Biomonitoring of coastal contamination using a biomarker approach: the use of *Pollicipes pollicipes* as a sentinel species

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O Presidente do Júri,

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Dissertação submetida à Faculdade de Ciências da Universidade do Porto, para a obtenção do grau de Mestre em Recursos Biológicos Aquáticos, da responsabilidade do Departamento de Biologia.

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Dedico esta dissertação a minha mãe pelo
pilar, pela amiga e pela guerreira que é

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Resumo

A poluição do ambiente aquático por contaminantes antropogénicos é um problema crescente, sobre o qual é necessário obter mais informação a fim de compreender as implicações deste tipo de poluição no ecossistema. A contaminação antropogénica tem origem em diversas atividades humanas, como a agricultura, a indústria, esgotos industriais e domésticos, e assume um papel ainda mais preocupante tendo em conta a ineficácia dos sistemas de tratamento de águas residuais. Assim, a avaliação do impacto da mistura de contaminantes antropogénicos no ambiente tornou-se uma necessidade urgente. Para a realização dessa avaliação, encontram-se já desenvolvidos vários ensaios ecotoxicológicos, mais especificamente testes com base em biomarcadores, que demostraram ser uma abordagem eficaz. Biomarcadores são ferramentas úteis para a avaliação do impacto de misturas de poluentes em condições reais no biota, permitindo a identificação, avaliação, avaliação comparativa e gestão dos riscos decorrentes de contaminantes no meio ambiente. O uso de biomarcadores é comumente associado a espécies sentinelas, espécies selvagens utilizadas em trabalhos de biomonitorização, uma vez que estas permitem uma avaliação mais realista dos efeitos biológicos causados pelos xenobióticos. Uma espécie pouco estudada, mas que apresenta características importantes para se tornar uma valiosa espécie sentinela é o percebe (*Pollicipes pollicipes*), um crustáceo filtrador interdidal. Tendo em conta os dados apresentados, o objetivo principal deste trabalho residiu na validação de *P. pollicipes* como uma espécie sentinela para a avaliação da poluição antropogénica nos ecossistemas aquáticos. Para esse efeito, dois estudos de biomonitorização foram realizados, cada um com um ano de duração. A primeira parte do estudo visou conhecer os padrões de variação de uma série de biomarcadores em função da sazonalidade e do padrão de contaminação em diferentes áreas geográficas. A recolha dos percebes foi realizada nas 4 estações do ano, em 3 praias distintas (praia da Aguda, de Lavadores e de Matosinhos) no litoral norte de Portugal. Nos cirros e pedúnculo de *P. pollicipes* foram quantificados diferentes marcadores bioquímicos com significados biológicos distintos, i) neurotoxicidade, através da medição da atividade da enzima colinesterase (ChE), ii) stresse oxidativo/fase II de destoxificação, pela quantificação da atividade das isoenzimas glutationa-S-transferases (GSTs) e iii) dano peroxidativo, através da avaliação dos níveis de substâncias reativas com o ácido tiobarbitúrico (TBARS). Os resultados obtidos mostram que a principal forma colinérgica presente no pedúnculo é a acetilcolinesterase (AChE). Em todos os biomarcadores testados foi encontrada uma variação significativa, entre os locais de amostragem, onde

Matosinhos e Lavadores (locais potencialmente poluídos) apresentaram maiores níveis de atividade nas enzimas metabólicas e aumento dos níveis de peroxidação lipídica, quando comparados com os resultados obtidos no possível local de referência (praia da Aguda). Os resultados mostraram também uma variação sazonal em todos os biomarcadores testados, com valores mais altos de atividade nas estações mais quentes (primavera e verão), sugerindo uma associação entre a presença de poluentes na água e flutuações nos parâmetros abióticos e bióticos. Assim, com o intuito de compreender a implicação real dos efeitos que os fatores abióticos e contaminação poderão ter nos resultados, foi realizado um segundo estudo apenas na praia de Lavadores. Ao longo de um ano de amostragem, e com uma periodicidade mensal, foram recolhidos 30 percebes durante a baixa-mar, para a avaliação da atividade da ChE, das GSTs e níveis de TBARS, nos cirros, pedúnculo e hemolinfa. Adicionalmente foram determinados os níveis de glicogénio no pedúnculo. Na hemolinfa, foi ainda efetuada a avaliação da variação do número de hemócitos presentes em cada amostra. A utilização da hemolinfa nos ensaios avaliados, teve o intuito de validar o seu uso como um tecido onde possam ser determinados biomarcadores de forma não invasiva, uma vez que é uma técnica não destrutiva, e que permite uma avaliação individual, podendo ser repetida ao longo do tempo no mesmo organismo. Os resultados mostram que a principal forma colinérgica presente nos cirros e hemolinfa é a acetilcolinesterase. Os resultados dos biomarcadores testados (AChE, GSTs e TBARS) revelaram um padrão semelhante entre todos os tecidos testados, evidenciando uma variação sazonal, com valores significativamente mais elevados durante os meses mais quentes, podendo indicar um maior impacto dos poluentes químicos nesses períodos, possivelmente potenciados pelas variações naturais (temperatura e salinidade). Os níveis de glicogénio mostraram uma potencial relação com o ciclo reprodutivo, registando-se os níveis mais baixos na primavera e verão. As variações no número de hemócitos mostram um padrão ambíguo, que pode estar relacionado com o aumento de contaminantes na água ou com variações naturais. *P. pollicipes* mostrou ser um organismo promissor, com potencial para ser uma espécie sentinela em programas de biomonitorização da zona costeira. A utilização do tecido hemolinfa mostrou ser vantajoso para a quantificação de biomarcadores, apresentando um padrão de resposta semelhante, em níveis mais baixos, quando comparado com restantes tecidos testados, permitindo ainda uma avaliação dos efeitos tóxicos ao longo do tempo.

Palavras-chave: *Pollicipes pollicipes*, espécie sentinela, biomonitorização, biomarcadores, hemolinfa, variações sazonais, contaminantes antropogénicos

Abstract

The pollution of the aquatic environment by anthropogenic contaminants is an emerging issue, about which more information is needed, in order to understand the implications of this kind of pollution on the ecosystem. Anthropogenic contamination is caused by several human activities, as agriculture and industrial and domestic wastewaters, which becomes an even more important issue taking into count the inefficiency of water treatment systems. Consequently, the evaluation of the impact of contaminant mixtures in biota became an urgent need. In order to perform this evaluation, several biomarker based ecotoxicological tests were already developed, which were shown to constitute a valid and efficient method for that assessment. Biomarkers are useful tools for the evaluation of impact in biota of pollutants mixtures under realistic conditions, allowing the identification, estimation, comparative assessment and management of the risks posed by contaminants in the environment. Biomarkers are frequently associated with sentinel species, wild species that fulfill several requirements, and are commonly used in biomonitoring studies, allowing more realistic assessments of biological effects caused by xenobiotics. One particular species, poorly studied so far, but presenting important characteristics to become a valuable sentinel species, is the crustacean gooseneck barnacle (*Pollicipes pollicipes*), an interdidal filter feeding species. Considering all these issues, the general aim of the present study is the validation of *P. pollicipes* as a sentinel species for the assessment of anthropogenic pollution in marine coastal ecosystems. For that purpose, two biomonitoring studies were conducted, each with one year of duration. In the first part of the study, the barnacle collection was made once per season, in 3 different beaches (Aguda, Lavadores and Matosinhos beaches) in the North coast of Portugal. In *P. pollicipes* cirri and peduncle different biochemical markers with distinct biological meanings were quantified: i) neurotoxicity, by measuring the activity of enzyme cholinesterase (ChE), ii) oxidative stress/phase II detoxification, by quantifying the enzymatic activity of glutathione-S-transferases (GSTs) and iii) peroxidative damage by assessing the levels of reactive substances to thiobarbituric acid (TBARS). The results obtained showed that the major cholinergic form present in peduncle is acetylcholinesterase (AChE). In all tested biomarkers, a significant variation between sampling sites was found, and animals collected at Matosinhos and Lavadores (potentially polluted sites) showed higher levels of metabolic enzymes, and increased levels of lipid peroxidation, when compared with the obtained results for organisms from the reference site (Aguda). The results also showed a seasonal variation in all

tested biomarkers, with higher activity values in hotter seasons (spring and summer), suggesting an association between the presence of water pollutants and abiotic and biotic fluctuations, which may interfere in biomarkers response. So, in order to understand the real implications of abiotic factors may have in the obtained results, a second study was developed only for organisms collected at Lavadores beach. Every month, 30 barnacles were collected, and the following biomarkers were evaluated: ChE activity, GSTs activity and TBARS levels in cirri, peduncle and also in haemolymph tissues. Additionally were measured the glycogen levels in peduncle. In haemolymph tissues the variation on haemocytes number was also monitored. The use of hemolymph in tests aimed to validate its use as a source tissue for the assessment of non-invasive biomarkers, since it involved a non-destructive technique that allows repeated individual evaluations over time in the same organism, allowing a compilation of a historical data. The results showed that the major cholinergic form present in cirri and haemolymph is acethylcholinesterase. The results of the tested biomarkers (AChE, GSTs and TBARS) showed a seasonal variation with significant higher values during the hotter months, indicating a greater impact of chemical contaminants in these periods, possibly enhanced by the natural variations (temperature and salinity). Glycogen levels showed a direct relation with the reproductive cycle, with lower levels in spring and summer. The variation in the haemocytes number showed an ambiguous pattern, which may be related with an increase of contaminants in the water or natural variations. The results also showed a similar pattern for all tested tissues. In conclusion, *P. pollicipes* showed promising responses that make it an important sentinel organism for coastal biomonitoring programs. Haemolymph showed to be a reliable tissue for ecotoxicology tests presenting a similar response pattern when compared with other tested tissues, allowing an assessment of toxic effects over time.

Keywords: *Pollicipes pollicipes*, sentinel species, biomonitoring, biomarkers, haemolymph, seasonal variations, anthropogenic contaminants

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ACh	Acetylcholine
AChEs	Acetylcholinesterases
BChEs	Butyrylcholinesterases
CAT	Catalase
CBs	Carbamates
CDNB	1-chloro-2,4-dinitrobenzene
ChEs	Cholinesterases
CHH	Crustacean hyperglycemic hormone
DTNB	Ditiobisnitrobenzoate
GPx	Glutathione peroxidase
GRed	Glutathione reductase
GSH	Glutathione (reduced form)
GSTs	Glutathione-s-transferases
H_2O_2	Hydrogen peroxide
LPO	Lipid peroxidation
MDA	Malondihaldeyde
MTs	Metallothioneins
O_2	Molecular oxygen
$O_2^{\bullet-}$	Superoxide anion radical
OCPs	Organochlorine pesticides
OH^+	Hydroxyl radical
Ops	Organophosphates
PAHs	Polycyclic aromatic hydrocarbons
PCBs	Polychlorinated biphenyls
PChEs	Pseudocholinesterases
POPs	Persistent organic pollutants
ROS	Reactive oxygen species
SOD	Superoxide dismutase
TBA	2-thiobarbituric acid
TBARS	Thiobarbituric acid reactive substances
UV	Ultraviolet

Chapter one

General introduction

During the last decades, levels of anthropogenic pollution in marine ecosystems have systematically increased, as a result of several human activities, including agriculture, industry, and release of treated and untreated domestic sewage, becoming an alarming situation for marine species and therefore for humans. For this reason, the development of novel methods for the identification, estimation, comparative assessment and management of the risks posed by pollutants in the environment has become essential (Cajaraville et al. 2000). Awareness about this scenario was first introduced during the 20th century, since a large number of new contaminants (including persistent organic chemicals such as polychlorinated biphenyls (PCBs), organochlorine pesticides (OCPs) and polycyclic aromatic hydrocarbons (PAHs) have been discharged into the environment. The aquatic compartment was immediately recognized as the final resting place of the mentioned compounds, due to the direct discharges, or hydrologic and atmospheric processes (Stegeman and Hahn 1994). Since then, the obtained knowledge concerning this issue allowed us to understand the long-term effects of most xenobiotics, and predict their interaction with other contaminants; however, the impact on aquatic life of a large number of chemical compounds (and their metabolites), has not yet been assessed, a factor that increases the importance of studying the impact of chemical pollutants in the aquatic environment.

Taking into account the necessity of evaluating aquatic pollution, the ecotoxicological tests based on biomarkers are considered a valid and realistic approach. Biomarkers are indicators of events in biological systems, allowing the assessment of chemical impact in exposed biota, and can function as a complimentary tool of chemical analysis. The use *per se* of analytic chemistry in environmental assessment cannot provide any indication of deleterious effects of pollutants on the organisms. Biomarkers are thus a valuable method supported by several authors (e.g., Timbrell 1998; Zelikoff 1998; Nunes et al. 2008), and is therefore a valid approach in ecotoxicological tests, allowing the regular monitoring and support in the evaluation of biological effects caused by xenobiotics. Biomarker responses are considered to be intermediates between contaminants sources and higher-level effects (Suter 1990), allowing the evaluation of their biological consequences not only in the analysed organism, but also at the ecosystem level, being a more relevant information than a mere quantification of the environmental levels of a given contaminant. Through the evaluation of a variety of biomarkers it is possible to understand the mechanisms and

effects of environmental contaminants, since each specific pollutant can trigger a cascade of biological responses. In field samples, the biomarker data can provide an important index of the total external load that is biologically available in the ecosystem (McCarthy et al. 1991), showing the relationship between the environmental presence of a compound, its uptake and biological effects.

Biomarker is a broad designation of a toolbox of methodologies that can be used and applied to a vast set of conditions, studies and organisms. Biomarkers can include alterations in different parameters, such as biochemical, physiological, cellular, morphological or behavioural changes (Timbrell 1998; van der Oost et al. 2003). According to the evaluated effect, biomarkers can be divided into classes, including i) exposure biomarkers, ii) biomarkers of effect and iii) biomarkers of susceptibility (Timbrell 1998; van der Oost et al. 2003). The impact of a chemical pollutant can be potentiated by its bioaccumulation, bioconcentration and biomagnification properties. In fact, the presence of an anthropogenic chemical in the tissue of an exposed organism can also serve as a biomarker. Chemicals may accumulate in aquatic organisms by: i) direct uptake from water by gills or skin (bioconcentration), ii) by uptake of suspended particles (ingestion) and iii) consumption of contaminated food (biomagnification) (van der Oost et al. 2003). The bioaccumulation of chemicals can ultimately induce adverse effects on ecosystems (Franke et al. 1994), by altering the exposed organisms (eg., biochemically, physiologically and/or behaviorally) (van der Oost et al. 2003).

Ecotoxicology can be understood as the science that relates ecology and toxicology. Ecology studies the interaction of living organisms with each other, and with the environment in which they live (Lindeman 1942); toxicology is the science that seeks to understand the types of effects caused by chemicals, and biological processes responsible for such effects, taking into account the sensitivity of different organisms to exposure to chemicals, and the relative toxicity of different substances (Klaassen 2008). The main goal of ecotoxicology is to understand and predict the effects of chemicals on living beings and natural communities (Chapman 2006). One of the main practical objectives of ecotoxicology is the capacity to predict toxic responses of organisms exposed via the environment to a multiplicity of toxicants (Figure 1). In order to attain this objective, a comprehensive set of tests has been developed. These ecotoxicological tests can be mainly used as an early diagnostic tool of deleterious effect on biota, and the obtained data can allow the regulation of certain substances, their production, uses and releases (van der Oost et al. 2003).

The standard toxicity tests are based on responses of an organism to a particular dose of compound during a defined and limited period of exposure, and are

based in processes that reflect responses from organisms exposed to one or more pollutants, allowing the measurement of the biological effects elicited by those pollutants (Walker et al. 2012). Toxicity tests have been routinely performed mostly based in standard guidelines published by international organizations (e.g., OECD, ISO, USEPA). These procedures are standardized methods, and are used to identify and characterise potential deleterious effects caused by chemical substances and/or chemical mixtures.

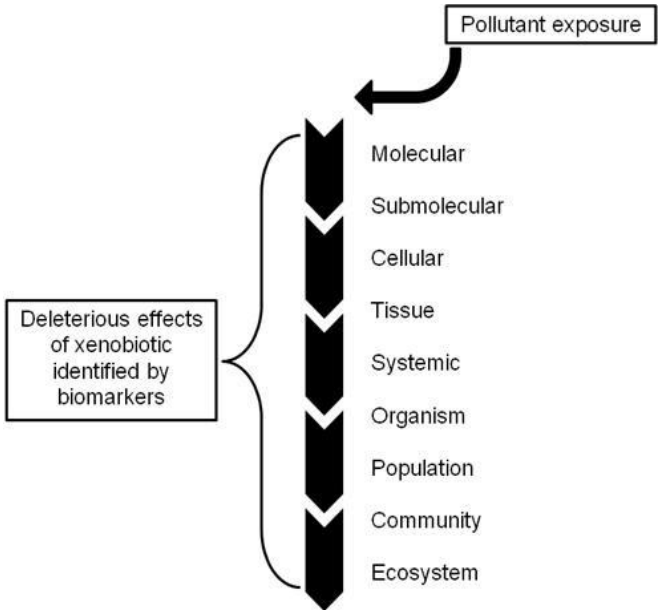

Figure 1- Representation of the response of a biological system in a polluted environment. Adapted from Bayne et al. (1985).

From the several types of biomarkers now available for monitoring purposes, two can be stressed: the biochemical, and the histological biomarkers. Biochemical biomarkers are sensitive tools which can be determined using simple and standardized procedures (van der Oost et al. 2003), and can be used in different organisms (fish, mammals, molluscs, plants, crustaceans and insects). The quantification of biomarkers has been widely used both *in vivo* and *in vitro*, for the evaluation of the effects caused by xenobiotics (Binelli et al. 2006).

One of the most commonly used effect biomarkers in ecotoxicology is the evaluation of the activity of the enzymes cholinesterases (ChEs). Different types of ChEs have been already described, and are differentiated by their substrate preference and by their specific inhibition profiles in the presence of particular inhibitors. One of the most significant subdivisions of cholinesterases include acetylcholinesterases (AChEs), which show higher hydrolytic affinity for acetylthiocholine; a second group is composed by the butyrylcholinesterases (BChE),

also known as pseudocholinesterases, with higher affinity for butyrylthiocholine (Nunes et al. 2005).

The importance of these biomarkers is high, especially in the case of AChE. AChE cleaves the neurotransmitter acetylcholine (ACh) into choline and acetic acid, during neurotransmission in the synaptic cleft of cholinergic synapses, preventing continuous nerve stimulation, protecting the normal neuromuscular function. AChE activity can be compromised by several substances commonly found in the environment, such as organophosphates (OPs) and carbamates (CBs) pesticides that operate as neurotoxic molecules, being thus designated as anticholinesterasics compounds. Inhibition of AChE causes the accumulation of the neurotransmitter ACh in the synaptic cleft, provoking an overstimulation of ACh receptors, and thus the blockage of neurotransmission, which may cause several alterations in exposed organisms (e.g., muscle weakness, hyperactivity, loss of coordination, paralysis, convulsions and death) (Hayden et al. 2010). Due to the inhibition of AChE by anticholinergic compounds, the evaluation of its activity can be used as a useful biomarker of pesticide exposure in most species. Besides organophosphates and carbamates pesticides, other compounds or matrices can be important sources of AChE inhibition, such as complex mixtures of pollutants including metals, pulp mill effluents, domestic sewage and PAHs (Payne et al. 1996; Bonacci et al. 2009). According to this background information, cholinesterase inhibition has been widely used as a biomarker of pesticide presence especially in aquatic environment (Schiedek et al. 2006) and has been considered a reliable and responsive marker of anticholinesterasic contamination of non-target organisms (Nunes et al. 2005).

Many pollutants (or their metabolites) can exert toxicity by triggering the onset of oxidative stress, and this outcome is established whenever the production of reactive oxygen species (ROS) induced by xenobiotics exceeds the endogenous protection constituted by the antioxidant defence. Reactive oxygen species, also referred as oxygen free radicals or oxyradicals, can cause injuries in tissues leading to enzyme inactivation, lipid peroxidation (LPO), DNA damage and cell death (Winston and Di Giulio 1991). The major ROS forms, reduction products of molecular oxygen (O_2), are the superoxide anion radical ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2) and the hydroxyl radical (OH^+). A diverse number of compounds can enhance intracellular oxyradical production through the process of redox cycling. In order to maintain homeostasis, organisms develop a series of defence systems that tend to inhibit oxyradical formation. The defence can be classified as enzymatic (if the antioxidants are enzymes, such as superoxide dismutase (SOD), catalase (CAT), glutathione-dependent peroxidase (GPx)

and glutathione reductase (GRed)) and non-enzymatic (glutathione (l-γ-glutamyl-l-cysteinyl-glycine- GSH), β-carotene (vitamin B), ascorbate (vitamin C), α-tocopherol (vitamin E)) (López-Torres et al. 1993).

The effects of chemical pollutants on aquatic organisms can also be observed in terms of their metabolic capacity. Considering that major routes of metabolism of toxicants involves phase I and phase II pathways, the activities of enzymes involved in these steps can serve as appropriate markers of toxicity. An important biomarker in environmental analysis is the activity of glutathione-s-transferases (GSTs). GSTs isoenzymes belong to the phase II of metabolism, and act by preventing the occurrence of oxidative stress, by enhancing the excretion of xenobiotic compounds. This occurs by catalyzing the nucleophilic attack by a sulphur atom of glutathione to an electrophilic group of metabolic products or xenobiotic compounds (Blanchette et al. 2007). This corresponds to a detoxification process, during which the toxicity is gradually reduced by the continued excretion of xenobiotics. The excretion of xenobiotic compounds from tissues by action of GSTs indirectly reduces the amount of ROS and prevents the occurrence of oxidative stress and cellular damage. GSTs also act in order to remove ROS from the organism (Gorrini et al. 2013). The activity of GSTs can be induced or inhibited by exposure to xenobiotics compounds like PAHs and PCBs (Kaaya et al. 1999) and metals (Zhang et al. 2012). The enzyme induction means an increase in the amount or activity of these enzymes. So, the variation in GSTs activity can be used as an important biomarker of pollution exposure (Fitzpatrick et al. 1997).

Despite the strong induction of the activity of enzymes involved in the antioxidant defense system, oxidative stress can occur, with the establishment of cellular damage, including lipoperoxidation. The evaluation of peroxidative damage using the thiobarbituric acid reactive substances (TBARS) test is also a common biomarker. LPO or the oxidation of polyunsaturated fatty acids, is a very important consequence of oxidative stress, since radical species occurring during LPO lead to deleterious biochemical reactions (Kappus 1987).

The energetic status of the organism is an important factor when addressing the most likely toxic outcome. One way of evaluating energetic balance is to analyze energetic reserves, such as glycogen, in tissues of exposed organisms. Glycogen is a carbohydrate, a multibranched polysaccharide of glucose, whose major function is energy storage in animals. Glycogen can be essentially found in liver and muscle tissue, and functions as an immediate reserve source of available glucose for muscle cells. Glycogen accumulation can increase during several alterations in the ecosystem,

including the reduction of food abundance (starvation periods), nitrogen or sulfur starvation, heat shock, or osmotic stress. This may mean that the changes in the accumulation of glycogen may act as protectors against stress (Silljé et al. 1999). The measurement of glycogen levels, in organisms from polluted waters, is described by several authors as a valuable biomarker for the assessment of deleterious impacts caused by the exposure to pollutants (e.g., Becker et al. 2009). The decrease in glycogen levels in organisms from a polluted ecosystem may indicate that the degradation of glycogen occurred to maintain the energy for the metabolic processes, counteracting the stress caused by environmental contamination (Becker et al. 2009).

Histological biomarkers are also a useful methodology of analysis for the determination of effects caused by water pollution, being used as a common indicator of the health condition of invertebrates (Au 2004; Bonacci et al. 2009) and fish (Fernandes et al. 2007). Aquatic organisms, when exposed to pollutants, may suffer significant structural damages in their bodies, with subsequent histological changes and possible impairment of the organ functionality (Costa et al. 2009). Histological changes can be quantified, by assessing qualitative data, namely by recording structural alterations caused by environmental pollutants (Ayas et al. 2007). Consequently, the onset of alterations may be a response of high sensitivity, reflecting previous changes, both at physiological or biochemical functions (Nero et al. 2006). In crustaceans, the alterations in haemolymph can be used as valuable histological biomarkers, presenting the advantage of being non-destructive biomarkers, not requiring the sacrifice of the organisms, and allowing a continuous assessment in the same organism over time. Alterations in the number of haemocytes can be used for the determination of pollutants in the ecosystem, since haematopoiesis can be affected by the presence of anthropogenic compounds, as described by Sami et al. (1993) and McCormick-Ray (1987). The haemocytes in crustaceans are involved in the detoxification process of metallic compounds, since they bind with xenobiotics in the haemolymph, and conduct them to endolisossomal system where these compounds are further detoxified (Cajaraville and Pal 1995).

However, the use of biochemical markers is not exempt of issues that must be addressed prior to its use, especially when these tools are used in biomonitoring programs, for the assessment of effects on organisms environmentally exposed to diffuse sources of pollution. Seasonality is one of the main driving forces of biological fluctuations, and for a correct interpretation of biomarker data, it is necessary to consider these variations, despite changes caused by contaminants (Cajaraville et al. 2000). The biomonitoring of parameters of sentinel species can be used as a valuable

method for the assessment of environmental contamination caused by anthropogenic activity, since they reflect the impact of biological/physiological changes in biota caused by pollutants. So, by means of quantifying the changes in the physiology of organisms through the use of biomarkers measured in autochthonous sentinel species, it is possible to infer the overall water quality (Livingstone 1993). Valbonesi et al. (2003) defended that the use of resident populations, as an alternative to caged animals, can be a valid tool for the evaluation of environmental health allowing the comparison of the results with ecological and biodiversity studies.

For the correct evaluation of the xenobiotics effects on biota, it is fundamental to develop a comprehensive set of toxicity markers. Namely, it is mandatory the involvement of toxicity tests that incorporate wild resident species, which can give more specific and relevant ecological data. The organisms used for this ecotoxicological evaluation are designated as sentinel species, and are commonly used in biomonitoring studies (Basu et al. 2007). For a wild species to be recognized as a sentinel species, it must fulfil several requirements, including a widespread distribution, ability to bioaccumulate pollutants, success while been maintained and studied in captivity, captured in sufficient numbers, restricted home range, well-known biology, and sensitive (as summarized by Basu et al. 2007). The bioaccumulation capacity is related with the feeding modality, normally being more commonly the occurrence of bioaccumulation in filter-feeders organisms (Rittschof and McClellan-Green 2005).

To attain this purpose, many aquatic organisms have been proposed for biomonitoring programmes. Marine invertebrates, for instance, are important bioindicators of coastal and estuarine pollution, and several authors have selected these as sentinel species. Livingstone (1998) described the relevance of using invertebrates in biomonitoring studies; among marine invertebrates, crustaceans seem had adequate response to most need of biomonitoring studies. Xuereb et al. (2007) advocates the validity of the use of a crustacean (e.g., *Gammarus pulex*) as a sentinel species for contamination of the aquatic environment by pesticides. Antó et al. (2009) work described the feasibility of using two deep-sea crustaceans (namely, *Aristeus antennatus* and *Nephrops norvegicus*) to investigate the changes in various biomarkers during seasonal variations. Devi et al. (1996) analysed the copper accumulation in a crustacean food chain (*Artemia salina*, *Carcinus maenas* and *Homarus americanus*).

A promising sentinel species is *Pollicipes pollicipes* (Gmelin 1790), also known as gooseneck barnacle, a marine species found in rocky shores of all northeast Atlantic Ocean and North Africa, from 48 ºN at Britain (France) to 14 ºN at Senegal (Barnes 1996). Gooseneck barnacle is composed by a beefy peduncle (muscle tissue) with a

thick integument covered with small and calcified scales, which contain the ovary and the adhesive gland. This structure is housed in the terminal part of the body, allowing its fixation to rocks. It also shows a rigid capitulum, composed by calcified plates where the functional organs, cirri and digestive systems, are protected from dehydration (Barnes 1996). In crustaceans with open circulatory systems, as is the case of *P. pollicipes*, the interstitial fluid is called haemolymph and is constituted by several types of cells called haemocytes (Renwrantz 1990). Cytologically, the haemocytes are classified into groups according to their morphology, structure and functional characteristics. The cellular groups found in crustacean haemolymph are agranulocytes (cells with few or no granules), granular or dense granulocytes (presence of many granules) and semi-dense granulocytes (Bauchau 1981). In the specific case of barnacles, a low number of haemocytes in the haemolymph was described.

The reproductive cycle of this organism in the Portuguese coast, according to Cruz and Hawkins (2009), is comprised by a brooding period, occurring mainly during spring and summer, and significantly related with the seawater temperature, and the peak of the reproductive season occurs from may to august. Gooseneck barnacle feeds by filtration, feeding on suspended particles that are collected with their cirri composed by six pairs of biramous thoracopods (Chan et al. 2008). Gooseneck barnacle lack of mobility prevents their escape from the contamination area and their filter-feeding behaviour favours the bioaccumulation of pollutants (Rittschof and McClellan-Green 2005). These are edible organism, intensively exploited in Spain and Portugal (Bernard 1988). *P. pollicipes* may thus be considered an interesting candidate to serve as sentinel species in biomonitoring studies. The studies published by Reis et al. (2012; 2013) refer *Pollicipes pollicipes* as an important species for biomonitoring purposes, specially of metal contamination in water.

Objectives

Having in account the present scenario of coastal pollution, and the described methodologies for the evaluation of xenobiotics effects in the marine environment, this study aimed to perform a biomonitoring assessment in the northern coastal area of Portugal, using different biomarkers quantified in specific tissues of the sentinel crustacean species *Pollicipes pollicipes*. In order to attain this general objective, a set of several specific secondary objectives were defined:

- To validate the use of *Pollicipes pollicipes* as a sentinel species for

ecotoxicological studies, by evaluating the biochemical effects from nonspecific sources of pollution in different tissues of *P. pollicipes*;

- To establish relationships between the effects of xenobiotics and biotic and abiotic seasonal variations, in the patterns/profiles of biomarker results;
- To validate the putative use of haemolymph as a tissue suitable for the development of non-destructive biomarkers, by comparing results of biomarker assays obtained in haemolymph and in other tissues, namely in terms of oxidative stress biomarkers.

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Chapter two

AS Ramos, SC Antunes, F Gonçalves, B Nunes (2014). The gooseneck barnacle (*Pollicipes pollicipes*) as a candidate sentinel species for coastal contamination. Archives of Environmental Contamination and Toxicology 66:317–326

The gooseneck barnacle (*Pollicipes pollicipes)* as a candidate sentinel species for coastal contamination

Abstract

The assessment of toxic effects caused by complex mixtures of pollutants in the marine environment requires previous validation of toxicological criteria, which may include biomarker end points with distinct biological meanings. This is the case of oxidative stress/phase II detoxification (glutathione-S-transferases activity), oxidative damage (thiobarbituric acid reactive substances), and neurotransmission (cholinesterase activity), which are likely to be affected after toxic insults by common marine pollutants. The main purpose of the present study was to assess potential biological alterations in the crustacean species *Pollicipes pollicipes* (gooseneck barnacle) caused by human contamination and seasonality, during a period of 1 year, in three different areas of the North Atlantic shore of Portugal. Our results indicate that fluctuations of the mentioned biomarkers were strongly related to seasonality, but they may also suffer influence by the already documented patterns of chemical pollution. Organisms collected in polluted sampling sites (urban areas and oil refinery) showed greater levels of metabolic enzymes and increased levels of lipid peroxidation. These alterations were more evident during the summer, and, in some cases, spring months, suggesting an association between the presence of chemical stressors and temperature-dependent seasonal physiological fluctuations, which contribute to the modulation of the toxic response. In general terms, *P. pollicipes* was shown to be a promising organism in coastal biomonitoring programs, with an adequate sensitivity toward pollution and/or seasonal fluctuations. However, it is of the utmost importance to consider seasonal fluctuations in physiological parameters that modulate the toxic response. These factors can ultimately compromise the development and interpretation of data from marine biomonitoring programs if a thorough characterization of biological responses is not previously performed.

Keywords: Gooseneck barnacle, sentinel species, biomonitoring, biomarker.

Introduction

The presence of anthropogenic contaminants in the aquatic environment, namely in marine ecosystems, is a major issue in modern ecotoxicological studies. The scientific evidence points to a large number of chemical classes already documented in the wild, and the combined biological effects of such compounds is not always simple to characterize (Ben-Khedher et al. 2013). It is not only important to identify and/or quantify these chemicals in water matrices, it is also of fundamental significance to fully characterize their interactions with biotic systems. The approach to measuring biological effects is the cornerstone of the use of biomarkers in ecotoxicological assessment being integrated into a broader approach that considers space and time, such as biomonitoring programmes. To evaluate relevant information on the types, amounts, availability, and effects of environmental pollutants, it is common to employ a sentinel species in biomonitoring, which allows us to evaluate the environmental pollution levels caused by anthropogenic action. Sentinel species are present in the study area and serve as proxies of pollution by reflecting the biological impact/adaptive physiological changes on biota caused by pollutants. The assessment of toxic effects may involve the use biomarkers, which consist of any measurable parameter related to the toxic effect that elucidates the toxic impairment caused to exposed organisms (Timbrell 1998). By quantifying these changes in the organism's physiology, it is possible to infer the overall quality of the water (Livingstsone 1993).

A putative marine species to be considered as a sentinel species of aquatic pollution is *Pollicipes pollicipes* (gooseneck barnacle), which is an intertidal sessile cirripede belonging to the crustacean group. The typical habitat of *P. pollicipes* is crowded waters, particularly in the intertidal zone of cliffs (Molares and Freire 2003). This species has a large spatial distribution and can be found along the rocky shores of the northeast Atlantic Ocean and North Africa from 48 °N at Britain (France) to 14 °N at Senegal (Barnes 1996). Gooseneck barnacle is a filter feeder that feeds on suspended particles that they collect with their cirri (six pairs of biramous thoracopods - Chan et al. 2008). Due to their lack of mobility, exposure to marine contaminants for large periods, and continuous availability (both spatially and temporarily), this particular species is an interesting candidate to serve as a sentinel species in biomonitoring studies.

To attain the objective of validating *P. pollicipes* as a suitable organism for biomonitoring purposes, we tested the feasibility of assessing several biomarker commonly used to evaluate the impact caused by human activities (namely, chemical pollution) in the ecosystem. Using these selected biomarkers, we aimed to assess the

toxic effects caused by a realistic mixture of pollutants, including (a) isoenzymes glutathione-S-transferases (GSTs; as a function of phase II metabolism/oxidative stress), (b) cholinesterase activity (ChE; indicative of neurotoxicity), and (c) lipid peroxidation (TBARS; as an indicator of oxidative damage).

GSTs belong to the family of phase II detoxification enzymes (Beckett and Hayes 1993) and catalyse the nucleophilic attack of an electrophilic group of metabolic products or xenobiotic compounds by a sulphur atom of glutathione (L-c-glutamyl-L-cysteinyl-glycine - GSH) (Nava et al. 2009), which results in the detoxification of reactive intermediates and oxygen radicals (van der Oost et al. 2003). GSTs acts in the defence against oxidative damage and peroxidative products of DNA and lipids. Due to this fact, GSTs have been proposed as a biomarker of pollution exposure by several investigators (Fitzpatrick et al. 1997). GSTs activity can by influenced by exposure to various xenobiotic compounds, such as polycyclic aromatic hydrocarbons (PAHs) and polychlorinated biphenyls (PCBs) (Kaaya et al. 1999) as well as metals (Zhang et al. 2012).

Malondihaldeyde (MDA) results from the degradation of lipids by peroxidation caused by overgeneration of free radicals (ROS). The evaluation of lipid peroxidation can be measured by quantifying the amounts of substances similar to MDA, which exhibit the property of reacting with thiobarbituric acid. These TBARS can thus be expressed as MDA equivalents and are routinely quantified by the TBARS test using thiobarbituric acid as a reagent (Oakes and Van Der Kraak 2003).

Before testing the feasibility of using cholinesterase activity as an environmental tool, we proceeded with the full characterization of the cholinesterasic forms present in tissues of *P. pollicipes*. The inhibition of cholinesterases (ChE) has been widely used as an environmental biomarker of exposure to organophosphate (OP) and carbamate (CB) pesticides. Nevertheless, diffuse contamination can also exert potential cholinesterase inhibition, and thus the integration of this parameter in biomonitoring studies is plausible. ChEs belong to the esterases family and are responsible for the hydrolysis of carboxylic esters. This enzyme is found in almost all invertebrates despite being in different forms, such as acetylcholinesterase (AChE) and butyrylcholinesterase (BChE). AChE is an enzymatic form present in the central nervous system and neuromuscular junctions of a large number of species, and it is responsible for catalyzing the hydrolysis of the neurotransmitter acetylcholine into choline and acetic acid, which allows the cholinergic neurons to resume its resting state after activation, thereby preventing excessive cholinergic transmission and consequently an excessive neuromuscular stimulation (Guilhermino et al. 2000; Nunes

et al. 2005). BChE can be found in most vertebrates (namely in plasma), but its physiological functions are not entirely known (Jbilo et al. 1994). The two cholinesterase forms can also be present in the same tissue at the same time (Garcia et al. 2000). Consequently, the determination of the most prominent form present is required before the onset of any toxicological determination. Different ChE isoforms may be present in the same tissue and may exhibit distinct sensitivities toward environmental pollutants.

Material and methods

Study Site

This biomonitoring study was performed in three different geographical areas of the North of Portugal: (1) an estuarine area (Douro river) adjacent to a large Portuguese city (Oporto)—Lavadores (41°7′53.41″N; 8°40′13.87″W), (2) in the vicinity of an oil refinery, Matosinhos (41°14′27.22″N; 8°43′39.51″W), and (3) a potential reference site - Aguda, ~30 km south of the Douro estuary (41°02′53.00″N; 8°39′24.01″W) (Figure 2). The selected areas have different population indices with different anthropogenic influence in the marine environment. The potential reference site, Aguda beach, belongs to Arcozelo village, which has a population density of approximately 1,585 hab/km^2 (INE 2001) and is characterized by a large sand beach area and a flat rocky shore. Lavadores is characterized by rocky shore without sand. This is near an estuarine area, with human pressure caused by a population density of ~2,949 hab/km^2 (INE 2001). Matosinhos beach has a population density of ~1,299 hab/km^2 (INE 2001) and is in the vicinity of an oil refinery. The sampling site was similar to Lavadores beach in terms of substrate. All sampling sites, with the natural exception of Lavadores (near the Douro river estuary), are free of major freshwater streams.

Animal Collection and Laboratory Procedures

A group of *P. pollicipes* was collected in every of the three study sites during 1 year once every season (autumn, winter, spring, and summer). Animals from all locations were collected in the same day during the low tide period using a spatula to remove the entire colony from the rock. After collection, animals were immediately transported to the laboratory in plastic boxes. In the laboratory 20 animals were selected from each collected group (sizes between 4 and 5 cm) to perform the enzymatic assays.

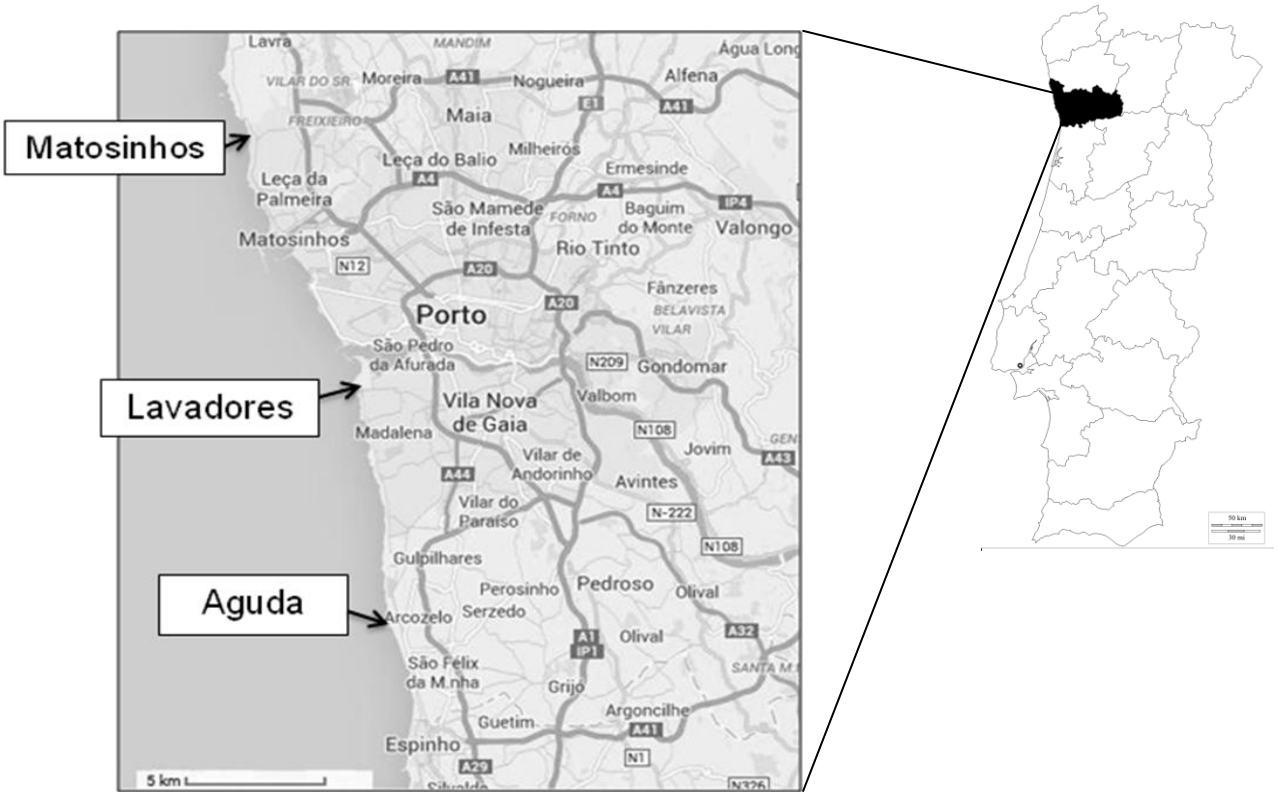

Figure 2 - Map of Continental Portugal, with the main focus on the coastal and sampling sites of the Porto district.

On arrival, animals were killed by dissection, and specific tissues were isolated (peduncle and cirri; each organism allowed collection of biological samples for all determinations, and each organism worked as a replicate, thus n = 20). After this procedure, tissues were individually homogenized in ice cold phosphate buffers. For glutathione-S-transferases (GSTs) and and TBARS quantifications, samples were homogenized in phosphate buffer with Triton X-100 0.1 % at pH = 7.0 and 50 mM. To quantify ChE activity, samples were homogenized with phosphate buffer at pH = 7.2 and 0.1 mM. Samples for GSTs and TBARS determinations were centrifuged at 15,000 x*g* for 10 min at 4 °C, and samples for ChE quantification were centrifuged at 3,800 x*g* for 3 min at 4 °C.

ChEs Characterization

Cholinesterase characterization on peduncle of *P. pollicipes* samples was performed using the following enzyme specific substrates: acetylthiocholine (specific for AChE), butyrylthiocholine (specific for BChE), and propionylthiocholine (specific for propionylcholinesterase- PChE). Substrate concentration varied from 0.005 to 20.48

mM. The following cholinesterasic inhibitors were used: eserine sulphate, BW284C51, and Iso-OMPA, which selectively inhibit total ChEs, AChEs, and BChEs, respectively. Inhibitor concentrations were 6.25 to 200 μM for eserine and BW284C51 and 0.25 to 8 mM for Iso-OMPA. Stock solutions of eserine and BW284C51 were prepared in ultrapure water, and Iso-OMPA stock solution was dissolved in ethanol. Each inhibitor solution (volume 5 μL) was mixed with 495 μl of homogenized and centrifuged sample (10 peduncles/sample in one total of three samples) and then incubated at room temperature for 20 min (as described by Nunes et al. 2005). Ultrapure water was used as a control, and an additional control was prepared with ethanol for the samples exposed to Iso-OMPA.

Biomarkers: GSTs, TBARS, and ChEs

GSTs activity

GSTs activity on cirri and peduncle of *P. pollicipes* was determined according to Habig et al. (1974). GSTs catalyze the conjugation of the substrate 1-chloro-2,4-dinitrobenzene (CDNB) with glutathione, thus forming a thioether (ε = 9.6 mM^{-1} cm^{-1}), whose formation can be spectrophotometrically followed by the increment of absorbance at a wavelength of 340 nm.

TBARS

The extent of lipid peroxidation on cirri of *P. pollicipes* was measured by the quantification of TBARS according to the protocol described by Buege and Aust (1978). This methodology is based on the reaction of lipid peroxidation by products, such as MDA, with 2-thiobarbituric acid (TBA).

The amount of TBARS was spectrophotometrically measured as a single determination at a wavelength of (ε = 156 mM^{-1} cm^{-1}), and results were expressed as nmol of MDA equivalents per milligram of sample protein.

ChEs activity

ChE determination on peduncle of *P. pollicipes* was performed by quantifying the degradation of acetylthiocholine (the substrate preferred by the cholinesterasic form in *P. pollicipes* tissues; see previous section) by enzymatic action, thus producing acetate and thiocholine; the latter product complexes with ditiobisnitrobenzoate (DTNB), producing a colored compound the formation of which was determined at a wavelength

of 412 nm as described by Ellman et al. (1961). The protein quantification was determined in triplicate according to the spectrophotometric (wavelength 595 nm) method described by Bradford (1976) adapted to microplate.

Statistical Analysis

Data of ChE characterization were analyzed with one-way analysis of variance (ANOVA) followed by Dunnett test ($p \leq 0.05$). Biomarkers data were analyzed with two-way ANOVA followed by a simple main effects analysis whenever local x season interaction occurred; post hoc Tukey tests were then used to test for differences among sites ($p \leq 0.05$).

Results and Discussion

ChEs Characterization

Results showed that acetylthiocholine was the preferential substrate, with greater rates of hydrolysis, followed by propionylthiocholine; butyrylthiocholine was poorly hydrolyzed (Figure 3). The rate of hydrolysis of acetylthiocholine was consistently greater than the rates observed for the other two substrates. We can thus conclude that the cholinesterasic form present in the tested tissues of *P. pollicipes* preferably hydrolyses acetylthiocholine. Furthermore, the esterasic activity was completely suppressed following incubation with eserine, even for the lowest concentrations ($F_{[6, 14]} = 7.21$; $p = 0.001$ - Figure 4). Considering specific inhibitors, BW284C51 was also capable of inhibiting ChE activity ($F_{[6, 14]} = 16.98$; $p < 0.001$ - Figure 4) even more effectively than eserine. Iso-OMPA, on the contrary, did not cause any significant inhibitory effect ($F_{[7, 16]} = 0.806$; $p = 0.595$ - Figure 4). These results showed that cholinesterases in this organism had typical acethylcholinesterasic (AChE) properties because they preferred acetylthiocholine as substrate and are simultaneously inhibited by eserine as well as BW284C51. Acetylcholinesterasic forms of cholinesterase are widespread in a large variety of organisms, and they assume a leading role compared with the other cholinesterasic forms (Nunes 2011). Similar results were found for other aquatic organisms, including crustacean species, such as *Palaemonetes pugio* (Key and Fulton 2002), *Palaemon serratus* (Frasco et al. 2006), *Litopenaeus vannamei* (Garcia-de la Parra et al. 2006) and *Gammarus pulex* (Xuereb et al. 2007). Fish species also show a similar tendency, i.e., AChE being the major cholinesterasic form in muscle, as was reported for *Dicentrarchus labrax* (Varó et al. 2003), *Gambusia holbrooki* (Nunes et al. 2005), *Limanda yokohama* (Jung et al. 2007), and *Lepomis*

gibbosus (Rodrigues et al. 2011).

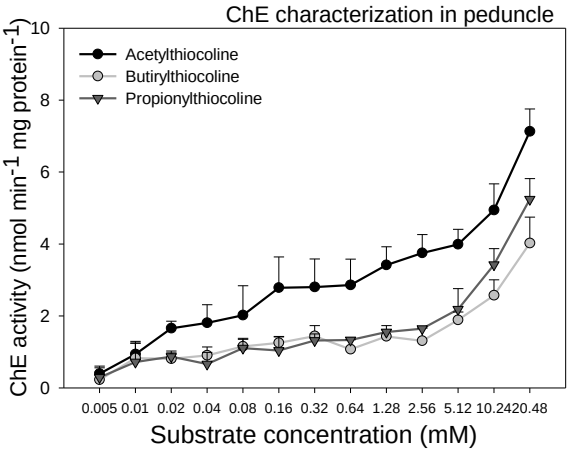

Figure 3 - Substrate preference of cholinesterases from peduncle homogenates of *P. pollicipes*.

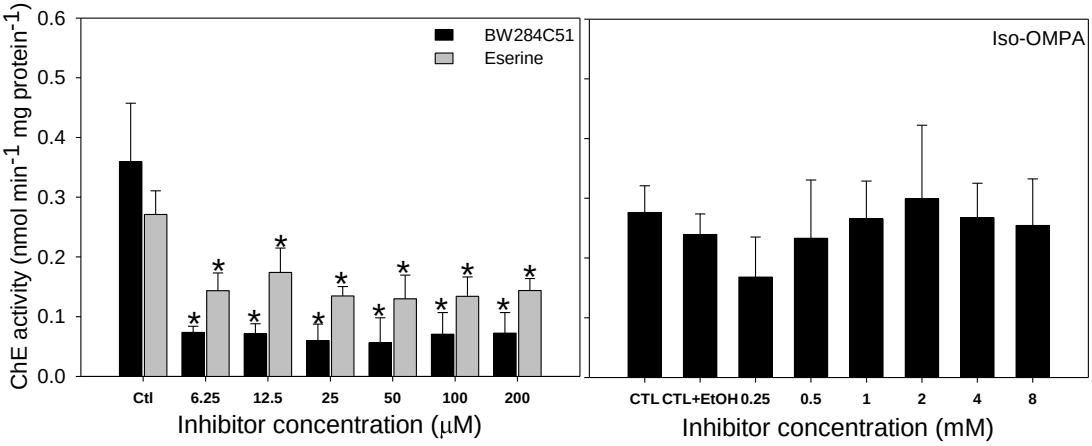

Figure 4 - Effects of specific inhibitors (eserine, BW284C51 and iso-OMPA) on cholinesterase activity of peduncle homogenates of *P. pollicipes*. Values are the mean of three replicate assays, each one with ten homogenized peduncles, and corresponding standard error bars. *- stands for significant differences, $p \leq 0.05$.

Biomarkers

GSTs Activity

GSTs activity in peduncle showed a marked difference between the Aguda sampling station (reference) and the other sites in summer. Consistently lower values of GSTs activity were obtained for animals from Aguda for all seasons (Table 1; Figure 5). In contrast, another pattern was determined because GSTs activities in cirri showed

greater values in Matosinhos (in the proximity of the Leça oil refinery) for all seasons except for summer (Figure 5) followed by organisms from Lavadores. Similar patterns of response, marked by increased GSTs activity, have been previously reported in a range of marine coastal crustacean species exposed to analogous forms of contamination, such as *Carcinus aestuarii* (Fossi et al. 1998), *Carcinus maenas* (Gowland et al. 2002), *Palaemonetes pugio* (Kuzmick et al. 2007), and several species of amphipods (Correia et al. 2003). In addition, mollusc species, such as *Mytillus galloprovincialis* and *Perna perna*, also responded to exposure to industrial and domestic untreated wastewater with GSTs induction as shown by Kaaya et al. (1999).

The obtained results, which depict induction of GSTs activity at sites located near the oil refinery at Matosinhos or the heavily populated estuarine area (Lavadores), can be related with exposure of the test organisms to several types of pollutants, including metals and different organic compounds. The meaning of the obtained results is in line with the rationale behind the use of GSTs as toxicological parameters for the detection of anthropogenic compounds in the aquatic compartment. GSTs belong to a major group of xenobiotic detoxifying enzymes (namely phase II metabolism by conjugation with the tripeptide glutathione), the increase of which occurs in the presence of a large number of electrophilic pollutants (including metals and organic chemicals). The presence of such compounds in both sampling stations has been previously reported; a clear signature of metallic anthropogenic contamination (e.g., metals such as zinc, copper, lead, and chromium) has been shown to be present in both sediment and water of Douro river (Mucha et al. 2003; Mucha et al. 2005). In addition, Douro river is also target of a diffuse pollution profile with human origin, which includes pharmaceutical drugs (Madureira et al. 2010), industry derived endocrine disruptors (Ribeiro et al. 2009), organochlorine pesticides and PCBs (Ferreira et al. 2005), and chemicals of domestic use (Quintaneiro et al. 2006).

Several previous studies have shown that Matosinhos coastal area is under the influence of hydrocarbons released from the Leça oil refinery (Moreira and Guilhermino 2005; Lima et al. 2007). From our results, it was possible to observe a consistent pattern as reflected by the greater GSTs activity values found in the sampling site of Matosinhos as well as Lavadores, but this was true only for hot seasons (spring and summer). This result may be related to the exposure of *P. pollicipes* to particular compounds derived from the oil refinery and domestic/industrial sewage. In fact, hydrocarbon exposure can result in a significant increase in this enzyme's activity.

Table 1 - Summary table of the two-way analysis of variance applied to tested biomarkers (*d.f.* degrees of freedom, MS - mean square, *F* - F statistic (MSfactor/MSresidual), *P* probability).

Parameter	Source of variation	*d.f.*	MS	*F*	*P*
GSTs-Peduncle	Site	2	$2.7x10^{6}$	162.6	<0.001
	Season	3	$3.5x10^{6}$	214.1	<0.001
	Site x Season	6	$4.1x10^{5}$	25.10	<0.001
	Residual	219	$1.6x10^{4}$	-	-
GSTs-Cirri	Site	2	$2.9x10^{6}$	176.3	<0.001
	Season	3	$8.7x10^{6}$	526.0	<0.001
	Site x Season	6	$8.5x10^{5}$	51.31	<0.001
	Residual	204	$1.7x10^{4}$	-	-
TBARS-Cirri	Site	2	$1.5x10^{-12}$	177.0	<0.001
	Season	3	$8.5x10^{-12}$	996.7	<0.001
	Site x Season	6	$1.6x10^{-13}$	18.18	<0.001
	Residual	211	$8.6x10^{-15}$	-	-
AChE-Peduncle	Site	2	16.21	24.27	<0.001
	Season	3	13.43	20.11	<0.001
	Site x Season	6	8.413	12.59	<0.001
	Residual	215	0.668	-	-

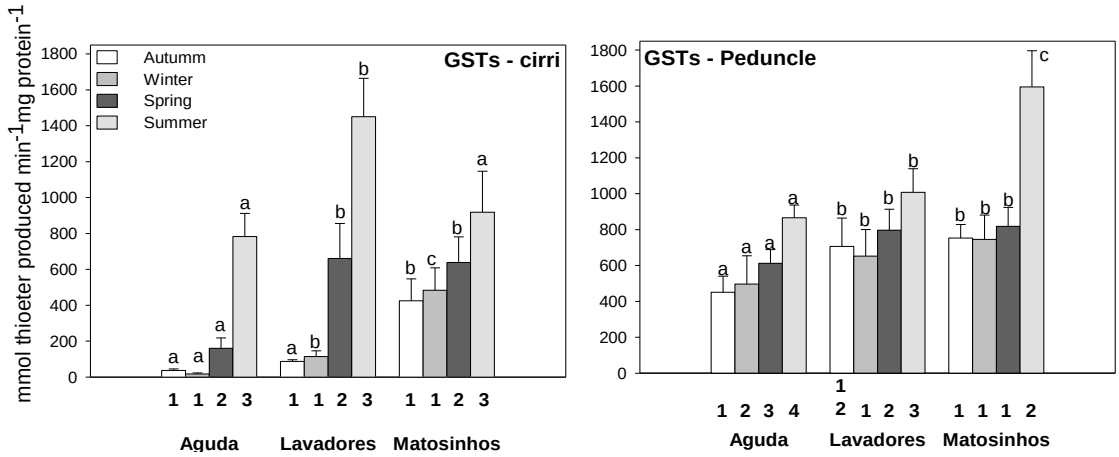

Figure 5 – Mean activity of specific biomarkers GSTs in cirri and peduncle of twenty *P. pollicipes* from the 3 study sites. The error bars correspond to the standard error. Different letters (a, b, and c) are used to describe differences among sites in each season (Tukey test, $p \leq 0.05$). Different numbers (1, 2, and 3) are used to describe differences among seasons in each site, $p \leq 0.05$.

The use of GSTs activity as a biomarker for PAH pollution has been described in several works, which shows the potential induction of this group of isoenzymes in aquatic organisms (Payne et al. 1996; Guilhermino et al. 2000; Moreira and Guilhermino 2005; Tim-Tim et al. 2009). Furthermore, the study by Cairrão et al. (2004) evidenced significant alterations of GSTs activity in the algal species *Fucus sp*. in several coastal sites, including in the proximity of Matosinhos. Similarly, the results obtained by Lima et al. (2007) showed that *Mytilus galloprovincialis* collected at Cabo do Mundo were clearly subjected to oxidative stress resulting from chronic exposure to hydrocarbons. These are noteworthy comparisons because the pattern of response is

coincident despite being assessed in distinct taxa.

It is possible to conclude that the marked differences observed between study sites may occur due to different profiles of pollution, with Lavadores being a site affected by unspecific pollution of urban origin (namely metals, and organic compounds, such as pesticides, drugs, and domestic/industrial wastes) capable of inducing significant metabolic alterations in *P. pollicipes*. However, considering the induction of GSTs activity during only spring and summer periods at Lavadores, one cannot discard the influence of abiotic factors (e.g., temperature), which can decisively modulate the metabolic response of aquatic organisms to pollutants, thus contributing to an additional increase in this biomarker activity. A somewhat similar outcome of GSTs induction was caused by chronic exposure to hydrocarbons, and the organisms collected in the vicinity of the Leça oil refinery, at Matosinhos, showed clear indications of this induction.

Our results also reflected a component of seasonal variability/fluctuation. As previously referred, abiotic factors can alter the toxic effects and biological adaptive responses of exposed organisms to chemical stressors. Some investigators relate the annual cyclical variations of biomarker activities of several sessile animal species (e.g., mussels) to the seasonal nature of their metabolism (Ahmad and Chaplin 1979) and to the complex interactions between exogenous and endogenous factors (Gabbott 1983). The degree of response to pollutants seems to differ among species in relation to their trophic level, habitat type, feeding habits, biotransformation capabilities, and abiotic factors (Barreira et al. 2007). An important factor to consider in biomonitoring programs includes physiological adaptations to cyclical temperature variations. Greater assimilation rates are associated with increased nutritional condition but also to increased water temperature (Urrutia et al. 1999). Greater temperature values can increase the accumulation and the toxicity of pollutants and consequently enhance the biomarker response (Vidal et al. 2002), including that of GSTs. This was one of the major observations in our study because hotter months (see Table 2), for all sampling sites, corresponded to the period during which GSTs activity was more increased. These data are in good alignment with previously published studies, such as the work by Robillard et al. (2003) with *Anodonta cygnea*, which showed a clear influence of temperature and pH in GSTs activity. Regoli et al. (1997) described several studies with *Adamussium colbecki* showing that GSTs activity was significantly increased of as a function of temperature. It becomes clear that temperature plays a central role in the definition of key features of metabolism, with potential outcomes occurring at the detoxification level (including GSTs activity), and the regulation of other aspects, such

as reproduction. Filho et al. (2001) established a relation among variations of temperature, GSTs activity, and reproductive cycle in *Perna perna*. Levels of organic pollutants in tissues can change during the seasons due the change of metabolism of pollutants and/or changes in lipid contents (Livingstone et al. 1995; Swaileh 1996), which are conditioned by temperature. It is thus possible to suggest that abiotic factors (with particular emphasis being attributed to temperature) may be, at least, contributors to seasonal variations in the levels/activity of specific enzymes, which are not related to the presence of chemical contaminants in water. Abiotic factors may be the cause of the significant increases of GSTs activity observed in spring and summer.

Table 2 - Mean sea water temperature in Leixões, in each month during the biomonitoring study (Instituto Hidrográfico 2010/2011).

Month (2010/2011)	sept	oct	nov	dec	jan	fev	mar	apr	may	Jun	jul	ago
Temperature (Cº)	18.6	17.3	15.6	13.9	14.2	14.0	14.2	15.7	17.5	16.5	15.7	16.8

TBARS

Several studies suggest that the major toxic outcomes for aquatic organisms occur as a consequence of free radical attack under oxidative-stress scenarios including environmental exposure to pro-oxidative compounds (Livingstone 2001; Oakes and Van Der Kraak 2003). Oxidative stress occurs when the formation of free radicals by xenobiotic metabolism exceeds the endogenous protection constituted by specific enzymes, antioxidant vitamins, and other radical scavengers, consequently resulting in cellular damage (Livingstone 2001), which in turn is frequently associated with membrane degradation. These results in the production of compounds, such as MDA, being formed by the degradation of membrane lipids by free radical attack. The presence of MDA is indicative of oxidative damage and functions as a proxy for the occurrence of oxidative damage. The reaction of MDA with TBA is one of the most widely used estimators of oxidative stress (Oakes and Van Der Kraak 2003). The results obtained in this study showed a clear seasonal variation in TBARS levels in all sites, with greater values occurring in spring and summer months (Table 1; Figure 6). These results potentially reflect the increased metabolic activity that encompasses the augment in average water temperature for the given periods (Table 2).

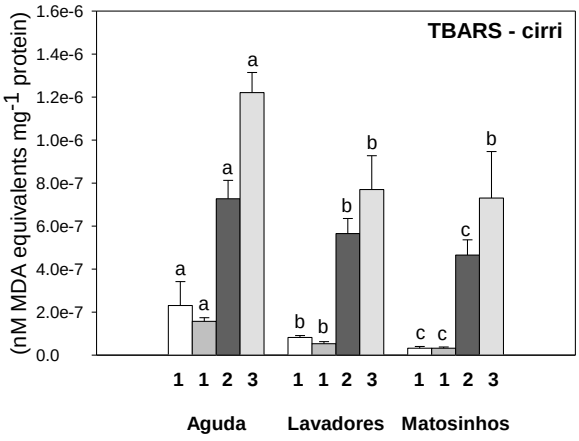

Figure 6 – Mean TBARS content, in cirri of twenty *P. pollicipes* from the 3 study sites. The error bars correspond to the standard error. Different letters (a, b, and c) are used to describe differences among sites in each season (Tukey test, $p \leq 0.05$). Different numbers (1, 2, and 3) are used to describe differences among seasons in each site, $p \leq 0.05$.

Altogether, results from GSTs and TBARS monitoring do indeed indicate that water temperature may play a decisive role in the metabolic activation and detoxification of chemical pollutants; in the presence of substances with oxidative properties, GSTs are not capable to cope with the excess of free radicals, which in turn cause peroxidative damage. In addition, the chemical pollution expected to occur for both polluted sites (Lavadores and Matosinhos) was shown to exert pro-oxidative effects because the pattern of response in terms of lipid peroxidation was comparable, even almost identical (with the exception of spring at the Matosinhos sampling site).

Another factor to take into account is desiccation because this species is subjected to long periods of direct exposure to the sun or hot air, which facilitates water evaporation and challenges water homeostasis. Being an intertidal organism, *P. pollicipes* is exposed to extremely adverse environmental conditions for 12 hours/day, which corresponds to low tide; these harsh conditions are even more challenging during the spring and summer months.

This fact may contribute to the establishment of oxidative stress, even more so during hotter months. Exposure to adverse conditions, including sunlight, is not only deleterious due to high temperature, which favors desiccation; ultraviolet (UV) radiation is also important because it can cause adverse effects (Vargas et al. 2010) at a subindividual or individual level, include the following: immunosuppression, DNA mutation, cancer, and production of ROS (Vargas et al. 2010), thus contributing to the onset of oxidative stress. UV radiation was also indicated to be a direct stimulator of lipoperoxidation levels in living organisms (e.g., crab *Neohelice granulat*- Vargas et al.

2010). Zeeshan and Prasad (2009) showed that UV-B induced the formation of MDA, indicating enhanced lipid peroxidation in three cyanobacteria species: *Nostoc muscorum*, *Plectonema boryanum*, and *Aphanothece sp.*

ChEs activity

The obtained results showed greater AChE activity in the potential reference site (Aguda); lower values were recorded in Matosinhos and Lavadores (Table 1; Figure 7). These findings may be related to the presence of anticholinesterasic compounds both in river water and oil refinery effluents. ChE inhibition in bivalves and fish has been mainly attributed to pesticides (organophosphate and carbamate classes) but also to a wide range of pollutants, including metals, pulp mill effluents, domestic sewage, and PAHs (Payne et al. 1996; Bonacci et al. 2009). PAHs are environmental pollutants with an important environmental impact and are toxic to several marine species (French 1998). The transfer of PAHs along marine food webs is explained by the bioaccumulation and bioconcentration exerted by zooplanktonic organisms (Barreira et al. 2007), thus allowing PAHs to reach other marine species to exert their toxic effects. Cholinesterasic inhibition is one of the potential toxic insults by PAHs, which may explain the lowest ChE activity registered in Matosinhos as a consequence of the proximity of this sampling site to the oil refinery; similar results have been published in the past (Wake 2005) relating hydrocarbon contamination and cholinesterasic impairment. As showed by Bonacci et al. (2009), ChE inhibition in *Adamussium colbecki* by complex mixtures of PAHs and PCBs may occur. Our data are also supported by similar results obtained in a field study by Tim-Tim et al. (2009), which showed that hydrocarbons resulting from the Prestige tanker oil spill could impair ChE activity in *Mytilus galloprovincialis*.

ChE activity may also be influenced by the presence of anticholinesterasic compounds, such as OPs and CBs (Mora et al. 1999; Nunes et al. 2005; Xuereb et al. 2007), which are usually employed as pesticides in agricultural practices. These compounds are typically used in combinations of more than one pesticide at the same location, thus contributing to the presence of enriched and varied mixtures of pesticides in surface waters in the proximity of crop fields (Cuppen et al. 2002). Pesticides used in agriculture practices are released into the environment, thus contaminating river water and in some cases reaching the ocean (Mora et al. 1999). Their effects may occur on marine organisms, and several published papers have point out this possibility. The study performed by Ben-Khedher et al. (2013) showed a decrease in AChE activity in crustacean tissues from lagoons contaminated with heavy metals and aquaculture

effluents. Bonacci et al. (2009) showed that ChE activity of *Adamussium colbecki* could be affected by in vitro exposure to OPs. The low AChE activity registered in Lavadores, near an estuarine area, may be related to the presence of anticholinesterasic compounds. This assumption is in line with the results obtained by Kopecka-Pilarczyk (2013) showing the impairment of AChE in the fish species (*Platichthys flesus*) captured at the Douro river mouth compared with control organisms coming from open waters.

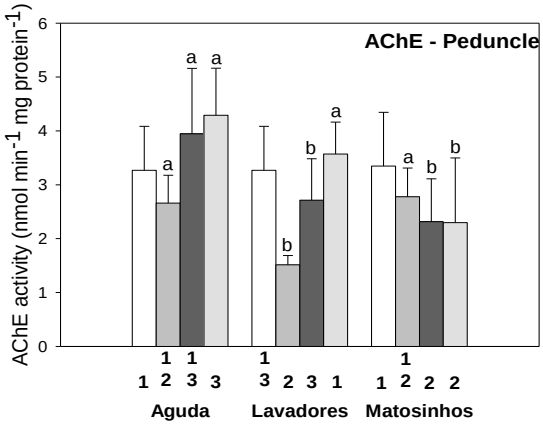

Figure 7 – Mean activity of specific biomarkers AChE, in peduncle of twenty *P. pollicipes* from the 3 study sites. The error bars correspond to the standard error. Different letters (a, b, and c) are used to describe differences among sites in each season (Tukey test, $p \leq 0.05$). Different numbers (1, 2, and 3) are used to describe differences among seasons in each site, $p \leq 0.05$.

ChE activity can be also affected by physiological (Frasco et al. 2010) and abiotic factors (e.g., temperature and pH- Mora et al. 1999; Frasco et al. 2010). Temperature may be identified as the natural most important factor affecting AChE activity (Hogan 1970). However, the somewhat erratic seasonal pattern of AChE activity observed during our campaign may also be related to abiotic variations, such as the reproductive cycle (which conditions the protein turnover of the organism), water pH, or, more importantly, water temperature.

At the reference site Aguda, greater levels of AChE activity occurred during spring and summer months. The same seasonal pattern was described by Leiniö and Lehtonen (2005), who described a significant increase of AChE activity in molluscs *Mytilus edulis* and *Macoma balthica*. Vidal et al. (2002) also observed greater AChE activity in *Corbicula fluminea* during the summer period when the temperature of water is naturally greater. These results may indicate that the seasonal variation in AChE activity may be influenced, at least in part, by temperature fluctuations. However, the variations in AChE activity that were observed for Lavadores and Matosinhos seem to

be more likely related with the presence of pollutants in water because a similar seasonal pattern was not observed in organisms collected at the reference sampling station.

Conclusion

The general conclusions of the present study show the importance of analyzing the patterns of response of a sessile organism to be included, in the future, in comprehensive monitoring studies of coastal areas. From the obtained results, it was possible to devise the potential effects of well-known, already reported profiles of chemical anthropogenic pollutants, the toxic response of which has been thoroughly characterized in other test species. For the first time, the crustacean *P. pollicipes* was equated as a sentinel species for biomonitoring purposes, and basal levels of commonly used biomarkers were assessed both in contaminated and reference sampling sites. In addition, the characterization of cholinesterases of this species was fully performed, and this represents a new tool to diagnose environmental exposure to pesticides and hydrocarbon derivatives in the marine environment. One of the most important conclusions of this study points to the influence of abiotic factors, which can significantly modulate the toxic responses to specific chemicals. In fact, abiotic factors are drivers of seasonal adaptations in the organism's physiology that can even surpass the biological responses to chemical stressors and thus function as strong confounding factors that consequently limit the interpretation of data from biomonitoring programs. However, through our approach, it is possible to distinguish the natural versus anthropogenic contributions in terms of response, thus opening new perspectives concerning the use of autochthonous species in marine ecotoxicological studies.

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Chapter three

AS Ramos, SC Antunes, B Nunes (*in preparation*). Biomonitoring of anthropogenic pollution in *Pollicipes pollicipes* in the northern cost of Portugal: validation of a non-destructive biomarker-based approach using haemolymph.

Biomonitoring of anthropogenic pollution in *Pollicipes pollicipes* in the northern cost of Portugal: validation of a non-destructive biomarker-based approach using haemolymph

Abstract

In the intertidal area, interactions between the responses to anthropogenic contaminants and the influence of natural variations (biotic and abiotic factors) in the chemical stressors concentration and assimilation are poorly understood. Consequently, there is a great need for new assessment procedures to characterize the biological responses occurring in organisms of this extreme environment. The main purpose of the present study was to assess the influence of seasonal variations in the toxic response elicited by anthropogenic compounds, by using a biomarker based approach, on a marine crustacean species. According to this purpose, the seasonal variations in the ecotoxicological response were investigated in the crustacean *Pollicipes pollicipes* from the Northern coast of Portugal; the biomarkers used were the activity of phase II biotransformation isoenzyme glutathione-S-transferases (GSTs), the activity of cholinesterases (ChEs), the levels of lipid peroxidation (TBARS), quantified in distinct tissues, cirri, peduncle and haemolymph. The glycogen content in peduncle and the variation in haemocyte number in haemolymph were also analysed. Samples were collected monthly, during a year, in Lavadores beach, which is located in the proximity of an estuarine area (Douro river). The haemolymph validation as a tissue for non-destructive biomarker quantification was successfully attained. The results showed a seasonal pattern in all tested biomarkers. The results also showed a significant increase in GSTs activities and in peroxidative damage, especially in months with higher temperature. The lowest AChE values were recorded during the rain seasons. Glycogen showed to be potentially related to the reproductive cycle, with lower values in spring and summer. The haemocyte count showed an increase in hotter months (exception for december and february). The results also showed a similar pattern among all tested tissues, validating the proposed use of the haemolymph as a source tissue for non-destructive biomarkers. The results pointed to an influence of the natural fluctuations in the impact of anthropogenic stressors in *P. pollicipes.*

Keywords: Gooseneck barnacle, haemolymph, biomonitoring, biomarkers, seasonal variations, anthropogenic pollution.

Introduction

Coastal zones have suffered the contamination by a large number and diversity of contaminants of anthropogenic origin (Venturini et al. 2008), acting simultaneously with quick and continuous alterations in abiotic factors such as pH, dissolved oxygen and temperature (Monserrat et al. 2007). This makes the intertidal area an extreme environment for autochthonous marine organisms. The major sources of contamination in coastal waters include agriculture and urban runoff, released directly in the coastal area or into the adjacent environment, contaminating the river water reaching the coast (Mora et al. 1999). Oporto coastal area (North of Portugal) is under the influence of Douro river, where several types of anthropogenic pollutants in water were already described (e.g., metals, pesticides and domestic chemicals- Cerejeira et al. 2003; Mucha et al. 2003; Quintaneiro et al. 2006). The large scale of contamination found in Douro river can be justified by the considerable number of domestic sewage and industrial effluents being discharged directly in the estuary (and its tributaries), in some cases without treatment (Ferreira et al. 2005). The contaminants from the river can then be dispersed along the adjacent coastal area. In the Oporto coast (Figure 8), a specific profile of anthropogenic contamination by hydrocarbons and its derivatives was previously described by other studies (Moreira and Guilhermino 2005). However, more studies are necessary to assess the ecotoxicological outcomes of anthropogenic contaminants on aquatic organisms in the northern coastal areas of Portugal.

Aquatic pollutants are commonly found in very complex mixtures, especially in marine environments, and the alterations that these mixtures can cause in biota difficult and even confound the evaluation of their effects. Xenobiotics can induce several physiological and morphological alterations in non-target organisms, since they interfere with specific metabolic pathways, as receptors, ion channels, and enzymes causing various adverse effects such as alterations in the redox cycle and impairment of the organ functionality (van der Oost et al. 2003; Costa et al. 2009).

Figure 8 - Map of Continental Portugal, with the main focus on the coastal and sampling zone of the Porto district.

The biological/physiological alterations elicited by xenobiotics can be assessed by the use of biomarkers, being this an adequate solution to overcome the difficulties in the evaluation of their effects in biota, allowing the performance of a global estimation of the simultaneous effects of a large set of substances on wildlife. However, exposure biomarkers alone may not provide the complete information for the development of a comprehensive set of data in a contamination scenario. To obtain more ecologically relevant data from monitoring studies, the incorporation of wild resident species in ecotoxicity tests is of fundamental significance, especially in the marine coastal environment. Wild species, being incorporated in ecotoxicological tests, can be adopted as sentinel species (Basu et al. 2007), reflecting the impact of biological/physiological changes in biota caused by pollutants (Livingstone 1993).

The combination of biomarkers with sentinel species is a common practice in the evaluation of deleterious effects caused in biota by anthropogenic pollution. One of the most common used biomarkers for evaluation of impacts of chemical stressors is the activity of glutathione-S transferases (GSTs), a phase II detoxifying group of isoenzymes. The quantification of lipid peroxidation levels, by the TBARS test, is also a reliable source to quantify the oxidative effects of pollutants in the aquatic ecosystem.

Is based on the reaction of MDA with 2-thiobarbituric acid (TBA), being one of the most widely used estimators of oxidative stress (Oakes and Van Der Kraak 2003; Nunes et al. 2006). The ChEs activity inhibition is also described as a sensitive biomarker in the identification of specific pollution scenarios, namely by organophosphate, carbamates and recently demonstrated alternative pollutants, such as metals and domestic sewage (Payne et al. 1996; Bonacci et al. 2009). Chemical stressors can unbalance normal energy metabolic processes, causing variations in glycogen levels, a major energetic reserve in almost species. An important method for evaluating the impact of biological changes in biota caused by pollutants can be the quantification of glycogen levels in the organisms. Glycogen is a carbohydrate being essentially found in liver and muscle tissue. In contamination scenarios a decrease in glycogen levels was already described by several authors (e.g., Becker et al. 2009), since glycogen degradation occurs to maintain the normal energy levels for the metabolic processes, reducing the stress caused by environmental contamination (Becker et al. 2009). This central role in the energetic homeostasis of living organisms makes the quantification of glycogen levels an important biomarker for the assessment of deleterious impacts caused by the exposure to pollutants.

Among the multiplicity of tissues that can be used for the assessment of toxic biological responses, haemolymph seems suitable as a tissue source for non-destructive markers of ecotoxicity. The use of haemolymph in biomonitoring studies can be also a valuable tool since it allows repeated individual evaluation over time, allowing a compilation of a set of historical data. In fact, changes in the biochemical composition of haemolymph has been used as an evaluation criteria of physiological and pathological conditions in crustaceans (Jayasree 1999), being a useful way to evaluate the alterations in marine ecosystems. Besides the nutritional status, the protein concentration in haemolymph can also be affected by variations in salinity (Ferraris et al. 1986). Changes in haemolymph can be influenced by physiological alterations, development phases, defence mechanisms and pollution levels (Jayasree 1999). Consequently, changes in haemolymph can be used as indicators of environmental alterations, including exposure to chemical pollution (Jayasree 1999). In haemolymph tissue, several antioxidant enzymes occur, for protection against the presence of reactive oxygen species (ROS) (Pipe et al. 1993), and their activity may be also affected by pollutants. This is another important feature of the haemolymph tissue, which shows the usefulness of assessing the antioxidant activity as a response to levels of anthropogenic contamination in aquatic ecosystems. The use of haemolymph

as a non-destructive tissue for biomarkers quantifications as been proposed and validated by Fossi (1994) and Fossi et al. (2000), in their works with the littoral crab species *Carcinus aestuarii* and by van Oosterom et al. (2010) in their study with the crab *Scylla serrata.*

According to this background information, the main objective of this biomonitoring study was to assess the influence of seasonal variations in the toxic response elicited by anthropogenic compounds in the barnacle *Pollicipes pollicipes*. For this propose a several biochemical parameters were quantified in different tissues: oxidative stress levels, with a quantification of the activity of glutathione-S-transferases (GSTs); peroxidative damage, analysed by means of lipid peroxidation levels (TBARS); evaluation of neurotoxicity with measuring the cholinesterase activity (ChE); and general fitness markers as the analysis of the variation in glycogen levels and the variations in haemocytes counts. A second objective was to validate the use of haemolymph as a source tissue for the determination of non-destructive biomarkers in *P. pollicipes*, by comparing the patterns of response in this tissue, with the biomarker responses obtained for other analysed tissues. Considering the previously information, it was mandatory to define comprehensive sets of biomarkers to be used under realistic environmental conditions.

Materials and methods

Study site and samples procedures

Lavadores is a village located in an estuarine area in the vicinity of Douro river, near to a large Portuguese city (OPorto) - 41°7'53.41"N; 8°40'13.87"W (Figure 8). This sampling site was chosen according to the previous results obtained from Ramos et al. (2014) study. Lavadores is a heavily populated estuarine area, with an extensive area of rocky beach where significant metabolic alterations in *P. pollicipes* were already described (Ramos et al. 2014). This biomonitoring study was conducted for a period of one year (from september 2013 until august 2014) and 30 specimens of *P. pollicipes* were monthly collected. Individuals with sizes between 5 and 6 cm were collected during low-tide period, and immediately transported in ice to the laboratory. For the performance of enzymatic determinations, peduncle, cirri and haemolymph from each organism were collected. Haemolymph was collected by percutaneous puncture of the peduncular sinus (as described by Petersen et al. 1974). One drop of haemolymph was used to obtain cellular smears for microscopic analysis (according to Mix and Sparks 1980). The remaining haemolymph was diluted 1:3 with distinct buffer solution (as

described by Moreira and Guilhermino 2005) and preserved in ice until performance of biochemical tests.

ChEs characterization and activity quantification

Cholinesterases (ChEs) are of the most diverse and conserved enzymes among living organisms, found in almost all invertebrates: ChEs can be found in different forms such acetylcholinesterases and butyrylcholinesterases. Different cholinesterasic forms can be present in the same organism, in the same tissue at the same time (Garcia et al. 2000), so before the onset of any toxicological determination, the determination of the predominant cholinesterase form of ChE in the tested tissue is required. The determination of the major cholinesterase form in tissues can be carried out by a set of sensitivity tests towards different substrates and inhibitors. According to Ramos et al. (2014) the predominant form found in peduncle tissue of *P. pollicipes* is acetylcholinesterase (AChE). Cholinesterase characterization on haemolymph and cirri of *P. pollicipes* samples was similarly undertaken, using enzyme-specific substrates and inhibitors. For this determination, three replicates were used, each with 10 tissue samples (haemolymph or cirri). The cirri of each replicate were homogenized in ice-cold phosphate buffer, 0.1 mM, pH = 7.2, and centrifuged at 3800 x*g* for 3 min, and the obtained supernatants of each replicate were used for the tests. The enzyme-specific substrates selected for the ChE characterization were: acetylthiocholine (specific for acetylcholinesterase- AChE), butyrylthiocholine (specific for butyrylcholinesterase- BChE), and propionylthiocholine (specific for pseudocholinesterases- PChE); the substrates concentrations varied from 0.005 to 20.48 mM. The inhibitors selected for the ChE characterization were: eserine sulphate (inhibitory of total ChEs), BW284C51 (inhibitory of AChEs) and iso-OMPA (inhibitory of BChEs); the inhibitors concentrations varied from 6.25 to 200 µM for eserine and BW284C51, and between 0.25 to 8 mM for Iso-OMPA. The quantifications were performed as described by Nunes et al. (2005).

ChE activity determination was performed in all selected tissues (haemolymph, cirri and peduncle). Cirri and peduncle preparation involved the sample homogenization on a specific phosphate buffer, 0.1 mM, pH = 7.2; follow by a centrifugation at 3800 x*g* for 3 min at 4°C.The haemolymph preparation for all biomarkers quantification did not require a mechanical homogenization and centrifugation ChEs determination on haemolymph, peduncle and cirri of *P. pollicipes* was performed by quantifying the degradation of acetylthiocholine (substrate) by enzymatic action, producing acetate and thiocholine; the latter product complexes with

DTNB (ditiobisnitrobenzoate), giving rise to a colour compound, whose formation can be determined at 412 nm, as described by Ellman et al. (1961).

GSTs activity and TBARS quantification

In each isolated tissue (peduncle, cirri and haemolymph – from the same animal) selected biomarkers were quantified (GSTs activities and oxidative damage using the TBARS test). Peduncle and cirri were individually homogenized in ice-cold buffer (phosphate buffer 50 mM, pH = 7.0, with Triton X-100 0.1%); for TBARS and GSTs determinations. After being mechanically homogenized, samples were centrifuged at 15000 x*g* for 10 min at 4°C.

Glutathione-*S*-transferases are a group of phase II detoxification isoenzymes, which conjugate reduced glutathione (γ-glutamyl-cysteinylglycine- GSH) with xenobiotic compounds. GSTs activity in haemolymph, cirri and peduncle was determined according to Habig et al. (1974). GSTs catalyse the conjugation of the substrate 1-chloro-2,4-dinitrobenzene (CDNB) with glutathione, forming a thioether (ε = 9.6 mM^{-1} cm^{-1}), whose formation can be spectrophotometrically followed by the increment of absorbance at a wavelength of 340 nm. The results were expressed as mmoles of thioeter produced per minute and per mg protein.

The lipid peroxidation can occur due the breakdown of fatty acids caused by the oxidation by reactive oxygen species (ROS). From this oxidative attack, an intermediate compound malondialdehyde (MDA) is formed, being its presence an indicative of oxidative damage. The extent of lipid peroxidation in haemolymph, peduncle and cirri of *P. pollicipes* was measured by the quantification of thiobarbituric acid reactive substances (TBARS), according to the protocol described by Buege and Aust (1978). This technique is based in the reaction of lipid peroxidation by-products (including the most abundant MDA) with 2-thiobarbituric acid (TBA). The amount of TBARS was spectrophotometrically measured as a single determination, at a wavelength of 535 nm (ε = 156 mM^{-1} cm^{-1}), and results were expressed as nmol of MDA equivalents per mg of sample protein.

Glycogen content

For the glycogen assay, a portion of 25 mg of muscle from *P. pollicipes* peduncle was used to assess glycogen content according to Lo et al. (1970). Samples were submerged in 30% NaOH with Na_2SO_4 and boiled until complete digestion of the tissue. Ethanol 95% was added to the suspension to precipitate the glycogen that

resulted from alkaline digestion. Samples were centrifuged at 500 x*g* for 30 minutes and the resulting pellet was collected. Then, H_2SO_4 96-98% was added, and samples were subjected to an incubation period on ice. After this period, the absorbance of each sample was spectrophotometrically measured as a single determination, at a wavelength of 490 mn (Lo et al. 1970). The results obtained were expressed as µg of glycogen mg protein^{-1}.

Total protein

Total protein was individually determined, as described by Bradford (1976), in order to express enzymatic activities and glycogen content taking into account the protein content of the analysed tissues.

Haemocyte count

For the haemocyte analysis, a smear of haemolymph from each *P. pollicipes* was obtained, according to Mix and Sparks (1980). The smears were obtained by physically dispersing a drop of haemolymph with one drop of anticoagulant (EDTA 10%), and air-dried at room temperature. The smears were fixed in methanol for 5 min, coloured with Giemsa 5% for 30 min, and rinsed with distilled water to remove dye excess. After the preparations dried, smears were observed by optical microscopy (Zeiss Imager.A2) at a magnification of 100x and images recorded using a USB CMOS camera (Zeiss AxioCam MRc). From each smears of haemolymph, 5 optical areas were randomly selected and all the cells of each area were counted. At the end of the counting process, a sum of the total number of cells of total organisms (n = 30) was calculated, and results were expressed by a total area observed (9950 nm^2).

Statistical analysis

Data of ChE characterization were analysed with one-way analysis of variance (ANOVA) followed by Dunnett test ($p \leq 0.05$). A repeated measures ANOVA was used to test for differences between the tissues analyzed (within-subjects effect) and sampling period (between-subjects effect). Because of deviations to multisample sphericity (measured by ε), the degrees of freedom were adjusted in the RM ANOVA procedure, using either the Greenhouse–Geisser (if $e < 0.75$) or the Huynh–Feldt (if $e > 0.75$) estimate (Quinn and Keough 2002). When a significant tissue x sampling period was found, we carried out one-way ANOVAs for each tissue, to assess seasonal

differences in each biomarker. A post-hoc Tukey test was used to assess differences among sampling periods. All analysis used a significance level of 0.05.

Results and discussion

ChEs Characterization

After assessing the levels of substrate hydrolysis in cirri and haemolymph tissue, acetylthiocholine was the substrate showing the most intense response, with high levels of hydrolysis in both tested tissues, followed by butyrylthiocholine and propionylthiocholine (Figure 9). These last two substrates were both poorly hydrolyzed, and in both tissues (Figure 9). So, with the obtained results, we can conclude that the major cholinesterasic form, present in cirrus and haemolymph of *P. pollicipes* preferably hydrolysis acetylthiocholine.

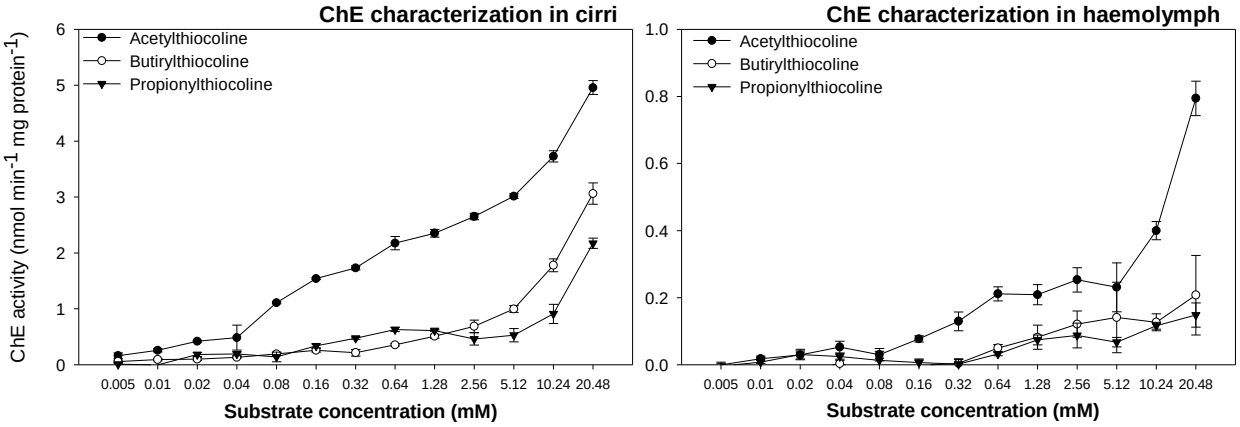

Figure 9 - Substrate preferences of cholinesterases from cirri and haemolymph of *P. pollicipes*

The results obtained for the tests with inhibitors showed a significant inhibition caused by eserine, which occurred even at low concentrations and for both tissues ($F_{[6, 14]}$ = 18.844; $p < 0.001$ - cirri and $F_{[6, 14]}$ = 109.068; $p < 0.001$ - haemolymph) (Figure 10). Esterasic activity was also considerably inhibited by BW248C51 in the two tested tissues in all tested concentrations ($F_{[6, 14]}$ = 6.243; p = 0.002 - cirri, and $F_{[6, 14]}$ = 42.467; $p < 0.001$ - haemolymph) (Figure 10). On the other hand, the results obtained for the other specific inhibitor, iso-OMPA, showed a complete absence of effects for all tested tissues ($F_{[7, 16]}$ = 0.533; p = 0.797 - cirri, and $F_{[7, 16]}$ = 1.822; p = 0.152 - haemolymph) (Figure 10). According to the results obtained, we can conclude that the major cholinesterasic form found in the tested tissues (cirri and haemolymph) of *P.pollicipes* is acethylcholinesterasic (AChE), since there was a preference for the

substrate acetylthiocholine and simultaneously a strong inhibition by eserine, and also by BW284C51. Similar results were found in the peduncle of *P. pollicipes*, described in the previous study of Ramos et al. (2014). In other crustacean species, the same pattern was already described by Antó et al. (2009), for the red shrimp *Aristeus antennatus* and the Norway lobster *Nephrops norvegicus*.

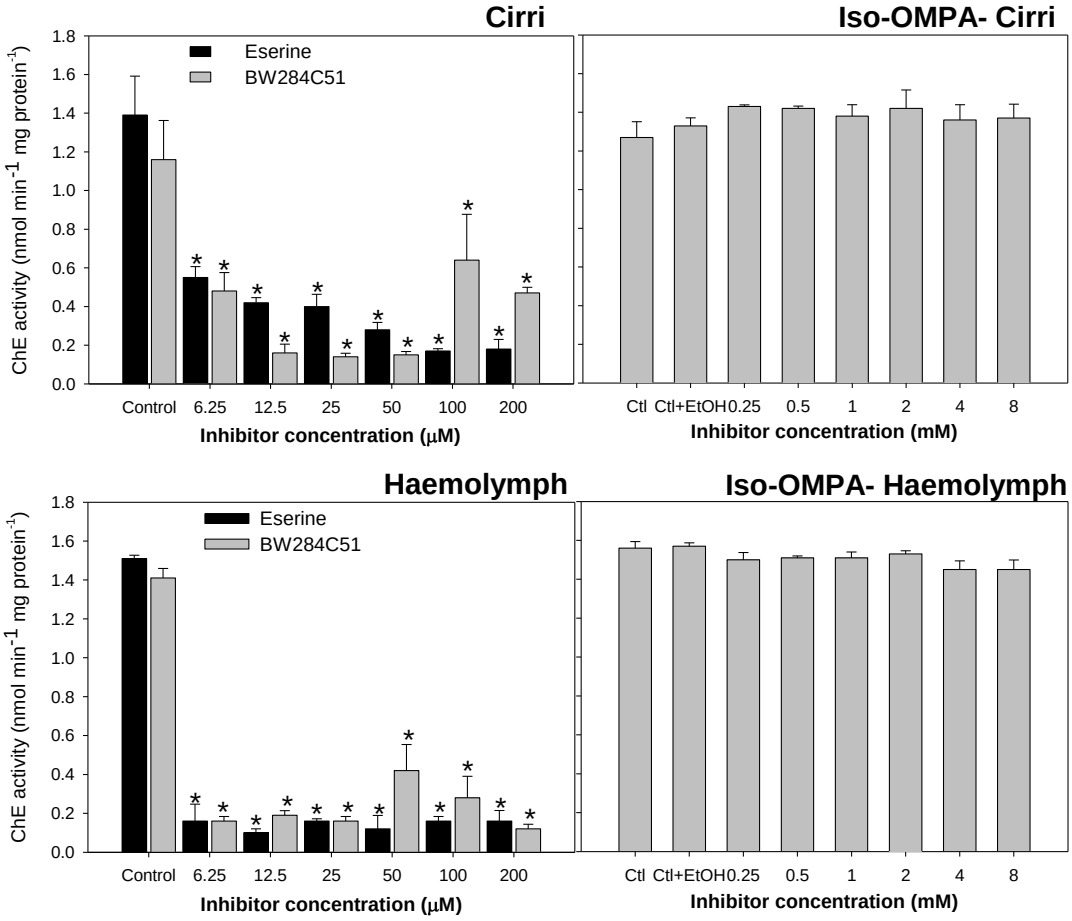

Figure 10 - Effects of specific inhibitors (eserine, BW284C51, and iso-OMPA) on cholinesterase activity of cirri and haemolymph of *P. pollicipes*. Values are the mean of three replicate assays, each one with 30 samples, and corresponding standard error bars. *Significant differences, $p \leq 0.05$.

Biomarkers

GSTs Activity

GSTs activity, in all tested tissues, varied significantly along the year (Table 3 and 4; Figure 11). All analysed tissues demonstrated a similar pattern in terms of GSTs activity (Figure 11), with consistently higher activity in hotter months (september, october, june, july and august). The evaluation of GSTs activity is commonly used as toxicological biomarker for the detection of anthropogenic compounds in the aquatic

ecosystem (Payne et al. 1996; Viñas et al. 2009). GSTs act to detoxify xenobiotics by conjugation with intracellular glutathione and its activity is enhanced in the presence of a large number of electrophilic pollutants (e.g., hydrocarbons and metals - Ben-Khedher et al. 2013). Being Lavadores beach located in the proximity of an estuary area (namely, of the Douro river), it is likely to be under the influence of several sources of unspecific contamination from urban origin, including domestic/industrial wastes, metals, PAHs and pesticides (Mucha et al. 2003; Cerejeira et al. 2003; Moreira and Guilhermino 2005; Quintaneiro et al. 2006). Consequently, it is possible to hypothesize that the levels of pollution in water were capable of inducing significant metabolic alterations in *P. pollicipes* (Reis et al. 2012; Reis et al. 2013; Ramos et al. 2014).

Among all chemical classes that may cause significant enhancement of GSTs activity, it is possible to identify hydrocarbons (Ben-Khedher et al. 2013). Among the most common sources of these compounds, one can identify the urban runoff, sewage disposal, industrial effluents, oil production and transportation (Kim et al. 1999). Different hydrocarbons, due to the different periods of permanence in the water column, may have different levels of absorption by the coastal filter feeding organisms (e.g., gooseneck barnacle), resulting in different biomarkers responses. In the northern Portugal coastal area (including Lavadores), the presence of several hydrocarbons compounds (Moreira and Guilhermino 2005) has been already described. Physiological alterations in most aquatic organisms can be related with seasonal variations in the concentrations of chemicals stressors concentrations in water (Barreira et al. 2007; Reis et al. 2013). A seasonal variation in PAHs concentrations, in sediments collected at the coastal area of Gaia (near to a submarine outfall), in the vicinity of Oporto (North of Portugal) and Lavadores was reported by Santos et al. (2011). In this study, higher PAHs concentrations were described during march and september when compared with february values. Barreira et al. (2007) have studied the variations in PAHs concentrations in soft tissues of *Ruditapes decussatus* collected from Ria Formosa lagoon, and, similarly, higher PAHs concentrations in tissues were described during summer and winter. In other countries, seasonal variations in hydrocarbons concentrations were also described (Khedir-Ghenim et al. 2009), and higher concentrations of these compounds were found in sediments especially during spring and summer. This seasonal pattern may justify the higher activities of the GSTs isoenzymes observed for organisms collected under the influence of hydrocarbon compounds, a reality occurring in the vicinity of the sampling area.

Metals are also capable of altering GSTs activity (Ben-Khedher et al. 2013).

The presence of these compounds was also previously described in Douro river water by Mucha et al. (2003). Similarly to what was hypothesized for PAHs, the fluctuations observed in GSTs activity along the year may be partly explained by the seasonal variations in metallic pollution. Reis et al. (2013) described a seasonal pattern of heavy metals contamination in seawater and in *P. pollicipes* soft tissues, both collected in the proximity to Lavadores beach. This study found that the highest concentrations of metals (Cd, Fe, Zn and Mn) were found in summer and autumn. This patter may explain the increase levels of activity of GSTs during hotter months (see Table 5) observed in the present study (Figure 11).

Different metals have varying toxic effects in the biota, and their bioavailability in water can be related with salinity and pH (Riba et al. 2004). Salinity can exert diverse impacts in the toxic profile of different metals, and in different contamination scenarios. In areas affected by chronic heavy metal contamination (namely, by Zn, Cu, and Pb), metals are more easily captured in sediments at low salinity values (Riba et al. 2004). For decades, the contamination by metals, in Douro estuarine area, has been described (Leal et al. 1997; Mucha et al. 2003), indicating a chronic contamination scenario. In estuarine areas and during the rainy season, low salinity values may occur (Bally and Branch 1986) as has been reported near to the Lavadores beach (Reboreda et al. 2014), leading to an increased metal retention by sediments, reducing its bioavailability (Riba et al. 2004). This phenomenon is coincident with the period where lower GSTs activity were recorded, suggesting a relationship between the reduction in metals in water and the reduction in the activity of the detoxifying enzyme GSTs.

The mobility of heavy metals from sediment to seawater increases when pH decreases. Variations in pH in water can be related with dilution by superficial runoff water, which may cause a small increase in pH (Saínz et al. 2002). This hypothesis may explain the lower metal concentrations during the winter months, when rainfall occurs more intensely. During summertime not only water pH is lower, due the low precipitation levels, but also higher evaporation favors the increase of metal concentrations (Olías et al. 2004), leading to a greater bioavailability of metals in water, and an enhanced GSTs response. In general terms, an increase in the concentrations of anthropogenic pollutants in water occurs during the dry season, thus causing more impact on exposed biota. On the other hand, a decrease in the concentration of contaminants is associated to the wet season, when the water discharges are augmented, a pattern that was already described by several authors (e.g., Olías et al. 2004; Qi et al. 2014). It is possible to hypothesize that, considering the higher concentration of pollutants in Douro river water during the summer months, a more

prominent toxic impact caused by waterborne xenobiotics may occur on exposed biota during this period. This was a major finding from our data, evidenced by the enhancement of GSTs activity in the same period, in all *P. pollicipes* tissues.

The variations in GSTs activity may also reflect the direct influence of abiotic factors, such as temperature. Our results evidenced an increase in GSTs activity in organisms collected from a polluted area during periods of higher temperature. A similar scenario was described by Kaaya et al. (1999) who recorded an increase in GSTs activity during summer and autumn, in *Mytilus galloprovincialis* and *Perna perna*. Giarratano et al. (2011) also reported a higher GSTs activity during the summer period, in caged mussels (*Mytilus edulis chilensis*), collected in an industrial zone where untreated wastes were discharged directly into the sea. These results are similar to ours, where increases in GSTs activity were recorded in the late spring, summer and also early autumn. Temperature is an important parameter that influences the variability of results of several biomarkers (including GSTs activity), since it enhances the biological response, by increasing the accumulation and the toxicity of pollutants (Filho et al. 2001). Having into account all these potential contributions, it is licit to suggest that variations in biomarker responses are not only related with the presence of xenobiotics in the aquatic ecosystem, but can also be influenced by variations of abiotic factors (Pfeifer et al. 2005).

Table 3 - Summary of repeated measure ANOVA applied to the biomarkers data (AChE, GST and TBARS) of *P. pollicipes* along the sampling period.

Source of Variation	**AChE**			**GST**			**TBARS**		
	d.f.	**MS**	***P***	***d.f.***	**MS**	***P***	***d.f.***	**MS**	***P***
Between subjects									
Month	11	$1.8E^{4}$	<0.001	11	$1.3E^{6}$	<0.001	11	$2.0E^{-9}$	<0.001
Residual	284	$5.8E^{2}$		274	$2.E^{4}$		282	$6.7E^{-11}$	
Within subjects									
Tissue	1.57	$1.6E^{5}$	<0.001	1.67	$1.5E^{7}$	<0.001	2.0	$1.1E^{-9}$	<0.001
Tissue x Month	17.28	$6.3E^{3}$	<0.001	18.4	$3.5E^{5}$	<0.001	22.0	$2.0E^{-10}$	<0.001
Residual	446.6	$8.6E^{2}$		457.5	$2.6E^{4}$		564.0	$6.9E^{-11}$	

Table 4 - Summary table of the one-way ANOVA applied to tested biomarkers (*d.f.* degrees of freedom, MS - mean square, *F* - F statistic (MSfactor/MSresidual), *P* probability).

Parameter	Tissue	*d.f.*	MS	*F*	*P*
AChE	Cirri	11, 323	6501.8	19.92	<0.001
	Peduncle	11, 326	21766	15.32	<0.001
	Haemolymph	11, 325	3169.6	15.24	<0.001
GSTs	Cirri	11, 330	1426808	36.84	<0.001
	Peduncle	11, 320	439755	32.45	<0.001
	Haemolymph	11, 315	431486	37.86	<0.001
TBARS	Cirri	11, 327	1,27E-09	21.08	<0.001
	Peduncle	11, 326	8,26E-10	11.08	<0.001
	Haemolymph	11, 323	7,86E-10	11.11	<0.001
Glycogen	Peduncle	11, 106	2239430	8.72	<0.001
Number of haemocytes	Haemolymph	11, 347	24804	13.34	<0.001

Table 5 - Mean sea water temperature in Leixões, in each month during the biomonitoring study (Instituto Hidrográfico 2013/2014).

Month (2013/2014)	sept	Oct	nov	dec	jan	fev	mar	Apr	may	jun	jul	ago
Temperature (Cº)	14.9	18.2	15.6	14.0	14.0	13.1	13.1	15.3	14.4	17.4	17.0	17.6

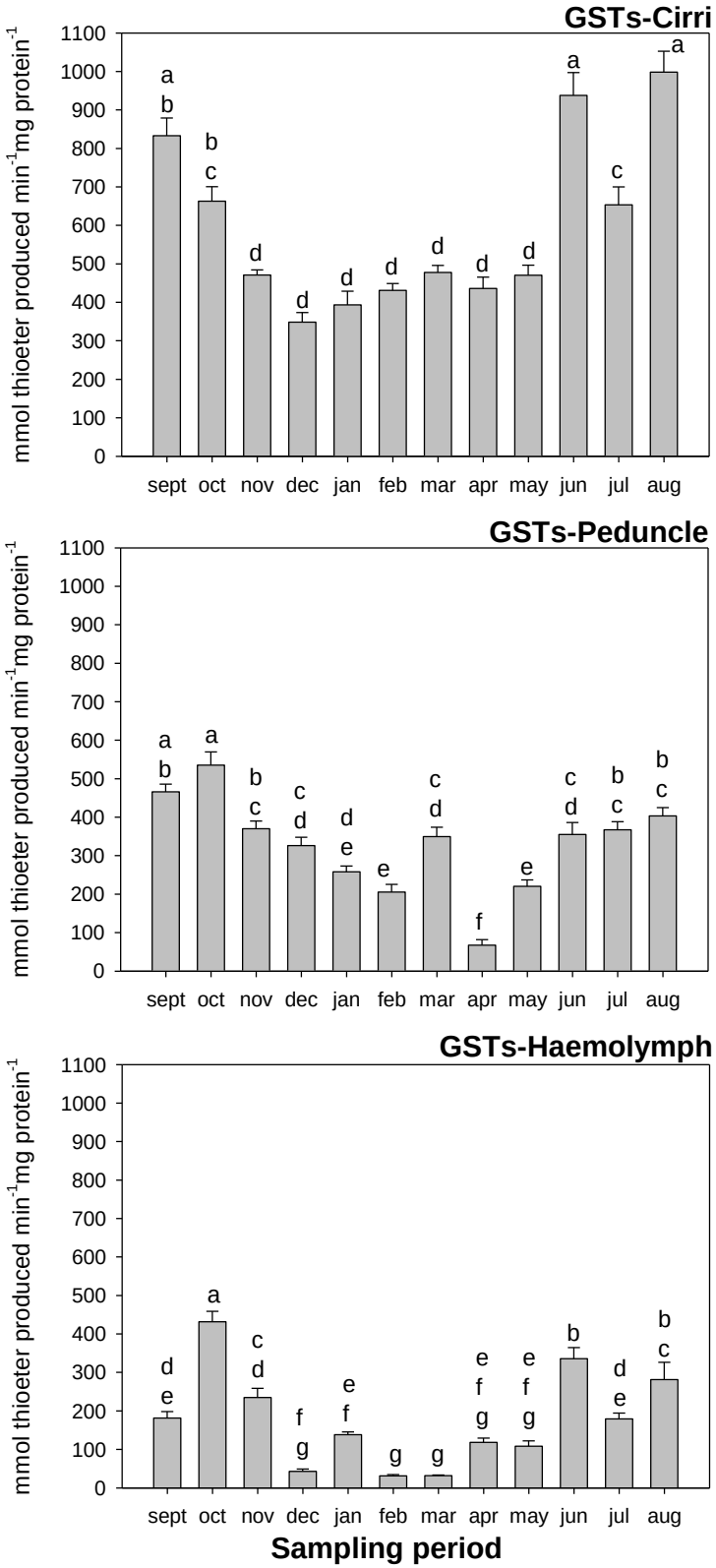

Figure 11 - Mean activity of the biomarker GSTs in cirri, haemolymph and peduncle of 30 *P. pollicipes*. Error bars correspond to the standard error. Different letters are used to describe differences among months, $p \leq 0.05$.

TBARS

Lipid peroxidation (LPO) profiles showed a seasonal variation in all tested tissues (Figure 12), with higher TBARS levels in late spring, summer and early autumn and are also recorded a similar pattern of response in all tested tissues (Table 3 and 4; Figure 12). Peroxidative damage occurs when the formation of free radicals by xenobiotic compounds exceeds the endogenous protection (Livingstone 2001), leading to membrane lipid degradation by oxidative mechanisms. The degradation of lipids leads to the production of compounds such as malondialdehyde (MDA), whose presence is indicative of oxidative damage (Nunes et al. 2006). The free radical attack is mainly directed to the polyunsaturated fatty acids and occurs in a chain reaction (Mylonas and Kouretas 1999). Our results showed an enhancement of the antioxidant mechanisms that coincided with the months with higher peroxidation levels, showing that the increased efficacy of defence mechanisms was not sufficient to avoid the occurrence of oxidative damage. The increased levels of TBARS in late spring, summer and early autumn may be related with the presence of the previously referred chemical stressors (PAHs and metals) in seawater near the collection area (Santos et al. 2011; Reis et al. 2013), and with their concentration increase during the dryer months (Olías et al. 2004; Qi et al. 2014).

The LPO not only occurs when the antioxidant defence is surpassed by pollutants toxic effects, and are not capable of detoxifying the xenobiotics in order to prevent ROS formation. In fact, LPO formation may be stimulated by abiotic variations typical from estuarine/intertidal areas in several parameters such as temperature, salinity and desiccation upon air exposure caused by tidal movement (Freire et al. 2011). Being an organism from an intertidal area, *P. pollicipes* are exposed to an extremely adverse environment, during and immediately after the low tide period (which can correspond to 12 hours per day). During this period, animals are exposed to wave action, and especially to desiccation, an intense sunlight and ultraviolet (UV) radiation, which is naturally more challenging during the spring and summer months. Several authors described an increase in TBARS levels after exposure to high UV levels (e.g., Vargas et al. 2010).

From all abiotic factors, temperature is perhaps the main responsible for LPO increased formation, influencing the pollutants accumulation in *P. pollicipes* tissues. The increase of temperature may cause changes in the metabolism of contaminants and/or changes in the organism's lipid contents (Livingstone et al. 1995; Swaileh 1996). In ectotherms organisms, temperature accelerates the ROS formation, by accelerating the mitochondrial respiration (Freire et al. 2011). In our study, the higher TBARS values

recorded during hotter months, in all tested tissues, may be related with higher temperatures (see Table 5), which may influence other abiotic factors, such as dissolved oxygen, pH and salinity (Freire et al. 2011). The same pattern was described by Giarratano et al. (2011) work where higher LPO levels in gills of *Mytilus edulis chilensis*, collected from a polluted area, during summer were observed. Damiens et al. (2004) studied the influence of salinity and temperature in TBARS values in *Crassostrea gigas* larvae, and described a significant increase in TBARS levels for higher temperatures and salinity concentrations.

Another natural variation that may enhance the peroxidation damage is the reproductive cycle. Filho et al. (2001) described an increase in TBARS content during the reproductive cycle of *Perna perna*. The reproductive cycle of *P. pollicipes* occurs between may and august, with a maximal annual gamete emission. During this period, higher metabolic activities occur, where the gonads have a higher lipid and carbohydrate mobilization and protein synthesis (Magalhães 1998). This situation causes a significant increase in TBARS levels, already described by other authors (Filho et al. 2001). The TBARS contents increased in july to may, as observed in our study, are in agreement with this reported pattern (Figure 12).

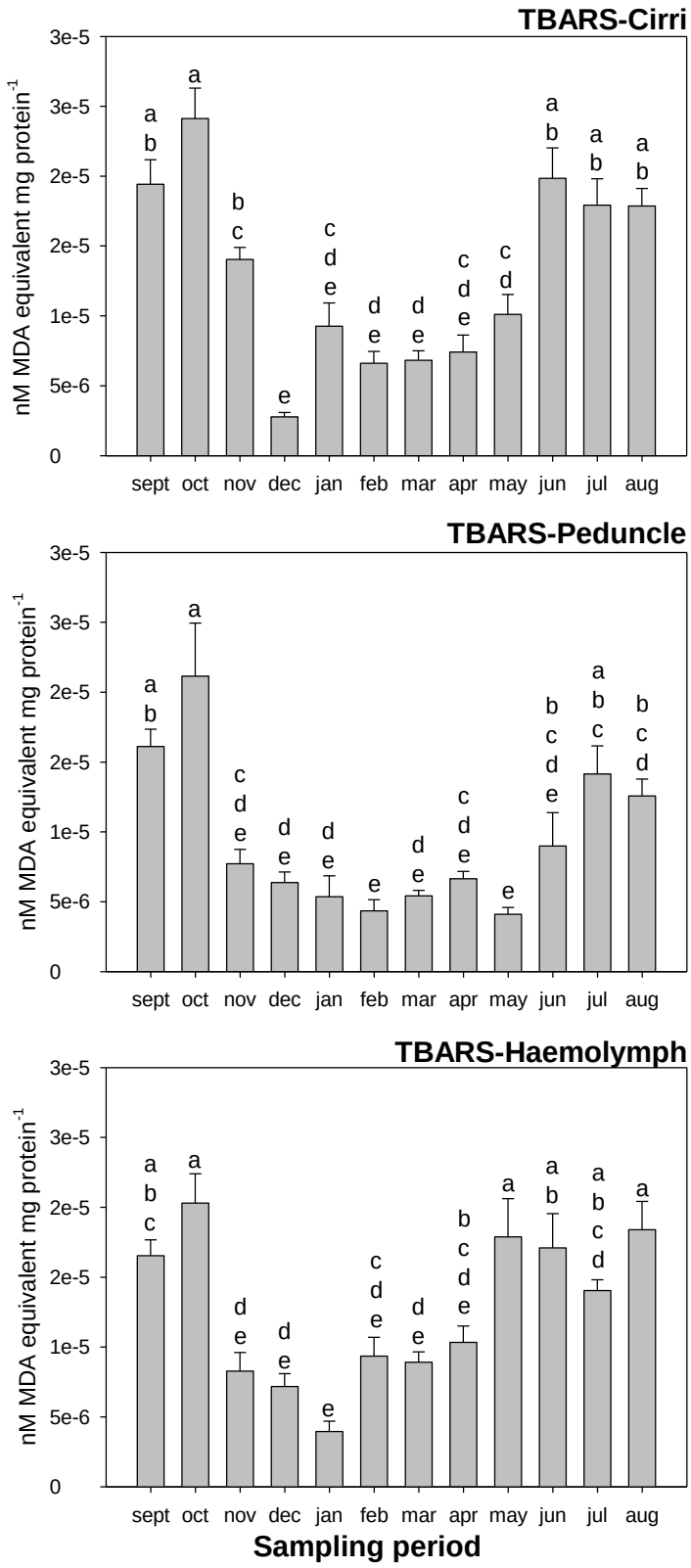

Figure 12 - Mean content of specific biomarker TBARS in cirri, haemolymph and peduncle of 30 *P. pollicipes*. Error bars correspond to the standard error. Different letters are used to describe differences among months, $p \leq 0.05$.

ChEs activity

The here-obtained results showed, similarly to what was observed for other tested biomarkers, a seasonal variation in all tested tissues (Table 3 and 4; Figure 13), with higher levels of AChE activity in hotter months. Evaluating changes in AChE activity is commonly used as an indicative of neurotoxicity in aquatic ecosystems. AChE inhibition by anthropogenic sources of pollution is extensively described by several authors (e. g., Mora et al. 1999; Nunes et al. 2005; Bonacci et al. 2009; Frasco et al. 2010). The most important and studied source of AChE inhibition is attributed to pesticides (organophosphate - OPs and carbamate - CBs, commonly used as pesticides in agricultural practices). These compounds are released into the environment, contaminating river waters and in some cases reaching the ocean (Mora et al. 1999) also affecting estuarine areas. AChE activity inhibition by anticholinergic pesticides was described by Rickwood and Galloway (2004), in their study with *Mytilus edulis* exposed to environmental realistic concentrations of chlorfenvinphos. Ramos et al. (2014) described a higher inhibition of AChE activity in *P. pollicipes* peduncle in an estuarine area, when compared with a non-polluted area. Other AChE inhibitors present in marine areas are polycyclic aromatic hydrocarbons (PAHs). PAHs can be found in the environment and are toxic to several marine species (French 1998). The AChE activity inhibition by PAHs was described by Bonacci et al. (2009), with *Adamussium colbecki*, exposed to a complex mixture of PAHs and PCBs. Metals are also described as AChE inhibitors, by altering the pathway of enzyme synthesis or by reducing enzyme production. The deleterious impact of metal pollution in AChE activity was reported by Liao et al. (2006) with *Oryzias latipes,* after a sublethal exposure to methylmercury chloride. Binelli et al. (2005) described the inhibition in AChE activity in *Dreissena polymorpha,* collected from several lakes affected by persistent organic pollutants (POPs).

The alterations in biomarkers activity can be related with the periodic variations in the use/release of chemicals by anthropogenic activities (e.g., different agricultural pesticides are used during the year in Portugal - Cerejeira et al. 2003). In the Douro region, in northern Portugal (an area corresponding to the hydrographic basin of Douro river), an intensive agricultural production was established centuries ago (Goveia et al. 2011). Vast areas are used to grow vineyards and olive trees (Geraldes 2012), in which specific types of pesticides are systematically applied (e.g., dimethoate, chlorpyrifos and copper sulfate, well established anticholinesterasics - Geraldes 2012), especially during the spring, summer and autumn (DGAV 2014). The use of such biologically active compounds may have consequences in terms of toxicity, in organisms exposed

to Douro river water. Other unspecific chemical contaminants may also be leached into the soils, until they reach the river. Makepeace et al. (1995) in his work observed that the majority of compounds transported by water runoff are toxic to wildlife and are a significant source of pollution for natural waters (Hoffman et al. 1995). However, from our results, it was possible to observe that cholinesterasic inhibition (most probably due to pesticide exposure) occurred during the winter and early spring, a finding that may be explained by the heavy rainfall that occurred during these periods. Heavy rainfall may favor agricultural runoffs, which bring influxes of pesticide-enriched water into the river. Qi et al. (2014) reported higher pesticide concentrations during the rainy season, and this fact may be explained by the higher water runoff from agricultural fields. Additionally, the reduction of treatment efficiency in the sewer systems due the high hydraulic loads also favours this scenario. In the Douro river, the presence of anticholinergic compounds was already described (e.g., Cerejeira et al. 2003), probably being this class of pesticides the major responsible for the AChE inhibition recorded in our data. The increase of anticholinergic compounds during the rainy season seems to have overcome the dilution factor caused by the increase of water entering the hydrographic basin, leading to the higher AChE inhibition in all tested tissues during the winter.

Besides the influence of xenobiotics on AChE activity, abiotic factors (such as temperature and pH) can influence the relation between the pollutants and AChE activity (Mora et al. 1999; Frasco et al. 2010). From all abiotic factors, temperature plays the most important role in the modulation of AChE activity (Hogan 1970), since water temperature increases the susceptibility of invertebrates to pesticides (Escartín and Porte 1997). In our study, a decrease in AChE activity was observed in winter and early spring. The same pattern was described by Ramos et al. (2014) in *P. pollicipes* collected in Lavadores beach. Beltran and Pocsidio (2010) described a lower AChE activity in the mussel *Corbicula fluminea* collected from a highly populated area surrounded by large rice and cornfields treated with OPs, during the coldest and wettest months. So, it is possible to conclude that beyond the chemical stressor impact on AChE activity, the natural variations may also have a modulation effect in the AChE activity variations.

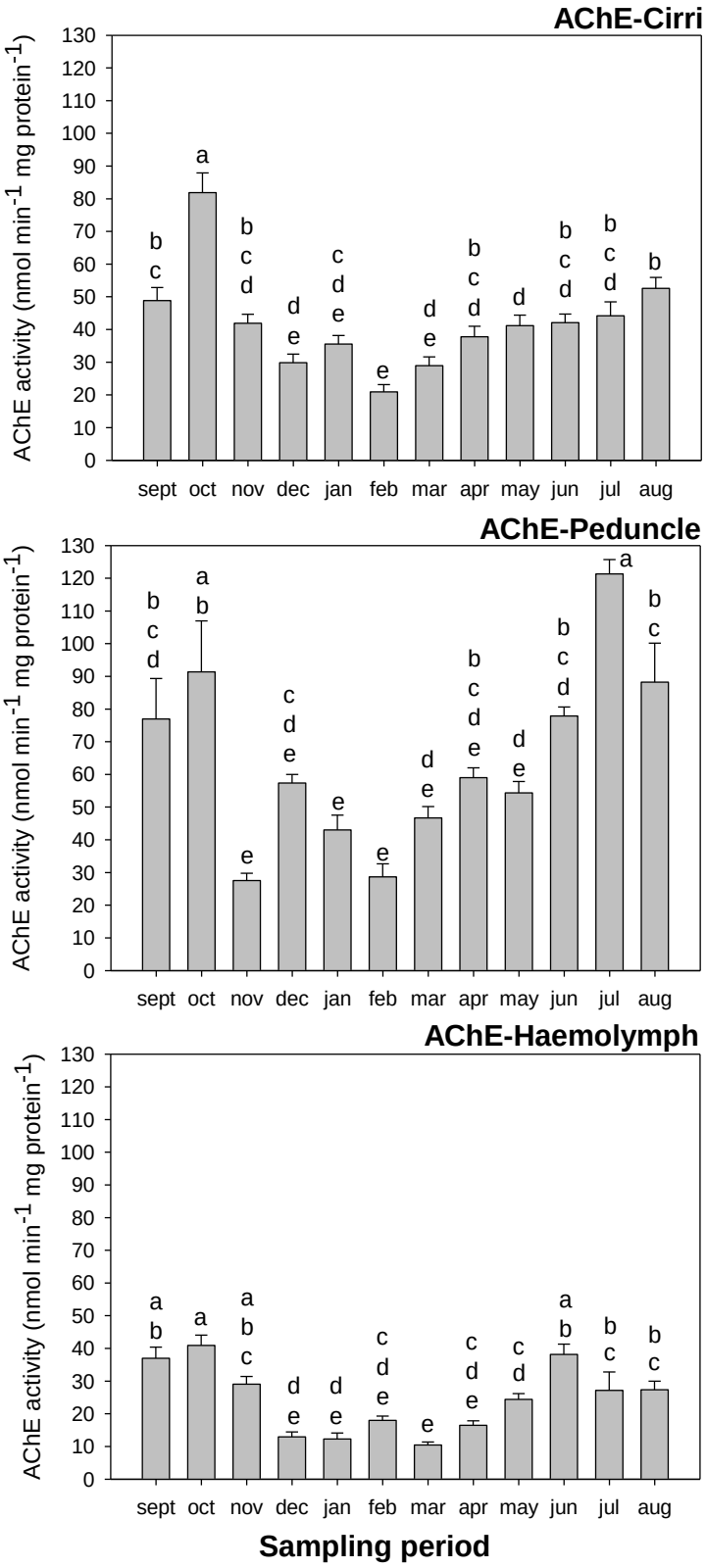

Figure 13 - Mean activity of specific biomarker AChE in cirri, haemolymph and peduncle of 30 *P. pollicipes*. Error bars correspond to the standard error. Different letters are used to describe differences among months, $p \leq 0.05$.

Glycogen

Glycogen levels showed a significant decrease in organisms sampled between may and august (Table 4, Figure 14). Several authors already described that animals exposed to different contaminated scenarios, show a significant decrease in their glycogen levels (Hamza-Chaffai et al. 2003). Becker et al. (2009) reported a significant decrease in kidney glycogen levels of *Rhamdia quelen* from the high anthropic activity site when compared with organisms from the low anthropogenically impacted areas. The glycogen variation in presence of pollutants may be a way to reduce the stress induced by environmental contamination; the degradation of glycogen probably occurs to help maintain energy in the metabolic process, since glycogen is one of the major sources of metabolic reserves for a variety of cell types. From our results, it was possible to observe a significant reduction in glycogen content occurring especially during late spring and summer (Figure 14). This is in agreement with the higher levels of metal contamination in seawater that were already reported by other studies (Santos et al. 2011; Reis et al. 2013). This suggests that the reduction in glycogen levels in *P. pollicipes* muscle can work as a protective response to the pollutants presence. The decrease of glycogen contents in chemical stress scenarios was already described by several authors (Pellerin et al. 1993; Hamza-Chaffai et al. 2003). Pellerin et al. (1993) described a decrease in glycogen content in *Mytilus edulis* and *Mya arenaria*, after exposure to pulp and paper mills effluents. Hamza-Chaffai et al. (2003) also described a decrease in glycogen content after zinc exposure in *Ruditapes decussatus*.

The consumption of glycogen not only occurs in the presence of pollutants, glycogen can be spent for osmoregulation and/or growth (Kucharski and Da Silva et al. 1991) and also during the reproductive cycle (Filho et al. 2001). The latter is described as the most representative cause for glycogen degradation (Filho et al. 2001). Our results showed that the most significant period of decrease in glycogen levels coincides with the period of reproductive cycle of *P. pollicipes* (spring and summer) (Figure 14). The stored glycogen releases energy in response to the increased energy needs during the reproductive cycle, occurring a significant decrease in glycogen content during this period. The variation in glycogen storage is controlled by crustacean hyperglycemic hormone (CHH), which has a significant role in reproduction. The lower CHH levels stimulate the hydrolysis of glycogen from muscle stores (Verri et al. 2001) providing the energy required for the maturation of the ovary, and the occurrence of the reproductive cycle. According to our data, it is possible to assume that the decrease in glycogen content in spring/summer months is related with the reproductive cycle, being the glycogen spent in order to provide energy for vitellogenesis. The same hypothesis

was described by previous studies (Oliveira et al. 2003; Buckup et al. 2008) in which the reduction of glycogen content was coincident with the reproductive cycle, suggesting that these reserves are mobilized for vitellogenesis.

Beyond the impact of the reproductive cycle in the decrease in glycogen content other factors may contribute for alterations of the glycogen levels, especially during the hotter seasons. The reduction in glycogen levels may be related with an increase in the use of energy for ATP synthesis due to the decrease in environmental oxygen levels (hypoxia), caused by an increase in water temperature values. The same assumption was described by da Silva-Castiglioni et al. (2007) after a study focusing in *Parastacus varicosus* hepatopancreas glycogen content. On the other hand, the highest energy reserves were recorded during the colder months, which may be a consequence of a slower metabolism, due to low temperature recorded (see Table 2). This hypothesis was already raised by Gismondi et al. (2012) in their work with *Gammarus roeseli*, where the higher energy reserves were measured in autumn and winter, and lower energy reserves were measured in spring and summer. The variation in glycogen content was thus related with the water temperature and the reproductive cycle. Considering these facts, it is possible to conclude that the chemical stressors presence in water and the natural fluctuations may be the major source of variation in glycogen levels in gooseneck barnacle.

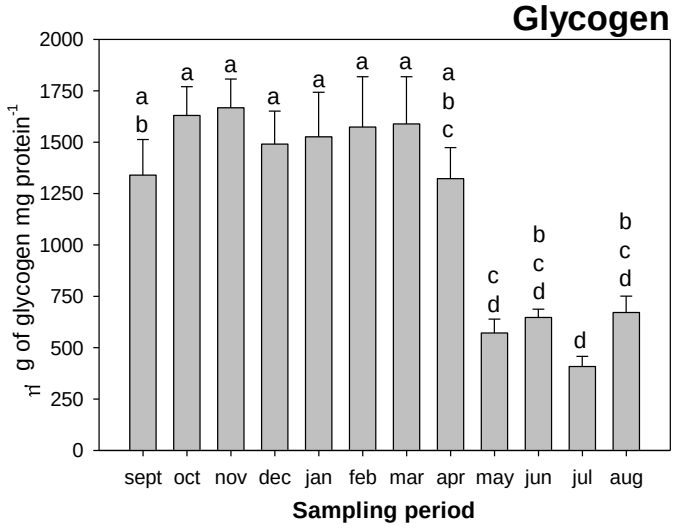

Figure 14 - Mean content of glycogen levels in peduncle of 10 *P. pollicipes*. Error bars correspond to the standard error. Different letters are used to describe differences among months, $p \leq 0.05$.

Haemocyte count

In the results obtained after counting the number of haemocytes in each sample, an apparent seasonal pattern was observed (Table 4; Figure 15). The variation in the haemocytes number may be affected by the presence of anthropogenic compounds, as PAHs (McCormick-Ray 1987; Sami et al. 1993; Pipe and Coles 1995), PCBs (Canesi et al. 2003), pesticides (Auffret and Oubella 1997) and heavy metals (Gagnaire 2004). Haemocytes in crustaceans are also involved in the detoxification and/or accumulation of metallic compounds in non-toxic forms (Viarengo and Nott 1993). Pipe and Coles (1995) described an increase in the total number of circulating haemocytes after pollutants exposure. This phenomenon resulted from proliferation and/or movement of the cells from tissues into circulation. So, the increase in circulatory haemocytes number may occur too in order to regulate the heavy metal concentration. One of the major heavy metal homeostasis mechanisms is the action of metal-binding proteins, the metallothioneins (MTs) that are involved in the detoxification of essential and non-essential metals, being present in circulatory haemocytes. MTs act in order to avoid the non-specific binding of non-essential metals within cells, reducing their toxic potential (Roesijadi 1992). Taking into account the described data, it is possible to assume that the increase in the cell haemocytes number, observed in our results, may be associated to a protective response to face the presence of anthropogenic pollution. The variations in haemocytes number after exposure to pollutants were also described by several authors (Cheng 1988; Coles et al. 1995; Pipe et al. 1999) for mollusc species. Pipe et al. (1999) in his study described an increase in the number of circulatory haemocytes in *Mytilus edulis* haemolymph, after exposure to environmentally realistic concentrations of copper. Coles et al. (1995) also described enhanced numbers in circulatory haemocytes of *Mytilus edulis* after cadmium exposure. Cheng (1988) reported an increase in haemocytes number in *Crassostrea virginica* after cadmium exposure. Gagnaire et al. (2006a) analysed the influence of several pollutants in the haemocytes of the oyster *Crassostrea gigas*, and described an increase in cell percentage after exposure to PAHs. The variation pattern obtained in our study showed an increase in the haemocyte counts during summer (exception for december and february), coincident with the periods for which the highest pollutants concentrations were observed in water (Santos et al. 2011; Reis et al. 2013). This association suggests that the increase in the haemocytes number is a protective response to the increase of anthropogenic pollutants in water.

Regarding the contribution of abiotic factors in the number of haemocytes, Gagnaire et al. (2006b) described that the temperature increase induces an increase of

haemocyte mortality. This phenomenon may explain the high cell number in december and february. Another factor to consider is salinity. The haemolymph of marine invertebrates is affected by the osmotic strength and ionic composition of the ambient water, being the haemocytes directly exposed to the salinity variations (Gilles, 1979). In the study of Gagnaire et al. (2006b) the authors reported a significant increase in *Crassostrea gigas* haemocyte mortality when the salinity decreases when compared with salinities of 32ppt. The decrease in salinity during the rainy seasons, may promote the decrease in haemocytes numbers. This assumption is in agreement with our study data, where a decrease in the haemocytes number during the rainy season (exception to december and february) was recorded. However, the pattern obtained may also be related with other factors such as the effects of bacteria (e.g., Vibrio), who may induce an increase in the haemocyte number in crustaceans (Alavandi et al. 2004).

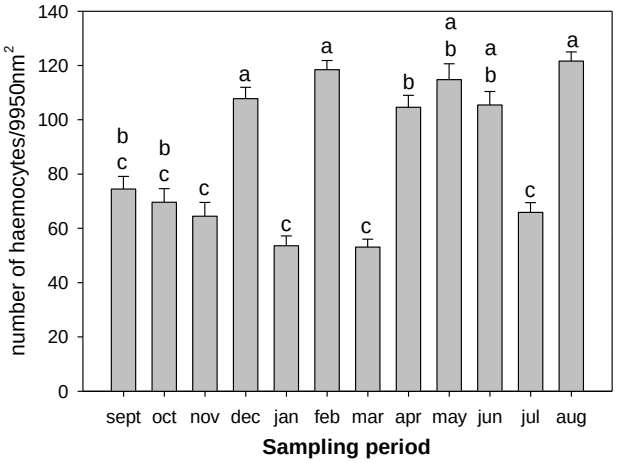

Figure 15 - Mean haemocytes number in haemolymph of 30 *P. pollicipes*. Error bars correspond to the standard error. Different letters are used to describe differences among months, $p \leq 0.05$.

Haemolymph as a source tissue for non-destructive biomarker determinations

The here-obtained results showed a similar pattern of response among all tested tissues for the distinct biomarkers quantifications (Figures 11, 12 and 13). These results support one of the major objectives of this study, which was the validation of haemolymph as a source tissue for non-destructive biomarker determinations. The use of haemolymph in ecotoxicological assays for evaluating deleterious effects of

xenobiotics was already described by other authors (Owen et al. 2002; Moreira and Guilhermino 2005, Pan et al. 2006; van Oosterom et al. 2010). Owen et al. (2002) described the inhibition of ChEs in the haemolymph of *Euvola ziczac* after exposure to organophosphate and carbamate pesticides. Moreira and Guilhermino (2005) used the haemolymph of *Mytilus galloprovincialis* to evaluate the seasonal variation on acetylcholinesterase activity, in animals collected at potentially polluted sites. van Oosterom et al. (2010) analysed the AChE and glutathione S-transferases (GSTs) activities in haemolymph of the crab species *Scylla serrata*, collected from several potential polluted rivers. Pan et al. (2006) study reported the increase in lipid peroxidation (LPO) levels in scallop *Chlamys ferrari* haemolymph after acute exposure to benzo(a)pyrene and benzo(k)fluoranthene. In our study, the biomarkers determined in haemolymph of *P. pollicipes* collected from a potentially polluted area, seemed to respond satisfactorily. A similar result was described by Blaise et al. (2002) in the scallop *Mya arenaria* haemolymph, collected from potentially polluted sites. Kaloyianni et al. (2009) also reported significant increases in malondialdehyde (MDA) levels in the haemolymph of molluscs exposed to different concentrations of heavy metals like zinc, cadmium and organic pollutants as PAHs. Lorenzon et al. (2007) described that the evaluation of haemolymph total protein can be used to assess the health and stress levels in the crustacean *Homarus americanus*. These data support the viability of using haemolymph as a source tissue for non-destructive biomarker determinations. The same conclusion was confirmed by comparing the response pattern of biomarkers in haemolymph with those determined in the two other tested tissues (cirrus and peduncle - Figures 11, 12 and 13). In all tissues, a very similar response pattern was obtained, but in different scales. For all biomarkers quantified in haemolymph, a lower activity range was obtained when compared with other tissues.

Conclusion

According to the results obtained in this study, the quantified biomarkers (namely GSTs activity and TBARS levels), showed a higher activity at specific periods, i.e. spring, summer and early autumn. Lower AChE activities were recorded in winter and early spring. In terms of glycogen levels, a significant decrease was observed in may and august. The seasonal variation pattern found in all biomarkers showed a modulation according the natural variations of abiotic factors such as temperature and salinity. Data fluctuations can also be related to the reproductive cycle of the selected organism. These factors can thus play an important role in the influence of

anthropogenic pollutants on biomarkers determined in *P. pollicipes* by modulating the toxic response. The higher GSTs activity values, and higher peroxidative damage observed for hotter months may be related with the seasonal pattern of pollution described in Douro river and in the estuarine seawater, and with the increase of contaminants concentration in water during the hotter and dryer months. The lower AChE activity in winter and early spring seems to be related with the increase in the anticholinesterasic compounds in water, due to the water runoff from agriculture fields. Glycogen levels decrease during the reproductive cycle period of the test organism seems to happen in order to provide energy for vitellogenesis. The variations in haemocytes counts showed an increase during summer (exception to december and february) that may be related with the increase in pollutants concentration in water. This set of results show the importance of natural fluctuations in the biological responses to the anthropogenic pollutants. Another important conclusion is the similar response pattern found between biomarkers quantified in haemolymph tissue and in the other tested tissues (cirri and peduncle). This conclusion supports that parameters quantified in haemolymph tissue, may constitute non-destructive biomarkers, showing to be a valuable tool in future ecotoxicological studies.

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Final Remarks

The results obtained from this study showed that the major cholinergic form present in all tested tissues (cirri, peduncle and haemolymph) is acethylcholinesterase, working as an important tool to diagnose environmental exposure to pesticides and hydrocarbons derivatives in the marine environment. Levels of commonly used biomarkers were assessed both in contaminated and reference sampling sites showing that *P. pollicipes* can be proposed as a valuable sentinel species for biomonitoring programs in the evaluation of anthropogenic stressors in marine coastal environment. From the obtained results, it was possible to ascertain the influence of natural parameters in the biodisponibility and variation in chemicals stressors concentration in water, which causes variations in anthropogenic pollutants impact on biomarkers responses. Intertidal area is an extreme environment where organisms are subjected to continuous alterations, triggering a series of adaptations in the biota for their own protection and survival. This assumption brings a new perspective about the use of biomarkers and limits the interpretation of data from biomonitoring programs, indicating the necessity to include seasonal factors for a correct interpretation of data obtained from organisms collected at intertidal areas. After analysing the seasonal variation profiles obtained in the two chapters of the present study (chapters 2 and 3), it is possible to conclude that the variation in biomarkers results may be explained by the seasonal pattern of pollution already described near the study area. This study brings new perspectives for the use of resident species (*P. pollicipes*) in marine ecotoxicological studies, and the use of haemolymph of *P. pollicipes* as a tissue for non-destructive biomarker determinations. This information bringing new possibilities for ecotoxicological tests where an historical assessment of contamination and response is required. In conclusion, the use of biomarkers for evaluation the impact of anthropogenic pollutants, and natural variation in *P.pollicipes*, seems to be a valuable tool for monitoring the quality of the coastal environment in Portugal North coast.