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Comparative analysis of biomarker responses to environmental contamination in estuaries: a multi-taxa approach

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Abstract

Estuaries are highly productive areas of high ecological and economic importance, providing various benefits and services for mankind. The estuarine watershed is a preferential location for human settlement and associated anthropogenic activities, such as industrial and agricultural development, resource exploitation and shipping activities, which result in continuous pressures inevitably leading to environmental degradation.

Biomarkers are considered early-warning signs able to provide a predictive perspective of the long-term effects of exposure to pollutants in organisms. Therefore, biomarkers are considered useful tools for environmental quality assessment that integrate biological responses and the degree of stressors, usually applied in a single taxa context.

Exposure to xenobiotics compounds and their metabolites lead to enhanced oxidative stress and potentially to major molecular damages such as oxidation of proteins, DNA and peroxidation of unsaturated lipids in cell membranes. Hence, defence mechanisms play a major role in preventing damages and include several enzymes such as antioxidant enzymes superoxide dismutase enzymes (SOD) and catalase (CAT), as well as phase I ethoxyresorufin-O-deethylase (EROD) and phase II glutathione S-transferase (GST) biotransformation enzymes. Accordingly, these enzymes activities as well as both biomarkers of effects lipid peroxidation (LPO) and DNA damage (DNAd) were determined in the present study aiming at assessing overall contamination impacts on various organisms.

In this context, the aim of this work is to quantify multiple biomarker responses in a multi-taxa approach, in order to assess the diversity in response patterns among species from two differently impacted estuarine systems.

Two Portuguese estuaries, Tejo and Ria de Aveiro, were sampled in two months, June and September 2015, specifically in two sites in each estuary: Alcochete (ALC) and Vila Franca de Xira (VFX) in Tejo and Mira channel (AVSUL) and Murtosa (MUR) in Ria de Aveiro. Several species were selected based on their abundance and estuarine occurrence as well as on their prior use as bioindicator species. Two fish species were considered, the European sea bass *Dicentrarchus labrax*, a marine migrant species whose juveniles use estuarine areas as nursery areas; and the common goby *Pomatoschistus microps* a resident estuarine species. Four invertebrate species were also sampled, two common infaunal species, the bivalve *Scrobicularia plana* and the ragworm *Hediste diversicolor* and two epibenthic crustaceans, the brown shrimp *Crangon crangon* and the green shore crab *Carcinus maenas*.

Overall, biomarker responses signaled environmental chemical exposure and some degree of deleterious effects for all species. Low variability among sites and months was observed in antioxidant enzymes responses for most species and no clear pattern was discernible amongst species. In fish species, induction of both biotransformation enzymes was observed with significant spatial variability, with lower variability of GST comparing to EROD activity, though with overall concordant higher levels in Tejo estuarine sites, especially in ALC. Less marked responses in biotransformation enzymes were observed in invertebrate species. A similar GST response pattern was observed for *H. diversicolor*, *S. plana* and *C. maenas*, identifying higher contamination levels in AVSUL, considered the least contaminated site in this study. Very low or even incipient spatial and temporal variability in EROD activity was observed in all invertebrates. Nevertheless, these species signaled mostly Tejo sites according to phase I enzyme responses, especially VFX, which is in agreement with previously reported environmental contamination levels. Concerning monthly variation in general all species showed higher enzyme activities and effects in September, which may reflect the effects of continuous exposure.

Notwithstanding the significant activity of detoxification enzymes, deleterious effects were reported for all species, suggesting an overall contamination level above the capacity of the molecular defence mechanisms to limit exposure effects in these species. Biomarkers of effects response patterns differed amongst species, yet akin LPO variability patterns were observed among species, namely between *C. crangon*, *C. maenas* and *P. microps*, signalling VFX and for *D. labrax*, *H. diversicolor* and *S. plana*, signalling MUR. Most species also showed higher mean DNAd values in VFX, except for *C. crangon* and *C. maenas* that signalled MUR. A positive correlation between biomarkers of effects was observed for all invertebrate species (except for *H. diversicolor*), highlighting site-specific contamination. IBR indices also varied throughout sampled sites for all species studied, yet the major pattern observed was the opposed response pattern between a highly mobile fish species *D. labrax* and both infaunal invertebrate species (*S. plana* and *H. diversicolor*).

Species-specific responses were evident from lack of concordance among species biomarker responses and IBR scores, most likely due to biological differences (in terms of physiology, vulnerability and overall capacity of defence mechanisms), but also to ecological differences such as differential habitat use, feeding habits, life-strategies and consequently differential contaminants exposure. Overall, Tejo sites were considered more impacted than Ria de Aveiro, yet significant responses were also found in the latter for all species studied.

In conclusion, this multi-biomarker and multi-taxa approach provided important insights into the variability of species responses to contaminants exposure in estuaries. The complexity of biomarker response patterns for all species in this study evidenced species differential response and differential exposure to environmental contamination, on top of the complex environmental stimuli, such as pollutants' mixtures and natural variability characteristic of the estuarine environment. This emphasizes the difficulties associated with effective multi-species ecological risk assessment, and application must carefully consider the potential added ecological value of a multispecific approach (similar to a multi-biomarker approach) versus more complex results interpretation and assessment of the environmental quality.

Keywords: Estuarine environmental quality; Biomarkers; Fish; Invertebrates; Integrated biomarker response index

Resumo

Os estuários são sistemas de elevada importância ecológica onde, por exemplo, muitas espécies encontram as condições favoráveis à sobrevivência e rápido desenvolvimento, bem como de elevado potencial económico, constituindo uma fonte importante de recursos e serviços para o Homem. Consequentemente, estes sistemas tornam-se locais preferenciais para o desenvolvimento de centros urbanos, de que resultam inúmeras pressões associadas às atividades antropogénicas, desde a pesca, aquacultura, à indústria, agricultura e navegação, o que inevitavelmente conduz à degradação da qualidade ambiental. Os biomarcadores são frequentemente utilizados com o intuito de avaliar de que forma as pressões nestes sistemas afetam os organismos que os habitam. Constituem desta forma uma ferramenta útil em estudos de avaliação da qualidade ambiental, ao fornecerem uma perspetiva integrada da pressão existente e das respostas ao nível biológico.

A exposição dos organismos a compostos xenobióticos resulta no aumento do stress oxidativo que propicia a ocorrência de danos ao nível da célula, nomeadamente a oxidação de proteínas e do DNA (DNAd) e peroxidação lipídica (LPO). Não obstante, os mecanismos de defesa das células desempenham um papel fundamental na prevenção desses efeitos, de que são exemplos: enzimas antioxidantes, como a catalase (CAT) e a superóxido dismutase (SOD), e de biotransformação, como a 7-etoxiresorufina-O-deetilase (EROD) e a glutationa-S-transferase (GST).

O objetivo deste trabalho consiste no estudo comparativo das respostas biológicas de várias espécies às pressões antropogénicas a que estão sujeitas no seu ambiente natural, através da quantificação de diferentes biomarcadores, em estuários com níveis de pressões distintos, o estuário do Tejo e a Ria de Aveiro. A amostragem foi realizada em dois meses, em junho e em setembro de 2015, e em dois locais em cada estuário: Alcochete (ALC) e Vila Franca de Xira (VFX) no estuário do Tejo e o canal de Mira (AVSUL) e Murtosa (MUR) na Ria de Aveiro, cujo nível de contaminação, fontes de pressão, função de viveiro e abundância de espécies foi previamente descrito. Para tal, foram selecionadas várias espécies que utilizam ambos os estuários e consideradas, em trabalhos anteriores, bioindicadoras da qualidade ambiental, nomeadamente: duas espécies de peixes, o robalo-legítimo *Dicentrarchus labrax* e o caboz-comum *Pomatoschistus microps*, e quatro espécies de invertebrados, nomeadamente duas espécies endobentónicas, o poliqueta *Hediste diversicolor* e o bivalve *Scrobicularia plana*, e duas espécies de crustáceos epibentónicos, o camarão-mouro *Crangon crangon* e o caranguejo-verde *Carcinus maenas*. As atividades das enzimas SOD, CAT, EROD e GST bem como os níveis de LPO e DNAd foram analisados nos tecidos apropriados para cada uma das espécies consideradas.

De um modo geral, as respostas dos biomarcadores evidenciaram a exposição a contaminantes no ambiente, passível de induzir alguns mecanismos de defesa e destoxificação e de produzir efeitos ao nível molecular em todas as espécies estudadas. A atividade das enzimas antioxidantes apresentou reduzida variabilidade entre locais e meses e não se observou nenhum padrão específico entre todas as espécies. As enzimas de biotransformação apresentaram maior variabilidade espacial e temporal que as antioxidantes, e maior nos peixes do que nos invertebrados. Os efeitos a nível molecular também foram evidentes, em todas as espécies, evidenciando um nível de contaminação global considerável que ultrapassa a capacidade de defesa e metabolização de xenobióticos destas espécies.

Na generalidade, as respostas dos biomarcadores sinalizaram maiores níveis de contaminação nos locais do estuário do Tejo, assim como uma tendência de respostas mais elevadas em setembro, entre todas as espécies, quer para as enzimas antioxidantes e de biotransformação como para os biomarcadores de efeitos, possivelmente evidenciando um efeito de exposição continuada a stresses ambientais. De um modo geral, as respostas biológicas bem como os valores obtidos através do índice de IBR variaram bastante entre espécies e de acordo com os locais amostrados, tendo no entanto sido

possível observar alguns padrões de resposta semelhantes entre espécies, particularmente no que diz respeito ao uso do habitat. Além disso, apesar dos locais do estuário do Tejo serem considerados mais impactados que os locais da Ria de Aveiro, nestes foram igualmente observados respostas significativas em várias espécies. A complexidade dos padrões de resposta dos vários biomarcadores analisados evidencia diferenças entre espécies em termos da sua fisiologia, vulnerabilidade aos contaminantes e capacidades dos mecanismos de defesa, bem como ao nível da ecologia, como o uso do habitat, e hábitos alimentares de que naturalmente decorrem diferentes níveis de exposição.

A aplicação de uma abordagem multiespecífica e de vários biomarcadores pode constituir uma ferramenta útil para a compreensão de diferentes vias de exposição e para uma análise mais completa dos efeitos em diferentes componentes biológicos do ecossistema. No entanto, apesar de pertinente a nível ecológico, é evidente a complexidade de interpretação que lhe está associada, e para a qual a aplicação em programas de monitorização e de avaliação da qualidade ambiental requer maior planeamento. Além disso, a seleção das espécies deverá ser ponderada de acordo com o objetivo do estudo, sendo que diferentes espécies irão gerar avaliações de impacto diferentes para os mesmos locais.

Palavras-chave: Qualidade ambiental estuarina; Biomarcadores; Peixes; Invertebrados; IBR

Resumo alargado

Os estuários e zonas costeiras são áreas de elevada importância ecológica e económica. Do ponto de vista ecológico, um exemplo é a função de área de viveiro para várias espécies de peixes marinhos. É nestes sistemas que os juvenis destas espécies encontram as condições necessárias ao seu desenvolvimento, como alimento abundante, temperaturas mais favoráveis para o crescimento, refúgio e menor densidade de predadores que aumentam as probabilidades de sobrevivência. Os estuários constituem igualmente uma importante fonte de recursos e serviços para o Homem, como por exemplo a pesca, aquacultura, atividades ligadas ao turismo, navegação, indústria e agricultura. Estes sistemas são também locais preferenciais para o desenvolvimento de centros urbanos, de que decorrem inúmeras pressões ambientais associadas às atividades antropogénicas, o que inevitavelmente conduz à degradação da qualidade ambiental.

Um biomarcador pode ser definido como uma qualquer alteração ao nível bioquímico, celular ou fisiológico que resulta da exposição de um organismo a um determinado stress e que é mensurável, constituindo desta forma um indicador de potenciais efeitos. Assim, os biomarcadores são considerados uma ferramenta útil na avaliação da qualidade ambiental ao permitirem a análise, numa perspetiva integrada, das respostas ao nível biológico e das condições ambientais.

A exposição dos organismos a xenobióticos contribui para o aumento do stress oxidativo e pode levar a danos ao nível celular como a oxidação de proteínas, do material genético (DNAd), bem como promover a peroxidação lipídica (LPO). No entanto, existem mecanismos de defesa que permitem minimizar os efeitos do stress oxidativo e que, como tal, desempenham um papel fundamental na prevenção desses efeitos nocivos. São exemplos as enzimas antioxidantes superóxido dismutase (SOD) e catalase (CAT), bem como as enzimas de biotransformação etoxiresorufina-O-deetilase (EROD) e glutathione-S-transferase (GST). Consequentemente, estas enzimas constituem bons indicadores da exposição a pressões ambientais, sendo frequentemente utilizadas como biomarcadores em estudos ecotoxicológicos.

Este trabalho tem como objetivo o estudo comparativo das respostas biológicas de várias espécies às pressões antropogénicas a que estão sujeitas no seu ambiente natural, através da quantificação de diferentes biomarcadores, em estuários com níveis de impactos distintos.

O estuário do Tejo e a Ria de Aveiro são dois sistemas com diferentes níveis de pressões antropogénicas, com elevada importância ecológica e económica. A amostragem foi realizada em dois meses, em junho e em setembro de 2015, e em dois locais em cada estuário: Alcochete (ALC) e Vila Franca de Xira (VFX) no estuário do Tejo e o canal de Mira (AVSUL) e Murtosa (MUR) na Ria de Aveiro, cujo nível de contaminação, fontes de pressão, função de viveiro e abundância de espécies foi previamente descrito. Foram selecionadas várias espécies que utilizam ambos os estuários e que já foram utilizadas, em outros trabalhos, como bioindicadoras da qualidade ambiental. Assim sendo, foram selecionadas duas espécies de peixes, o robalo-legítimo *Dicentrarchus labrax*, uma espécie marinha cujos juvenis utilizam os estuários como áreas de viveiro e o caboz-comum *Pomatoschistus microps*, uma espécie residente em estuários. Paralelamente foram escolhidas quatro espécies de invertebrados, nomeadamente duas espécies endobentónicas, o poliqueta *Hediste diversicolor* e o bivalve *Scrobicularia plana*, e duas espécies de crustáceos epibentónicos, o camarão-mouro *Crangon crangon* e o caranguejo-verde *Carcinus maenas*. Assim, foram determinadas as atividades das enzimas CAT, SOD, EROD e GST bem como os níveis de DNAd e LPO nos tecidos apropriados para cada uma das espécies consideradas, nomeadamente o fígado em ambas as espécies de peixes, o hepatopâncreas em *C. maenas*, a glândula digestiva em *S. plana*, o músculo em *C. crangon* e o corpo inteiro em *H. diversicolor*.

Na generalidade, as respostas dos biomarcadores evidenciaram a exposição a contaminantes químicos no ambiente em todas as espécies estudadas. A atividade das enzimas antioxidantes apresentou reduzida variabilidade entre locais e meses e não se observou nenhum padrão específico entre todas as espécies. As enzimas de biotransformação apresentaram maior variabilidade espacial e temporal que as anteriores. Para ambas as espécies de peixes, as respostas das enzimas de biotransformação apresentaram variabilidade espacial, tendo no entanto a GST apresentado menor variabilidade relativamente à EROD. Contudo, ambas enzimas sinalizaram, maioritariamente, os locais do estuário do Tejo como os mais contaminados, sobretudo ALC. De um modo geral, as respostas dos biomarcadores de biotransformação nos invertebrados foram menos evidentes que nos peixes tendo, no entanto, sido possível distinguir um padrão de resposta de GST semelhante entre *H. diversicolor*, *S. plana* e *C. maenas*, que indica maiores níveis de contaminação em AVSUL. No entanto, os reduzidos níveis de atividade observados nos locais do estuário do Tejo, onde os níveis de contaminação são mais elevados, poderão ter origem na presença de compostos inibitórios. Ao contrário da GST, a variabilidade das respostas da enzima EROD foi muito reduzida, ou mesmo incipiente nas espécies de invertebrados. Contudo, atividades mais elevadas da enzima EROD foram observadas para o estuário do Tejo, especialmente em VFX, o que vai de encontro com os níveis de contaminação previamente descritos para estas áreas. Foi também possível observar uma tendência de respostas mais elevadas em setembro, entre todas as espécies, quer para as enzimas antioxidantes e de biotransformação como para os biomarcadores de efeitos, possivelmente evidenciando um efeito de exposição continuada a stresses ambientais.

Os efeitos a nível molecular foram também evidentes em todas as espécies e a sua variabilidade espacial e temporal foi significativa, evidenciando a existência de um nível de contaminação global considerável que ultrapassa a capacidade de defesa e metabolização de xenobióticos celular destas espécies. As respostas ao nível dos biomarcadores de efeitos variaram entre espécies, tendo sido possível observar padrões de LPO semelhantes, onde *C. crangon*, *C. maenas* e *P. microps* sinalizaram VFX e *D. labrax*, *H. diversicolor* e *S. plana* sinalizaram MUR, como os locais com maior nível de contaminação. Relativamente aos danos ao nível do DNA, a maioria das espécies apresentou valores mais elevados em VFX, à exceção de *C. maenas* e *C. crangon*, que assinalaram MUR. Ainda nos invertebrados, observaram-se correlações positivas entre os biomarcadores de efeitos, à exceção de *H. diversicolor*, evidenciando os efeitos da contaminação característicos dos locais amostrados.

De um modo geral, as respostas dos biomarcadores bem como os valores obtidos através do índice IBR variaram de acordo com os locais amostrados, sendo possível observar diferenças entre os dois locais de um mesmo estuário, bem como entre estuários. Da análise dos padrões de IBR obtidos, observaram-se correlações negativas entre *D. labrax* e duas espécies de invertebrados, *S. plana* e *H. diversicolor*, evidenciando diferenças ao nível das respostas biológicas relativas às características inerentes das espécies bem como a diferenças ao nível do uso do habitat, que naturalmente resultam em diferentes níveis de exposição.

Globalmente, as respostas variaram bastante entre espécies, contudo foram observados alguns padrões de resposta semelhantes entre espécies, particularmente no que diz respeito à sua ecologia, nomeadamente ao uso do habitat. Além disso, apesar de os locais do estuário do Tejo serem considerados mais impactados que os da Ria de Aveiro, nestes foram igualmente observadas respostas significativas em várias espécies.

A complexidade dos padrões de resposta dos vários biomarcadores analisados evidencia as diferenças entre espécies, nomeadamente em termos da sua fisiologia, vulnerabilidade aos contaminantes e capacidades dos mecanismos de defesa, bem como ao nível da ecologia, como o uso do habitat, hábitos alimentares ou estratégias de vida. Adicionalmente é necessário considerar também os efeitos que múltiplos estímulos presentes no ambiente têm nas respostas biológicas, desde a

variabilidade natural às complexas misturas de poluentes, e que contribuem para a complexidade na interpretação dos padrões de resposta.

Apesar do contexto ecologicamente mais relevante, uma abordagem multiespecífica aumenta a complexidade de interpretação e requer maior esforço de planeamento para aplicação em programas de monitorização e de avaliação da qualidade ambiental. Contudo, uma abordagem multi-taxa pode constituir uma boa ferramenta para a compreensão de diferentes vias de exposição e uma avaliação mais completa dos impactos em vários componentes biológicos do ecossistema. A seleção das espécies deverá igualmente ser pensada de acordo com o objetivo do estudo, visto que diferentes espécies poderão gerar avaliações de impacto diferentes para os mesmos locais.

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Symbols and Abbreviations

BHT	Butylated hydroxytoluene
CAT	Catalase
CDNB	Dinitrochlorobenzene
DNAd	DNA damage
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
EROD	Ethoxyresorufin-O-deethylase
GSH	Glutathione
GST	Glutathione S-transferase
H₂O₂	Hydrogen peroxide
KCl	Potassium chloride
LPO	Lipid peroxidation
MDA	Malondialdehyde
NADPH	Dihyronicotinamide-adenine dinucleotide phosphate
NaOH.	Sodium hydroxide
O₂^{•-}	Superoxide anion radical
OH[•]	Hydroxyl radical
PAHs	Polycyclic aromatic hydrocarbons
PCBs	Polychlorinated biphenyls
PMS	Post-mitochondrial supernatant
PMSF	Phenylmethylsulfonyl fluoride
ROS	Reactive oxygen species
SDS	Sodium dodecyl sulfate detergent
SOD	Superoxide dismutase
TBA	Thiobarbituric acid
TCA	Trichloroacetic acid

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CHAPTER 1

General Introduction

General Introduction

In the late 1960s, the concern on the effects of chemical contaminants in organisms and the environment starts to grow, and the urge to understand those effects in biological and ecological systems gives rise to ecotoxicology science. Rather than the chemical contamination analysis, the most important feature of contaminant pollutants is its biological significance, and the need to evaluate those effects in the organisms culminated in the developing of measurements capable of predict long-term effects of pollutants: biomarkers. The term biomarker can be defined as any biological measurement, at the biochemical, cellular or molecular level, that results from the interaction between the organism and any environmental chemical, physical or biological agent (NRC 1987; WHO 1993). Biomarkers can be divided in three classes, according to their characteristics: biomarkers of exposure, effect and susceptibility. Biomarkers of exposure, resulting from the exposure to a particular exogenous substance, comprehend the direct detection of the substance or the products of their metabolism in the organism. Biomarkers of effect reflect the alterations in the organisms that may be associated to internal exposure damages, and biomarkers of susceptibility reflect individual susceptibility to respond to the exposure to xenobiotic substances (Benford et al. 2000, van der Oost et al. 2003). Thus, biomarkers are considered early-warning signs that provide a predictive perspective of the effects from pollution that in long-term can lead to deleterious effects at higher biological levels organization (Bucheli & Fent 1995, van der Oost et al. 2003). In fact, the important role of biomarkers for environmental risk assessment is recognised, and proposed as important tools in monitoring programs for Marine Strategy Framework Directive 2008/56/EC (MSFD) (Hagger et al. 2008; Sanchez & Porcher 2009; Schettino et al. 2012).

The exposure of animals to xenobiotics compounds and their metabolites contribute to enhanced oxidative stress when the generation of reactive oxygen species (ROS) overcomes their elimination capacity from the cell. Reactive oxygen species are naturally produced, as the result of aerobic metabolism, when instead of water, the partial reduction of oxygen in the electron transport chain, generates reactive intermediates such as superoxide ($O_2^{\bullet-}$) and hydroxyl (OH^{\bullet}) radicals or hydrogen peroxide (H_2O_2). However, since ROS are highly reactive and damaging, they are under tight control in the cell, whereas constant production and elimination mechanisms allows the maintaining the steady-state ROS concentrations and consequently the stability of the redox status (Martínez-Álvarez et al. 2005, Valavanidis et al. 2006, Lushchak 2011). Additionally, unstable environmental parameters such as temperature, salinity and oxygen concentrations may contribute to enhancement of ROS concentrations in the cell. To overcome major damages resulting from the exceeded ROS production such as oxidation of proteins, DNA, as well as peroxidation of unsaturated lipids in cell membranes (LPO), primary defence mechanisms such as antioxidant enzymes are triggered in the redox cycle (Figure 1.1), including superoxide dismutase enzymes (SOD) and catalase (CAT), that act as scavengers, intercepting and inactivating the reactive intermediates in the cell (Giulio et al. 1989, van der Oost et al. 2003, Lushchak 2011).

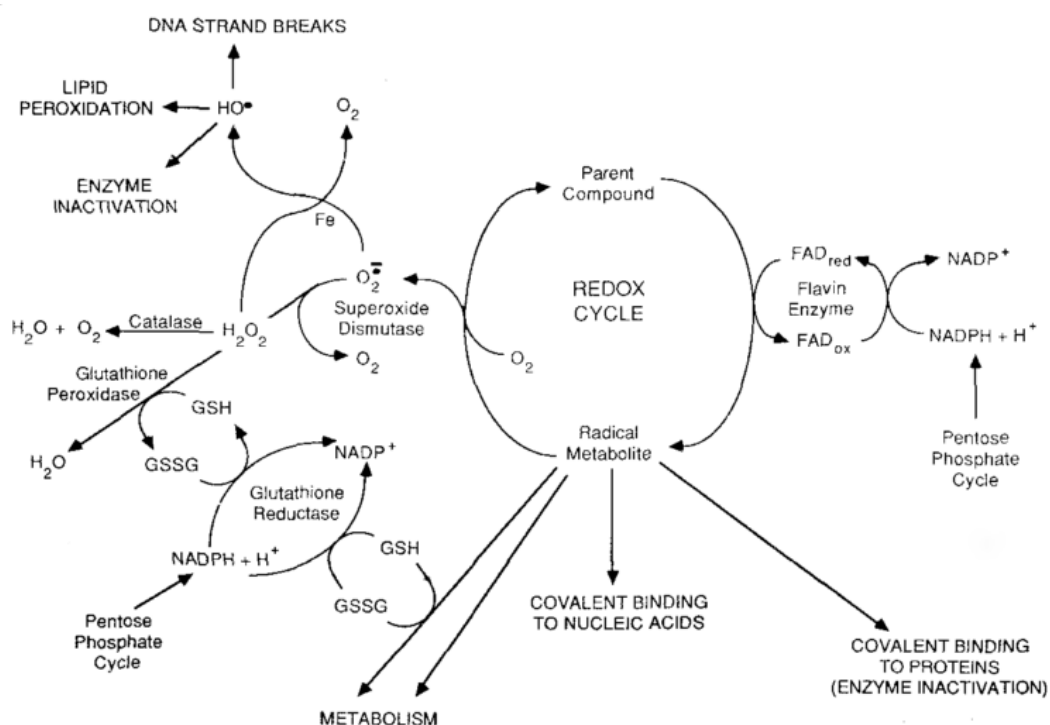


Figure 1.1 Illustration of the redox cycle of xenobiotic metabolism, associated antioxidant defence mechanisms and possible resultant damages. Adapted from Giulio et al. (1989).

SODs are metalloenzymes responsible for the conversion of highly toxic superoxide anions into H_2O_2 , which is later then reduced into oxygen and water by catalase or other glutathione dependent peroxidases (Figure 1.1). Since antioxidant enzymes are responsible for preventing the effects of ROS production through multiple environmental factors such as temperature, salinity variations or the presence of xenobiotics, they are considered non-specific biomarkers, with ubiquitous responses to stressors. Both SOD and CAT enzymes were found to be induced or inhibited by several compounds, such as PCBs, PAHs and metals, although some studies couldn't find any significant differences in laboratory assays, but in field studies, mostly SOD and CAT induction have been reported (van der Oost et al. 2003). Additionally, in the natural environment, marine organisms are exposed to tide variations in the intertidal areas, with severe temperature, salinity and oxygen fluctuations that have been associated with enhanced ROS production leading to increased oxidative stress and related damages (van der Oost et al. 2003, Martínez-Álvarez et al. 2005, González et al. 2015). Especially in ectotherms, temperature factor critically contributes to alterations in the equilibrium of steady-state ROS concentration, as higher temperatures increases metabolic processes and enhance oxygen consumption, increasing ROS production (Lushchak 2011). Salinity variations have also been associated to increased ROS production and enhanced antioxidant defence, as well as hyperoxia and anoxia periods (Storey 1996, Lushchak 2011, Canesi 2015).

Binding of xenobiotics to AhR, a cytosolic aryl hydrocarbon-receptor, induce a cascade of events that in the end leads to the expression of several genes, including CYP1A. CYP1A is a diverse family of cytochrome P450 proteins, localized in mitochondria and endoplasmatic reticulum membranes that reacts with several compounds, both endogenous and exogenous, in what is known as phase I reactions. Oxidation, hydrolyze and reducing reactions allow the organism to minimize the effects of xenobiotic compounds, altering their structure to more water-soluble compounds, now suitable for further metabolism or excretion (Bucheli & Fent 1995, Whyte et al. 2000, van der Oost et al. 2003).

Ethoxyresorufin-O-deethylase (EROD) activity describes the formation of resorufin, mediated by the CYP1A enzymes, whereas allowing the inference of the amount of this enzymes present in the cells. Since CYP1A concentrations tend to increase with input of xenobiotic compounds in the cells, EROD activity provides an indirect measure of exposition (Whyte et al. 2000, van der Oost et al. 2003). If after phase I, the compound is not yet able to be excreted, or secondary toxic metabolites are formed, then conjugation with phase II enzymes, such as GSTs (glutathione S-transferases), occurs in order to facilitate their excretion. Furthermore, some xenobiotics contain functional groups that allows direct metabolism by secondary phase enzymes therefore also playing an important role in detoxification. GSTs are responsible for the cytosolic conjugation of electrophilic compounds and metabolites with reduced glutathione (GSH), preventing oxidative damage (Sherratt & Hayes 2002, van der Oost et al. 2003).

EROD activity is known to be induced by several xenobiotic compounds, such as organic PAHs, PCBs and metals, but it is also known that environmental factors, such as temperature and pH, can affect EROD activity, though they must be taken in consideration (Whyte et al. 2000). Additionally, inhibition of EROD activity have also been reported in the presence of organic, organometallic, and metallic compounds (Bucheli & Fent 1995, van der Oost et al. 2003). Induction of GST is reported in several studies (e.g. Monteiro et al. 2007, Ahmad et al. 2008), as well as inhibition, probably due to GST direct binding to the xenobiotic compound or its metabolites (Almeida et al. 2012), or by the presence of antagonistic compounds in the environment (Quintaneiro et al. 2008, Maria, Ahmad, et al. 2009, Fonseca et al. 2011, Serafim et al. 2012).

Despite the enhanced defence mechanisms, when exposure to environmental contamination is constant and significant, organisms may be unable to completely inhibit the damages resulting from oxidative stress (Figure 1.1).

Lipid peroxidation (LPO) is a commonly used biomarker of effect, as it is directly related to oxidative stress and consists in the oxidation of polyunsaturated fatty acids mostly present in cellular membranes (Storey 1996, van der Oost et al. 2003). Lipid peroxidation comprises a cascade of reactions that start with the attack of lipids (LH) by a radical, creating an unstable carbon atom (L•). Prompt reaction of this molecule with oxygen will lead to the formation of a peroxy radical (LOO•) that will attack the adjacent lipid, creating an hydroperoxy radical (LOOH) as well as a radical oxygen species, establishing the LPO cycle (Storey 1996, Devasagayam et al. 2003, Valavanidis et al. 2006). The degradation of hydrocarbons will result in the formation of numerous products, such as malondialdehyde (MDA), usually used to quantify LPO levels in the cell. Membranes play a fundamental role in the cell, acting as a barrier in numerous organelles in the cell, allowing the maintenance of cellular processes. Though, the peroxidation of membrane lipids compromise the integrity and normal function of membranes, as well as their proteins (such as P450) with consequent disturbed homeostasis and cell death (Devasagayam et al. 2003).

The exposure to contaminants and oxidative stress can further lead to DNA damage, hence it is also frequently assessed as biomarker of effects. Several pollutants are known to form adducts, i.e. to covalently bind to DNA molecule, whilst other chemicals and free radicals induce the break of phosphodiester linkages of the DNA molecules, which is called strand breaks (Figure 1.1) (Shugart 2000, van der Oost et al. 2003, Monserrat et al. 2007). Additionally, changes in DNA base composition, creation of abasic sites (i.e. deletion of a base, resulting in a momentary break in the DNA chain) and interference with DNA processing, such as repair or replication, are also known effects (Shugart 2000). Generally, the defence mechanisms of the cell allow the repair of the mentioned alterations in DNA structure, although in some cases mutations can occur and become permanent with consequent adverse effects (Shugart 2000, Monserrat et al. 2007).

Estuaries sustain valuable ecologic resources, as highly productive systems and nursery areas for numerous marine species, providing favorable conditions for species survival and development such as abundant food resources and habitat refuge (Costa & Cabral 1999, Cooper 2003, Vasconcelos

et al. 2007). However, these systems are subjected to various anthropogenic pressures as the result of human settlement in the surrounding areas, inevitably spurring environmental degradation of these systems quality. Additionally, the presence of several economically valuable species leads to considerable resource exploitation in estuarine areas (Vasconcelos et al. 2007, França et al. 2012).

In this study, two Portuguese estuaries, Tejo and Ria de Aveiro, were selected based on their ecological importance and on the known degree of anthropogenic pressures. Tejo is one of the largest estuary in Europe, and the largest in Portugal, with a total area of approximately 320 km², with an important intertidal area of about 40%, 50 km long and mean depth of 5 meters (Vasconcelos et al. 2007). Surrounded by the populated city of Lisbon and two mainly important industrial areas, on the north, in Vila Franca de Xira and in the south in Barreiro, Tejo estuary receives the discharged domestic effluents from approximately 2.5 million habitants and effluents from different industries types such as chemical, petrochemical and metallurgic as well as agriculture. Additionally, important harbor and fishing activities, as well as construction of dams and infrastructures have contributed over the years to the increased degradation of the system (Cabral et al. 2001; Costa & Cabral 1999; Caçador & Duarte 2012; França et al. 2005). Despite recently reduced industries activities and effluent disposal in the estuary (Costa & Cabral 1999, França et al. 2005), as well as water treatment improvement, anthropogenic organic and metal contaminants are still present in the water and sediments (Fonseca et al. 2011, 2015; Serafim et al. 2012; Vale et al. 2008). Notably, several studies reported higher metal concentrations with anthropogenic origins in the industry nearby areas, as the result of metal accumulation in the sediments and retaining in salt marshes areas, especially Pb, Zn, Cu, As and Hg (Figueres et al. 1985; França et al. 2005; Vale et al. 2008). Furthermore, recent studies have reported significant metal contamination in tissues of several species, including significant concentrations in *S. plana*, *C. maenas* and *H. diversicolor*. Fish species, although with lower concentrations, also presented accumulated metals, with higher concentrations in *P. microps* than in *D. labrax* (França et al. 2005, Caçador et al. 2012).

Ria de Aveiro is a coastal lagoon with permanent contact with the sea, with 74km² of estuarine area, with a highly important 87% of intertidal area and low mean depths of 2 m (Vasconcelos et al. 2007). This lagoon has been impacted by several anthropogenic pressures, especially by discharges of chlor-alkali and pulp/paper plants industries, as well as shipbuilding activities, which contributed to the metal and organic contamination (Abreu et al. 2000, Oliveira et al. 2009, Pereira et al. 2009). Furthermore, the study of the anthropogenic impacts in this system, reported significant exploitation by fishing, aquaculture as well as intense agriculture in the nearby areas (Vasconcelos et al. 2007).

The interest in anthropogenic impacts in these systems is mainly associated to the important role of this estuaries as nursery areas for several fish and invertebrate species with ecological and economic value, which is the example of both Tejo and Ria de Aveiro, recognized nursery areas for several marine species, such as seabreams (e.g. *Diplodus vulgaris*), common and Senegalese sole (*Solea solea* and *Solea senegalensis*, respectively) and sea bass (*Dicentrarchus labrax*) (Cabral et al. 2001, França et al. 2005, Vasconcelos et al. 2010). A review of the anthropogenic stressors affecting these systems was conducted (Vasconcelos et al. 2007), and revealed several pressures in both systems, such as urban and industrial discharges, harbor activities, as well as resources exploitation, with the Tejo estuary characterized as highly pressured, whilst in Ria de Aveiro pressures were mostly related to resource exploitation (fishing, aquaculture and agriculture) and was comparatively less impacted. Nevertheless previous studies on chemical contamination of metals and PAHs were found in both Tejo sites, especially in the northern area, and to a lesser degree in the upper part of Ria de Aveiro (Fonseca et al. 2011, 2015). Furthermore, the Mira channel, a southern branch of Ria de Aveiro lagoon, has been considered the site with the lower sediment contamination and used as reference site in several studies (e.g. Ahmad et al. 2011; Serafim et al. 2012). In fact, metal

contamination levels were found to be significant and considered as potentially toxic to biological systems (Fonseca et al. 2011, 2015; França et al. 2005).

In order to understand how overall contamination present in these systems affects their inhabiting organisms, several species were selected based on their concomitant use of these estuaries. Thus, two fish species were selected, the European sea bass *Dicentrarchus labrax* (Linnaeus, 1758) which is a marine migrant species, abundant in the European coasts, whose juveniles use estuarine areas as nursery grounds and the common goby, *Pomatoschistus microps* (Krøyer, 1838), a resident estuarine species. Additionally, four invertebrate species were selected, two infaunal species, the bivalve *Scrobicularia plana* (da Costa, 1778) and the ragworm *Hediste diversicolor* (O.F. Müller, 1776) and two macroinvertebrates, the brown shrimp *Crangon crangon* (Linnaeus, 1758) and the green shore crab *Carcinus maenas* (Linnaeus, 1758). All species considered are common in Portuguese estuaries, and play important ecological roles in the community, and are considered suitable bioindicators of habitat quality that have been used in several ecotoxicological studies (e.g. Fonseca et al. 2011; Gomes et al. 2013; Maria et al. 2009; Quintaneiro et al. 2006; Silva et al. 2012). Carnivorous *P. microps*, and omnivorous *C. crangon* and *C. maenas* feed on a variety of epibenthic meiofauna organisms such as crustaceans, molluscs and annelids (Oh et al. 2001, Salgado et al. 2004, Baeta et al. 2006). These species live mostly in permanent contact with the sediment, and *P. microps* and *C. crangon* share burrowing habits that allow avoidance of predators. Carnivorous *D. labrax*, on the other hand, contacts with the sediment mostly in searching for food. *Scrobicularia plana* is a filter-feeding bivalve, that live mostly buried in the sediment and which siphons raise into the surface to filter the suspended matter present in the water (Hughes 1969). The annelid *H. diversicolor* also lives buried in the sediment and is an omnivorous species with multiple feeding modes that feed mostly on the organic content of the sediment (Costa et al. 2006).

Furthermore, an interesting overview of the effects of contamination is aimed to be achieved with a multi-taxa approach, where differences in taxa and consequently in physiology, as well as in habitat use or feeding habits, may be determinant of different levels of toxicity and responses. In fact, toxicity is known to be associated to several factors including inherent different physiology of the species, as well as lifestyle characteristics including habitat use, feeding behaviour, nutrition and reproduction (Lagadic et al. 1994, Bucheli & Fent 1995, Livingstone 1998, Hyne & Maher 2003, Martínez-Álvarez et al. 2005, Monserrat et al. 2007, Baun et al. 2008).

The aim of this study is to assess habitat quality of the mentioned estuarine Portuguese sites through an holistic approach, integrating a multi-biomarker response in a multi-taxa context, aiming at understanding the diversity in responses patterns among these species regarding differently impacted areas.

CHAPTER 2

Comparative analysis of biomarker responses to environmental contamination in estuaries: a multi-taxa approach

Comparative analysis of biomarker responses to environmental contamination in estuaries: a multi-taxa approach

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Abstract

Estuaries sustain valuable ecologic and economic resources, however they are subjected to numerous anthropogenic pressures with consequent environmental quality degradation. In this study, various biomarker responses were determined in various species: two fish species (*Dicentrarchus labrax* and *Pomatoschistus microps*), and four invertebrate species (*Carcinus maenas*, *Crangon crangon*, *Hediste diversicolor* and *Scrobicularia plana*); collected in two Portuguese estuaries (Ria de Aveiro and Tejo). Two sites in each estuarine system were selected based on previous characterization of sources of anthropogenic pressures (e.g. industrial, agricultural and shipping activities) and magnitude of environmental contamination. Multiple biomarker responses were determined, namely: superoxide dismutase (SOD), catalase (CAT), ethoxyresorufin O-deethylase (EROD) and glutathione S-transferase (GST) activities, as well as lipid peroxidation (LPO) and DNA damage (DNAd), in appropriate tissues for each species. Biomarker responses signaled environmental chemical exposure and some degree of deleterious effects in all species, especially in Tejo estuarine sites. Spatial variability in species responses was observed, likely due to both site contamination levels and to species-specific differences, such as habitat use, feeding habits and life-strategies. Overall, this multi-biomarker and multi-taxa approach provided important insights into the complexity of species response patterns to contaminants exposure and natural variability characteristic of the estuarine environment, and highlighted the difficulties associated with effective multi-species ecological risk assessments.

Keywords: Estuarine environmental quality; Biomarkers; Fish; Invertebrates; Integrated biomarker response index

2.1. Introduction

Coastal and estuarine areas are highly productive systems and play an important role as nursery areas for numerous marine species, providing favorable conditions for survival and development such as abundant food resources and habitat refuge, which is of great ecological value (Cooper 2003, Vasconcelos et al. 2010). The presence of several economically valuable species leads to significant resource exploitation in estuarine areas (Vasconcelos et al. 2007, França et al. 2012). Additionally, these systems are preferential locations for human settlement and associated anthropogenic activities (e.g. industries and tourism) which results in constant pressures and inevitably leads to environmental degradation of water and sediment quality.

The effects of chemical contaminants in aquatic organisms have been extensively studied, since aquatic systems are frequently impacted with discharges of human's activities (Livingstone 2003, van der Oost et al. 2003). Biomarkers are defined as measurable cellular, biochemical or molecular alterations in the organism in response to stressors, which signal early-on the interaction between organisms and contaminant compounds and are thus considered sensitive indicators of

primary exposure to environmental pollutants (van der Oost et al. 2003, Gagné 2014). Recently, the value of biomarker determination in environmental quality assessment has been recognized at the management level, with their inclusion as an indicator of health status in the Marine Strategy Framework Directive (MSFD) (Sanchez & Porcher 2009, Capela et al. 2016). Biomarker responses have been frequently used to assess environmental quality for several species, although the vast majority of works focus solely on the responses of one species, with few exceptions (e.g. Fernandes et al. 2002; Fonseca et al. 2015; Martín-Díaz et al. 2008). Akin to the use of a multi-biomarker approach in order to achieve a more comprehensive assessment of the biological effects of exposure to stressors; a multi-taxa approach should provide a more integrative view of environmental health, by encompassing species with diverse forms of biological integration of the environmental, accounting for multiple exposure routes and different species sensitivities (e.g. Fonseca et al. 2011; Solé et al. 2009).

Nonetheless, biomarker responses may be influenced by several factors such as environmental conditions, as well as on ecological and physiological features of the species considered; which is a significant source of variability in any assessment that needs to be addressed (van der Oost et al. 2003, Fonseca et al. 2015). Considering the high variability of abiotic conditions, characteristic of the estuarine environment, it is particularly important and yet extremely difficult, to discern between biological responses to natural variability and to anthropogenic stressors such as contaminants exposure (Elliott & Quintino 2007).

In this context, a multi-taxa approach could improve our understanding of the factors determining differences among species responses to contamination, particularly if integrating species with different habitat use and physiological features. Therefore, in the present study multiple biomarker responses of vertebrate and invertebrate species inhabiting the same estuarine areas were analyzed. Two fish species were considered, namely the European sea bass *Dicentrarchus labrax* which is a marine migrant species, whose juveniles use estuarine areas as nursery grounds; and the common goby *Pomatoschistus microps*, a resident estuarine species. Additionally, four invertebrate species were selected, two common infaunal species, the bivalve *Scrobicularia plana* and the ragworm *Hediste diversicolor* and two epibenthic crustaceans, the brown shrimp *Crangon crangon* and the green shore crab *Carcinus maenas*. These species are common in Portuguese estuaries and have been previously considered suitable bioindicators of habitat quality, subsequently used in ecotoxicological studies (e.g. Fonseca et al. 2011; Gomes et al. 2013; Maria et al. 2009; Quintaneiro et al. 2006; Silva et al. 2012).

In order to improve understanding of the biological impact of differently contaminated estuaries on the various species selected, a multi-biomarker approach was applied, with biological responses selected according to the anthropogenic pressures previously described for the sites considered. For each species the following biomarker responses were determined: antioxidant enzymes superoxide dismutase (SOD) and catalase (CAT) activity, which are critically involved in the reactive oxygen species (ROS) detoxification processes, avoiding oxidative stress; phase I ethoxyresorufin-O-deethylase (EROD) and phase II glutathione S-transferase (GST) biotransformation enzymes activity, that metabolize xenobiotics or their metabolites facilitating their excretion; and biomarkers of effects namely lipid peroxidation (LPO) and DNA damage (DNAd), that ultimately link exposure to contaminants to deleterious effects at the cellular level. In order to integrate all biomarker responses for the different species, Integrated Biomarker Response indexes were calculated for each species, allowing a species-specific classification of the different estuarine sites and enabling intraspecies comparisons of response patterns. Overall, the purpose of this work is to assess habitat quality of estuarine sites through an holistic approach integrating multiple biomarker responses in a multi-taxa context, aiming at understanding the diversity in responses patterns among species on differently impacted areas.

2.2. Materials and Methods

2.2.1. Study area and sampling survey

Two Portuguese estuaries were considered in this study, Ria de Aveiro and Tejo. Site selection was based on previous knowledge on major anthropogenic stressors (Vasconcelos et al. 2007), as well as on available information on species diversity and abundance; including the fact that these areas function as major nursery areas for various fish species (Cabral et al. 2007, Vasconcelos et al. 2010).

Tejo is the largest estuary in Portugal, delimited by the most populated city in the country and subject to a wide array of impacts, including domestic discharges, nearby industries, large shipping, port and dredging activities, all contributing to the classification of the most impacted Portuguese estuary according to Vasconcelos et al. (2007).

On the other hand, Ria de Aveiro is a coastal lagoon connected to the sea also with nearby urban and industrial areas, but mainly impacted by agriculture, fishing and aquaculture activities, thus it is considered less impacted compared with the Tejo estuary (Vasconcelos et al. 2007). The presence of complex contaminant mixtures in estuarine systems is well known, and has been described for both estuaries, including the presence of organic, metal and organometallic compounds (Vasconcelos et al. 2007, Fonseca et al. 2011, Serafim et al. 2012).

Two sites in each estuary were sampled in early June and late September 2015: in Ria de Aveiro, MUR - Murtosa and AVSUL - Mira Channel; in Tejo estuary, ALC – Alcochete and VFX – Vila Franca de Xira (Figure 2.1). The months considered allowed the sampling of juveniles of early recruits (before June) and after a period of growth and further exposure to contamination in the estuarine environment. Three replicate sediment samples were also collected from each site, for sediment chemical characterization (data not yet available).

Table 2.1- Mean (and standard deviation) temperature and salinity in Tejo (ALC and VFX) and Ria de Aveiro (AVSUL and MUR) estuarine sites, during sampling events in June and September 2015.

Month	Site	Temperature (°C)	Salinity
June	AVSUL	22 ± 0.0	10.8 ± 0.0
	MUR	24.5 ± 2.0	25.3 ± 1.8
	ALC	-	19.4 ± 0.0
	VFX	23.4 ± 1.0	14.7 ± 2.5
September	AVSUL	17 ± 0.0	21.0 ± 0.6
	MUR	18 ± 0.0	12.2 ± 1.5
	ALC	18 ± 0.0	22.7 ± 0.1
	VFX	18 ± 0.1	9.0 ± 2.4

Table 2.2 – Number (n), length, Lt (mm) and weight, Wt (g) (mean and standard deviation) of individuals from the six species collected in estuarine sites, for biomarker determination.

Site		Month	Species													
			<i>D. labrax</i>			<i>P. microps</i>			<i>C. maenas</i>		<i>C. crangon</i>		<i>S. plana</i>		<i>H. diversicolor</i>	
			n	Lt	Wt	n	Lt	Wt	n	Lt	n	Lt	n	Lt	n	Wt
ALC		June	25	73.5 ± 11	4.2 ± 1.8	25	32.8 ± 3.8	0.3 ± 0.1	25	38.2 ± 7.6	25	34.3 ± 1.8	29	33.9 ± 3.6	25	0.3 ± 0.1
		September	30	105.1 ± 9.9	11.4 ± 3.3	25	35 ± 2.7	0.4 ± 0.1	25	42.6 ± 4.6	25	34 ± 3.5	25	35.6 ± 2.1	28	0.4 ± 0.1
VFX		June	25	69.6 ± 7.7	3.4 ± 1.2	25	37.8 ± 3.9	0.5 ± 0.1	25	40.9 ± 8.3	25	34.6 ± 4.1	25	36.3 ± 2.3	25	0.3 ± 0.1
		September	28	115.3 ± 8.7	14.4 ± 3.1	25	37.2 ± 4.4	0.5 ± 0.2	25	39.4 ± 6.4	25	35.6 ± 5.4	25	39 ± 2.0	25	0.4 ± 0.1
AVSUL		June	31	49.9 ± 3.7	1.2 ± 0.2	25	40.4 ± 4.0	0.7 ± 0.2	27	38.3 ± 4.3	25	31 ± 4.7	30	32.6 ± 2.7	27	0.4 ± 0.1
		September	25	100.2 ± 5.6	11.2 ± 2.5	25	38.2 ± 2.6	0.6 ± 0.1	25	40.5 ± 8.6	30	33.3 ± 3.8	25	31.4 ± 2.2	25	0.4 ± 0.1
MUR		June	4	51.5 ± 1.5	1.3 ± 0.5	25	34.7 ± 4.2	0.4 ± 0.1	29	40.2 ± 5.6	25	34.4 ± 6.2	25	31.5 ± 4.8	28	0.2 ± 0.1
		September	25	97.9 ± 5.2	10.3 ± 1.9	25	36.1 ± 4.6	0.5 ± 0.2	25	41.1 ± 5.4	25	31.9 ± 4.1	25	34.5 ± 3.7	25	0.4 ± 0.1

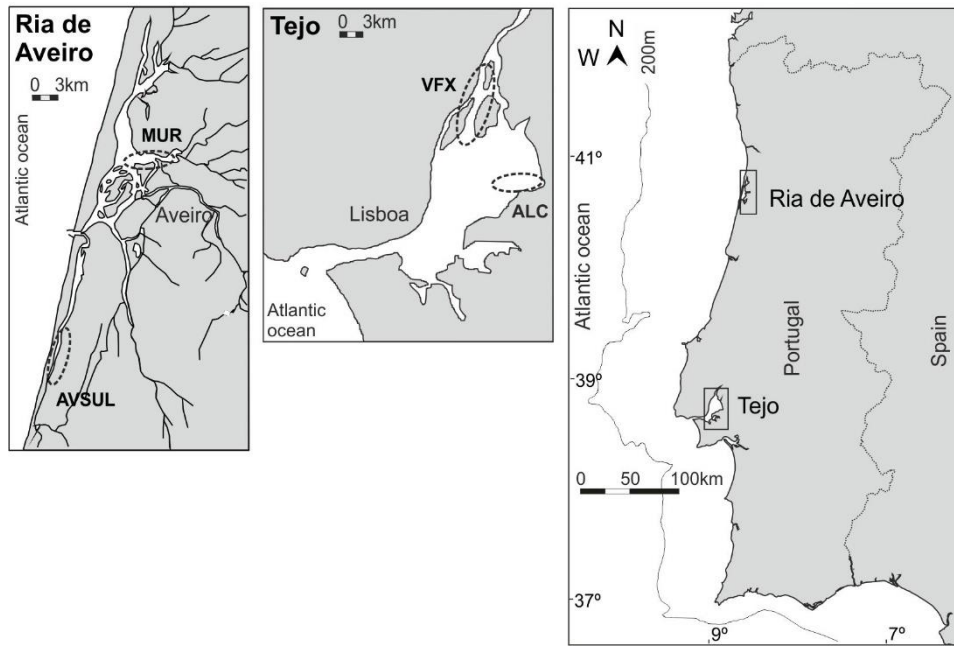


Figure 2.1 - Sampling sites at the two Portuguese estuaries, Ria de Aveiro lagoon (sites AVSUL and MUR) and Tagus estuary (sites ALC and VFX).

In order to produce a multi-taxa design, two fish species – *Dicentrarchus labrax* and *Pomatoschistus microps*, and four invertebrate species - *Crangon crangon*, *Carcinus maenas*, *Hediste diversicolor* and *Scrobicularia plana*, were targeted due to their abundance and previous use in ecotoxicological studies (e.g. Fonseca et al. 2010, 2011; Maranhão et al. 2014; Quintaneiro et al. 2006; Rodrigues et al. 2013; Solé et al. 2009). Fish and epibenthic invertebrate species' sampling was performed using a beam trawl in the selected estuarine areas during ebb-tide. Hauls were performed at a constant speed, for 10 min, and GPS coordinates registered. The two other invertebrate species were collected by hand at low tide in the intertidal areas of the sampling sites. Water temperature and salinity per site were determined (see Table 2.1) during fish sampling surveys using a multi-parameter probe.

Upon collection, individuals (ca. 25 per species and site) were transported to the laboratory where total length (Lt, with 1 mm precision) and/or weight (Wt, with 1 g precision) were recorded (see Table 2.2), and samples from muscle, brain (or head), liver (or digestive gland) or the whole individual were collected and immediately stored at -80°C .

2.2.2. Sampling procedure and biomarkers quantification

Biomarkers quantification was performed on three to eight replicate samples of suitable tissues, per sampling site and month, according to the invertebrate or fish species considered. Hence, individually collected samples of liver (*D. labrax*, *P. microps*), digestive gland (*C. maenas*, *S. plana*), abdomen (*C. crangon*) and portion of whole individual (*H. diversicolor*) were pooled per replicate (ca. 250-300 mg tissue) in order to determine all biomarkers considered in this study (catalase (CAT), superoxide dismutase (SOD), ethoxyresorufin-O-deethylase (EROD), glutathione S-transferase (GST), DNA damage (DNAd) and lipid peroxidation (LPO)).

Given small liver size in *P. microps*, pooled samples required more than 25 individuals (ca. 80 individuals). Concerning *C. crangon*, the antennae and exoskeleton surrounding the abdomen were removed prior to tissue homogenization, and only the soft tissue was used in the assays. Tissues were homogenized in 1:5 (w/v) of cold 100mM monobasic potassium phosphate/dibasic potassium phosphate (K_2HPO_4/KH_2PO_4) buffer (pH 7.4) containing 0.15 M KCl (potassium chloride), 0.1mM PMSF (phenylmethylsulfonyl fluoride), 1mM DTT (dithiothreitol) and 1mM EDTA (ethylenediaminetetraacetic acid), to avoid protein degradation.

Following homogenization, aliquots were separated for lipid peroxidation (LPO) and DNA damage (DNAd) assays. BHT (butylated hydroxytoluene) (1:15 (v/v sample)) was added to each LPO aliquot in order to prevent further lipid peroxidation until analysis.

The remaining homogenate was then centrifuged at 12000xg for 20 minutes at 4°C, and the post-mitochondrial supernatant (PMS) was aliquoted for superoxide dismutase (SOD), catalase (CAT), ethoxyresorufin-O-deethylase (EROD) and glutathione S-transferase (GST) assays.

In each step, additional aliquots were separated for the analysis of protein content.

All biomarker responses were determined in a microplate reader (Biotek Synergy HT), and each reading was done in triplicate.

i. Superoxide dismutase (SOD)

Superoxide dismutase activity was measured according to Mccord & Fridovich (1969), with slight modifications. Briefly, 50 mM phosphate buffer (pH 7.8) containing 0.1 mM EDTA, 1.5 mM Hypoxanthine, 0.15 mM cytochrome c and 30 mU/mL xanthine oxidase was used and SOD activity was determined by measuring the absorption of the reduction of cytochrome c by the xanthine oxidase/hypoxanthine system at a wavelength of 550 nm. One unit of SOD is the amount of the enzyme that inhibits the reduction of cytochrome c by 50%. SOD activity was expressed as U mg⁻¹ of total protein concentration.

ii. Catalase (CAT)

Catalase activity was determined according to Aebi (1974), measuring the consumption of the substrate, hydrogen peroxide (30 mM H₂O₂), that follows a decrease in absorbance at 240 nm. CAT activity was calculated as the difference in the absorbance per unit of time ($\epsilon=0.04 \text{ mM}^{-1}\text{cm}^{-1}$) and expressed as $\mu\text{mol min}^{-1}\text{mg}^{-1}$ of total protein concentration.

iii. Ethoxyresorufin-O-deethylase (EROD)

EROD activity was determined following the method described by Burke & Mayer (1974), with few modifications. The reaction was initiated by adding 100 mM phosphate buffer (pH 7.4), NADPH (8.33 mgmL⁻¹) and 7-ethoxyresorufin (0.1 mgmL⁻¹) to the microsomal fraction, and change in absorbance was followed at 550 wavelength, at 30°C. Activity was calculated as the amount of resorufin (pmol) generated per milligram of protein per minute of reaction time.

iv. *Glutathione S-transferase (GST)*

GST activity was measured according to Habig et al. (1974), following the conjugation of glutathione (GSH) with 1-chloro-2,4-dinitrobenzene (CDNB) in a final reaction mixture containing 100 mM phosphate buffer (pH 6.5), 20 mM CDNB and 20 mM reduced glutathione. The change in absorbance was recorded at 340nm, and the enzyme activity expressed as nmol CDNB conjugate formed per milligram of total protein per minute of reaction ($\epsilon=9.6 \text{ mM}^{-1}\text{cm}^{-1}$).

v. *Lipid peroxidation (LPO)*

Lipid peroxidation was determined according to Ohkawa et al. (1979), in which the products of the degradation of polyunsaturated fatty acid peroxides of membrane lipids, thiobarbituric acid reactive substances (TBARS), react with 2-thiobarbituric acid (TBA). The amount of TBARS was measured at 535 nm ($\epsilon = 1.56 \times 10^5 \text{ M}^{-1}\text{cm}^{-1}$) after the reaction occurred in a final reaction mixture containing 60mM Tris-HCl (pH 7.4), 0.1mM EDTA, TCA 12% and TBA 0.73%. LPO was expressed as nmol of TBARS formed per mg of protein.

vi. *DNA damage (DNAd)*

DNA damage was assessed through the DNA alkaline precipitation assay Olive (1988), which consists in DNA-protein precipitation followed by the determination of the damaged DNA concentration, which remains in the supernatant (strand breaks).

Samples were mixed with 2% SDS containing 10 mM EDTA, 10 mM Trisbase (pH 12.4) and 50 mM NaOH. After 1 min, 0.12 M KCl was gently added and the mixture incubated at 60°C for 10 min. Samples were cooled on ice for 15 min, and then centrifuged at 8000xg for 5 min (4°C). The supernatant was removed and DNA concentration was determined following the addition of Hoescht dye (1 $\mu\text{g}/\text{mL}$ in 0.1M K-phosphate buffer, pH 7.4), with fluorescence readings at 360 and 460nm of excitation and emission wavelengths, respectively. Fluorescence values were compared to a DNA standard curve and expressed as μg DNA per mg of protein.

vii. *Protein*

Samples' protein content (in mg) was determined following Bradford's procedure, using bovine serum albumin as standard. To each replicate sample, 250 μL of Sigma Bradford solution was added and the absorbance was read at 595 nm after 15 min of incubation.

2.2.3. Data analysis

Correlation analyses were performed to test possible relations between biomarker responses and individuals' biometrics, for each species. Few correlations were found to be significant and data was transformed, by subtracting the slope of the linear regression multiplied by the species length or weight (Vasconcelos et al. 2009). Despite the transformation, the resulting data was very similar to the initial, therefore raw data was used instead.

For each biomarker, normality and homogeneity of variances were verified and a log10 transformation was applied if necessary. Variation of biomarker responses between sites was analysed through a two-way analysis of variance (ANOVA). Whenever differences were significant, Tukey post-hoc test was applied to identify specific differences between sites, and months. Correlations between biomarker responses in each species were tested with Spearman rank correlation coefficient.

IBR (Integrated Biomarker Responses) indices were calculated according to Devin et al. (2013). The IBR is the sum of the area defined by the k biomarkers arranged in a radar diagram,

following a prior step of biomarker responses standardisation. Mean values (m) and standard deviations (s) for all sites in each biomarker were calculated. Mean values for a specific site (X) were then standardised through the equation: $Y=(X-m)/s$. According to the type biological process considered, either activation or inhibition, Y is transformed $Z=Y$ or $Z=-Y$, respectively. Afterwards, the biomarker score (S) is calculated by the addition of the absolute value of the minimum obtained for all sites to Z . Finally, IBR values for all biomarkers (k) result from the sum of A_i , calculated by the formula: $A_i = S_i \times S_{i+1} \times \sin(2\pi/k)/2$. Concordance among all IBR results for the six species was tested with a Kendall coefficient of concordance. A Spearman rank correlation coefficient was used to determine the relationship between all possible IBR pairs for all species. Differences were considered at the significance level of 0.05, in all tests, and analyses performed using R (RStudio Team, 2015).

2.3. Results

D. labrax antioxidant enzymes activity did not vary among sites ($F < 1.94$, $P > 0.05$), whereas biomarkers of effects and biotransformation enzymes responses evidenced spatial differences at the site level ($F > 7.29$, $P < 0.001$, Fig. 2.2). Higher LPO levels in *D. labrax* from MUR differed significantly from all other sites. A similar pattern was observed in sea bass GST activity, with significant higher levels in MUR compared to other sites, except ALC. *D. labrax* EROD activity was only similar in sites from Ria de Aveiro, with higher mean values in Tejo sites. The degree of liver DNAd was also higher in Tejo sites, yet the only significant spatial difference occurred between ALC and MUR (Table 2.3). Most *D. labrax* biomarker responses were consistently higher in September; except for EROD, where mean activity was significantly higher in June for ALC and AVSUL, and for CAT activity which did not vary between months (Table 2.3). Interaction effects between sites and months were observed for all biomarker responses in *D. labrax* ($F > 3.55$, $P < 0.05$, Table 2.3). Several correlations were found between biomarkers responses in *D. labrax*: GST was positively correlated to LPO ($r = 0.54$, $P < 0.001$) and SOD ($r = 0.38$, $P < 0.05$), and negatively correlated to both EROD and CAT ($r = -0.38$ and $r = -0.32$, respectively, $P < 0.05$). LPO and EROD showed a strong negative correlation ($r = -0.66$, $P < 0.001$), while DNAd was positively correlated to SOD ($r = 0.47$, $P < 0.001$) and EROD ($r = 0.31$, $P < 0.05$).

In general, higher mean values of biomarker responses in *P. microps* were observed in Tejo estuarine sites, particularly considering biotransformation enzymes activities (Fig. 2.3). Spatial variability was observed for *P. microps* CAT, EROD, GST, LPO and DNAd levels ($F > 3.09$, $P < 0.05$), but not for SOD activity ($F < 2.59$, $P > 0.05$) (Table 2.3). *P. microps* biomarker responses were significantly different between months considering LPO and CAT activity, in which September mean values were higher, and for SOD activity for which the opposite was observed ($F > 25.22$, $P < 0.001$). As for *D. labrax*, significant interaction effects between sites and months were observed for all biomarkers analysed in *P. microps* ($F > 6.02$, $P < 0.01$). Various correlations were found to be significant between biomarker responses in *P. microps*. Biotransformation enzymes, EROD and GST, evidenced a strong positive correlation ($r = 0.69$, $P < 0.001$), whilst antioxidant enzymes, CAT and SOD, were negatively correlated ($r = -0.48$, $P < 0.01$). GST was positively correlated with DNA damage ($r = 0.39$, $P < 0.05$), yet EROD and LPO showed the inverse trend ($r = -0.40$, $P < 0.05$). CAT activity was negatively correlated with both GST and DNA damage ($r = -0.44$, $P < 0.05$ and $r = -0.65$, $P < 0.001$, respectively).

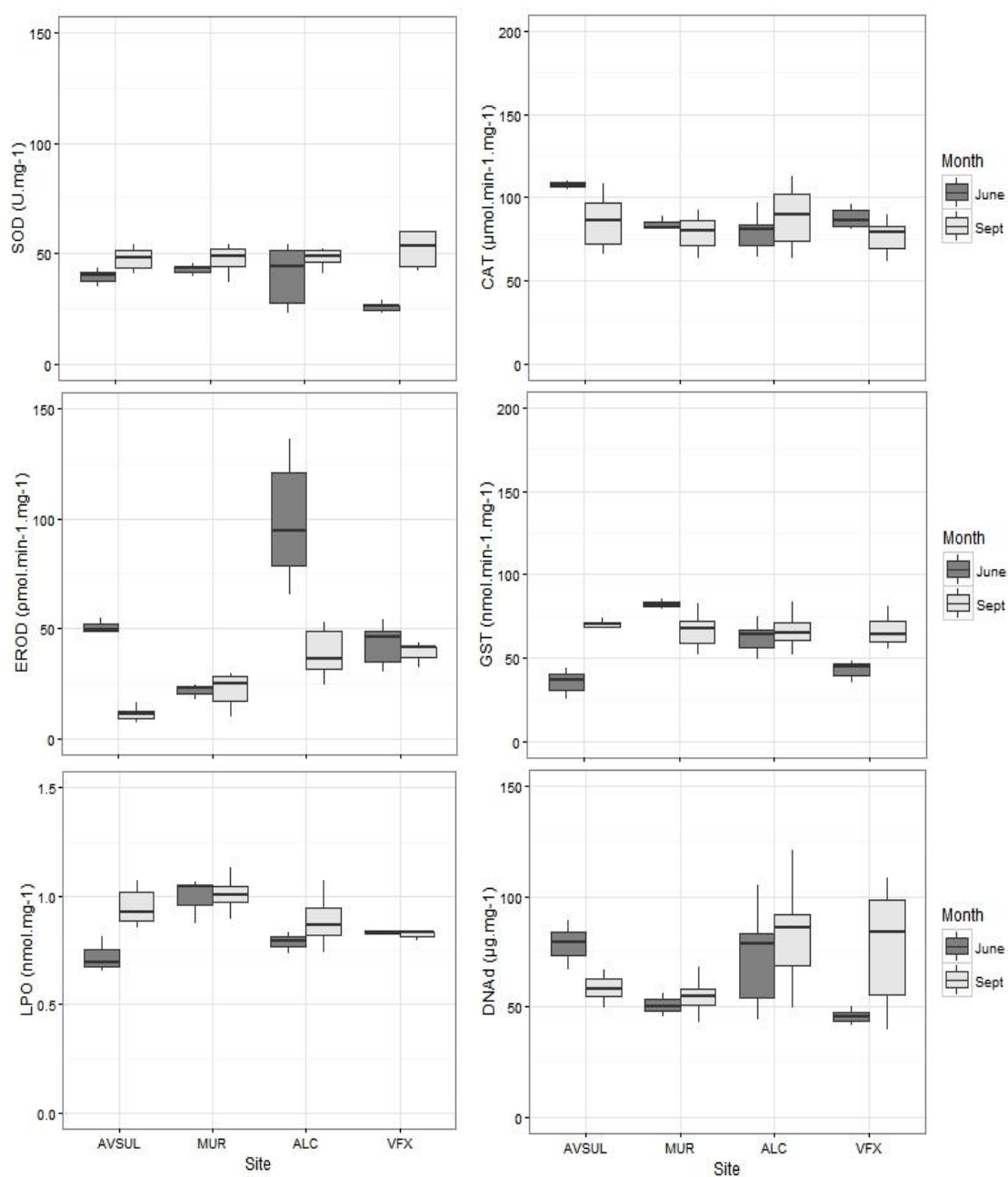
Dicentrarchus labrax

Figure 2.2 - Biomarker responses of *D. labrax* (boxplot with mean, 25th and 75th percentiles; whiskers represent minimum and maximum values) collected in June and September 2015 in two Portuguese estuaries: Ria de Aveiro (AVSUL and MUR) and Tejo (ALC and VFX).

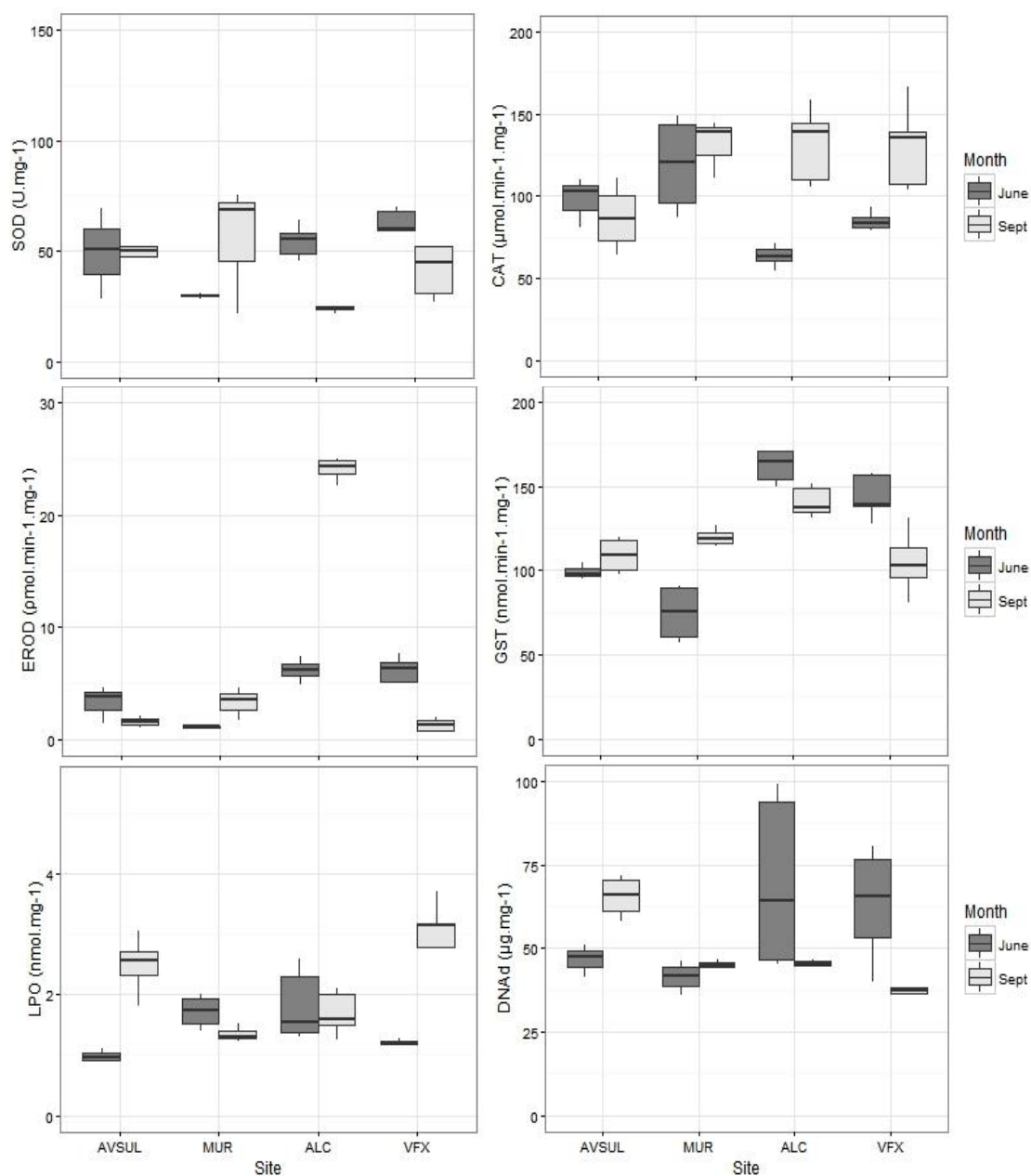
Pomatoschistus microps

Figure 2.3 - Biomarker responses of *P. microps* (boxplot with mean, 25th and 75th percentiles; whiskers represent minimum and maximum values) collected in June and September 2015 in two Portuguese estuaries: Ria de Aveiro (AVSUL and MUR) and Tejo (ALC and VFX).

In *C. maenas* all biomarkers varied among sites, except for SOD activity, with frequent differences found between MUR and Tejo estuary sites (Fig. 2.4). CAT activity and DNA damage levels were higher in MUR, which exhibited the lowest mean values for GST and LPO (Fig. 2.4, Table 2.3). Nonetheless, only GST activity differed between months ($F=4.23$, $P<0.05$), with higher values in September. Additionally, interaction effects were only significant for three biomarkers, namely LPO, DNA damage and SOD activity ($F>4.42$, $P<0.01$, Table 2.3). Significant positive correlations were found between biomarkers of effects, DNAd and LPO ($r=0.28$, $P<0.05$), and between each one independently with SOD ($r=0.31$, $P<0.05$ and $r=0.53$, $P<0.001$, respectively). GST was negatively correlated with DNA damage ($r=-0.47$, $P<0.001$), CAT ($r=-0.33$, $P<0.05$) and with EROD activity in *C. maenas* ($r=-0.30$, $P<0.05$).

Carcinus maenas

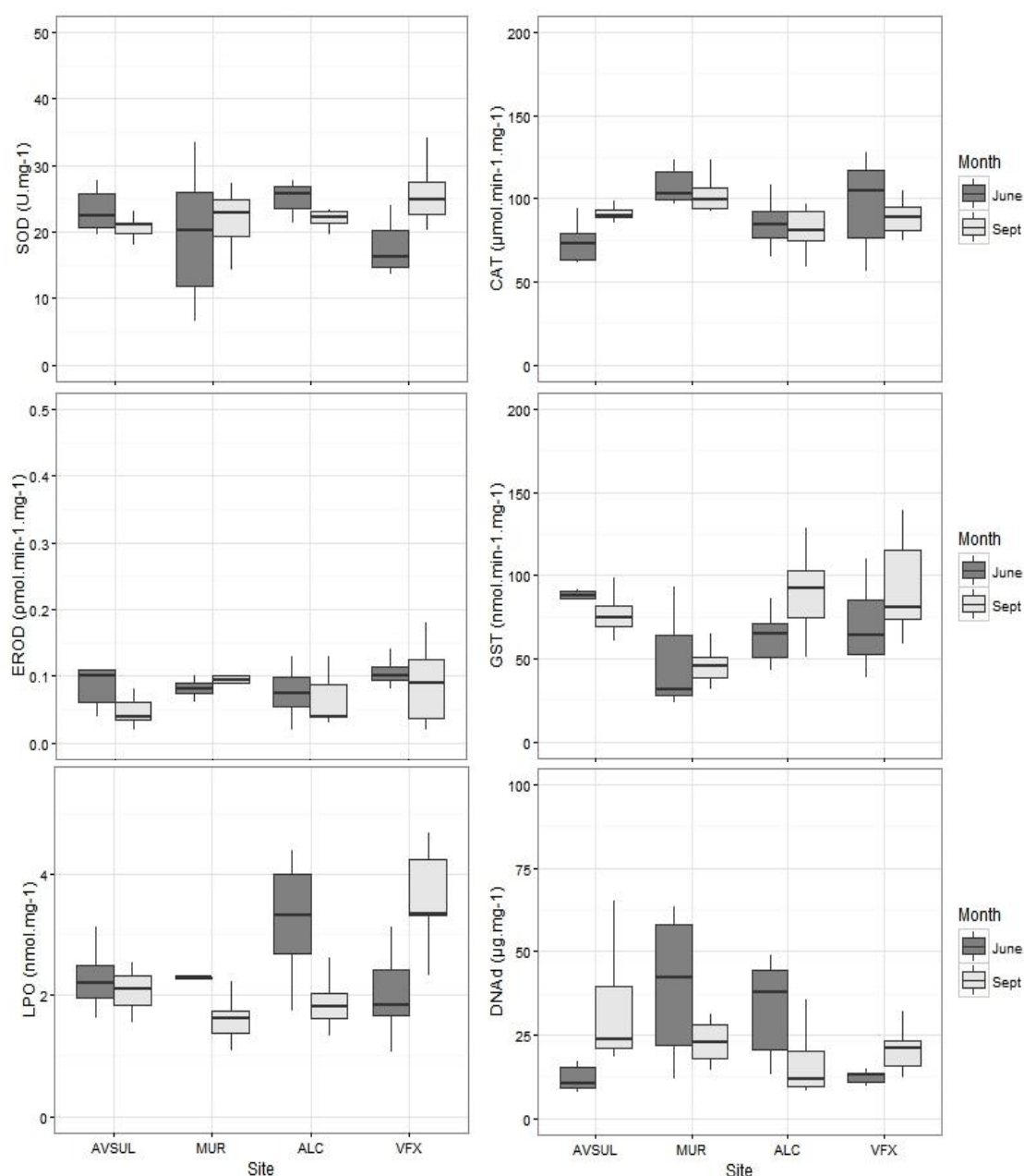


Figure 2.4 - Biomarker responses of *C. maenas* (boxplot with mean, 25th and 75th percentiles; whiskers represent minimum and maximum values) collected in June and September 2015 in two Portuguese estuaries: Ria de Aveiro (AVSUL and MUR) and Tejo (ALC and VFX).

For *C. crangon* no variability between sites in LPO levels and between sites and months for biotransformation enzymes activity were observed ($F < 3.09$, $P > 0.05$, Table 2.3). Also, interaction effects between sites and months were only evident for DNAd ($F = 4.36$, $P < 0.01$). Nevertheless, differences between sites were significant for both antioxidant enzymes and biomarkers of effects (Table 2.3). *C. crangon* biomarker responses in September tended to be higher than those in June (Fig. 2.5), although significant differences were only observed for CAT, LPO and DNA damage ($F > 4.30$, $P < 0.05$, Table 2.3). A similar variation pattern was observed for almost all biomarkers, with persistently higher mean values in MUR compared to Tejo sites, especially in September (Fig. 2.5). Positive correlations were found between SOD and both GST ($r = 0.43$, $P < 0.01$) and EROD activities ($r = 0.32$, $P < 0.05$), and between LPO and DNAd ($r = 0.37$, $P < 0.01$).

Crangon crangon

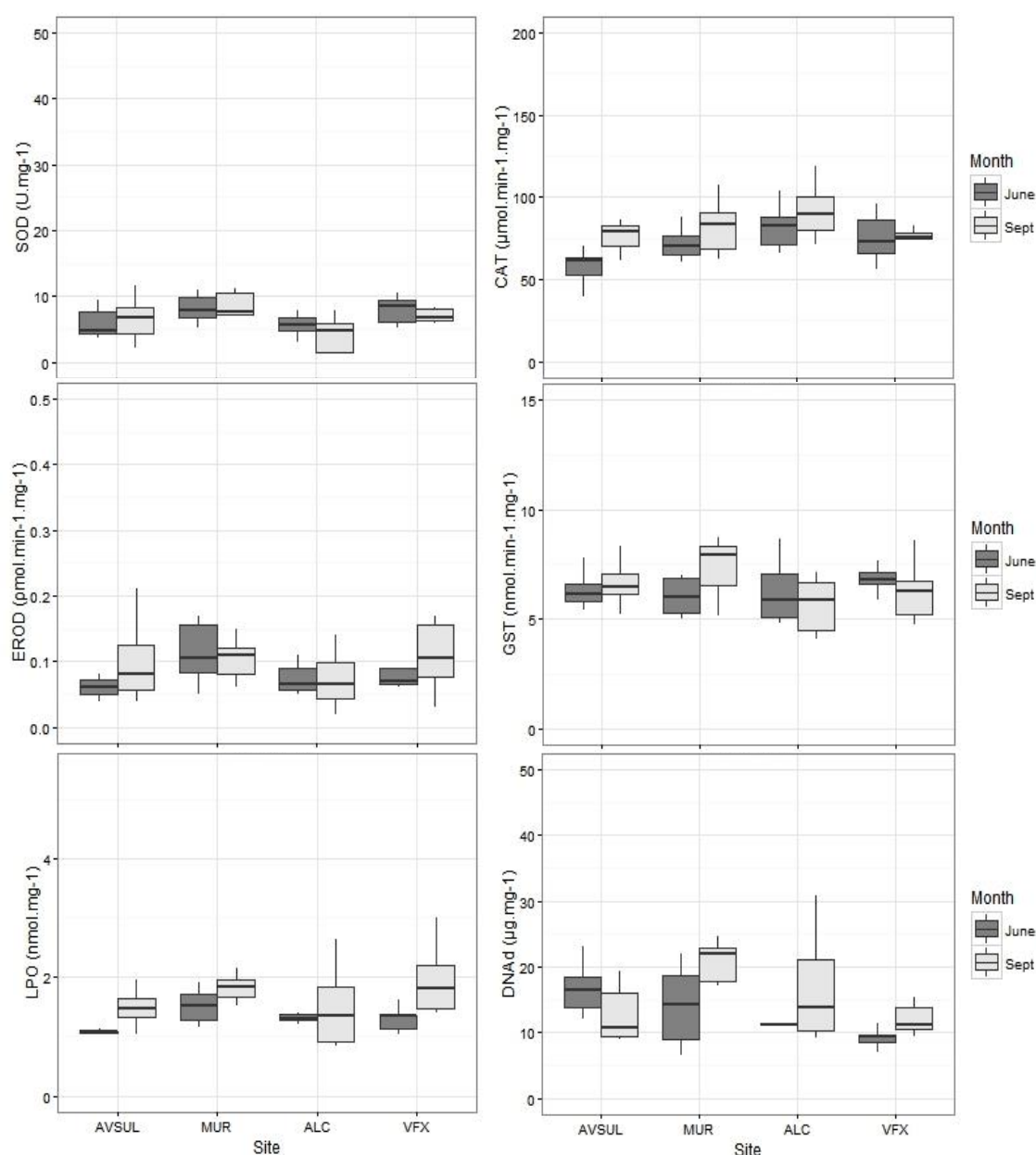


Figure 2.5 - Biomarker responses of *C. crangon* (boxplot with mean, 25th and 75th percentiles; whiskers represent minimum and maximum values) collected in June and September 2015 in two Portuguese estuaries: Ria de Aveiro (AVSUL and MUR) and Tejo (ALC and VFX).

For *H. diversicolor*, spatial variability was observed in SOD, GST, LPO and DNAd responses ($F > 7.16$, $P < 0.001$). LPO levels showed a clear distinction between Tejo and Ria de Aveiro sites ($F = 7.16$, $P < 0.001$), with higher values in the latest, while for DNA damage VFX was significantly higher than all sites ($F = 7.60$, $P < 0.001$). Both antioxidant enzymes and GST responses followed a similar pattern, with constantly higher mean values in AVSUL, and lower in MUR (Fig. 2.6). Differences between months were found significant only for CAT responses ($F = 4.03$, $P < 0.05$) and interaction effects between sites and months were significant for all biomarkers ($F > 2.80$, $P < 0.05$), except for SOD ($F = 1.65$, $P > 0.05$) (Fig. 2.6, Table 2.3). In *H. diversicolor* GST activity responses were found to be positively correlated to SOD ($r = 0.52$, $P < 0.001$), and negatively correlated to DNAd ($r = -0.30$, $P < 0.05$).

Hediste diversicolor

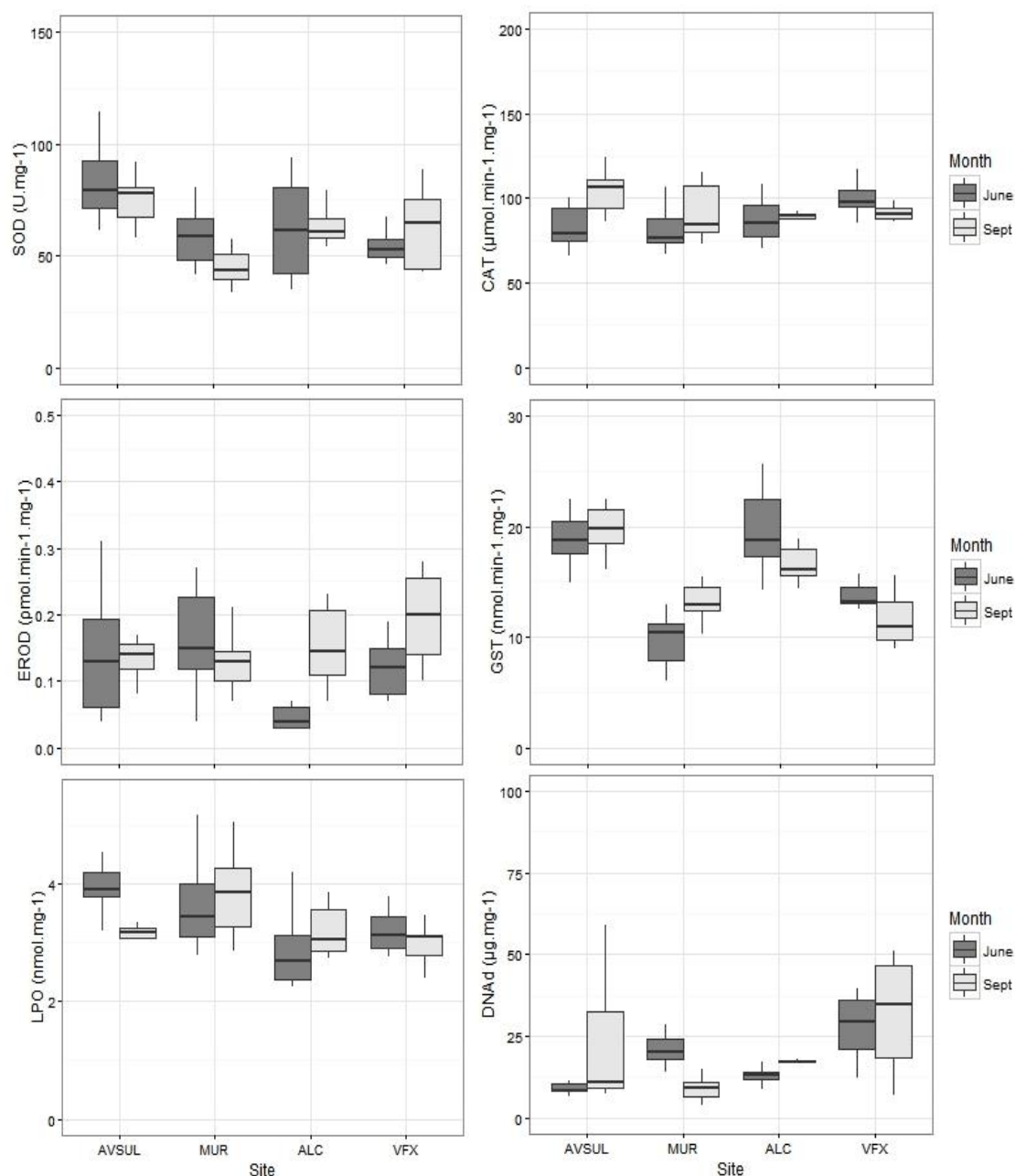


Figure 2.6 – Biomarker responses of *H. diversicolor* (boxplot with mean, 25th and 75th percentiles; whiskers represent minimum and maximum values) collected in June and September 2015 in two Portuguese estuaries: Ria de Aveiro (AVSUL and MUR) and Tejo (ALC and VFX).

In *S. plana*, several biomarker responses varied among sites ($F>6.43$, $P<0.01$), whilst significant differences between months were only observed for antioxidant enzymes activity, with higher values in September (Table 2.3, Fig. 2.7). DNA damage was considerably higher in both Tejo sites (Fig. 2.7). Significant interaction effects between sites and months were also observed for all biomarkers except for EROD activity ($F>4.11$, $P<0.05$, Table 2.3). EROD activity in *S. plana* did not vary also among sites and months considered ($F=0.29$, $P>0.05$). DNA damage values in *S. plana* were positively correlated with LPO ($r=0.35$, $p<0.05$) and CAT ($r=0.36$, $p<0.05$) and negatively with GST ($r=-0.42$, $p<0.05$). Also, SOD stands positively correlated to EROD ($r=0.37$, $p<0.05$).

Scrobicularia plana

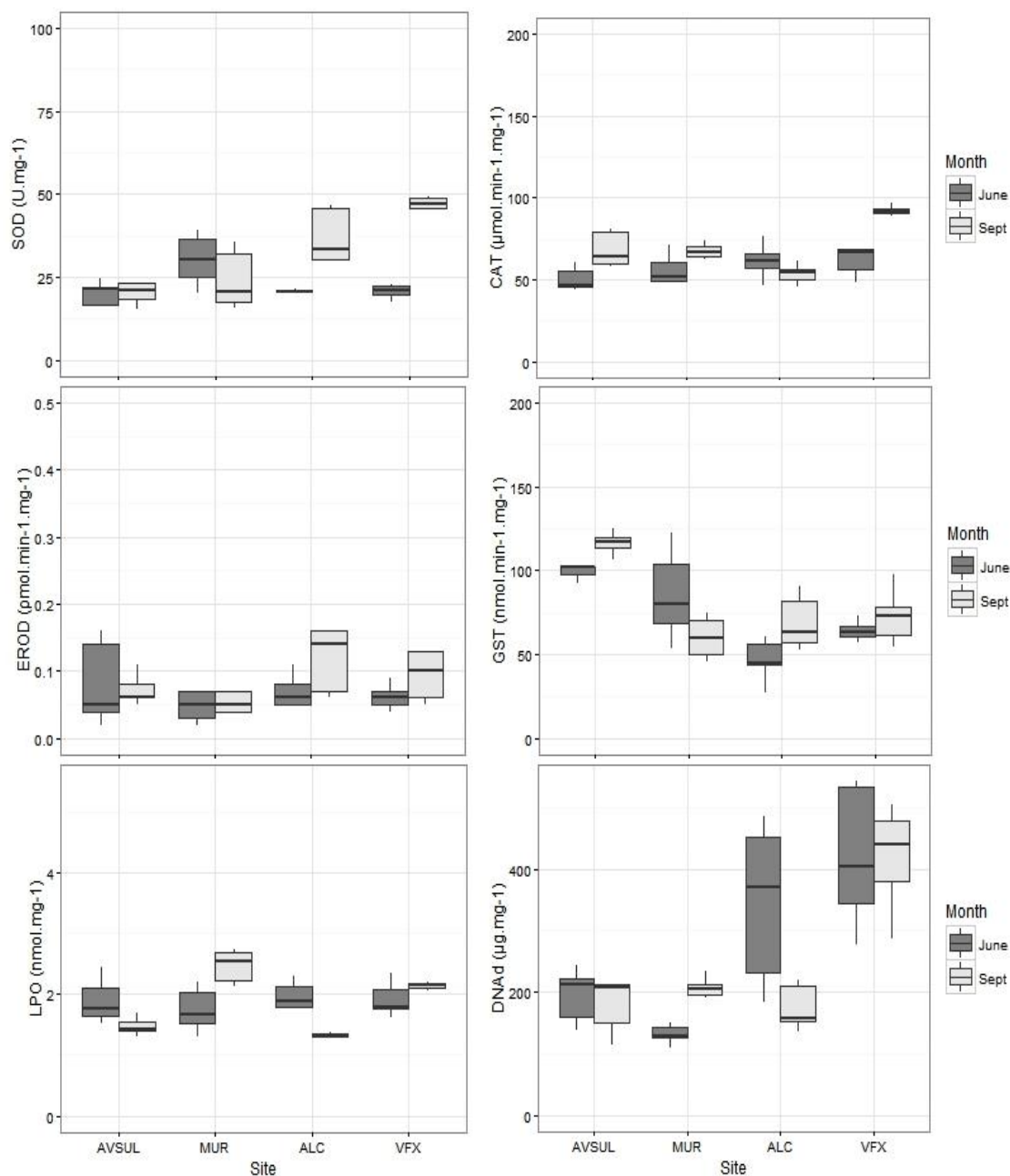


Figure 2.7 - Biomarker responses of *S. plana* (boxplot with mean, 25th and 75th percentiles; whiskers represent minimum and maximum values) collected in June and September 2015 in two Portuguese estuaries: Ria de Aveiro (AVSUL and MUR) and Tejo (ALC and VFX).

Integrated biomarker response indexes (IBR), calculated for each of the six species, were not concordant across taxa (Kendall coefficient of concordance = 0.08; $P > 0.05$). Nevertheless, significant negative correlations between species were observed, namely between *D. labrax* and *H. diversicolor* and *D. labrax* and *S. plana* (Spearman rank correlation coefficient -0.71 and -0.76, respectively, $P < 0.05$).

Despite overall IBR values' variation among sites for each species (Fig. 2.8), the estimated high impacted sites differed considerably among the species considered. In *D. labrax*, IBR values were quite similar among sites, with lower scores found in VFX. On the contrary, *P. microps* presented high spatial variability, with the highest score in ALC in September. In *C. maenas*, responses varied among sites and sampling events, yet VFX and MUR were considered the most impacted areas, although in different months. *C. crangon* showed consistently higher scores in September, with MUR signalled as the most impacted site, followed by ALC. *S. plana* and *H. diversicolor* showed spatial variability, with higher IBR levels in VFX, especially in June.

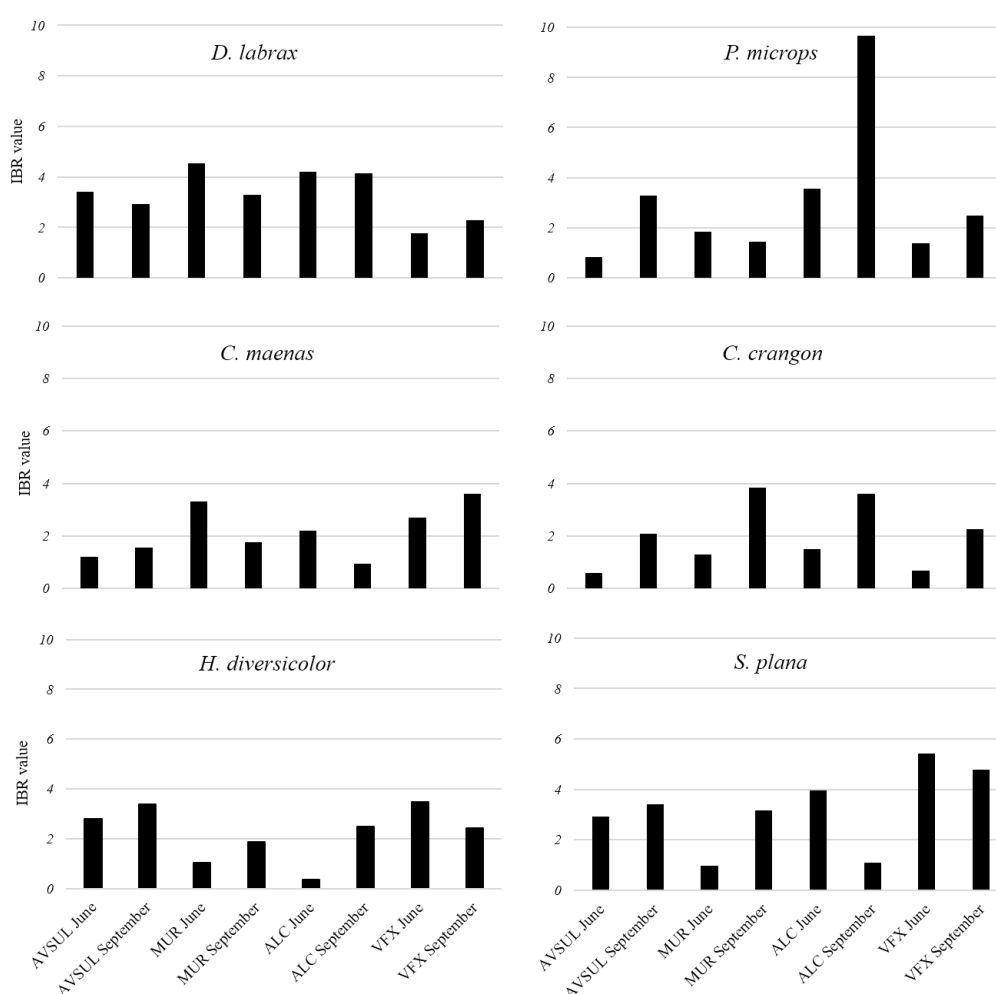


Figure 2.8 - Integrated biomarker responses (IBR) calculated for all sites in June and September, for each species: *D. labrax*, *P. microps*, *C. maenas*, *C. crangon*, *H. diversicolor* and *S. plana*.

S. plana and *H. diversicolor* also showed the same pattern for all sites with consistently higher values in September, except in ALC. *C. crangon* and *P. microps* also shared similar patterns among sites, except in MUR, where the highest value was observed for *C. Crangon* in September.

From the analyses of the radar plots produced for the IBR determinations (figures provided as supplementary material), biomarker contributions to final IBR values differed among species and sites. In general, for *D. labrax* and *P. microps*, GST and CAT responses seem to be determinant, while for

H. diversicolor, biotransformation enzymes and SOD activity are the highest contributing responses. IBR scores in *S. plana* depended mainly on SOD and GST responses, while for *C. crangon* most important responses were from biomarker of effects and CAT activity. *C. maenas* showed great variability in response patterns, with IBR scores deriving from different biomarkers for each site.

Table 2.3 – Different letters indicate significant differences, from post hoc comparison Tukey tests (a,b,c) following a two-way analysis of variance for each biomarker response per species. Asterisks indicate significant interaction effects (*, **, *** correspond to $P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively); and ‘-’ indicates no significant differences observed.

		Site				Month		Interaction site
		AVSUL	MUR	ALC	VFX	June	Sept.	x month
<i>D. labrax</i>								
	SOD	-	-	-	-	a	b	**
	CAT	-	-	-	-		-	*
	EROD	a	a	b	c	a	b	***
	GST	a	b	-	a	a	b	***
	LPO	a	b	a	a	a	b	*
	DNAd	-	a	b	a	a	b	**
<i>P. microps</i>								
	SOD	-	-	-	-	a	b	***
	CAT	a	b	a	-	a	b	**
	EROD	a	a	b	a		-	***
	GST	a	a	b	c		-	***
	LPO	-	a	-	b	a	b	***
	DNAd	-	-	-	-		-	**
<i>C. maenas</i>								
	SOD	-	-	-	-		-	**
	CAT	a	b	a	-		-	-
	EROD	a	-	-	b		-	-
	GST	a	b	a	a	a	b	-
	LPO	-	a	b	b		-	***
	DNAd	-	a	-	b		-	***
<i>C. crangon</i>								
	SOD	-	a	b	a		-	-
	CAT	a	-	b	-	a	b	-
	EROD	-	-	-	-		-	-
	GST	-	-	-	-		-	-
	LPO	-	-	-	-	a	b	-
	DNAd	a	a	-	b	a	b	**
<i>H. diversicolor</i>								
	SOD	a	b	b	b		-	-
	CAT	-	-	-	-	a	b	*
	EROD	-	-	-	-		-	*
	GST	a	b	a	b		-	**
	LPO	a	a	b	b		-	*
	DNAd	a	a	a	b		-	***
<i>S. plana</i>								
	SOD	a	-	b	b	a	b	***
	CAT	a	a	a	b	a	b	***
	EROD	-	-	-	-		-	-
	GST	a	b	b	b		-	*
	LPO	a	b	a	-		-	***
	DNAd	ab	b	a	c		-	***

2.4. Discussion

Previous environmental chemical characterizations indicated that Tejo and Ria de Aveiro are differently impacted, with higher impacts reported for the Tejo estuary, following Vasconcelos et al. (2007) which extensively assessed multiple anthropogenic pressures in estuarine systems along the Portuguese coast. Regarding metal contamination, however, both estuaries were considered equally impacted by Vasconcelos et al. (2007), while other authors reported higher chemical and metal contamination in Tejo sites in comparison to the Ria de Aveiro sites sampled in this study (Fonseca et al., 2015; Serafim et al., 2012). These studies reported major metal contribution from Zn and Pb in Tejo (ca. 202-269 mg/kg and 57-128 mg/kg, respectively) and Zn and Cr in Ria de Aveiro (ca. 96-258 mg/kg and 42-50 mg/kg, respectively), as well as major petrogenic but also pyrolytic PAHs. Additionally the Mira channel (AVSUL) has been described as reference or a low impacted site based on sediment chemical characterization (Ahmad et al., 2011; Fonseca et al., 2011; Serafim et al., 2012). According to the contamination profile described above, it would be expected to observe significantly different and generally higher biomarker responses in organisms collected in the Tejo estuarine sites when compared to Ria de Aveiro sites, particularly considering AVSUL as the least impacted site.

In general, biomarker responses signaled environmental chemical exposure and some degree of deleterious effects in all species studied. Overall, biomarker responses were within range or slightly higher compared to previously reported values in both field and laboratory assays for all species (e.g. Ben-Khedher et al. 2013; Buffet et al. 2011; Durou et al. 2007; Fonseca et al. 2015; Fonseca et al. 2011; Quintaneiro et al. 2006; Serafim et al. 2012; Silva et al. 2012). However, in this study there are some first records for *C. crangon*, which to our knowledge have not been previously analyzed in this species, namely LPO and DNA damage, although it has in other shrimp species such as *Litopenaeus vannamei* and *Xiphopenaeus kroyeri*, with different methodology (Rocha et al. 2012; Zenteno-Savín et al. 2006). Nevertheless, increases in both biomarkers of effects were observed along with the exposition to different stresses in those species.

Antioxidant enzymes responses showed low variability among sites and months for most species. Generally high antioxidant enzymes responses were observed in both fish species, although with low variability among sites and months, suggesting a general response to oxidative stress. Yet, different sites were signaled as higher impacted sites considering oxidative stress responses for each fish species. Among invertebrate species, low spatial variability was also found in antioxidant enzymes responses, although no clear patterns could be found among species. In spite of low variability, all species showed higher antioxidant activities in September. Higher water temperatures can induce higher ROS production, through increased metabolism and oxygen consumption (van der Oost et al. 2003). Nevertheless, during September mean water temperatures were lower, whereas salinity variation was similar between sampling months, suggesting that higher antioxidant responses in this period could reflect added exposure to other stressors (e.g. contaminants).

In fish species, induction of both biotransformation enzymes was observed with significant spatial variability, with overall concordant higher levels in Tejo estuary sites for both species, particularly in ALC. Fonseca et al. (2011) also reported higher EROD and GST activity in the Tejo estuary when compared to Ria de Aveiro for both fish species, which they associated to the presence of high molecular weight PAHs, known to induce biotransformation enzymes (Almeida et al. 2010, 2012; Danion et al. 2014). Furthermore, in *P. microps* positive correlation between biotransformation enzymes suggests simultaneous induction, signaling xenobiotics exposure. Yet, in *D. labrax* and *C. maenas* a negative correlation was found between EROD and GST, highlighting species-specific differences, from a biological point of view or from differences in exposure related to different habitat use (Fonseca et al. 2011). Phase II enzymes response patterns, such as GST, are generally less evident

than CYP1A (Livingstone 1998, van der Oost et al. 2003). Accordingly, lower variability of GST activity was found when comparing to EROD activity in fish species, which may be related to the influence of natural variability, such as temperature and nutrition, as well as by the presence of inhibitor compounds (Livingstone 1998, van der Oost et al. 2003).

Biotransformation enzyme responses in invertebrate species were less marked than in fish species. Biotransformation and elimination mechanisms are known to have considerably lower efficiency in invertebrate species in comparison to most fish species (Hyne & Maher 2003; Lagadic et al. 1994; Livingstone 1998). Accordingly, either incipient or very low spatial and temporal variability in EROD activity was observed in all invertebrate species. These significantly low EROD activity levels may result from inherent low enzyme expression that consequently leads to technical constraints in quantification, considering generally low expression levels of these proteins in those species. Nevertheless, these species signaled mostly Tejo sites according to phase I enzyme responses, especially VFX, which is in agreement with previously reported overall contamination levels. Consequently, higher variability in GST activities in invertebrates was observed among sites when compared to EROD activities, which was also observed in previous studies in molluscs and annelids (Sheehan & Power 1999, Pérez et al. 2004). A similar GST response pattern was observed for *H. diversicolor*, *S. plana* and *C. maenas*, identifying higher contamination levels in AVSUL. However, lower levels of activity in highly contaminated Tejo sites could be related to the presence of known inhibitory compounds, such as metals (e.g Goodrich & Basu 2012; Brüschweiler et al. 1996; Oliveira et al. 2004). In general, despite all four invertebrate species presented low temporal variability, higher values could be found in September months for all of them, which may reflect the effects of continuous exposure in those species.

Several significant correlations were observed between biotransformation enzymes and biomarkers of effects for both invertebrate and fish species. EROD activity was negatively correlated with LPO levels in both fish species, apparently contributing towards preventing oxidation damage in the cell. However, higher DNA damage was concordant with higher EROD and GST activities in *D. labrax* and *P. microps*, respectively, due to overt toxicity or a delay in the detoxification response which didn't prevent deleterious molecular effects. Negative correlations between GST and DNA damage were found in *C. maenas*, *H. diversicolor* and *S. plana*, revealing the contribution of GST in preventing the attack of free radicals to DNA, and consequent oxidative damage. Notwithstanding the significant activity of detoxification enzymes, deleterious effects were reported for all species, suggesting an overall contamination level above the capacity of these molecular defence mechanisms in order to completely constrain exposure effects in these species.

Biomarkers of effects response patterns differed amongst species, yet akin LPO variability patterns were observed among groups of species, namely between *C. crangon*, *C. maenas* and *P. microps*, signalling VFX and *D. labrax*, *H. diversicolor* and *S. plana*, signalling MUR. Most species also showed higher mean DNA damage values in VFX, except for *C. crangon* and *C. maenas* that signalled MUR. These similarities may be related to inherent physiological features and similar habitat use, as discussed above, but also to feeding habits. Among other factors, feeding behaviour (which is directly related to contaminant uptake) is known to influence biomarker responses in both fish and invertebrate species (Livingstone 1998; Martínez-Álvarez et al. 2005; Sheehan & Power 1999).

A positive correlation between biomarkers of effects was observed for all invertebrate species (except *H. diversicolor*), highlighting site-specific contamination. Since sediment can function as a sink, where contaminants are retained and concentrate, sediment-dwelling species as well as epibenthic species with low mobility are more likely to accumulate higher levels of contaminants (Monserrat et al. 2007, Baun et al. 2008). This could contribute to greater levels of deleterious effects observed in these species, as contaminants exposure is quite inevitable and more persistent than for mobile species. Additionally, infaunal species are exposed to tide variations in intertidal areas, with

severe temperature, salinity and oxygen fluctuations, which has been associated with enhanced ROS production, leading to increased oxidative stress and related damages (van der Oost et al. 2003, Martínez-Álvarez et al. 2005, González et al. 2015).

The complexity of biomarker response patterns for all species in this study was evident, implying that defence mechanisms are being modulated by the complex stimuli in the environment, such as complex mixtures of chemical pollutants and variable abiotic parameters. In this context, the results adhere to previous field studies considering these species, which reported induction of antioxidant and biotransformation enzymes, increased molecular damages (e.g. Durou et al. 2007; Fonseca et al. 2011; Gomes et al. 2013; Maria et al. 2009), as well as inhibition of enzyme activities in polluted sites (e.g. Ahmad et al. 2008; Fonseca et al. 2011; Quintaneiro et al. 2008).

The IBR (Integrated Biomarker Response) index proposed by Beliaeff & Burgeot (2002) comprises an integrative analysis of multiple biomarkers through standardization of the different biological responses, allowing direct comparisons among areas, and has been frequently applied in field studies, some of them in Portuguese estuaries (Serafim et al. 2012, Rodrigues et al. 2014).

Only two significant correlations were found between IBR scores amongst species, namely negative correlations between IBR for *D. labrax* and both *S. plana* and *H. diversicolor*. This trend emphasizes species-specific differences in biological responses to environmental stressors as well as differential habitat use which should result in different exposure conditions. *D. labrax* is a highly mobile fish species with demersal behavior, whose exposure to contaminants would be less severe comparing to both infaunal species that live in constant contact with sediments where many contaminants are deposited. In fact, a negative trend was observed, although not significant, between IBR values for all invertebrate species and *D. labrax*, which further suggests the different impacts on species according to their habitat use.

Overall, biomarker responses and IBR indices varied throughout sampled sites, within and between estuaries, for all species studied. Although some similarities among species response patterns were found, most likely related to habitat use and consequent differential exposure to contaminants, responses varied considerably among species. Furthermore, although Tejo sites are considered more impacted than Ria de Aveiro, significant responses were also found in the latter for all species studied.

Therefore, monitoring programs and environmental quality assessment studies must carefully consider species selection according to the purpose of the study, since the use of different species will produce different impact assessments for the same sites, as observed in this study. Likewise, a multi-taxa approach could be useful to account for multiple exposure routes and impacts. Yet results interpretation is not straightforward considering species-specific differences in terms of physiology, vulnerability to contaminants and overall capacity of defence mechanisms, as well as in terms of ecology, specifically life-strategies, habitat use and feeding habits.

Moreover, further research on the effects of the multi-stressors on biomarker responses, as well as on the specific response mechanisms of different species are needed to improve field application of these tools and to produce more reliable assessments of the overall environmental quality.

CHAPTER 3

Final remarks

Final remarks

In general, laboratorial ecotoxicological studies rely on the use of biomarkers to establish relations between contaminants dosage and biological responses, under controlled conditions. Although the ideal would be the exact extrapolation of those dose-response relations to the natural environment, natural variability of innumerable parameters make it difficult. In the natural environment, several factors can influence biomarker responses in both fish and invertebrate species, either abiotic such as temperature, salinity, dissolved oxygen and contaminants, or endogenous physiological features such as metabolic rates, reproductive status, habitat use, feeding behavior and nutrition (Hyne & Maher 2003, van der Oost et al. 2003, Martínez-Álvarez et al. 2005).

The measurement of several species biomarker responses allowed the recognition of how the complex stimuli in the environment can influence biological systems. The natural variability of abiotic features coupled with complex mixtures of chemical pollutants contribute to variable patterns in species biological responses and specie-specific responses and habitat use can also be responsible for considerable differences in response patterns.

For the same habitats, different responses amongst species were found, although overall higher biomarker responses could be found in Tejo estuarine sites, considered the most impacted sites in this study, yet significant high responses were also observed in the least contaminated site in Ria de Aveiro, previously characterized as a reference site (AVSUL).

Antioxidant enzymes activity varied scantily among sites and months, particularly when compared to biotransformation enzymes activities. These enzymes are the primary defence response of organisms to enhanced oxygen reactive species that result from exposure and uptake of contaminants as well as from natural variation in environmental parameters such as temperature, salinity and oxygen. Induction of these enzymes occurred in all species studied, regardless of the site considered, yet no clear pattern was discernible among responses when considering all species.

Considering both biotransformation enzymes, whose response is more specific to chemical contaminant exposure, and biomarkers of effects, some interspecies patterns could be found. Higher spatial and temporal variability was observed in these enzymes activity compared to antioxidant enzymes, underlining the presence of chemical contamination in the various sampled estuarine sites. Variability patterns found in this study also point to species-specific differences related to differential habitat use. Similarities could be found among sediment-dwelling species, namely *S. plana* and *H. diversicolor*, as well as for species living in permanent contact with the sediment; in clear contrast with biomarker responses from the species with higher mobility and use of the water column (*D. labrax*). For example, similar GST response patterns were observed between *S. plana*, *H. diversicolor* and *C. maenas*, while divergent responses were observed between *D. labrax* (a demersal and highly mobile species) and *P. microps* (a benthic species with burrowing habits). Also, LPO levels allowed the grouping of *C. maenas*, *C. crangon* and *P. microps*, signalling VFX as the most impacted site, whereas *H. diversicolor* and *S. plana* LPO levels highlighted MUR. Regarding DNA damage, most species signalled VFX, while *C. crangon* and *C. maenas* evidenced higher DNAd in MUR.

The combined action of biotransformation and antioxidant enzymes seems to be efficient in preventing or minimizing cellular damage, since overall lower effects were observed when enzymes activities were higher. Nevertheless, significant LPO and DNA damage were found in all species, despite general induction of defence mechanisms, suggesting that contamination was high enough for organisms' ability to avoid molecular damages. In particular, invertebrate species presented positive correlations between biomarkers of effects, supporting the idea that environmental contamination had a negative impact on those species at the molecular level.

In general, species responses signalled Tejo estuarine sites considering both defence mechanisms and effects, especially in September, which as suggested may reflect the effects of continuous exposure in these species. However, different responses were also found amongst species, which does not allow a straightforward interpretation when assessing contamination impacts through biomarker responses in a multi-taxa approach. As referred, differences in enzymes activities and effects levels among invertebrate and vertebrate species may be due to various factors. Differences in physiology are noteworthy, as it is known that antioxidant defences and biotransformation as well as elimination mechanisms are considerably less efficient in invertebrate species, when comparing to most fish species (Livingstone 1998, Hyne & Maher 2003, Martínez-Álvarez et al. 2005). Furthermore, differences in habitat use, which are related to route of exposure to contaminants, feeding habits and to the uptake of contaminants, are also important features regarding species contamination (Livingstone 1998; Martínez-Álvarez et al. 2005). Thus, it is important to recognise the complexity of this study and contextualize the results considering these multiple influential factors.

The methodologies and responses analysed in this study have been mostly applied and developed for quantifying fish species biomarkers. Although several ecotoxicological studies have targeted invertebrate species (e.g. Aguirre-Martínez et al. 2014; Solé et al. 2009) it is important to emphasize that some constraints in detection and quantification of some enzymes activities and metabolites may exist, as a result of evolutionary processes resulting in differences in physiology between fish and invertebrates, as well as in the enzyme presence in the different tissues considered.

Previous studies have characterized the major sources of anthropogenic pressures on both estuarine systems, including extensive chemical characterisation of the sites studied, specifically sediment metal and PAHs concentration (e.g. Vasconcelos et al. 2007, Fonseca et al. 2011). Noteworthy, major contributions of Zn, Pb and Cr metals and petrogenic and pyrolytic PAHs were reported, as well as seasonal variations in contamination levels which is known to occur in estuarine systems (Fonseca et al. 2014, Rodrigues et al. 2014). Undoubtedly, contaminants characterization concomitant to biological sampling could have provided further insight into species biomarker responses and improve understanding of the variation patterns observed, possibly clarifying the causes of higher responses observed in sites expected to be of lower contamination based on previous reports.

As for biomarker responses, spatial and temporal variability in IBR indices for all species was observed. IBR yield a simplified interpretation of the multiple biomarker responses and a generic assessment of environmental pressure in different habitats (Beliaeff & Burgeot 2002). Previous studies applying the IBR index reported its effectiveness in the assessment of habitat quality, as concordance between IBR values and contamination levels was observed (Tsangaris et al. 2011, Serafim et al. 2012, Ben-Khedher et al. 2013). However, IBR application is associated with some limitations, specifically concerning the number and disposition of the biomarkers used in the index calculation (Beliaeff & Burgeot 2002, Serafim et al. 2012). Additionally, IBR values cannot be directly compared with previous classifications reported in the same sites, since the score is the result of the values obtained in each study. Still, IBR is useful as a qualitative measure of the effects of environment contamination on biota, allowing an integrative analysis of all biomarker responses.

Biomarker responses are useful tools in environment quality assessment, as they enable biological and physico-chemical integration of the environment, with the potential of detecting early adverse effects on organisms inhabiting poor quality areas (Hagger et al. 2008, Capela et al. 2016). It is widely acknowledged that ecotoxicological studies must, whenever possible, integrate different biomarker responses, in order to avoid misinterpretation of environmental health status if assessed only by a few responses. In fact in this study, often biomarkers with similar functions (e.g. antioxidative response or xenobiotics biotransformation) did not respond in the same way, which

supports the relevance of a multi-biomarker approach. For example, in the same site, high GST activities were observed together with low EROD activity and vice-versa, and the same was observed for both antioxidant enzymes. Consequently, if considered independently, those results would probably lead to misinterpretation of the biological responses.

However, biomarker responses may be influenced by several factors such as environmental conditions, as well as on ecological and physiological features of the species considered; which is a significant source of variability in any assessment that needs to be addressed (van der Oost et al. 2003, Fonseca et al. 2015). Estuaries are naturally highly dynamic systems, where abiotic conditions fluctuate considerably, such as variable salinity, temperature, oxygen and pH levels, and even sediment flows. Furthermore, these systems are subjected to various forms of anthropogenic pressures that inevitably lead to increased stress in estuarine organisms. Estuarine communities are typically composed by few species with high densities, specifically species that have adapted to the natural variability of these ecosystems, which allows them to tolerate stressful conditions, survive and thrive (Elliott & Quintino 2007). However, this resilience may also contribute to some degree of tolerance towards anthropogenic stressors. Therefore, an important question that needs to be addressed is to what extent are organisms' responses a consequence of natural variability or due to human-induced stress in estuaries. Dauvin (2007) defined this issue as the Estuarine Quality Paradox, which refers to the difficulty with discerning between biological responses to natural inherent variability and contaminants exposure. In this context, laboratorial experimental assays on the effects of abiotic variations, namely temperature and salinity, with site-representative contaminants load should be considered in future studies as an important contribute for understanding the relations between natural and contaminant-induced biomarker responses in these species, and consequently an advantage toward the improvement of environmental quality assessments. Nonetheless, the study of natural populations constitute an essential approach in this matter, allowing the assessment of biological responses regarding species natural ecological features.

Naturally, the induction of defence mechanisms comprises energetic and physiological costs, which may compromise other biological processes such as growth (Fonseca et al. 2008). Accordingly, growth and condition indices (e.g. RNA:DNA), which have been previously considered an integrated response to environmental conditions (Vasconcelos et al. 2009, Fonseca et al. 2015), as well as the energy expenditure of organisms when facing contamination (e.g. De Coen & Janssen 1997) could also be considered in future studies as indicators of habitat quality, individuals' health and metabolic costs of coping with contaminants. Furthermore, interesting studies on biomarker responses have been conducted concomitant with behavioural changes, to understand rather than biochemical and physiological, the behavioural effects that contaminants may induce in organisms and their ecological implications (Vieira et al. 2009, Almeida et al. 2010, Buffet et al. 2011).

Moreover, when considering a multi-taxa approach, several interspecies differences must be addressed such as species-specific differences, for example in the overall capacity of defence mechanisms and/or in vulnerability to contaminants; as well as ecological differences, such as feeding habits or habitat use, all contribute to different responses patterns in similar contamination scenarios.

In conclusion, future environmental quality and risk assessment studies as well as monitoring programs should consider biomarker responses as a valuable indicator of the impacts on biota, but also carefully interpret the different species patterns, since divergent outcomes may arrive for the same habitat, increasing uncertainty in quality assessment and leading to inaccurate ecological status evaluation.

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Appendix

Dicentrarchus labrax

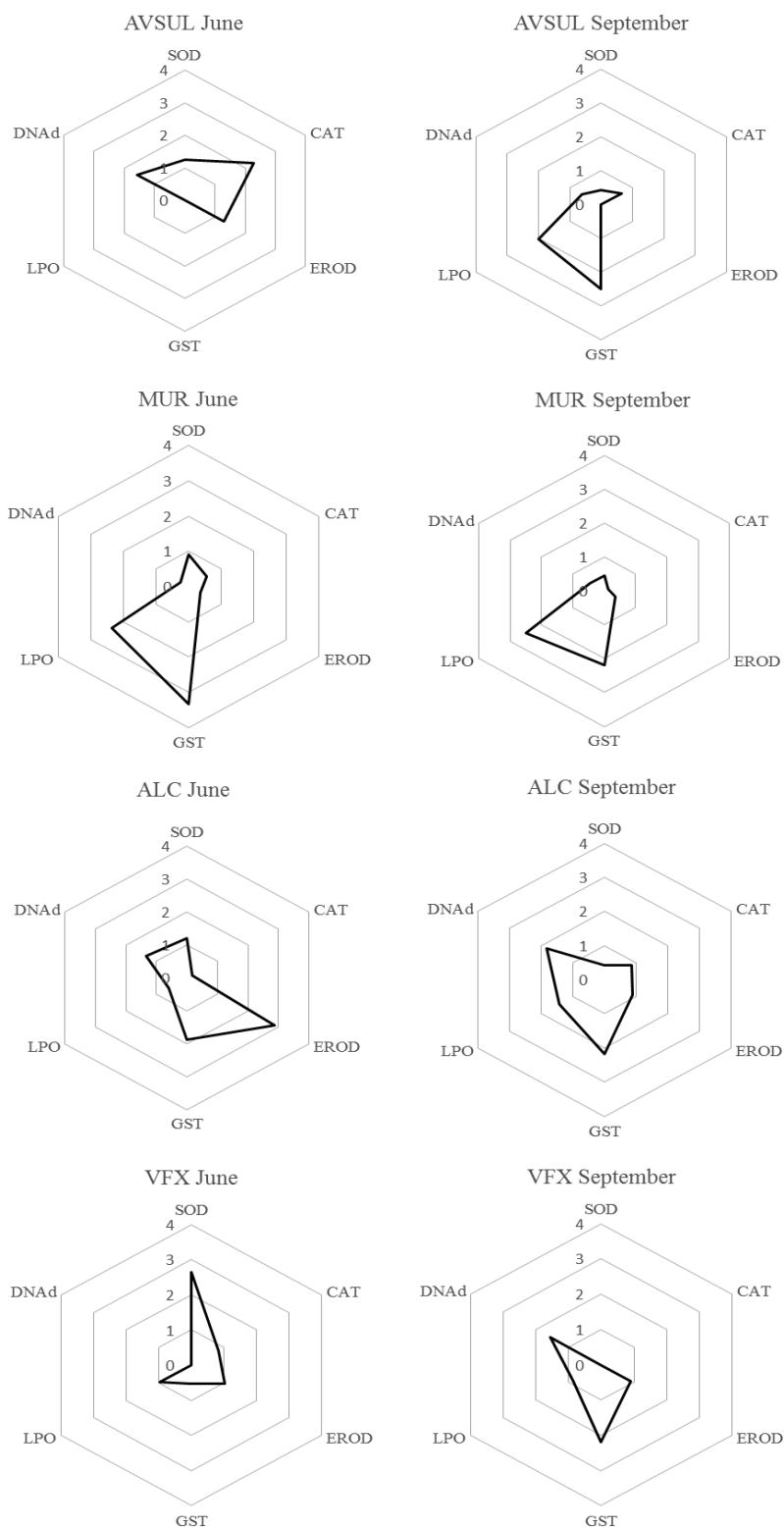


Figure 1 – Radar plots of *D. labrax*' IBR determinations. Scores range between 0 and 4, and represent biomarker contribution to final IBR value: SOD (superoxide dismutase), CAT (catalase), EROD (ethoxyresorufin-O-deethylase), GST (glutathione S-transferase), DNAd (DNA damage) and LPO (lipid peroxidation).

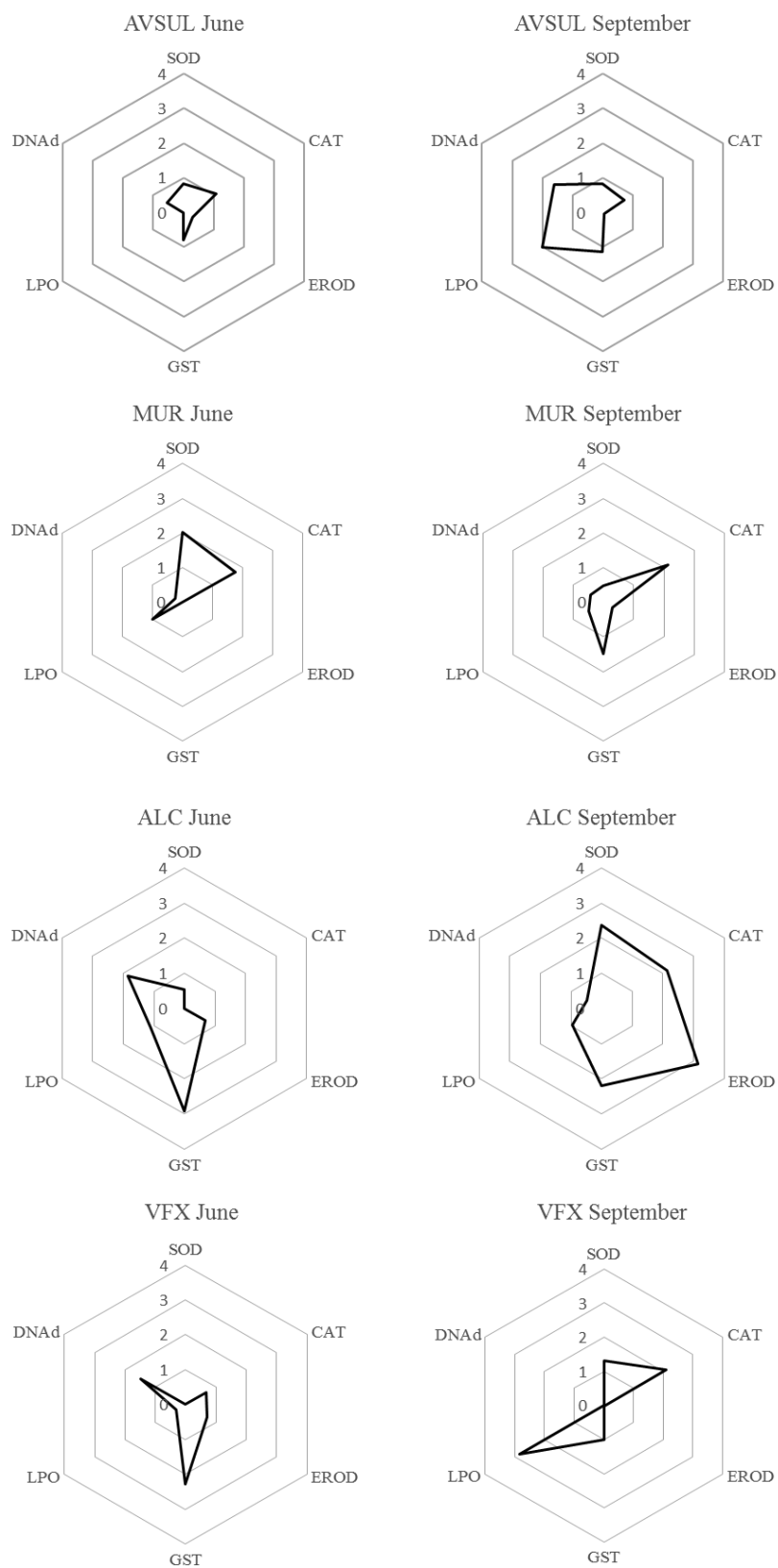
Pomatoschistus microps

Figure 2 – Radar plots of *P. microps*' IBR determinations. Scores range between 0 and 4, and represent biomarker contribution to final IBR value: SOD (superoxide dismutase), CAT (catalase), EROD (ethoxresorufin-O-deethylase), GST (glutathione S-transferase), DNAd (DNA damage) and LPO (lipid peroxidation).

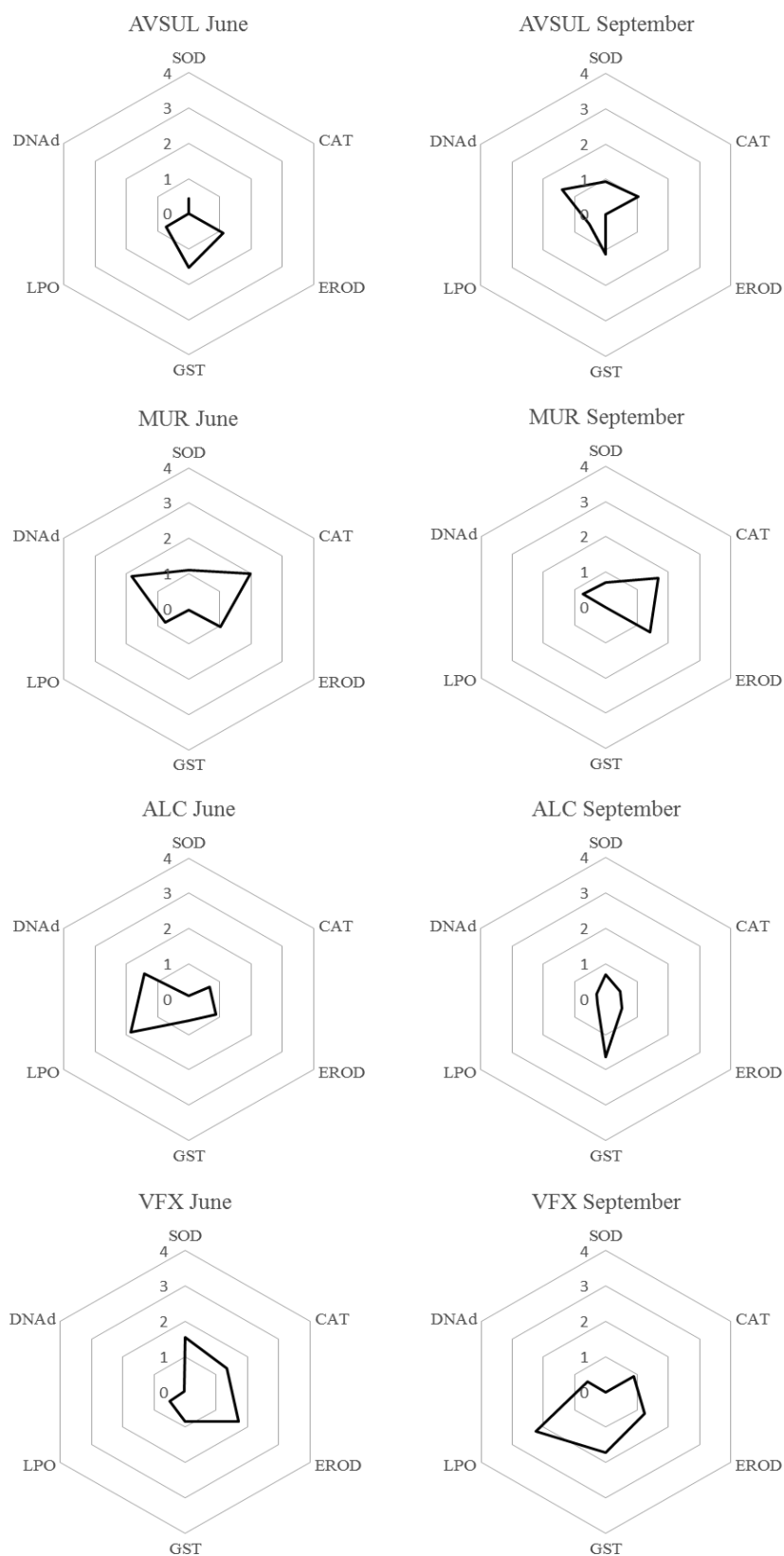
Carcinus maenas

Figure 3 – Radar plots of *C. maenas*' IBR determinations. Scores range between 0 and 4, and represent biomarker contribution to final IBR value: SOD (superoxide dismutase), CAT (catalase), EROD (ethoxyresorufin-O-deethylase), GST (glutathione S-transferase), DNAd (DNA damage) and LPO (lipid peroxidation).

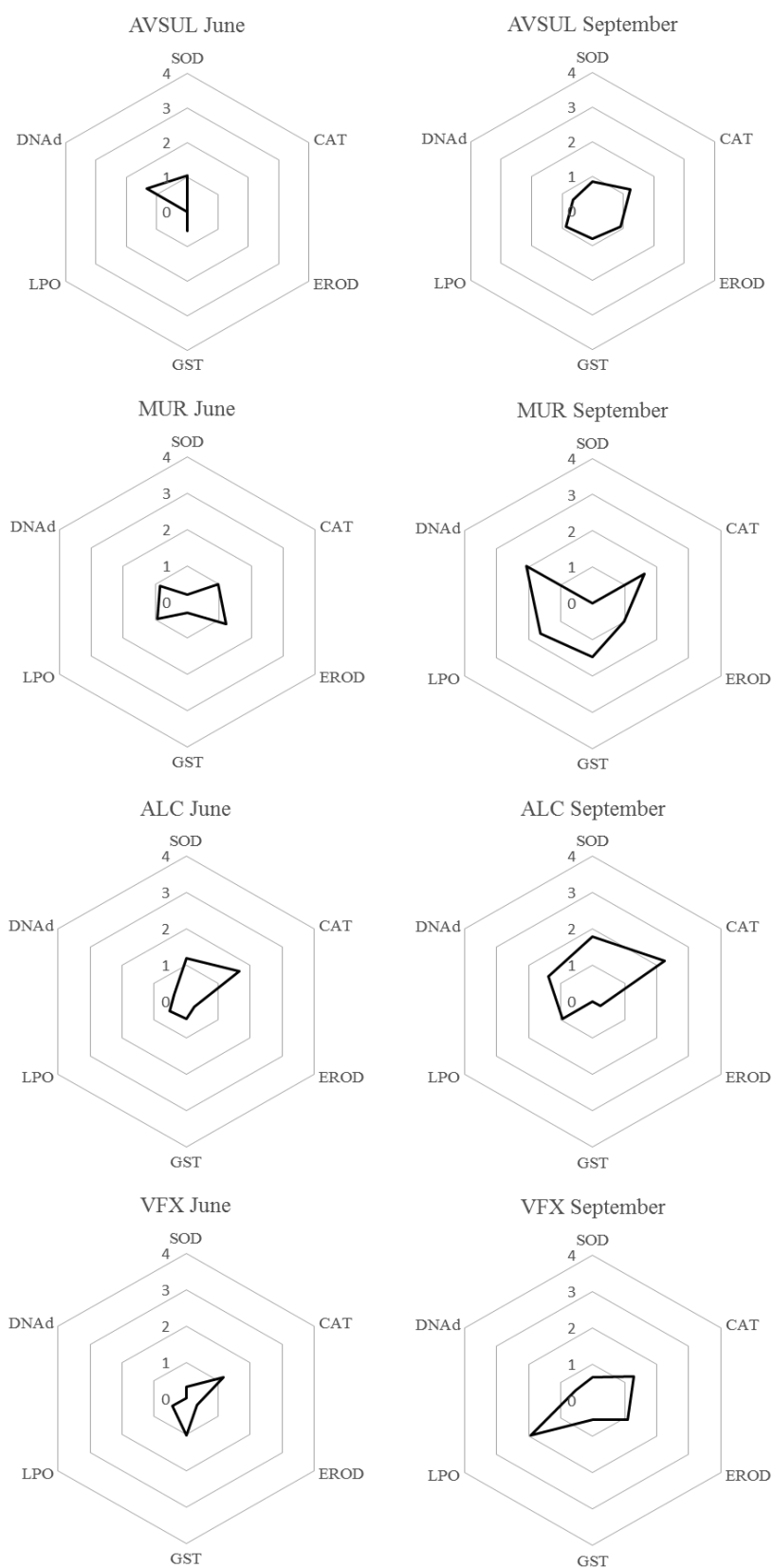
Crangon crangon

Figure 4 – Radar plots of *C. crangon*' IBR determinations. Scores range between 0 and 4, and represent biomarker contribution to final IBR value: SOD (superoxide dismutase), CAT (catalase), EROD (ethoxyresorufin-O-deethylase), GST (glutathione S-transferase), DNAd (DNA damage) and LPO (lipid peroxidation).

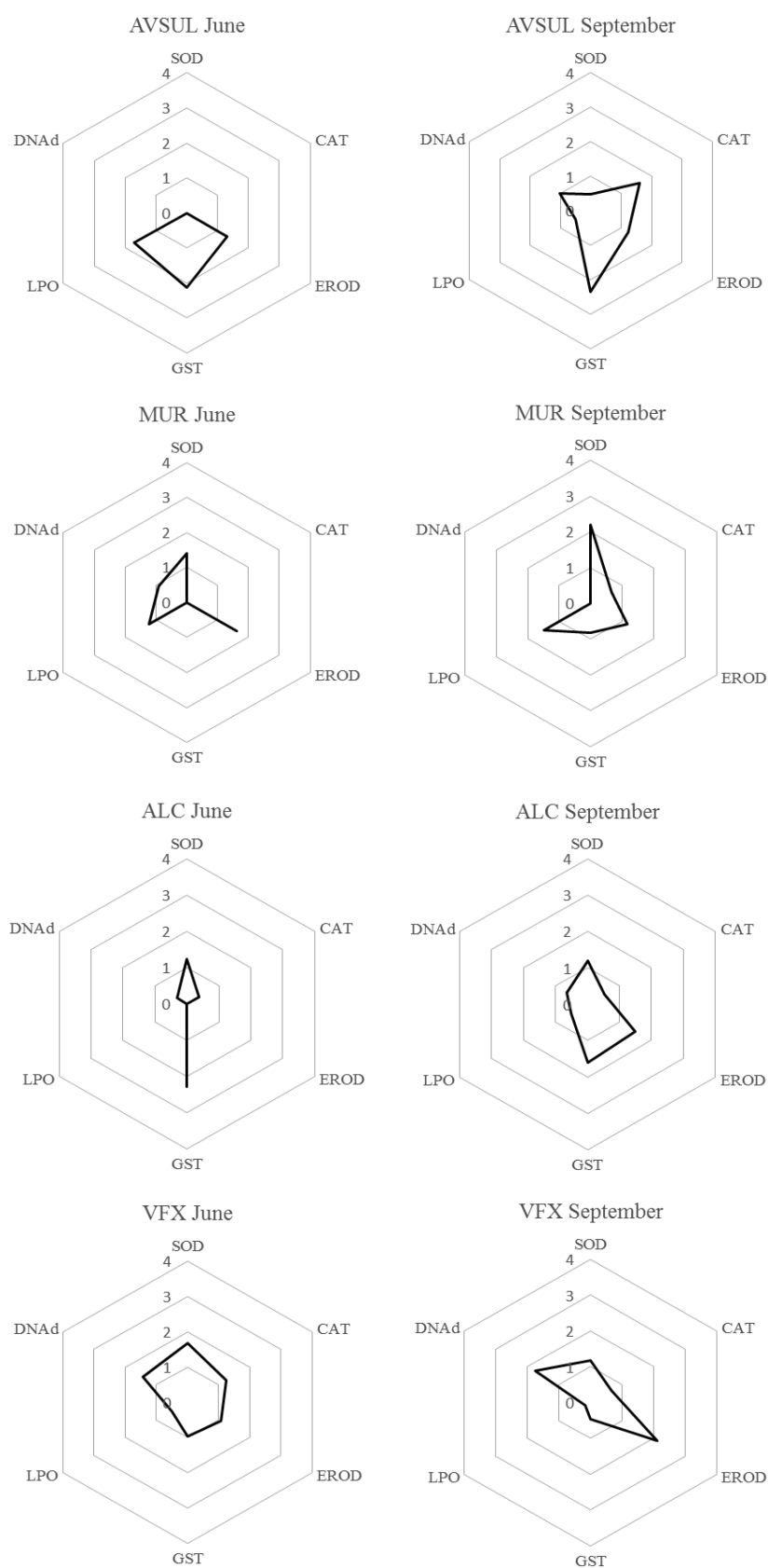
Hediste diversicolor

Figure 5 – Radar plots of *H. diversicolor* IBR determinations. Scores range between 0 and 4, and represent biomarker contribution to final IBR value: SOD (superoxide dismutase), CAT (catalase), EROD (ethoxyresorufin-O-deethylase), GST (glutathione S-transferase), DNAd (DNA damage) and LPO (lipid peroxidation).

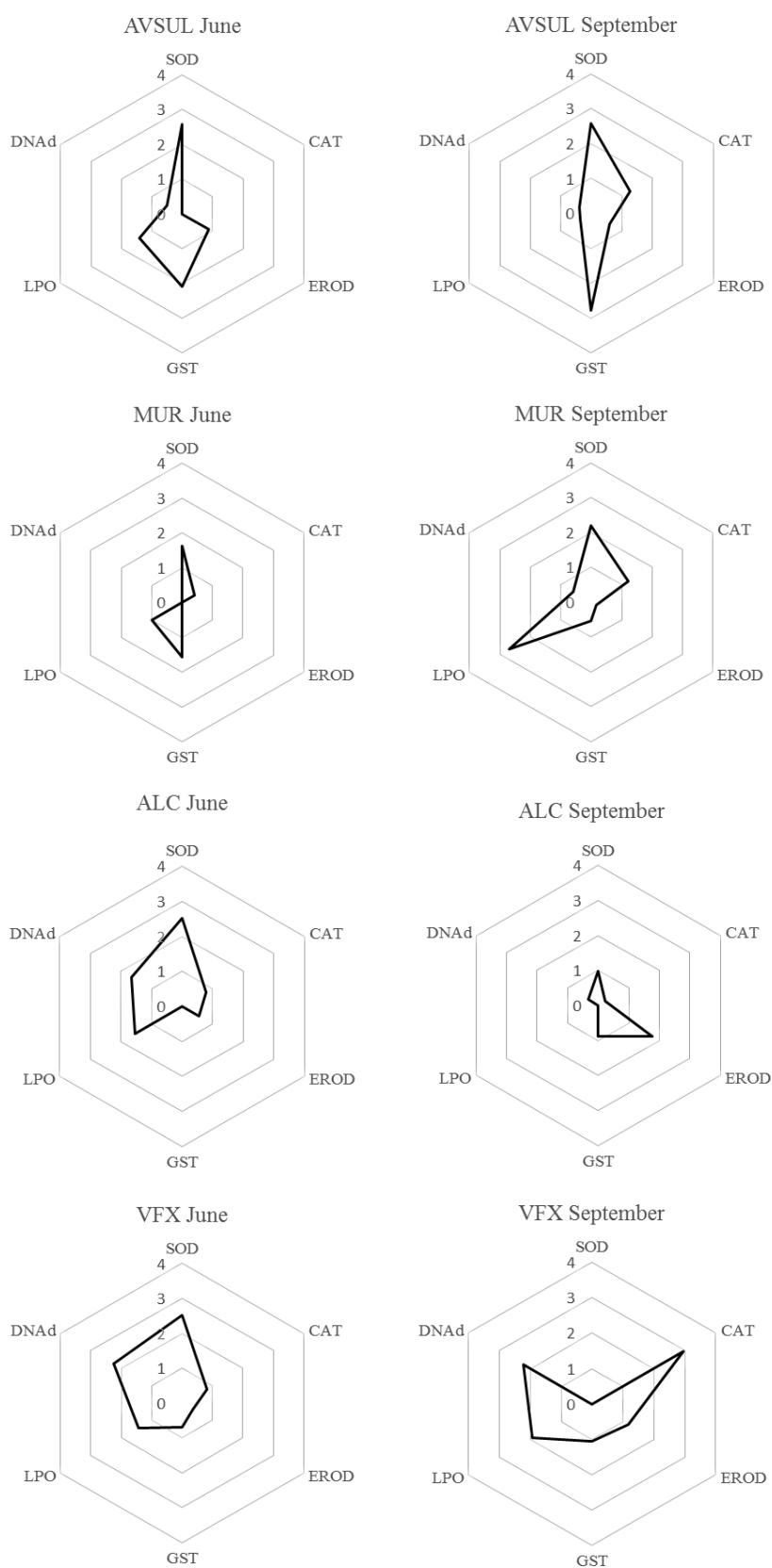
Scrobicularia plana

Figure 6 – Radar plots of *S. plana*' IBR determinations. Scores range between 0 and 4, and represent biomarker contribution to final IBR value: SOD (superoxide dismutase), CAT (catalase), EROD (ethoxyresorufin-O-deethylase), GST (glutathione S-transferase), DNAd (DNA damage) and LPO (lipid peroxidation).

