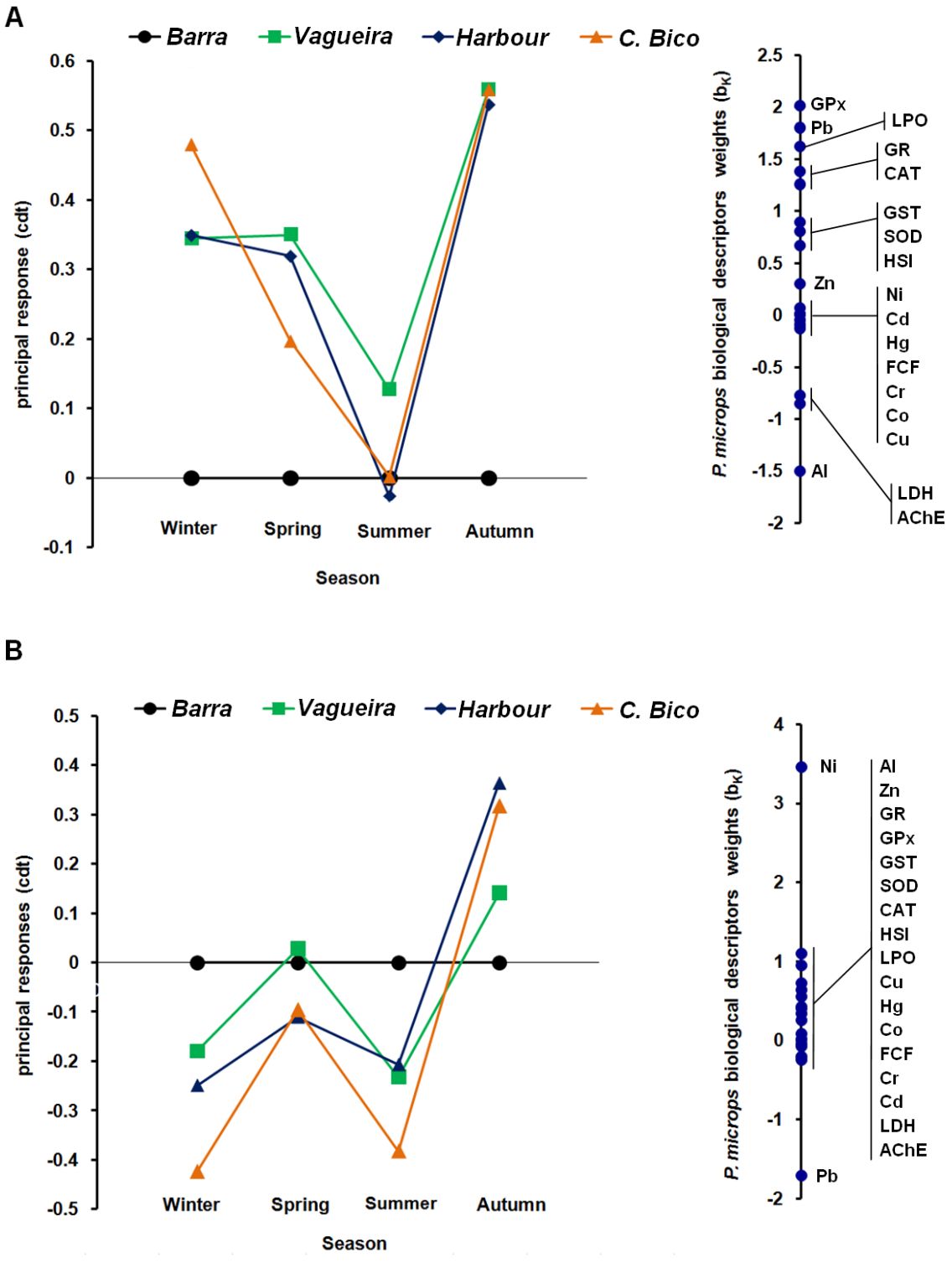


Figure 4.4. – PRC resulting from the analysis of the biological descriptors of *P. microps* (biomarkers, condition indexes and metals). (A) First axis; (B) Second axis. The lines represent the course of each sampling site levels in time. The descriptors weight (b_k) can be interpreted as the affinity of each described parameter with the Principal Response Curves.



4.5. DISCUSSION

4.5.1. Characterization and comparison of sampling sites

The highest values of nitrites, ammonia, and phosphates, found at *Vagueira*, indicate a higher pollution by organic materials at this site relatively to the others. This was expected since this site is located in the Mira channel, which receives agriculture (fertilizers and pesticides) and animal farming effluents.

In addition to water column, the sandy bottom is an important habitat for many fish species, including *P. microps*, and it provides suitable conditions for developing young and small fishes away from larger predators (Waligóra-Borek *et al.*, 2005). Therefore, the sediment characterization is very important, especially when species living in close association with sediments, such as *P. microps*, are used as sentinel species. In fact, the type of sediment may influence fish distribution due to preferences for a particular kind of habitat (e.g. sandy, muddy), availability of food and other factors. Moreover, particle size and organic matter content are important factors regarding the bioavailability of several chemicals, including metals (Van der Oost *et al.*, 2003). In the present study, the higher mean concentrations of chlorophylls and phaeopigments were found at *Vagueira*, suggesting a higher primary productivity at this site, therefore in good agreement with the increased concentrations of nutrients in the water. Increased turbidity at this site may induce the resuspension of pollutants accumulated in the sediment.

The concentrations of metals in sediments determined in the present study are in the range of those found by other authors in the Aveiro lagoon (Pereira *et al*., 1997; Abreu *et al*., 2000; Monterroso *et al*., 2003b). Considering the sum of all the metals (Annex 4.2.), the most contaminated sediments are those of *Vagueira*, followed by *C. Bico*, *Barra* and *Harbour*. The most abundant metal was Al at all the sites and the less abundant was Hg. Relatively to the other sites, *Vagueira* shows higher contamination by Al, Cr, Co and Ni, while *C. Bico* sediments are particularly contaminated with Pb, Zn and Hg.

4.5.2. Bioaccumulation of metals

In the case of metals, sediments may act as a ‘‘sink’’ that removes metals from the water column (Mota *et al.*, 2005). However, benthonic animals living in close association with the sediment, such as juvenile gobies that always stay close to the bottom (Hampel *et*

al., 2003) or feeding from benthonic preys may be particularly exposed to metals and often these chemicals are found in their body. In the present study, eight metals were found all over the year in fish from all the sampling sites with mean concentrations ranging from 142 to 258 µg/g for Al, 1.0 to 2.1 µg/g for Pb, 1.3 to 1.4 µg/g for Cr, 0.7 to 0.8 µg/g for Co, 5.5 to 6.2 µg/g for Cu, 1.5 to 3.9 µg/g for Ni, 84 to 120 µg/g for Zn and 0.2 to 0.4 for Hg (Annex 4.3.). These values were found to be in good accordance with corresponding concentrations that have been reported for several fish species from the Aveiro lagoon (Lima, 1986; Lucas *et al.*, 1986; Eira *et al.*, 2009) and are in the range of concentrations found in *Pomatoschistus* from other European estuaries. For example, Miramand *et al.* (1998) found concentrations of copper of 2.5 ±1 µg/g (dry weight) in *P. microps* from the Seine estuary, while Geffen *et al.* (1998) reported Hg concentrations between 29.8 ± 18 .29 and 43.93 ± 13.43 µg/g (wet weight) in *Pomatoschistus minutus*.

In the present study, *P. microps* was found to bioaccumulate Zn, Hg and Cu at all the sampling sites (Table 4.4.); Ni at *Barra*, *Vagueira* and *C. Bico*; Cr at *Vagueira*, *Barra* and *Harbour*; and Co at *Harbour*. No bioaccumulation was found for Al despite the high concentrations of this metal found in sediment samples from all the sites, nor for Pb. The higher BAF values were found for Zn, mainly at *Harbour* area (20.96 ± 6.052). It is also interesting to note that despite the sediment concentrations of Al, Cr, Co and Ni significantly higher at *Vagueira*, relatively to the other sites, fish from *Vagueira* had were concentrations of these metals similar or lower (Al) than those from the remaining sites and they were only accumulating Ni and less than fish from *Barra*, where the concentrations of Ni in sediments were lower. This suggests that the bioaccumulation of metals in *P. microps* does not depend only of the sediment concentrations. For example, it was possible that the organic matter at *Vagueira*, or other particles of sediments, is sequestering metals and making them not available for fish. A similar situation seems to be occurring with Pb and Zn at C. *Bico*. However in relation to Hg, *C. Bico* sediments were found to be the most contaminated, the fish from this site had the highest concentrations of Hg and fish were found to be accumulating Hg (second highest BAF for Hg). Still it is interesting to note that fish from *Vagueira* seem to be bioaccumulating more Hg (highest BAF) than those from *C. Bico*.

4.5.3. Condition indexes and biomarkers in fish

The FCF has been demonstrated to be a measure of the energy reserves of fish (Lloret *et al.*, 2002) and can provide information on potential pollution impacts (Van der Oost *et al.*, 2003). In fact, healthy fish are expected to have higher FCF than animals living in polluted environments. However, this is not always the case. For example, in polluted environments where the food is abundant exposed fish may accumulate a considerable amount of fat together with lipophilic toxicants that may decrease the reproductive success or even cause death if the fat is mobilised in a short period of time (Guimarães *et al*., 2009). In the present study, FCF was not a suitable parameter in relation to pollution, since no significant differences in this index were found among fish from distinct sites. Another commonly used index is HIS that can be related to the accumulation of energy stores in the liver, to the accumulation of toxic substances in this organ and to exposure to chemicals causing liver injury (Guimarães *et al*., 2009; Chan, 1995). Here, the fish collected at *Harbour, Vagueira* and *C. Bico* showed significantly higher HSI levels relatively to those from the reference site, suggesting that fish may be accumulating local contaminants in liver or that they are exposed to chemicals inducing liver weight increase.

P. microps collected at *Vagueira*, *Harbour* and *C. Bico* showed significantly inhibition of AChE activities suggesting exposure to anti-cholinesterase agents since the inhibition found (24%, 25%, 27%, respectively) is above 20%, the threshold usually considered as indicative of exposure to these agents (Ludke *et al.*, 1975). These agents may be metals but since known anti-cholinesterase agents of this group (e.g. Cu, Zn, Hg, Cr) were found also in fish from *Barra*, it is more likely that other chemicals (e.g. pesticides, PAHs, detergents) are causing the inhibition. Significantly inhibition of LDH activity was also observed in fish from *Vagueira*, *Harbour* and *C. Bico* relatively to those from *Barra*, suggesting that contamination is inducing changes in the pathways of cellular energy production. At contaminated sites, fish also showed increased activities of GST suggesting exposure to xenobiotics with electrophilic centers (e.g. PAHs, some pesticides), increased activities of anti-oxidant enzymes indicating exposure to oxidative agents and higher levels of LPO indicating oxidative damage.

4.5.4. Integrated data analysis

Concerning RDA analysis output, the first constrained axis (horizontal) was found to be strongly associated with a seasonality gradient suggesting a variation of environmental conditions along the year. It is interesting to note a group of environmental parameters including silica, iron, turbidity, pH, phenol and ammonia in the water, and Pb, Co, Cr, Zn and Al in sediments are associated with the autumn at *Vagueira*, *Harbour* and *C. Bico* (positive part of the axis). This association of some metals (Al, Pb and to a less extent Cr, Zn and Co) in sediments and the levels of turbidity and silica suggests their re-suspension from sediments after the first autumnal rains. It is also important to highlight the presence of iron within this group since the chemistry of aqueous iron has a major role in controlling the availability of many contaminants in re-suspended sediment (Jones-Lee and Lee, 2005).

The second constrained axis (vertical) was found to be particularly influenced by contamination gradient since the reference site is separated from the contaminated sites, and no data, related with the *Barra* (circles), was found in the negative side of axis. Concerning the integrated analysis, the results of the vertical axis suggested that the levels of biomarkers are mainly affected by the contamination gradient, being separated in two groups: (1) AChE and LDH being associated with the reference, especially in the autumn and (2) LPO, GST and antioxidant enzymes, more associated with the negative part of the second axis, more related with the contaminated sites. Here, it is interesting to note the association between these parameters and Cu (both in fish and sediments) a metal known to cause lipid peroxidation, and the association of LPO and anti-oxidant enzymes with high levels of nutrients. These results are in good agreement with the findings of Ognjanonovic *et al*. (2008) that lead to the conclusion that high concentrations of nitrites and nitrates in the water may have been a direct result of oxidative stress and loading of LPO in fish. In the figure it is also evident an opposite relation between two biomarkers (AChE and LDH) and metals in sediments, especially Zn, Co, Pb, Al, and Cr suggesting that the presence of these metals may have negative effects on neurotransmission and energy metabolism. It should be noted that RDA displays absolute comparisons, including several biological and environmental descriptors and covariates, which are positioned in function of each parameter weight. In this distribution and despite the seasonal variability found,

biomarkers were found to respond to a contamination gradient, separating the reference site from the contaminated sites, especially in the autumn and summer.

The two groups of descriptors (biological and environmental) integrated in RDA were also analysed separately, in terms of relative comparisons, in a PRC analysis for both RDA axis (Figures 4.3. and 4.4.). The differences found in environmental descriptors (Figure 4.3.) between the reference and contaminated sites were mainly associated with Al, Zn and Cr concentrations in sediments, for the first axis. For the second axis the differences were found to be mainly associated with turbidity. In relation to biological descriptors, the main differences between reference and remaining sites were associated with GPx, LPO, GR, CAT, LPO levels and Pb and Al in fish, in PRC1. In the PRC2 results, the observed differences were found to be mainly associated with Ni and Pb levels in *P. microps* body.

4.6. CONCLUSIONS

The present study used a field monitoring approach, including ecological and ecotoxicological parameters, and multivariate statistics (redundancy analysis and principal response components) to integrate all the data. Overall, the results of RDA indicated that the battery of biomarkers used, the concentrations of metals in both sediments and fish body, and water quality variables were valuable parameters discriminating different pollution scenarios and seasonal effects, also providing complementary information. On the contrary, the FCF was not a good discriminatory parameter. Furthermore, PRC analysis provided useful information regarding the discriminating power of parameters inside both biological and environmental descriptors groups.

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Annex 4.1. – Mean values of the environmental data in water and sediments from seasonal sampling at the four selected sites at the Aveiro lagoon. The mean values, per year, for each site are shown in the grey column. Minimum and maximum (min-max) values are shown within brackets.

	Barra					*Vagueira*					*Harbour*					*C. Bico*				
Water	**WINTER**	**SPRING**	**SUMMER**	**AUTUMN**	**Annual mean**	**WINTER**	**SPRING**	**SUMMER**	**AUTUMN**	**Annual mean**	**WINTER**	**SPRING**	**SUMMER**	**AUTUMN**	**Annual mean**	**WINTER**	**SPRING**	**SUMMER**	**AUTUMN**	**Annual mean**
Temperature (ºC)	**12.1** (9.00 - 16.3)	**20.9** (19.0 - 22.9)	**27.6** (26.6 - 28.5)	**18.3** (14.9 - 22.1)	**19.7** (9.00 - 28.5)	**12.9** (9.10 - 16.7)	**21.4** (18.0 - 23.1)	**27.6** (26.7 - 28.5)	**18.5** (14.8 - 21.2)	**20.1** (9.10 - 28.5)	**12.8** (9.80 - 15.5)	**20.7** (19.0 - 21.07)	**26.0** (25.7 - 26.6)	**17.6** (14.1 - 23.3)	**19.3** (9.80 - 15.5)	**12.2** (8.80 - 17.3)	**21.9** (20.0 - 23.3)	**27.0** (25.7 - 29.5)	**18.0** (15.3 - 22.0)	**19.8** (8.80 - 29.5)
Conductivity (m/S)	**45.9** (40.1 - 50.2)	**39.4** (22.6 - 48.8)	**43.5** (35.9 - 51.1)	**33.5** (29.8 - 38.8)	**40.6** (22.6 - 51.1)	**28.1** (16.8 - 39.4)	**30.4** (24.5 - 37.3)	**41.2** (33.2 - 49.1)	**12.14** (9.37 - 19.2)	**27.9** (9.37 - 49.1)	**38.7** (29.8 - 48.4)	**39.4** (32.8 - 49.9)	**41.2** (36.0 - 51.6)	**16.9** (12.4 - 28.9)	**34.0** (12.4 - 28.9)	**17.8** (15.7 - 19.8)	**30.4** (6.35 - 43.5)	**47.5** (45.3 - 51.3)	**19.2** (11.2 - 31.8)	**28.7** (6.35 - 51.3)
Salinity (‰)	**18.2** (17.0 - 24.4)	**18.1** (15.5 - 21.8)	**22.7** (20.6 - 27.7)	**20.0** (18.1 - 24.9)	**19.8** (15.5 - 27.7)	**14.2** (11.6 - 15.9)	**17.9** (16.4 - 19.6)	**22.3** (20.3 - 23.5)	**16.93** (15.7 - 17.9)	**17.8** (11.6 - 23.5)	**16.9** (12.8 - 20.7)	**18.0** (12.7 - 21.9)	**20.3** (15.7 - 23.7)	**15.1** (12.1 - 16.9)	**17.6** (12.1 - 23.7)	**13.4** (9.30 - 15.8)	**15.4** (5.80 - 20.4)	**19.8** (12.1 - 23.1)	**16.2** (12.3 - 23.1)	**16.2** (5.80 - 23.1)
pH	**8.09** (8.04 - 8.16)	**7.02** (5.80 - 7.95)	**8.05** (7.52 - 8.46)	**7.66** (7.09 - 8.19)	**7.70** (5.80 - 8.46)	**7.73** (7.04 - 8.35)	**6.82** (5.95 - 7.91)	**7.94** (7.42 - 8.36)	**7.73** (7.50 - 8.07)	**7.56** (5.95 - 8.36)	**7.99** (7.75 - 8.18)	**7.23** (6.24 - 7.92)	**8.00** (7.28 - 8.43)	**7.34** (6.74 - 8.45)	**7.64** (6.24 - 8.45)	**10.3** (7.18 - 15.7)	**7.26** (5.91 - 7.95)	**7.97** (7.23 - 8.37)	**6.95** (6.36 - 7.80)	**8.12** (5.91 - 15.7)
Dissolved O_2 (%)	**91.10** (38.1 - 110)	**81.4** (56.5 - 119)	**59.7** (36.8 - 98.1)	**78.1** (44.2 - 98.5)	**77.6** (36.8 - 119)	**80.7** (38.9 - 112)	**64.3** (42.8 - 110)	**62.5** (47.5 - 70.4)	**90.9** (48.6 - 97.2)	**74.6** (38.9 - 112)	**57.3** (30.2 - 93.5)	**68.1** (44.8 - 110)	**48.9** (33.1 - 78.1)	**79.7** (44.6 - 96.1)	**63.5** (30.2- 110)	**80.2** (45.7 - 101.5)	**85.5** (48.6 - 106.8)	**40.2** (34.9 - 99.8)	**85.7** (40.9 - 71.6)	**72.9** (34.9 - 111)
Nitrates (mg NO_3/L)	**0.41** (0.22 - 0.58)	**0.24** (0.05 - 0.34)	**0.22** (0.20 - 0.24)	**0.61** (0.30 - 0.90)	**0.37** (0.05 - 0.90)	**0.67** (0.44 - 1.06)	**0.41** (0.36 - 0.50)	**0.32** (0.26 - 0.38)	**2.94** (0.48 - 4.45)	**1.09** (0.26 - 4.45)	**1.55** (1.02 - 2.60)	**0.61** (0.44 - 0.74)	**0.24** (0.23 - 0.24)	**1.11** (0.68 - 1.94)	**0.88** (0.23 - 2.60)	**2.26** (0.90 - 3.80)	**0.71** (0.60 - 0.86)	**0.27** (0.24 - 0.32)	**1.69** (0.54 - 3.10)	**1.23** (0.24 - 3.80)
Nitrites (mg NO_2/L)	**0.01** (0.00 - 0.01)	**0.00** (0.00 - 0.00)	**0.00** (0.00 - 0.00)	**0.07** (0.05 - 0.09)	**0.02** (0.00 - 0.09)	**0.06** (0.01 - 0.11)	**0.00** (0.00 - 0.00)	**0.01** (0.01 - 0 .02)	**0.17** (0.14 - 0.28)	**0.06** (0.00 - 0.17)	**0.30** (0.05 - 0.55)	**0.02** (0.01 - 0.03)	**0.02** (0.01 - 0.02)	**0.05** (0.04 - 0.07)	**0.10** (0.01 - 055)	**0.03** (0.01 - 0.06)	**0.04** (0.02 - 0.07)	**0.04** (0.04 - 0.05)	**0.10** (0.08 - 0.11)	**0.05** (0.01 - 0.11)
Ammonia (mg NH_4/L)	**0.28** (0.25 - 0.40)	**0.21** (0.05 - 0.39)	**0.48** (0.24 - 0.61)	**0.86** (0.48 - 1.25)	**0.46** (0.05 - 1.25)	**0.46** (0.05 - 0.68)	**0.76** (0.32 - 1.08)	**0.37** (0.22 - 0.73)	**0.87** (0.23 - 1.42)	**0.61** (0.05 - 1.42)	**0.47** (0.24 - 0.89)	**0.69** (0.23 - 0.92)	**0.47** (0.23 - 0.73)	**0.73** (0.30 - 1.05)	**0.59** (0.23 - 1.05)	**0.31** (0.12 - 0.52)	**0.52** (0.16 - 1.15)	**0.56** (0.47 - 0.67)	**0.74** (0.65 - 0.80)	**0.53** (0.12 - 1.15)
Phosphates (mg PO_4/L)	**0.12** (0.10 - 0.16)	**0.25** (0.07 - 0.37)	**0.05** (0.04 - 0.07)	**0.20** (0.15 - 0.27)	**0.16** (0.04 - 0.37)	**0.21** (0.11 - 0.25)	**0.28** (0.05 - 0.45)	**0.16** (0.12 - 0.24)	**0.92** (0.20 - 2.40)	**0.39** (0.05 - 2.40)	**0.80** (0.13 - 2.10)	**0.86** (0.19 - 2.10)	**0.06** (0.03 - 0.07)	**0.32** (0.23 - 0.40)	**0.51** (0.03 - 2.10)	**0.38** (0.10 - 0.75)	**0.17** (0.06 - 0.29)	**0.15** (0.08 - 0.21)	**1.15** (0.34 - 2.10)	**0.46** (0.06 - 2.10)
Silica (mg Si/L)	**1.03** (0.50 - 1.45)	**1.10** (0.60 - 1.45)	**0.27** (0.20 - 0.34)	**2.63** (2.45 - 2.80)	**1.26** (0.20 - 2.80)	**1.07** (0.62 - 1.70)	**1.90** (1.55 - 2.25)	**1.06** (0.88 - 1.40)	**2.50** (0.19 - 3.90)	**1.63** (0.19 - 3.90)	**1.92** (1.40 - 2.80)	**1.03** (0.84 - 1.35)	**0.30** (0.29 - 0.30)	**3.55** (2.20 - 4.54)	**1.70** (0.29 - 4.54)	**2.15** (0.96 - 2.80)	**1.07** (0.60 - 1.45)	**1.09** (0.78 - 1.40)	**2.50** (2.10 - 2.75)	**1.70** (0.60 - 2.80)
Phenol (mg C_6H_5OH/L)	**0.17** (0.10 - 0.28)	**0.08** (0.06 - 0.10)	**0.07** (0.00 - 0.14)	**0.16** (0.06 - 0.22)	**0.12** (0.00 - 0.28)	**0.05** (0.01 - 0.10)	**0.05** (0.03 - 0.10)	**0.12** (0.07 - 0.17)	**0.62** (0.28 - 1.70)	**0.21** (0.01 - 1.70)	**0.09** (0.07 - 0.11)	**0.10** (0.05 - 0.16)	**0.11** (0.08 - 0.14)	**0.21** (0.11 - 0.30)	**0.13** (0.05 - 0.30)	**0.14** (0.09 - 0.20)	**0.06** (0.05 - 0.09)	**0.14** (0.11 - 0.17)	**0.13** (0.10 - 0.17)	**0.12** (0.05 - 0.20)
Iron (mg Fe/L)	**0.17** (0.00 - 0.50)	**0.00** (0.00- 0.00)	**0.00** (0.00 - 0.00)	**0.17** (0.00 - 0.30)	**0.08** (0.00 - 0.50)	**0.27** (0.00 - 0.75)	**0.19** (0.00 - 0.55)	**0.03** (0.00 - 0.05)	**0.68** (0.35 - 1.15)	**0.29** (0.00 - 1.15)	**0.03** (0.00 - 0.05)	**0.42** (0.00 - 0.90)	**0.01** (0.00 - 0.01)	**1.47** (0.15 - 2.40)	**0.48** (0.00 - 2.40)	**0.44** (0.00 - 1.30)	**0.59** (0.01 - 0.90)	**0.03** (0.01 - 0.05)	**0.72** (0.45 - 1.20)	**0.44** (0.00 - 1.30)
Hardness (mg $CaCO_3$ /L)	**423** (330 - 510)	**337** (210 - 550)	**810** (710 - 830)	**683** (630 - 730)	**563** (210 - 830)	**310** (190 - 390)	**203** (110 - 390)	**617** (530 - 690)	**290** (250 - 330)	**355** (110 - 690)	**283** (130 - 390)	**250** (130- 330)	**457** (290 - 510)	**607** (430 - 690)	**399** (130 - 690)	**250** (130 - 330)	**197** (102-230)	**510** (410 - 580)	**337** (330 - 350)	**323** (130 - 580)
Turbidity (FTU)	**2.67** (0.00 - 6.00)	**2.00** (0.00 - 4.00)	**0.00** (0.00 -0.00)	**5.67** (4.00 - 10.0)	**2.58** (0.00 - 10.0)	**3.00** (0.00 - 6.01)	**3.00** (0.00 - 6.00)	**4.00** (0.00 - 6.00)	**11.1** (6.00 - 20.0)	**5.28** (0.00 - 20.0)	**3.00** (1.00 - 4.00)	**4.00** (2.00 - 7.00)	**6.00** (2.00 - 9.00)	**23.0** (15.0 - 25.0)	**9.00** (1.00 - 25.0)	**2.33** (1.00 - 5.00)	**2.67** (0.00 - 4.00)	**10.67** (2.00 - 15.0)	**10.7** (8.00 - 20.0)	**6.58** (0.00 - 20.0)
Sediment																				
Chlorophyll *a* (mg/m^3)	**0.105** (0.032 - 0.268)	**0.246** (0.145 - 0.355)	**0.176** (0.097 - 0.259)	**0.128** (0.111 - 0.182)	**0.164** (0.032 - 355)	**0.355** (0.173 - 0.571)	**0.226** (0.125 - 0.335)	**0.217** (0.119 - 0.321)	**0.177** (0.157 - 0.189)	**0.244** (0.119 - 0.571)	**0.256** (0.167 - 0.378)	**0.212** (0.113 - 0.302)	**0.221** (0.119 - 0.321)	**0.089** (0.086 - 0.094)	**0.195** (0.086 - 0.378)	**0.180** (0.142 - 0.247)	**0.162** (0.086 - 0.231)	**0.062** (0.032 - 0.086)	**0.070** (0.067 - 0.074)	**0.119** (0.032 - 0.247)
Chlorophyll *b* (mg/m^3)	**0.030** (0.001 - 0.109)	**0.073** (0.023 - 0.101)	**0.049** (0.038 - 0.064)	**0.031** (0.027 - 0.037)	**0.046** (0.001 - 0.109)	**0.096** (0.015 - 0.204)	**0.060** (0.023 - 0.096)	**0.029** (0.012 - 0.055)	**0.037** (0.033 - 0.044)	**0.055** (0.012 - 0.204)	**0.054** (0.032 - 0.082)	**0.050** (0.029 - 0.075)	**0.019** (0.014 - 0.019)	**0.026** (0.021 - 0.028)	**0.037** (0.014 - 0.082)	**0.035** (0.016 - 0.062)	**0.059** (0.035 - 0.079)	**0.007** (0.006 - 0.009)	**0.014** (0.011 - 0.015)	**0.029** (0.006 - 0.079)
Chlorophyll *c* (mg/m^3)	**0.013** (0.001 - 0.038)	**0.135** (0.055 - 0.243)	**0.136** (0.094 - 0.159)	**0.060** (0.057 - 0.069)	**0.086** (0.001 - 0.243)	**0.288** (0.113 - 1.289)	**0.101** (0.048 - 0.146)	**0.152** (0.136 - 0.161)	**0.059** (0.045 - 0.072)	**0.150** (0.045 - 1.289)	**0.131** (0.067 - 0.208)	**0.108** (0.043 - 0.138)	**0.149** (0.131 - 0.156)	**0.056** (0.045 - 0.065)	**0.111** (0.043 - 0.208)	**0.069** (0.034 - 0.089)	**0.056** (0.041 - 0.083)	**0.109** (0.099 - 0.118)	**0.024** (0.019 - 0.028)	**0.065** (0.019 - 0.118)
Phaeopigments (mg/m^3)	**1.922** (1.154 - 2.945)	**7.128** (5.454 - 9.454)	**4.031** (2.754 - 5.703)	**1.178** (1.065 - 1.536)	**3.564** (1.065 - 9.454)	**8.523** (8.193 - 8.743)	**5.492** (3.554 - 7.995)	**1.628** (1.202 - 1.753)	**2.426** (2.126 - 2.919)	**4.517** (1.126 - 8.743)	**3.218** (2.421 - 3.961)	**3.942** (3.195 - 6.349)	**0.628** (0.202 - 0.753)	**0.867** (0.759 - 1.042)	**2.164** (0.202 - 6.349)	**1.897** (0.409 - 3.732)	**3.322** (2.449 - 3.988)	**2.496** (2.258 - 3.256)	**2.004** (1.904 - 2.119)	**2.430** (0.409 - 3.988)
Silt clay fraction (%)	**22.94** (9.363 - 37.56)	**14.20** (5.975 - 18.54)	**6.37** (2.879 - 9.843)	**7.68** (3.522 - 11.74)	**12.797** (2.879 - 37.56)	**11.12** (8.164 - 16.39)	**7.73** (4.872 - 12.31)	**13.97** (11.33 - 17.95)	**9.82** (6.777 - 12.53)	**10.66** (4.872 - 17.95)	**8.12** (3.138 - 11.64)	**5.64** (3.436 - 9.118)	**4.77** (2.691 - 9.001)	**6.01** (3.035 - 9.972)	**6.13** (2.691 - 11.64)	**11.43** (4.861 - 15.18)	**24.05** (16.81 - 34.18)	**4.52** (1.474 - 8.182)	**7.22** (5.178 - 9.115)	**11.81** (1.474 - 34.18)
Organic matter (%)	**0.504** (0.372 - 0.687)	**0.844** (0.069 - 1.441)	**0.620** (0.362 - 0.989)	**0.725** (0.551 - 0.993)	**0.673** (0.069 - 1.441)	**2.007** (1.224 - 2.851)	**0.985** (0.677 - 1.792)	**1.127** (0.758 - 1.481)	**1.063** (0.797 - 1.241)	**1.295** (0.677 - 2.851)	**0.724** (0.173 - 0.963)	**0.782** (0.141 - 1.429)	**0.718** (0.535 - 1.101)	**0.867** (0.653 - 1.075)	**0.773** (0.141 - 1.429)	**2.689** (1.554 - 4.706)	**1.860** (1.409 - 2.177)	**0.861** (0.683 - 1.127)	**0.911** (0.671 - 1.109)	**1.580** (0.671 - 4.706)

Annex 4.2. – Local and seasonal mean concentrations (µg/g) of the 9 selected metals measured in sediments, at the four selected sites. The grey column represents the mean values, per year, for each and total of metals (∑metals). Values indicate the mean ± S.E.M., with exception of the total metal concentrations (µg/g) per season, shown at the bottom of table. "<DL" – value below detection limit.

	Barra					*Vagueira*					*Harbour*					*C. Bico*				
	WINTER	**SPRING**	**SUMMER**	**AUTUMN**	**Annual mean**	**WINTER**	**SPRING**	**SUMMER**	**AUTUMN**	**Annual mean**	**WINTER**	**SPRING**	**SUMMER**	**AUTUMN**	**Annual mean**	**WINTER**	**SPRING**	**SUMMER**	**AUTUMN**	**Annual mean**
Al	**1067** ± 156.9	**2840** ± 332.4	**1028** ± 34.59	**2602** ± 160.2	**1885** ± 267.7	**4972** ± 169.5	**2508** ± 107.3	**3388** ± 100.1	**3667** ± 51.06	**3634** ± 270.7	**1024** ± 54.13	**813.0** ± 71.33	**1610** ± 75.71	**1121** ± 108.3	**1142** ± 94.45	**4258** ± 99.16	**2818** ± 658.8	**672.3** ± 31769	**3661** ± 210.3	**2852** ± 435.9
Pb	**0.993** ± 0.017	**2.943** ± 0.214	**2.110** ± 0.291	**2.440** ± 0.291	**2.122** ± 0.237	**3.307** ± 0.073	**2.347** ± 0.088	**3.193** ± 0.082	**3.230** ± 0.334	**3.019** ± 0.414	**2.137** ± 0.288	**1.467** ± 0.126	**3.210** ± 0.187	**1.783** ± 0.083	**2.149** ± 0.213	**4.753** ± 0.143	**3.903** ± 0.914	**1.863** ± 0.234	**4.250** ± 0.091	**3.693** ± 0.383
Cd	**< DL**	**< DL**	**< DL**	**< DL**	**< DL**	**0.210** ± 0.200	**< DL**	**< DL**	**< DL**	**0.052** ± 0.051	**< DL**	**< DL**	**< DL**	**< DL**	**< DL**	**< DL**	**< DL**	**< DL**	**< DL**	**< DL**
Cr	**2.190** ± 0.238	**3.730** ± 0.520	**0.787** ± 0.034	**2.433** ± 0.237	**2.285** ± 0.341	**6.523** ± 0.301	**3.290** ± 0.115	**3.800** ± 0.175	**1.773** ± 0.306	**3.847** ± 0.527	**1.643** ± 0.082	**0.196** ± 0.188	**1.397** ± 0.131	**0.883** ± 0.191	**1.029** ± 0.179	**5.113** ± 0.199	**2.837** ± 0.794	**< DL**	**4.007** ± 0.351	**2.990** ± 0.604
Co	**0.341** ± 0.168	**0.320** ± 0.311	**< DL**	**1.011** ± 0.070	**0.416** ± 0.133	**2.150** ± 0.037	**0.807** ± 0.014	**1.197** ± 0.066	**0.860** ± 0.060	**1.253** ± 0.163	**0.333** ± 0.013	**< DL**	**0.217** ± 0.181	**0.263** ± 0.228	**0.190** ± 0.071	**1.697** ± 0.046	**1.120** ± 0.372	**< DL**	**1.083** ± 0.352	**0.883** ± 0.214
Cu	**3.547** ± 0.525	**2.900** ± 0.741	**< DL**	**3.060** ± 0.693	**2.378** ± 0.485	**6.123** ± 0.532	**2.260** ± 0.776	**0.477** ± 0.441	**1.877** ± 0.590	**2.677** ± 0.683	**0.797** ± 0.054	**1.727** ± 0.276	**2.657** ± 0.183	**1.453** ± 0.344	**1.658** ± 0.226	**4.680** ± 0.142	**3.197** ± 0.841	**1.223** ± 1.188	**3.927** ± 0.302	**3.249** ± 0.504
Ni	**2.330** ± 0.401	**2.747** ± 0.598	**0.233** ± 0.198	**2.530** ± 0.339	**1.953** ± 0.354	**4.953** ± 0.076	**3.683** ± 0.943	**1.043** ± 0.128	**1.993** ± 0.235	**2.918** ± 0.501	**1.200** ± 0.055	**< DL**	**2.230** ± 0.257	**1.340** ± 0.127	**1.194** ± 0.246	**4.277** ± 0.179	**2.167** ± 0.462	**1.697** ± 0.72	**2.207** ± 0.191	**2.587** ± 0.321
Zn	**8.800** ± 0.812	**14.77** ± 1.722	**6.027** ± 0.137	**17.50** ± 1.126	**11.77** ± 1.457	**23.43** ± 1.304	**12.83** ± 1.047	**14.23** ± 0.887	**15.87** ± 0.841	**16.59** ± 1.131	**5.737** ± 0.438	**4.193** ± 0.712	**11.33** ± 0.504	**5.733** ± 1.053	**6.749** ± 0.875	**38.53** ± 1.125	**24.27** ± 4.299	**7.10** ± 0.026	**30.20** ± 1.873	**25.03** ± 3.623
Hg	**0.110** ± 0.026	**0.070** ± 0.005	**< DL**	**0.060** 0.001	**0.061** ± 0.012	**0.183** ± 0.038	**0.053** ± 0.003	**0.057** ± 0.018	**0.060** ± 0.004	**0.085** ± 0.019	**0.187** ± 0.056	**0.067** ± 0.006	**0.063** ± 0.021	**< DL**	**0.077** ± 0.024	**0.280** ± 0.033	**0.157** ± 0.028	**0.070** ± 0.015	**0.120** ± 0.05	**0.157** ± 0.025
Σmetals	**1086**	**2867**	**1037**	**2631**	**1905** ± 490.1	**5019**	**2533**	**3412**	**3692**	**3664** ± 515.3	**1036**	**820.6**	**1631**	**1132**	**1155** ± 172.1	**4317**	**2855**	**684.3**	**3707**	**2891** ± 794.4

Annex 4.3. – Local and seasonal mean concentrations (μg/g) of the nine selected metals measured in *P. microps*, collected at the four sites of Aveiro lagoon. The grey column indicates the mean values, per year, for each and total of metals (∑metals). Values indicate the mean ± S.E.M., with exception of the total metal concentrations (μg/g) per season, shown at the bottom of table. "<DL" – value below detection limit.

	Barra					*Vagueira*					*Harbour*					*C. Bico*				
	WINTER	SPRING	SUMMER	AUTUMN	Annual mean	WINTER	SPRING	SUMMER	AUTUMN	Annual mean	WINTER	SPRING	SUMMER	AUTUMN	Annual mean	WINTER	SPRING	SUMMER	AUTUMN	Annual mean
Al	**636.3** ± 73.45	**248.7** ± 31.92	**74.50** ± 3.879	**72.70** ± 4.611	**258.1** ± 71.37	**243.0** ± 36.11	**239.3** ± 32.63	**82.20** ± 22.47	**45.10** ± 4.172	**152.4** ± 29.38	**334.1** ± 79.41	**139.9** ± 26.12	**74.70** ± 13.65	**19.17** ± 2.338	**141.9** ± 40.12	**351.0** ± 15.01	**385.0** ± 24.82	**151.3** ± 13.54	**26.50** ± 7.703	**228.5** ± 44.82
Pb	**< DL**	**0.460** ± 0.451	**6.223** ± 1.571	**< DL**	**1.673** ± 0.867	**< DL**	**1.893** ± 0.453	**3.027** ± 1.617	**3.513** ± 2.118	**2.102** ± 0.714	**< DL**	**< DL**	**< DL**	**3.995** ± 2.018	**1.003** ± 0.675	**2.630** ± 0.902	**< DL**	**1.273** ± 1.238	**3.827** ± 1.903	**1.704** ± 0.665
Cd	**< DL**	**< DL**	**0.320** ± 0.026	**< DL**	**0.081** ± 0.080	**< DL**	**0.180** ± 0.059	**0.343** ± 0.054	**< DL**	**0.129** ± 0.081	**< DL**	**< DL**	**< DL**	**< DL**	**< DL**	**< DL**	**< DL**	**< DL**	**< DL**	**< DL**
Cr	**0.303** ± 0.291	**0.720** ± 0.242	**2.530** ± 0.049	**2.093** ± 0.342	**1.350** ± 0.313	**0.880** ± 0.291	**0.643** ± 0.052	**2.797** ± 0.518	**1.700** ± 0.552	**1.359** ± 0.342	**0.270** ± 0.261	**0.850** ± 0.283	**2.773** ± 0.228	**2.020** ± 0.209	**1.407** ± 0.328	**0.197** ± 0.188	**0.563** ± 0.074	**2.487** ± 0.127	**1.947** ± 0.371	**1.298** ± 0.297
Co	**< DL**	**< DL**	**1.167** ± 0.645	**1.970** ± 0.323	**0.783** ± 0.293	**< DL**	**< DL**	**1.343** ± 0.551	**1.687** ± 0.875	**0.756** ± 0.318	**< DL**	**< DL**	**1.077** ± 0.082	**2.022** ± 0.205	**0.777** ± 0.257	**< DL**	**< DL**	**1.047** ± 0.605	**1.623** ± 0.291	**0.666** ± 0.253
Cu	**7.187** ± 0.280	**9.140** ± 3.480	**1.470** ± 0.872	**4.003** ± 0.381	**5.446** ± 1.176	**9.347** ± 2.639	**7.950** ± 0.228	**2.957** ± 0.433	**4.513** ± 0.735	**6.192** ± 0.974	**4.910** ± 0.275	**8.667** ± 0.816	**5.813** ± 0.916	**4.210** ± 0.603	**5.901** ± 0.591	**5.287** ± 0.466	**4.717** ± 0.768	**9.700** ± 4.266	**4.193** ± 0.157	**5.974** ± 1.139
Ni	**< DL**	**2.413** ± 0.973	**1.883** ± 0.262	**4.990** ± 1.179	**2.323** ± 0.630	**2.145** ± 0.854	**1.363** ± 0.261	**2.930** ± 0.608	**1.743** ± 0.141	**1.867** ± 0.301	**3.035** ± 1.032	**2.353** ± 0.443	**0.923** ± 0.452	**0.547** ± 0.511	**1.451** ± 0.362	**9.567** ± 3.521	**1.590** ± 0.034	**3.760** ± 0.490	**0.820** ± 0.785	**3.927** ± 1.293
Zn	**89.03** ± 2.747	**111.6** ± 13.44	**60.37** ± 0.895	**75.37** ± 2.351	**84.10** ± 6.415	**110.9** ± 14.81	**147.3** ± 5.547	**86.87** ± 6.570	**75.00** ± 0.404	**105.0** ± 9.099	**167.0** ± 8.66	**147.0** ± 4.582	**105.8** ± 8.565	**59.37** ± 4.941	**119.8** ± 12.79	**138.0** ± 4.041	**138.7** ± 1.333	**91.03** ± 3.071	**65.17** ± 6.688	**108.2** ± 9.588
Hg	**0.437** ± 0.067	**0.313** ± 0.0617	**0.177** ± 0.003	**0.160** ± 0.028	**0.272** ± 0.039	**0.480** ± 0.068	**0.263** ± 0.053	**0.147** ± 0.006	**0.147** ± 0.008	**0.259** ± 0.45	**0.347** ± 0.023	**0.240** ± 0.028	**0.201** ± 0.020	**0.143** ± 0.031	**0.233** ± 0.025	**0.550** ± 0.030	**0.423** ± 0.056	**0.313** ± 0.143	**0.307** ± 0.085	**0.398** ± 0.048
Σmetals	**733.3**	**373.3**	**148.6**	**161.3**	**354.1** ± 136.5	**366.8**	**399.0**	**182.6**	**133.4**	**270.1** ± 66.01	**509.6**	**299.0**	**191.3**	**91.5**	**272.5** ± 89.57	**507.2**	**531.0**	**260.9**	**104.4**	**350.6** ± 102.4

Chapter 5. Concluding Remarks

5.1. CONCLUDING REMARKS

This last chapter synthesizes the main conclusions derived from the studies performed in this thesis, namely: the acute effects of selected polycyclic aromatic hydrocarbons (PAHs), water accommodated fraction of a fuel oil (#4 WAF) and metals on selected enzymatic biomarkers of *P. microps* as effect criteria; linking biomarkers responses to behaviour, using a device specifically developed for epibenthic fish (SPEDE); the validation of an integrated approach, including ecological and ecotoxicological parameters, to evaluate the effects of pollution on estuarine fish in real scenarios.

In a first phase of the study (chapter 2), the acute effects of BaP, anthracene and #4 WAF on wild *P. microps* were investigated in laboratory controlled conditions using sub-individual parameters as endpoints, namely: the activity of the enzymes AChE (involved in cholinergic neurotransmission), LDH (involved in the energy production), GST (detoxification and anti-oxidative stress defences), GR, GPx, SOD and CAT (antioxidant defences). All these enzymes have been widely used as biomarkers and effect criteria in studies with fish. The protocols for measuring the enzymes were adapted to this species. The values determined in fish from the control group of the three bioassays compare to correspondent values that have been reported in the literature for fish (Table 2.1).

The mechanisms of toxicity and detoxification of fuel oils and of PAHs in fish are not fully understood and contradictory effects on enzymes commonly used as biomarkers have been reported in the literature. Species differences, distinct experimental conditions, and different composition of the fuel oils tested, among other factors, seem to contribute to the observed differences. Therefore and despite the considerable effort that has been put on this matter, more research is still needed to increase the knowledge on the toxic effects of these compounds especially in wild fish from non commercial species. In the present study, BaP and anthracene were selected as test substances because they are included in the list of priority pollutants of the US Environmental Protection Agency (US EPA), their high use and common environmental occurrence, and toxicity. The tested concentrations of BaP (1 - 16 μg/L) and anthracene (0.25 - 4 μg/L) were considered to be ecologically relevant since they have been found in sediments, water column and organisms from estuaries polluted with petrochemical products (e.g. Viguri *et al*., 2002; Maskaoui *et al*., 2002; Buet

et al., 2006). Both PAHs and the fuel-oil where found to significantly inhibit common goby AChE, indicating that they may be responsible for the disruption of cholinergic transmission by inhibition of AChE. Fish exposed to BaP and anthracene showed a significant induction of LDH activity, suggesting that fish are getting additional energy from the anaerobic pathway in an attempt to support the processes needed to face chemical exposure (e.g. detoxification mechanisms). Inversely, significant inhibition of LDH was found in fish exposed to #4 WAF, suggesting a distinct effect of the mixture. The results of this study also indicated that care should be taken when analysing AChE and LDH inhibitions by petrochemical mixtures since several metals potentially present in fuel oils and other petrochemical mixtures have the potential to inhibit these enzymes in several species (Gill *et al*., 1990; Osman *et al*., 2007). The two isolated PAHs and the fuel-oil increased CAT activity, BaP and anthracene also significantly increased SOD, GR and GPx activities, while #4 WAF did not caused significant alterations on these enzymes. Thus all the substances may induce oxidative stress on *P. microps*, with BaP and anthracene apparently having more oxidative stress potential than #4 WAF. The results of this study also highlighted that care should be taken when using GST assay in ecosystems contaminated with different PAHs and or/petrochemical mixtures because they might have opposite effects on its activity. The results of this work are important since the tested substances are common contaminants of several estuarine and coastal ecosystems where *P. microps* plays an important ecological function and where it may be used as sentinel species.

Since enzymes and other molecules are measured at sub-individual level, it is important to know if and when effects on these parameters may have reflexes at higher levels of biological organization when considering their use in ecological risk assessment contexts. Therefore, in chapter 3, the information provided by parameters widely used as biomarkers was studied in relation to the information provided by behaviour which is considered an ecological relevant parameter in fish since it is determinant for predator avoidance, prey capture, sexual performance and other functions with direct effects on the intrinsic rate of population increase. For measuring *P. microps* behaviour, a new device was developed: the Swimming Performance Device (SPEDE) (Figure 3.2). It allows measuring the swimming resistance to water-flow (swimming resistance) and the covered

distance while swimming against water-flow (covered distance). In addition to the sub-individual endpoints previously used (chapter 2), the activity of EROD, an enzyme of the P450 system was also determined to go further into the biotransformation mechanisms, and LPO levels were measured to evaluate the oxidative damage. Copper, an essential metal, and mercury, a non-essential metal, were selected as test substances since they are common contaminants of estuaries and coastal areas and they are toxic to fish at ecological relevant concentrations.

Based on LC_{50} values, mercury was found to be more toxic to *P. microps* than copper, with a difference of about nine fold. The tested ionic concentrations of copper (25 - 400 µg/L) and of mercury (3.1 - 50 µg/L) were ecologically relevant since they compare to those found in sediments, water and organisms from several estuaries, including some were *P. microps* naturally occurs (e.g. Fernandes *et al.*, 2007; Pyle *et al.*, 2005; Miramand *et al.*, 1998; Guilherme *et al.*, 2008; Sellanes *et al.,* 2002). The results indicate that both tested metals may induce negative effects on exposed *P. microps*: (1) both metals cause neurotoxic effects through the inhibition of AChE, therefore, neurologic and neuromuscular functions were impaired at least at the highest concentrations tested; (2) increase of the use of the anaerobic pathway of energy production, by induction of LDH activity; (3) a decrease in the capability of phase I biotransformation in the presence of these metals, by inhibition of EROD activity; (4) induction of GST enzyme activity which may be interpreted as a first attempt to overcome metal stress and (4) induce of oxidative stress (significant inductions of liver CAT, SOD, GR and GPx activities, and LPO levels). Significant and positive correlations between the activities of AChE and EROD and behavioural parameters were found. On the contrary, negative correlations were found between all the other biomarkers and behavioural parameters.

The integrated analysis of data associated AChE, EROD and swimming performance of fish by opposition to LDH, GST, LPO and anti-oxidant enzymes. These results suggest that tested metals may be responsible for critical loss of swimming capability, which may have serious repercussions on its feeding, reproduction and survival. Moreover, the new device SPEDE was proven to be efficacious against metals, allowing the link of biochemical responses with behavioural alterations at ecological relevant concentrations. The results of chapters 2 and 3 indicate that *P. microps* is a suitable species to be used as test organism in laboratorial bioassays.

Laboratory results need to be extrapolated to the field and often this extrapolation is difficult since environmental conditions may be considerable different, pollutants use to occur as complex mixtures and thus toxicological interactions are likely to occur, and contaminants may interact with components of environmental compartments. Therefore, it is necessary to develop and validate methodologies to be used in real scenarios, preferentially with autochthonous organisms to increase the ecological relevance of the assessments.

In chapter 4, a field approach including ecological and ecotoxicological parameters was investigated in relation to its potential to discriminate between sites with different types and levels of environmental contamination. The common goby was selected as bioindicator and the Aveiro lagoon (NW coast of Portugal) as case study area. Four sampling sites, a reference and three other sites, with different types and levels of main contamination, were selected for study. The information provided by fourteen water quality variables, characteristics of sediments, two condition indexes, eight biomarkers and the concentrations of nine metals in sediments and in the fish body was integrated using two multivariate statistical analysis (RDA and PRC). All sites were found to be contaminated with metals, fish were found to accumulate Zn, Hg and Cu at all the sampling sites, and other metals in some of the sites. Overall, the results of RDA and PRC indicated that the selected battery of biomarkers, the concentrations of metals in both sediments and fish body, and water quality variables were valuable discriminating parameters. The results also indicate that *P. microps* is a suitable species for use as bioindicator (sentinel species).

5.2. REFERENCES

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