Universidade de Aveiro Departamento de Biologia
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Combined use of chemical data and biomarkers in aquatic key species

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Doutor em Biologia, realizada sob a orientação científica do Doutor Carlos Alberto Garcia do Vale, Investigador Coordenador do IPIMAR-INRB, Instituto Nacional dos Recursos Biológicos, e do Doutor Mário Guilherme Garcês Pacheco, Professor Auxiliar do Departamento de Biologia da Universidade de Aveiro

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Dedico esta tese ao Mateus

o júri

presidente

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Bem hajam!

palavras-chave

Ulva sp.; *Carcinus maenas*; *Liza aurata*; Stresse oxidativo; Metais; Eutrofização; Lagoa de Óbidos

resumo

As lagoas costeiras têm sido sujeitas a fortes pressões antrópicas capazes de causarem stresse nos organismos residentes. Neste contexto, o presente trabalho constitui um estudo de biomonitorização investigativa na Lagoa de Óbidos (Portugal) dado que este sistema representa um paradigma de lagoa costeira com sintomas de eutrofização e de contaminação moderada por metais. Foi adoptada uma abordagem ampla que combinou a avaliação dos níveis de exposição externa com marcadores de bioacumulação e efeitos bioquímicos em três espécies-alvo – *Ulva* sp. (alface do mar), *Carcinus maenas* (caranguejo verde) e *Liza aurata* (taínha-garrento). Foi investigado em que medida estas espécies-alvo reflectem a contaminação ambiental, tendo sido consideradas três áreas de amostragem: braço da Barrosa e braço do Bom-Sucesso, ambos localizados em áreas confinadas na porção superior da Lagoa, e ainda uma área seleccionada como referência, situada mais próximo da embocadura, na porção central/inferior da Lagoa. Foram quantificados, sazonalmente e numa escala dia-noite, parâmetros de qualidade da água, nomeadamente oxigénio dissolvido, nutrientes e metais na coluna de água. Determinaram-se igualmente as concentrações de metais nos sedimentos superficiais das referidas áreas da Lagoa. As espécies-alvo foram amostradas sazonalmente (*Ulva* sp., *C. maenas*) ou, no mínimo, no Inverno e Verão (*L. aurata*) de forma obter a uma representatividade de períodos de condições ambientais contrastantes, em particular no que concerne às fontes de metais e à disponibilidade de nutrientes. Foram medidos os níveis de peroxidação lipídica (LPO) e respostas antioxidantes (catalase – CAT, glutationa peroxidase – GPx, glutationa reductase – GR, glutationa-S-transferase – GST, glutationa total – GSH_t) em *Ulva* sp., hepatopancreas de caranguejo, e órgãos-chave (brânquias, fígado e rim) de taínha. A capacidade de biotransformação - actividade da etoxiresorufina-*O*-desetilase (EROD) - foi igualmente avaliada em hepatopancreas de *C. maenas*. As concentrações de metais (Mn, Cu, Ni, Cr, Pb e Cd) foram determinadas em *Ulva* sp., *C. maenas* e *L. aurata* e investigadas as associações entre a respectiva acumulação e os efeitos bioquímicos. Os dados de respostas bioquímicas de *Ulva* sp., *C. maenas* e *L. aurata* foram integrados de forma a calcular um índice geral de stresse ("Integrated Biomarker Response" – IBR).

Os resultados em termos de níveis de metais nos sedimentos indicaram uma contaminação moderada da Lagoa. Para além disso, foram registadas apenas pequenas diferenças entre ambos os braços e o corpo central da Lagoa, enquanto diferenças mais acentuadas foram detectadas entre os braços e a Lagoa inferior. Todavia, registaram-se aumentos de metais na coluna de água do braço da Barrosa em períodos húmidos devido às entradas de água doce. Por outro lado, os níveis de metais aumentaram na coluna de água do braço

da Barrosa durante a noite, apontando para a importância crucial dos processos de remobilização na disponibilidade de metais em períodos estivais. Estes processos foram considerados particularmente relevantes no braço da Barrosa dado que esta zona foi classificada neste estudo como eutrófica. De facto, esta área confinada apresentou os níveis mais elevados de nutrientes (amónia, nitrato+nitrito, fosfato), em especial no Inverno. Por conseguinte, os organismos aquáticos residentes no braço da Barrosa estarão expostos a metais no Inverno e Verão, provenientes de entradas de água doce e da remobilização do sedimento, respectivamente.

De um modo geral, a *Ulva* sp. (Outono, Primavera, Verão) apresentou concentrações de metais mais elevadas no braço da Barrosa, tal como as brânquias e fígado de *L. aurata* (Inverno). As diferenças espaciais de metais acumulados no rim de *L. aurata* (Inverno e Verão) e hepatopancreas de C. maenas (Outono, Inverno e Verão) foram menos evidentes. As correlações de Pearson entre metais acumulados e os níveis na água foram investigadas nas três espécies, sendo significativas apenas para *Ulva* sp. Porém, as brânquias e o fígado da *L. aurata* apresentaram aumentos de metais no braço da Barrosa no Inverno, em concordância com a maior disponibilidade ambiental. Os metais acumulados nas três espécies-alvo variaram entre o Inverno e o Verão. No braço da Barrosa, apenas os níveis de Mn em *Ulva* sp. registaram máximos no Verão em comparação com o Inverno, eventualmente em resultado de uma maior incorporação durante a noite devido à remobilização de Mn do sedimento. Contrariamente à macroalga, os metais nos caranguejos e peixes aumentaram no Inverno (comparativamente com o Verão) e, portanto, a fonte externa de metais (água doce) sobrepôs-se à sua libertação intermitente durante as noites de Verão.

O braço da Barrosa revelou a presença de compostos que estimularam a produção de H_2O_2, expressa nos aumentos de CAT e GPx em *Ulva* sp. (Outono e Verão), no hepatopancreas de *C. maenas* (Outono) e nas brânquias, no fígado e no rim de *L. aurata* (principalmente no Verão). Para além disso, a exposição a compostos pró-oxidantes no braço da Barrosa provocou um aumento de GST na *Ulva* sp. (Outono), nos caranguejos (Outono e Verão) e no rim dos peixes (Inverno). Ademais, a GSH_t diminuiu nos animais (hepatopancreas de caranguejos no Verão e fígado de peixes no Inverno), tendo aumentado pontualmente em *Ulva* sp. (Outono). As espécies-alvo mostraram incapacidade para combater eficazmente as espécies reactivas de oxigénio (ROS) produzidas ao nível celular, tendo-se observado aumento de LPO em *Ulva* sp. (Outono e Primavera), *C. maenas* (Outono), bem como em brânquias e fígado de *L. aurata* (Inverno). Os resultados de LPO enfatizaram a degradação ambiental do braço da Barrosa relativamente a outras áreas da Lagoa. A comparação sazonal das respostas bioquímicas nas três espécies-alvo revelou que apenas os caranguejos e peixes apresentaram diferenças entre o Inverno e Verão ao nível das respostas de stresse oxidativo. Os resultados de IBR corroboraram a indicação de que o braço da Barrosa é a área mais deteriorada da Lagoa. Com base na análise de Pearson, a actividade de GPx na *Ulva* sp, bem como nas brânquias e no rim de *L. aurata*, foi correlacionada com os níveis de metais acumulados (Mn, Zn e Cu, respectivamente). Por outro lado, foram identificadas correlações significativas entre GST em *Ulva* sp. e o Cd acumulado. De igual modo, a GST nas brânquias, no fígado e no rim de *L. aurata* foi correlacionada com os níveis acumulados de Cu, Pb e Cu, respectivamente. Apesar das correlações encontradas, outros factores relacionados com o processo de eutrofização foram putativamente associados aos efeitos bioquímicos registados no braço da Barrosa. Por exemplo, neste trabalho sugeriu-se que a grande flutuação do oxigénio dissolvido registada no Verão induz a activação de defesas antioxidantes nas brânquias das taínhas. Por outro lado, no Inverno, as elevadas concentrações de amónia e nitrito estarão eventualmente associadas às respostas bioquímicas nas macroalgas, caranguejos e peixes.

O presente trabalho contribuiu com novas perspectivas para a biomonitorização, através da identificação de espécies e órgãos sensíveis. De facto, a *Ulva* sp., que tem sido pouco empregue como sentinela, demonstrou capacidade de traduzir os níveis de metais na água da Lagoa de Óbidos

através dos níveis acumulados. Por outro lado, a sua capacidade de resposta em termos de parâmetros de stresse oxidativo foi comparável à dos caranguejos e dos peixes. Adicionalmente, os níveis de metais e efeitos bioquímicos nas brânquias de peixes reflectiram a condição ambiental da Lagoa de Óbidos, tendo-se este órgão de interface revelado uma importante via de absorção de metais. Com base nestas evidências, as brânquias de *L. aurata* foram consideradas uma "porta espelhada" no contexto da contaminação aquática. Os resultados desta tese também recomendam a separação de géneros em programas de monitorização com *C. maenas*. Padrões espaciais e temporais comuns às diferentes espécies-alvo foram difíceis de identificar e os biomarcadores (acumulação e efeitos bioquímicos) nas taínhas mostraram padrões específicos para cada órgão. Porém, através da análise combinada de níveis de exposição, concentrações acumuladas e respostas bioquímicas foi possível confirmar a existência de condições de risco no braço da Barrosa. De facto, a contaminação da Lagoa de Óbidos, ainda que em concentrações sub-letais, induziu alterações ao nível bioquímico nas espécies-alvo estudadas. Por conseguinte, a estratégia adoptada demonstrou a sua fiabilidade na avaliação do estado de saúde ambiental. Tendo isto em consideração, este estudo constituiu uma contribuição para a avaliação ambiental da Lagoa de Óbidos, fornecendo informação base às entidades de gestão locais. Adicionalmente, as conclusões desta tese poderão ser particularmente úteis para futuros programas de monitorização investigativa em lagoas costeiras, à escala mundial, afectadas por processos de eutrofização e contaminação moderada por metais.

keywords

Ulva sp.; *Carcinus maenas*; *Liza aurata*; Oxidative stress; Metals; Eutrophication; Óbidos lagoon

abstract

Costal lagoons are still under a major human pressure that can cause stress on inhabitant organisms. An investigative biomonitoring study was carried out in the Óbidos lagoon (Portugal) since this system represents a prototype of coastal lagoon with eutrophication symptoms and moderate contamination by metals. A comprehensive approach was adopted combining the evaluation of external levels of exposure with bioaccumulation markers and biochemical effects in three target species - *Ulva* sp. (sea lettuce), *Carcinus maenas* (shore crab) and *Liza aurata* (golden grey mullet). It was searched in what extent these target species reflected environmental contamination. For those proposes three sites were considered: Barrosa branch and Bom-Sucesso branch located in confined areas of the upper lagoon; and middle/lower lagoon closer to the lagoon inlet and thus selected as reference area. Water quality parameters, including dissolved oxygen, nutrients and metals were surveyed in the water column over a day-night scale and on a seasonal basis. Surface sediments were analyzed to assess the pool of metals in several areas of the lagoon. Target species were collected seasonally (*Ulva* sp., *C. maenas*) or at least in winter and summer (*L. aurata*) in order to cover periods of contrasting environmental conditions regarding metal sources and nutrients availability. Lipid peroxidation (LPO) and antioxidant responses (catalase - CAT, glutathione peroxidase - GPx, glutathione reductase - GR, glutathione-S-transferase – GST, total glutathione - GSH_t) were measured in *Ulva* sp., crabs' hepatopancreas and key organs (gills, liver and kidney) of mullets. Additionally, hepatopancreas of *C. maenas* were analyzed for biotransformation capacity (ethoxyresorufin-*O*-deethylase (EROD) activity). Levels of metals (Mn, Cu, Ni, Cr, Pb and Cd) were measured in *Ulva* sp., *C. maenas* and *L. aurata* and associations between metals accumulation and biochemical effects were searched. A general stress index (Integrated Biomarker Response – IBR) was applied to data on biochemical responses of *Ulva* sp., *C. maenas* and *L. aurata*.

Metal levels in surface sediments pointed to a moderate contamination of the lagoon. Moreover, only slightly differences were recorded among both branches and the middle lagoon, whereas spatial differences were more accentuated between both branches and the lower lagoon. Despite that, metals in the water column enhanced at Barrosa in wet period due to freshwater inputs. Additionally, metal concentrations increased in the water column at Barroasa branch during the night, pointing to the main importance of remobilization processes for metals availability in warm conditions. This was considered particularly relevant at Barrosa branch since in the current study this area was classified as eutrophic. In fact, this confined area registered the highest concentrations of nutrients (ammonium, nitrate+nitrate, phosphate)

particularly in winter. Accordingly, aquatic organisms living at Barrosa branch seemed to be exposed to metals both in winter and summer that would be eventually provided respectively by freshwater inputs and sediment remobilization.

In general, *Ulva* sp. (autumn, spring and summer) presented higher metal levels at Barrosa branch, as well as gills and liver of *L. aurata* (winter). Spatial differences concerning accumulated metals were less pronounced for kidney of *L. aurata* (winter and summer) and hepatopancreas of *C. maenas* (autumn, winter and summer). Pearson correlations between accumulated metals and water levels were searched for the three species but only found for *Ulva* sp. Despite that, gills and liver of *L. aurata* exhibited increases of metals at Barrosa branch in winter in line with the higher environmental availability. Accumulated metals in the three target species varied between winter and summer. At Barrosa, only Mn levels in *Ulva* sp. peaked in summer in comparison to winter, eventually related with an enhanced incorporation during the night due to the remobilization of that element from the sediment. Differently from macroalgae, metal levels in crabs and fish increased in winter (in comparison with summer) and thus, external metal levels (freshwater inputs) superimposed intermittent release of summer nights.

Barrosa branch also presented compounds that stimulated the production of H_2O_2 as depicted by the increase of CAT and GPx in *Ulva* sp. (autumn and spring), hepatopancreas of *C. maenas* (autumn), as well as gills, liver and kidney of *L. aurata* (mainly in summer). Moreover, the exposure to pro-oxidant stressors at Barrosa branch led to an elevation of GST in *Ulva* sp. (autumn), crabs (autumn and summer) and fish' kidney (winter). Additionally, GSH_t decreased in animals (crabs' hepatopancreas in summer and fish' liver in winter), whereas a punctual increase was recorded in *Ulva* sp. (autumn). Target species did not cope efficiently with reactive oxygen species and LPO was recorded for *Ulva* sp. (autumn and spring), *C. maenas* (autumn) and *L. aurata* gills and liver (winter). Results on LPO highlight the environment state degradation at Barrosa branch relatively to other areas of the lagoon. The seasonal comparison of biochemical responses in the three target species revealed that only crabs and fish exhibited differences between winter and summer on oxidative stress responses. Current IBR results substantiated the evidence that Barrosa branch is the most impacted site in the lagoon. Based on Pearson analysis, GPx activities in *Ulva* sp., as well as in gills and kidney of *L. aurata* were correlated with accumulated metal levels (Mn, Zn and Cu, respectively). Moreover, significant correlations were obtained between GST in *Ulva* sp. and accumulated Cd. Similarly, GST in gills, liver and kidney of *L. aurata* were correlated respectively with accumulated Cu, Pb and Cu. Despite the obtained correlations, other factors related with eutrophication process were hypothesised to be on the basis of biochemical effects at Barrosa. For instance, the broad fluctuation of dissolved oxygen recorded in summer was suggested to lead to the activation of antioxidant defences in mullet's gills. Additionally, in winter the higher levels of ammonium and nitrite were eventually associated with biochemical responses in macroalgae, crabs and fish.

The work brought new perspectives to biomonitoring by the identification of sensitive species and organs. In fact, *Ulva* sp., that had been underemployed as sentinel, displayed the ability to closely reflect the metal levels in water of the Óbidos lagoon on its body burden. Additionally, the responsiveness of oxidative stress parameters in *Ulva* sp. was comparable to that of crabs and fish. Furthermore, metal levels and biochemical effects in fish gills reflected the environmental status of the Óbidos lagoon, showing this interface organ as an important route of entry for metals. Based on both evidences, *L. aurata* gills were considered as a "mirror door" in the context of water contamination assessment. Moreover, data of this thesis recommend genders separation in biomonitoring programs using *C. maenas*. Additionally, common spatial and temporal patterns to the different target species were difficult to discern and biomarkers (accumulation and biochemical effects) showed to be organ-specific in mullets. Despite that, through a combined analysis of external levels of exposure, accumulation levels and biochemical responses it was possible to confirm the existence of hazardous conditions at Barrosa branch. In fact, the

presence of contamination at the Óbidos lagoon, even at sublethal concentrations, induced changes at biochemical levels in the target species. Hence, the adopted strategy had demonstrated its suitability for environmental health assessment. In view of that, this study is a contribution for Óbidos lagoon health assessment and offered the baseline information for local management entities. Moreover, conclusions of the thesis may be particularly useful for designing future investigative monitoring programs in coastal lagoons worldwide suffering from eutrophication and moderate contamination by metals.

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CHAPTER I

General Introduction

1 General Introduction

1.1 Aquatic coastal environment - degradation and restoration

The transitional ecosystems between land and sea, a narrow strip at the edge of both environments, contains some of the most productive and valuable habitats of the world (Valiela, 1995). Coastal lagoons are among these important ecosystems, since several organisms use lagoon habitats for nesting, feeding, reproduction or sheltering (Barnes, 1980). Despite a general decrease in the anthropogenic pressure on coastal ecosystems observed recently in developed countries, coastal lagoons are still undergoing major human impact (Lotze et al., 2006). The most important environmental concerns are associated with unplanned development (haphazard urbanization and industrialization), unregulated discharges (municipal sewage and industrial waste) and depletion of resources (over-fishing and bad use of agricultural land). Those activities could lead to the enhancement of contaminant availability and massive algal growth due to eutrophication, associated with an increase in the duration of intermittent periods of lower oxygenation (Rabouille et al., 2007). In fact, the deficient water renewal in coastal lagoons may slow down the dilution process and enhance sediment accumulation and/or retention of contaminants that are transferred to the biota (Mucha et al., 2004). Moreover, the fate of waterborne contaminants in shallow waters is regulated by resuspension and deposition, two physical processes that strongly depend on tidal currents and wind, which have low expression in some lagoon systems.

The major aim of environmental science is to make robust, practical and relatively low cost procedures for risk assessment, and to predict consequences of toxic compounds (Rice, 2003). The protection, improvement and sustainable use of Europe's water resources are a major goal of current European Water Policy. A key piece of legislation introduced to transform the way in which this is achieved across all European states is the European Union's Water Framework Directive (WFD). The key environmental objective of the WFD is to achieve and ensure "good ecological" status for all water bodies throughout Europe by 2015. The emphasis given by WFD to ecological elements has been widely welcomed by scientists and environmental managers as it focuses management efforts on restoring impacted ecosystems to achieve "good ecological status". However, coastal systems are complex and fluctuating entities and, thus, the development of adequate ecological assessment and classification systems is one of the most technically challenging aspects of the legislation (EU, 2003). Three

modes of monitoring are specified in the directive and form part of the management plans (Allan et al., 2006):

- Surveillance monitoring aims to assess long-term water quality changes and provide baseline data on river basins allowing the design and implementation of other types of monitoring.
- Operational monitoring aims to provide additional and essential data on water bodies at risk of failing environmental objectives of the WFD.
- Investigative monitoring aims to assess causes of such failure.

Until recently, monitoring of water quality had generally relied in the collection, at prescribed periods of time, of spot water samples followed by chemical analysis. However, considerable limitations are associated with spots sampling to determine total pollutant concentrations, since an important number of factors are not accounted. For instance, intermittent chemical releases associated with industrial/urban wastewater effluents and diffuse pollution (e.g. runoff from periodic application of pesticides in agriculture) (Marques et al., 2008). Moreover, sediment remobilization of stored compounds leads to spatial-temporal variation in a water body's physic-chemical characteristics (Thouzeau et al., 2007). In this way, the WFD provides an important legislative opportunity to promote and implement an integrated approach to risk assessment of chemicals, combining chemical measures, with biomonitoring (including biomarkers) and ecological appraisals.

1.1.1 Contamination of coastal systems and the importance of sediment-water exchanges

An increasing variety of industrial and agricultural chemicals are introduced in coastal ecosystems. Among the best studied contaminants are chemicals of both longstanding and more recent concern, such as: polycyclic aromatic hydrocarbons (PAH); organochlorine pesticides (e.g. DDT - dichlorodiphenyltrichloroethane); industrial products (e.g. PCB – polychlorobiphenyls); dioxins; nitroaromatic compounds; organometallic compounds; pesticides; estrogenic compounds; and many metals including Cd, Cr, Cu, Fe, Hg and Zn (Livingston, 2001). Metals are of great environmental concern, since they tend to concentrate in aquatic organisms, are virtually non-degradable, and thus produce long lasting effects upon the environment even after their major sources have been removed.

Metals are introduced into coastal systems through fluvial inputs, direct effluent discharges and by the atmosphere. After discharged metals are mostly adsorbed on suspended particles and finally accumulate in the sediment, which can serve as sink or source of metals to the overlying water. Many aquatic organisms spend a major portion of their life in or on sediment with the possibility to take up those metals. Moreover, numerous studies have shown that sediment-water interaction in aquatic systems play an important role on controlling metals transport processes (Gomez et al., 1999; Point et al., 2007; Thouzeau et al., 2007). Indeed, the mineralization of organic matter plays a fundamental role in the sediment-water exchanges of metals. Iron- and Mn-oxides in sediment are important scavengers for metals. However, during the mineralization of organic matter, Mn^{2+} and Fe^{2+} and associated trace metals (Cu, Ni, Pb, Zn and Cd) can be released into the pore-water and diffuse upward due to concentration gradients. This process is largely affected by Eh and pH changes (Miao et al., 2006). Cadmium remobilization from sediments to the water column was recorded in the Elbe Estuary (Germany) and attributed to the effects of lowering of the pH and fluctuating Eh conditions as a result of seasonal changes in the oxygen regime above sediments. Moreover, sediments of Thau lagoon could act also as source of Cd to the overlying water (Metzger et al., 2007). Identical observations were recorded for Pb, Ca, Mg, Al and Zn in a Louisiana coastal freshwater lake (Miao et al., 2006). The processes governing the exchange between the sediment and the water column are associated with two types of flux. The diffusive flux corresponds to the natural diffusion of trace elements between the water column and pore-waters, with a direction and range depending on the depleted (or enriched) compartment. Second, the advective flux corresponds to the exchange of metals between the sediment and water driven by and external factor (e.g. water currents; bioturbation) (Point et al., 2007).

Phosphate is strongly bounded to Fe-oxides that are deposited at the oxidised boundary layer at the sediment surface (Gomez et al., 1999). As this layer becomes reduced due to oxygen consumption during organic matter decomposition, dissolution of Fe-oxides occurs, releasing phosphate to the overlying waters. Phosphate regeneration is a temperature dependent process and phosphate efflux from sediment obeys to diffusion laws (Lillebø et al., 2004).

Nutrients are essential for organisms but excessive amounts of nitrogen and phosphorus can be detrimental to ecosystem health causing eutrophication problems

(Valiela, 1995). When nutrient levels remain too high, ecological effects can be devastating in shallow systems with restrict circulation. Eutrophication processes have been studied in freshwater systems for more than 40 years but in coastal waters this is a more recent concern and its scientific understanding is still in progress (Pérez-Ruzafa et al., 2005). The increase of turbidity and light attenuation (due to accelerated phytoplankton growth) as well as a shift from a seagrass-dominated to a phytoplankton- or macroalgae-dominated system are common features of eutrophic environments (Kennish, 2000; Pardal et al., 2004; Lillebø et al., 2007). The decrease of dissolved oxygen in bottom waters caused by bacterial decomposition and benthic organisms' respiration is also a worry problem. In some cases, anoxic conditions were recorded during the night since the consumption of oxygen is not compensated by photosynthesis. In theory, these conditions will favour the release of metals and nutrients from the sediment to the overlying water. In fact, several studies concerning sediment-water exchanges were performed in the eutrophic Thau lagoon both by the measurement of benthic fluxes but also using *in situ* probes (Metzger et al., 2007; Point et al., 2007; Thouzeau et al., 2007). In spite of that, studies performed in eutrophic environments concerning metal contamination associated with internal sediment inputs continues to be scarce.

1.1.2 Toxicokinetic of metals

A number of metals are used by living organisms to stabilize protein structures, facilitate electron transfer reactions, and are essential cofactors for oxidative phosphorylation, in gene regulation and free-radical homeostasis. For example, Cu, Zn and Fe are essential as constituents of the catalytic sites of several enzymes (Siegel, 1973). Nevertheless, other metals like Pb, Hg and Cd may displace or substitute essential metals and interfere with the proper functioning of enzymes and associated cofactors. Trace elements may accumulate in aquatic organisms through different mechanisms: directly from water, via uptake from suspended particles and sediment, or by the consumption of lower trophic level organisms. The former is an essential point to consider in evaluating adverse effects on ecosystems (Van der Oost et al., 2003). In view of that, there are several works that use the accumulation of metals in organisms as mean to assess the environmental health status (Fernandes et al., 2007; Morrison et al., 2007; Pereira et al., 2009).

The absorption of metals by aquatic animals involves their transfer to the circulatory system by epithelial barrier of gills, digestive organs or integument. Dissolved metals are

mainly taken up by exposed body surfaces such as the gills, whereas particulate metals are mostly ingested and then taken up after solubilization in the gut. Uptake of essential metals such as Ca, Cu, Fe and Zn, often involves specific pathways - calcium channels and specific membrane carriers for Fe and Cu (Sunda and Huntsman, 1998). For nonessential metals (e.g. Cd and Hg) specific uptake mechanisms are not known and, thus appear to follow existing pathways for essential metals (Sunda and Huntsman, 1998).

Sequestration of metals in an immobilized form occurs throughout the various organs involved in pathways for metal uptake, transport, utilization and release. One of the best studied intracellular structures are the metallothioneins. These are low-molecular-weight cytosolic proteins rich in SH groups, with high affinity for IB and IIB metal ions, known to be involved in metal homeostasis and over-expressed in organisms experiencing high metal contamination (Viarengo et al., 1998). Their expression in tissues is regarded as an indicator of metal contamination and widely used as a tool for biomonitoring programs (Viarengo et al., 1998). In vertebrates, plasma proteins play a main role in metal transport. For example, Fe is transported by transferrin and Cu by ceruloplasmin, while serum albumin carries out Ca, Ni and Zn. In invertebrates, hemocytes are the primary vehicles for transporting metals. Many types of these cells are highly mobile and with phagocytic activity.

Aquatic organisms utilize a variety of mechanisms to eliminate metals. The kinetic of metal release is complex and reflects the diverse compartments from which metals must be mobilized. Additionally, physical and chemical parameters, such as temperature and salinity, may affect the rate of release in aquatic animals, which can use several pathways to release metals: renal, digestive or diapedesis (in molluscs).

1.2 Biomonitoring aquatic coastal environments – relevant target species and tools

In the early 20th century, researchers proposed the use of living organisms in parallel with physical-chemical analyses to evaluate the health state of the aquatic system (Amiard et al., 1998). In this context, biomonitoring was defined as the systematic use of biological responses to evaluate changes in the environment (Cairns and Van der Schalie, 1980). This approach is based on the knowledge that chemicals which have entered the organisms leave markers reflecting exposure. The marker may be the chemical itself, a breakdown product or a biological change in the organism as a result of the action of the chemical. Bivalve molluscs, particularly mussels, have been elected as “sentinel” organisms in international

environmental monitoring programs as part of the “Mussel Watch Programme” (Goldeberg et al., 1993). Sentinel species can be defined as biological indicators that accumulate a pollutant in their tissues (Beeby, 2001), offering a potentially simple solution to both the problem of measuring bioavailability and of summarizing complex patterns of contamination.

Sentinel species in monitoring can have different uses (Beeby, 2001):

- as accumulators - to increase analytical sensitivity for a contaminant; to compare the scale of contamination between sites; to summarize a complex pollution signal;
- as integrators – to provide a running mean over time and space;
- as a measure of exposure – to quantify bioavailability of a pollutant from a particular source.

Sentinel species should be widely distributed, easy to identify in the field, abundant and large enough to provide material for analysis (Phillips, 1977; Beeby, 2001; Galloway et al., 2004). The assumption that a sentinel's tissues accurately reflect differences between sites or sampling dates has rarely been tested in any attempt at validation (Beeby, 2001). Nowadays, by using species available in ecosystems already impacted, it is likely that a shifting baseline may have occurred. This common problem to monitoring efforts could be reduced by choosing species with different feeding strategies, and a diverse range of habitats in coastal areas (Galloway et al., 2004). Since the “Mussel Watch Programme” many other organisms beside mussels have been employed as relevant tools in environmental programs. In the aquatic monitoring, the used sentinel species belong to several groups as plankton, plant, crustacean, molluscs and fish, among others. Each sentinel species shows the special merits for biomonitoring when compared to the others.

Despite the role of macroalgae in ecosystems, they have been underemployed for the diagnosis or prediction of the negative consequences of human activities. Macroalgae are sedentary, sensitive to environmental variations and react, as first stages of food chain, more rapidly to the presence of pollutants than organisms of higher stages (Rainbow and Phillips, 1993). The condition and abundance of algae species can directly reflect the water quality (Lopes et al., 2007). For example, the massive development of chlorophyceae (e.g. *Ulva* sp. and *Enteromorpha* sp.) observed in littoral or sheltered bays have lead to these organisms being considered as eutrophication sentinel species (Lazaridou et al., 1997). Moreover, macroalgae are able to absorb pollutants from the aquatic environment, biotransforme

organic compounds and immobilize metals to make them less toxic (Pflugmacher et al., 1999). Several studies used *Ulva* sp. in the biomonitoring of trace metal contamination (Vasconcelos and Leal, 2001; Villares et al., 2002; Morrison et al., 2007) but very few studies are available on biological responses to contaminant exposure. Ultimately, the combination of metal measurements and biological responses under real field scenarios is almost inexistent.

Invertebrates are major components of all ecosystems and their populations are often numerous, so samples can be taken for analysis without significantly affecting population dynamics (Martín-Díaz et al., 2008a). Increasing knowledge of the biochemistry of invertebrates now permits reasonable interpretation of the responses of biomarkers with respect to ecological risk assessment (Livingstone, 1991; Depledge and Fossi, 1994). Despite the determination of alterations in the different tissues of invertebrates starting to receive more attention, invertebrates are not widely employed as fish species. Responses determined in invertebrate species have not been considered to be strong enough and statistically significant for their use in environment management plans. Nevertheless, invertebrates have been found to be suitable species for monitoring studies. In fact, several studies in field and in laboratory employed *Carcinus maenas* as a model organism (Bjerregaard et al., 2005; Stentiford and Feist, 2005; Martín-Díaz et al., 2008a; 2008b; Morales-Caselles et al., 2008).

Fish play a major role in aquatic food-webs, occupy different habitats in the same ecosystem and have different feeding behaviours. Thus, the understanding of toxicant uptake, behaviour and responses in fish may have a high ecological relevance (Van der Oost et al., 2003). Despite the limitation of mobility, fish are generally considered to be the most feasible organisms for pollution monitoring in aquatic systems (Van der Oost et al., 2003). In fact, international monitoring protocols include measurements in fish species (WHO, 1993). Mullets (e.g. *Liza aurata*, *Liza saliens*, *Liza ramada* and *Mullus barbatus*) in particularly, have been recommended as key indicators by MED POL (FAO/UNEP, 1993) and have been extensively used in the assessment of fish health and pollution in aquatic systems (Pacheco et al., 2005; Fernandes et al., 2007; Guilherme et al., 2008; Zorita et al., 2008). Mullets inhabit tropical and temperate waters, presenting an extreme salinity tolerance. Their feeding behaviour is characterized by a regular contact with the sediment by filtering its superficial layer, as well as particles of the water column. Mullets play an important role in the coastal trophic webs as consumers. Indeed, they present a wide range of feeding adaptations, from zooplankton, benthic organisms to detritus (Boglione et al., 2006). In Portugal, mullet species

have no commercial value. However, they are very important in many parts of the world being widely cultivated in freshwater ponds in other Europe countries and Southeast Asia.

1.2.1 Biomarkers

A biomarker is defined as a change in biological response (ranging from molecular through cellular and physiological levels to behavioural changes), which can be related to exposure or toxic effects of environmental chemicals (Peakall, 1994). According to the World Health Organisation (1993), biomarkers can be sub-divided into three classes:

- Biomarkers of exposure - cover the detection and measurement of an exogenous substance, its metabolites, or the product of an interaction between a xenobiotic agent and target molecules or cells, in a compartment within an organism;
- Biomarkers of effect - include measurable biochemical, physiological or other alterations within tissues or body fluids of an organism that can be recognized or associated with and established or possible health impairment or disease;
- Biomarkers of susceptibility - indicate the inherent or acquired ability of an organism to respond to the challenge of exposure to a specific xenobiotic substance, and include genetic factors and changes in receptors that alter the susceptibility of an organism to exposure.

In general, effects are more visible at cellular level than at higher levels of biological organization, consequently biomarkers may be similar in a large variety of organisms. Biochemical responses may be used to elucidate cause-effect and dose-effect relationships in health risk assessment, in clinical diagnosis and for monitoring proposes (Van der Oost et al., 2003). Generally, biomarker responses are considered to be intermediates between pollutant sources and higher-level effects. One of the most compelling reasons for using biomarkers is the fact that the information obtained is on the biological effects of pollutants, rather than a mere quantification of their environmental levels. By screening multiple biomarker responses, information will be obtained regarding mechanisms of chemicals toxicity (Van der Oost et al., 2003). For field samples, data can provide an important index of the total external load that organisms are exposed.

1.2.1.1 Biotransformation enzymes

Biotransformation can be defined as an enzyme-catalysed conversion of a xenobiotic compound into a more water-soluble form, which can be more easily excreted from the body than the parent compound. The toxicity of a foreign compound may be affected by metabolism, which can either be beneficial (detoxification) or harmful (bioactivation) to an organism (Figure 1.1). Biotransformation determines the activity of a compound, the duration of its activity and its half-life in the body (Timbrell, 1992). Xenobiotics can be biotransformed in highly metabolic organs (e.g. liver, kidney, digestive gland) by enzymes from phase I and II. Phase I is a non-synthetic alteration (oxidation, reduction or hydrolysis) of the original foreign molecule, which can be conjugated in phase II (Williams, 1959). Generally, the most sensitive effect biomarkers are alterations in levels and activities of biotransformation enzymes (Van der Oost et al., 2003).

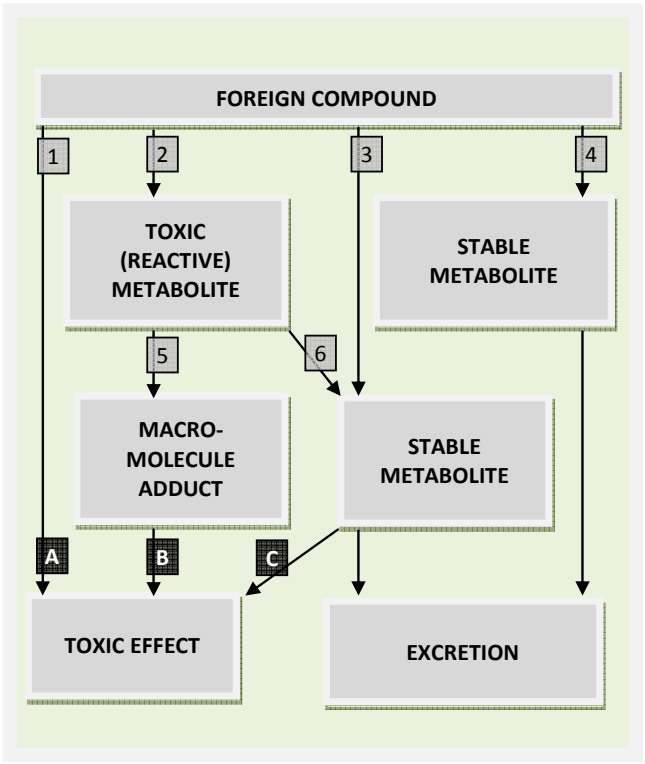

Figure 1.1. Possible toxication and detoxication pathways of xenobiotics. (1) Direct toxic effect (A); (2) Metabolic activation; (3) Formation of a stable metabolite that may cause a toxic effect (C); (4) Detoxification. The reactive metabolite formed by bioactivation (2) may cause a toxic effect (B); through reaction with critical targets (5) or be detoxified through reaction with a protective agent (6). Adapted from Van der Oost et al. (2003).

For the majority of xenobiotics the phase I reactions are catalysed by microsomal monooxygenase (MO) enzymes, also known as the mixed-function oxidase (MFO) system. This enzymatic system is associated with the membranes of the smooth endoplasmatic reticulum (Stegeman et al., 1992), where it catalyses the oxidation of lipophilic substances by utilizing O_2 and NADPH. The MFO activity is usually found at a low level, since it is related to the metabolism of endogenous lipophilic compounds. However, in organisms exposed to xenobiotics such as PAH or PCB certain iso-enzymes may be enhanced 10-100 fold (Bucheli and Fent, 1995).

Cytochrome P450 is the terminal component of the MFO system. It exists in many isoforms having different functions in the metabolism of endogenous and xenobiotic compounds (Stegeman et al., 1992). Among these, the CYP1A1 isoform has been studied in a number of species being the quantification of EROD (7-ethoxyresorufin-*O*-deethylase) activity the most widely used parameter for its measurement. In addition to examine the responses of the CYP1A isoenzyme catalytic activity, there are other approaches to evaluate the phase I induction, like CYP1A and mRNA levels.

CYP1A activity is greatly up-regulated by the presence of organic xenobiotics, so an assessment of EROD activity has usually been reported as a biomarker capable of detecting biological effects of many aromatic xenobiotics in water and accumulated in the organisms' tissues (Stegeman et al., 1992; Bucheli and Fent, 1995). Biological factors (e.g. animal age and sex) and environmental parameters (e.g. water temperature) are believed to affect EROD activity (Goksøyr, 1995). Moreover, heavy metals (including Cd, Cu and Hg) (Viarengo et al., 1997; Sorrentino et al., 2005), oxidative stress (Barouki and Morel, 2001), and excess of substrate/contaminants (Goksøyr, 1995), significantly inhibit MFO activity. CYP1A catalytic activity may be used in the assessment of exposure as an early warning sign for potential harmful effects of many pollutants. Indeed, increases in EROD activity have been reported in many species of vertebrates and invertebrates, after exposure to organic contaminants (Pacheco et al., 2005; Martín-Diaz et al., 2007).

The second phase of metabolism involves a conjugation of the parent compound or its metabolites (produced in phase I) with an endogenous ligand (Figure 1.1). Some xenobiotics contain the functional groups for direct metabolism by conjugative phase II enzyme systems, while others are metabolized by integrated steps involving prior action of the phase I enzymes (Sijm and Opperhuizen, 1989; Van der Oost et al., 2003). Conjugations are addition

reactions in which large and often polar molecules (e.g. amino acids, glutathione - GSH and glucuronic acid - GA) are covalently added to xenobiotics. The phase II type enzymes catalyze conjugation reactions, thus facilitating the excretion of chemicals by the addition of those groups to the xenobiotic or metabolites. The major pathway for electrophilic compounds and metabolites is the conjugation with GSH. Phase II enzymes can play an important role in homeostasis as well as in detoxification and clearance of many xenobiotics.

The conjugation of phase I metabolites with GSH is catalyzed by the glutathione S-transferases (GSTs), a multigene superfamily of dimeric, multifunctional and soluble enzymes. Apart from their essential functions in intracellular transport and the biosynthesis, GSTs have a critical role in defence against oxidative damage and peroxidative products of DNA and lipids (George, 1994). These enzymes are mainly located in the cytosolic fraction.

The toxicity of many exogenous compounds can be modulated by induction of GSTs. An increase in GST activity has been reported in several studies after exposure of fish to dioxins (Guosheng et al., 1998), PAH (Gravato e Santos, 2003), heavy metals (Ahmad et al., 2005), tributylin (TBT) (Wang et al., 2006) and pesticides (Peixoto et al., 2006). Contrarily, a significant decrease in GST activities was also observed in fish exposed to pesticides or PAH (Otto and Moon, 1996; Van der Oost et al., 1996). Alterations in GST were also largely recorded in invertebrates (Martín-Díaz et al., 2008a; 2008b; Morales-Caselles et al., 2008) and in less extent in plants (Ferrat et al., 2003). Moreover, native organisms from polluted aquatic systems presented also alterations in GST activities. For example, Ahmad et al. (2008) and Guilherme et al. (2008) observed changes in GST activities in fishes from a Portuguese coastal lagoon, whereas Nimptsch and Pflugmacher (2007) in an aquatic macrophyte.

1.2.1.2 Oxidative stress

Oxygen free radicals are essential in the physiological control of cell function and are continuously produced in living cells (Halliwell and Gutteridge, 1999). Basic cellular metabolism in aerobic organisms involves the production of oxygen free radicals and non-radical reactive species (referred to as Reactive Oxygen Species - ROS). There are numerous studies showing that living organisms use ROS, such as superoxide anion radical ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), peroxyl radical ($ROO^{\bullet}$) among others for advantageous biological effects. Important physiological functions that involve free radicals include the following: regulation of vascular tone, sensing of oxygen tension and regulation of functions that are

controlled by oxygen concentration, enhancement of signal transduction from various membrane receptors, and oxidative stress responses that ensure the maintenance of redox homeostasis (Dröge, 2003). $O_2^{\bullet-}$ is formed through one-electron reduction of O_2, while H_2O_2 can be produced by the dismutation reaction of $O_2^{\bullet-}$ (catalysed by superoxide dismutases) via the hydroperoxyl radical ($HO_2^{\bullet-}$) (Figure 1.2). The most reactive and toxic form of oxygen is probably the $^{\bullet}OH$ being produced by the metal ion (e.g. iron or copper) catalysed decomposition of H_2O_2. The mitochondria consume over 90% of the cellular oxygen in unstressed cells and are considered the major site of aerobic cellular ROS production (Han et al., 2001). Moreover, ROS generation occurs also by microsomal systems of the endoplasmatic reticulum (Winston et al., 1996).

Regulated production of free radicals and maintenance of ‘‘redox homeostasis’’ are essential for the physiological health of organisms (Ames et al., 1993). However, during metabolic processes, a small proportion (2–3%) of free radicals may escape from the protective shield of antioxidant mechanisms, causing oxidative damage to cellular components, like DNA, proteins and lipids (Halliwell and Gutteridge, 1999). The imbalance between generation and neutralization of ROS by antioxidant mechanisms within an organism is called oxidative stress (Davies, 1995). Biological systems have developed during their evolution adequate enzymatic and non-enzymatic antioxidant mechanisms to protect their cellular components from oxidative damage. These include antioxidant enzymes, such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GR) and GST (Figure 1.2), as well as some molecules with antioxidant action such as glutathione, uric acid and ascorbate.

There are many natural sources of oxidative stress, namely UV radiation, heat shock and inflammation. Other endogenous sources of ROS within cells are several oxidizing enzymes as cytochrome P450 reductase, which can produce $O_2^{\bullet-}$, while enzymes like guanyl cyclase and glucose oxidase generate H_2O_2 (Vigo-Pelfrey, 1990). Cytochrome P450 involvement in the production of ROS is of additional interest in toxicology because it is involved in the metabolism of xenobiotics (Zangar et al., 2004). Moreover, rates and amounts of ROS production can be increased by the presence of a wide range of anthropogenic compounds as PAH, PCB, dioxins and metals (Halliwell and Gutteridge, 1999). In view of that, oxidative stress has become an important subject for aquatic toxicology (Livingstone, 2001; Viarengo et al., 2007).

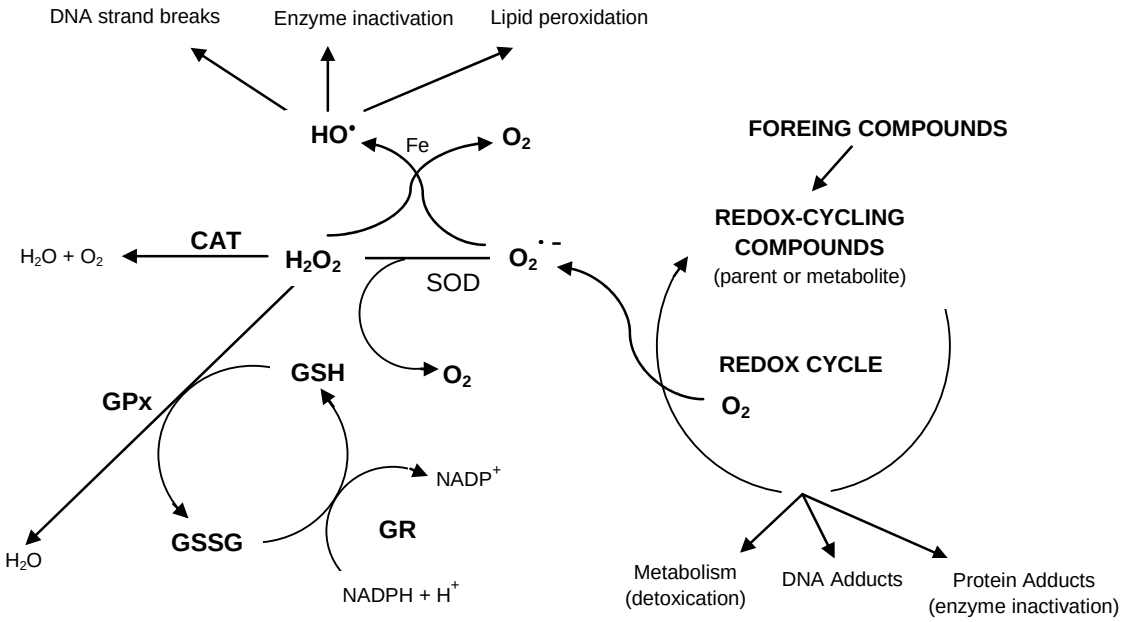

Figure 1.2. Antioxidant defences against ROS production due to the presence of pollutants. SOD: superoxide dismutase; CAT: catalase; GPx: glutathione peroxidase; GR: glutathione reductase; GSH: reduced glutathione; GSSG: oxidised glutathione (adapted from Stegeman et al., 1992).

1.2.1.2.1 Antioxidant Enzymes

CAT is a heme-containing enzyme that facilitates the removal of H_2O_2, which is decomposed to O_2 and water (Figure 1.2) (Schrader and Fahimi, 2006). Because CAT is localised in the peroxisomes of most cells and is involved in the fatty acid metabolism, changes on its activities may often be difficult to interpret (Stegeman et al., 1992). While CAT employs one molecule of H_2O_2 as donor in the reduction of another H_2O_2, peroxidases use other reductants. In animals, the principal peroxidase is a selenium-dependent tetrameric cytosolic enzyme (GPx) that employs GSH as a cofactor. GPx catalyses the metabolism of H_2O_2 to water with the concomitant conversion of reduced glutathione (GSH) to its oxidized form - glutathione disulfide (GSSG) (Figure 1.2) (Halliwell and Gutteridge, 1999). Similarly, GPx also catalyses the reduction of organic hydroperoxides to their corresponding alcohols, being this considered an important mechanism for halting lipid peroxidizing chain reactions (Van der Oost et al., 2003).

GSTs may play a dual protective role associated to their activity on conjugation of electrophilic compounds (or phase I metabolites) with GSH (Van der Oost et al., 2003), and can also employ GSH in the reduction of a broad range of organic hydroperoxides, but cannot

reduce H_2O_2 (Wang and Ballatori, 1998).This peroxidatic activity by GST is sometimes referred to as “selenium-independent peroxidase”, although GST is not a true peroxidase.

GR catalyses the transformation of GSSG to GSH with the concomitant oxidation of NADPH to $NADP^+$ (Figure 1.2). Therefore, GR maintains the GSH/GSSG homeostasis under oxidative stress conditions (Winston and Di Giulio, 1991).

All of those enzymes have showed increases and decreases in activities, in several organisms (from plants to animals) exposed to a variety of contaminants. Under field conditions, those enzymes measured in fish (Fernandes et al., 2007; Ahmad et al., 2008; Guilherme et al., 2008), invertebrates (Lima et al., 2006; Damiens et al., 2007; Pytharopoulou et al., 2008) and plants (Cairrão et al., 2004; Ferrat et al., 2003; Nimptsch et al., 2005) showed their ability to reflect pollution.

1.2.1.2.2 Non-enzymatic antioxidants

Glutathione represents the bulk of the non-protein thiols of the cells, but it was demonstrated that other thiols, namely N-acetyl-L-cysteine, can also play an important role as antioxidant. Reduced GSH, a tripeptide consisting of glutamine acid, cysteine and glycine, has received considerable attention in terms of its biosynthesis, regulation and various intracellular functions (George, 1994; Commandeur et al., 1995). Among these functions are two roles in detoxifications: (i) as a key conjugate of electrophilic intermediates, principally via GST activities in phase II metabolism, previously described; (ii) as an important antioxidant (Stegeman et al., 1992). GSH reacts with electrophilic compounds and replaces hydrogen, chlorine and nitro-groups. Increased fluxes of ROS can impose a drain on intracellular reducing equivalents with consequences on a variety of metabolic processes. The consumption of GSH due to the direct scavenging of ROS or as a cofactor for GPx and GST may represent such a drain – NADPH must be oxidized by GR to maintain GSH levels (Di Giulio et al., 1995). More indirectly, oxidative stress can impose a drain on the reductant pool as a consequence of the energetic costs of mounting a defence against an increased flux of ROS, i.e. biosynthesis of antioxidants (Winston and Di Giulio, 1991). Perhaps the most obvious direct effect of certain compounds is a decrease in the proportions between reduced and oxidized glutathione (GSH:GSSG), due to either direct radical scavenging or increased peroxidase activity (Stegeman et al., 1992).

Changes in GSH levels were observed in laboratory studies with fish exposed to a variety of contaminants, including PAH, PCB and metals (Van der Oost et al., 2003; Oliveira et al., 2004; 2007). Studies were also performed although in less extent with invertebrates (Livingston, 2001; Barata et al., 2005) and plants (Rama Devi and Prasad, 1998; Nimptsch and Pflugmacher, 2007). Moreover, field studies in metal-polluted environments demonstrated a significant increase in GSH levels of fish (Ahmad et al, 2008; Guilherme et al., 2008), but decreases were also recorded (Otto and Moon, 1998; Van der Oost et al., 1998). High concentrations of GSH were found in macroalgae from highly contaminated sites by metals (Pawlik-Skowrońska et al., 2007).

1.2.1.2.3 Oxidative damage – Lipid peroxidation

Membrane phospholipids of aerobic organisms are continually subjected to oxidant challenges from endogenous and exogenous sources, and thus peroxidized membranes and lipid peroxidation products represent a constant threat to cells. The process of lipid peroxidation is composed by a set of chain reactions, especially for polyunsaturated fatty acids (PUFA) which are very sensitive to reactions by ROS because of their double bonds. Lipid peroxidation products are formed with the abstraction of a hydrogen atom from an unsaturated fatty acid, and double bounds are rearranged to form dienes. Attack by molecular oxygen produces a lipid peroxyradical that can abstract a hydrogen atom from an adjacent lipid to form a lipid hydroperoxide (LOOH) (Almroth et al., 2005). The resulting LOOH can easily decompose into several reactive species including lipid alkoxyl radicals, aldehydes (malondialdehyde), alkanes, lipid epoxides and alcohols. Most of these products are toxic and active mutagens (Porter et al., 1995). Lipid peroxidation products may form DNA adducts giving rise to mutations and altered patterns of gene expression (Marnett, 1999). Peroxidized membranes become rigid and lose permeability and integrity. Lipid peroxidation is initiated mainly by hydroxyl radicals, especially in transition metal-catalyzed reactions.

Metals in the presence of H_2O_2 can produce $^{\bullet}OH$ through Fenton-like redox cycling reactions. Metals, such as Cu, Cr, Ni, and Cd are implicated in lipid peroxidation and subsequently in the promotion of carcinogenesis (Kasprzak, 1995). Several studies demonstrated enhancement of lipid peroxidation in various aquatic organisms exposed to metals (Hamoutene et al., 2000). High concentrations of Cu and Zn significantly reduced

antioxidant enzymatic activities and increased lipid peroxidation in fish tissues (Radi and Matkovics, 1998). The increase in oxidative damage with exposure to non-directly redox cycling compounds possible indicates that other pro-oxidant mechanisms may be occurring, such as biotransformation of PAH to redox-cycling quinines, induction of CYPs by PAH and PCB, auto-oxidation of CYPs in the presence of poorly metabolized PCB and disruption of membrane systems by lipophilic compounds (Livingston, 2001).

1.2.1.3 Limitations of biomarkers and the contribution of the "Integrated Biomarker Response" in the environmental assessment

Biomarkers are sensitive and allow the early detection of environment' degradation. However, a successful implementation of biomarkers in environmental monitoring programmes requires a good understanding of the mechanisms underlying the responses (Van de Oost et al., 2003). Many non-pollution variables can modulate a range of enzymes, and thus interfere with biomarker responses when experimental conditions are not thoroughly controlled. Examples of confounding factors are the organism condition, sex, age, nutritional status, metabolic activity, reproductive and developmental status. There are also a number of relevant extrinsic factors like season, temperature, heterogeneity of the environmental pollution (Van er Oost et al., 2003). A further limitation is the fact that various substances may affect the same biomarkers due to the lack of specificity for individual compounds. A paradigmatic example is the co-occurrence of inhibitors and inductors of CYP1A activity such as metals and PAH. Previous studies showed the reduction of EROD activity in fish exposed simultaneous or sequential to those classes of contaminants (Sorrentino et al., 2005). Moreover, levels extremely low of EROD activities were observed in fishes from environments polluted both by PAH and metals (Romeo et al., 1994). In this case, the low EROD activities should be interpreted as lack of expression rather than the absence of inductors. To prevent misinterpretations of biomarkers information their use should never be in isolation (Van der Oost et al., 2003). Therefore, to allow comparisons of the "health status" of different areas and the detection of temporal trends, the integration of several biomarkers is a basic requirement.

It is widely accepted that in ecotoxicology one key challenge is to integrate individual biomarker responses into a set of tools and indices capable of detecting and monitoring the degradation of the organisms' health (Damiens et al., 2007). The Integrated Biomarker

Response (IBR), in their original publication included only biochemical markers (GST, acethylcholinesterase and CAT enzyme activities, as well as DNA adducts) in mussels (Beliaeff and Burgeot, 2002). Thereafter, also histochemical biomarkers of toxic effects and mutagenic damage in the flounder and eelpout were applied to IBR estimation (Broeg and Lehtonen, 2006). In this study, metallothionein induction, metabolites of PAH in bile measured as fluorescent aromatic compounds and EROD activity were also considered. Recently, IBR was employed for the integration of biomarker responses of the mussels *Mytilus galloprovincialis*, demonstrating a clear distinction between sites and allowing classification of a pollution gradient (Pytharopoulou et al., 2008). In the previous studies, IBR index was compared visually with accumulated contaminants in organisms by star plot graphics. This visual comparison allowed establishing cause-effect relationships between biomarkers and contaminants.

1.3 Integration of environmental data and biomarkers in environmental monitoring

Monitoring the state of coastal systems has traditionally been almost entirely based on measurements of contaminants concentrations in sediment, water column and organisms. However, the presence of chemical compounds in sediment or water does not, by itself, indicate injurious effects on organisms, as bioavailability of these compounds should also to be taken into account. The monitoring of water column generally relied on spot samplings that could not cover the pulse release of contaminants. This is particularly important in systems subjected to diffuse pollution or intense remobilization from sediments. Moreover, with the large and continuously increasing number of potentially toxic substances present in and released into ecosystems, this chemical monitoring alone is no longer considered meaningful or cost-effective. It has also become evident that measuring concentrations in the tissues does not provide the information needed to assess biological effects that contaminants may cause in organisms on different levels of biological organization (molecular, cellular, tissue, individual, population). In fact, contaminants usually appear in the environment as very complex mixtures that can cause interactive effects on the biota impossible to evaluate only by means of chemical analyses.

During the past decades, molecular, biochemical, cytological, immunological and physiological parameters (so-called biomarkers) have been under dynamic development for the detection of effects of contaminants in aquatic organisms (review: Van der Oost et al.,

2003). Meanwhile, a wide range of such biomarkers has been suggested for application in monitoring environmental health and assessing deleterious effects in biological systems (Ferrat et al., 2003; Allan et al., 2006; Viarengo et al., 2007). Depledge and Galloway (2005) considered that the only realistic way to improve our understanding concerning environmentally harmful levels of contaminants is to learn more about their biological effects in a given ecosystem. Despite biomarkers have proved to be crucial tools in investigative monitoring, their usage have some limitations, as previously mentioned. Therefore, it is recommended their combination with other monitoring approaches, such as the assessment of environmental contaminants in sediment, water and organisms or the application of bioassays (Figure 1.3). In fact, this was the philosophy of many international projects like the ECOMAN, HELCOM or BEEP.

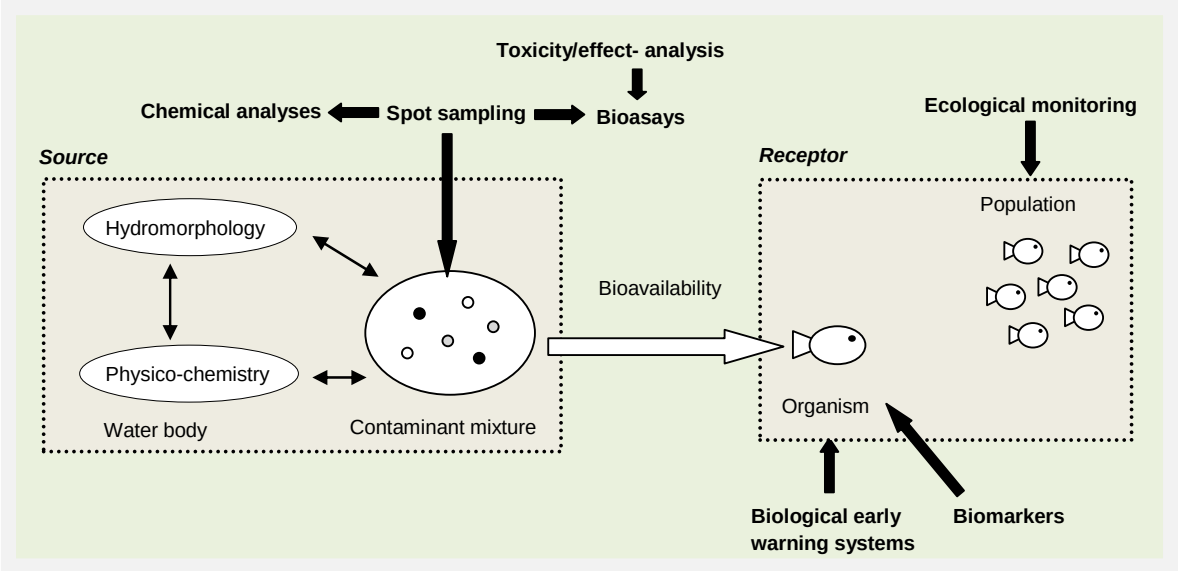

Figure 1.3. Techniques and methods for water quality monitoring under the Water Framework Directive. Scheme adapted from Allan (2006).

1.4 The Óbidos lagoon case study

The Óbidos lagoon is a shallow coastal lagoon with a mean depth of 1 m and a wet area of 7 km^2, located on the west coast of Portugal. The lagoon is permanently connected to the sea through a narrow inlet. Areas with different morphological and sedimentary characteristics were identified in the lagoon (Oliveira et al., 2006): several sand banks, narrow

channels and strong currents in the lower and middle lagoon; and muddy bottom sediments in the inner branches Barrosa and Bom-Sucesso. Óbidos lagoon is potentially exposed to nonpoint sources of contamination associated with agriculture and livestock. Moreover, during the past decades wastewaters from the town "Caldas da Rainha" (50,000 inhabitants) had been discharged in the Cal River, which enters into the lagoon at Barrosa branch. Nowadays, these loads are release directly in the coastal zone adjacent to the lagoon through a submersed outfall. Nevertheless, Cal River continued to have deteriorated quality conditions according to the Portuguese classification of freshwater systems (IST/IPIMAR, 2008) and consequently Barrosa branch. In fact, this coastal lagoon is facing serious environmental problems, mainly related with the higher input of nutrients that accelerate eutrophication processes. Those problems are substantiated by the difficulties of water renewing, particularly in the branches (Santos et al., personal communication) leading to a high accumulation of organic matter in sediments (Carvalho et al., 2006). In fact, the morphological characteristics of the lagoon induce a distinct hydrodynamic behaviour inside the system with velocities of 2 m s^{-1} in the inlet and less than 0.5 m s^{-1} in the inner branches.

Only very few studies were performed in this Portuguese aquatic system (Gordo and Cabral, 2001; Carvalho et al., 2005; 2006; Oliveira et al., 2006) and no ecotoxicological evaluation has been carried out before. Despite the lagoon had received loadings of anthropogenic discharges, its trophic status was never address. This is also the case of the pulse releases of metals and nutrients from sediments associated with the lagoon eutrophic conditions. Moreover, the risks to the aquatic biota associated with those pressures need to be assessed. In this complex contamination scenario the combined use of chemical data and biomarkers can provide crucial information to evaluate human impacts on the lagoon keeping in view a sustainable managing of biotic resources.

1.5 Aims and Structure of the Thesis

An ecotoxicological study was performed at the Óbidos lagoon. This system represents a prototype of coastal lagoons that are naturally stressed systems with frequent environmental disturbances and fluctuations. Their shallowness, restricted water exchange and the dependence from their watershed generally with a strong input of nutrients, make them vulnerable to eutrophication processes. Under these conditions sediment-water exchanges could be important for water quality. In view of that this thesis aims to:

- Identify the major environmental pressures in the lagoon focusing on nutrients and metals analysis on the different environmental matrices.
- Provide a better understanding of the cause-effect relationships to the pulse metal release (eutrophic areas) in several target organisms with distinct roles in trophic webs - macroalgae, invertebrate and fish - through the integrated assessment of bioaccumulation and biomarkers.
- Evaluate a battery of biomarkers, identify the sensitive responses, and propose a suitable biomonitoring tool to the environmental health status, stretching the relationship with the metal accumulation in tissues.
- Achieve a mechanistic knowledge concerning the metals toxicodynamics (uptake, distribution, accumulation) and organ-specific responses.
- Assess the efficacy of a strategy integrating chemical, biochemical and biological parameters.

At a national level, this study may provide important data to help the implementation of restoration and management decisions.

This thesis is composed by Chapters II, III, IV, V, VI, VII and VIII corresponding to articles that were submitted to SCI journals.

Chapter II – The classification of a trophic state of a system requires information about the spatial and temporal dynamics of nutrients. Nutrients were quantified seasonally in water column of five sites at Óbidos lagoon between 2004 and 2006 to achieve the trophic state of the ecosystem.

Chapter III – One of the major problems in eutrophic areas is the decline of dissolved oxygen concentrations during the night and the possible pulse release of nutrients and metals from the sediments. Nutrients, redox-sensitive elements (Fe, Mn) and metal contaminants (Cu, Ni, Cr, Pb, Cd) were measured in water and suspended particles each two hours over 24-hours to examine the pulse input from the sediments in a confined branch of the lagoon under summer conditions.

Chapters IV and VIII – Strategies combining external levels of exposure, bioaccumulation markers and oxidative stress responses are required in the monitoring of aquatic systems. Therefore, peroxidative damage and antioxidant responses were measured

in *Ulva* sp. on a seasonal basis and their relationship with metal accumulation, as well as with environmental chemical data (metals and nutrients) was assessed. A general stress index termed “Integrated Biomarker Response” (IBR), combining all the oxidative stress endpoints, was used to summarize the biomarker responses (Chapter IV).

Moreover, peroxidative damage, antioxidants and biotransformation responses were measured seasonally in hepatopancreas of *Carcinus maenas* and their relationship with metal accumulation, as well as with environmental chemical data (metals and nutrients) were assessed (Chapters V and VI).

Additionally, gills, liver and kidney of *Liza aurata* were monitored for metals accumulation and oxidative stress in two contrasting seasons (winter and summer). Peroxidative damage and antioxidant (enzymatic and non-enzymatic) responses were measured in *L. aurata*’ organs and their relationship with metal accumulation as well as with environmental chemical data (metals and nutrients) were assessed (Chapters VII and VIII).

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CHAPTER II

Spatial and seasonal variation of water quality in an impacted coastal lagoon (Óbidos Lagoon, Portugal)

2 Spatial and seasonal variation of water quality in an impacted coastal lagoon (Óbidos Lagoon, Portugal)

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Abstract

The spatial distribution of silicate, ammonium, nitrate, nitrite, phosphate, chlorophyll *a* and dissolved oxygen in Óbidos lagoon was obtained by surveying five sites in eight campaigns, between October 2004 and October 2006. A confined inner branch of the lagoon showed higher availability of ammonium (1.2-81 µmol L^{-1}), phosphate (1.9-17 µmol L^{-1}), silicate (0.85-86 µmol L^{-1}) and chlorophyll *a* (0.30-18 µg L^{-1}) than other sites (0.47-25 µmol L^{-1}, 0.10-3.9 µmol L^{-1}, 0.47-25 µmol L^{-1}, 0.25-11 µg L^{-1}, respectively). According to several trophic classification tools, that branch is considered eutrophic to polytrophic, emphasising its deteriorated conditions, while the rest of the lagoon is of better quality. In autumn/winter nutrients were inversely correlated to salinity ($r>0.93$) reflecting the freshwater inputs enriched in nitrogen and phosphorous compounds to the inner branch. In warmer periods, dissolved oxygen concentrations dropped during the night, and sediments of the branch become an important source of ammonium and phosphate. The low DIN:P ratio (median=10) obtained in the branch, which suggests an excess of phosphate that increased in warmer periods and changed the limiting nutrient in the entire lagoon. These results emphasize the spatial heterogeneity of water quality in Óbidos lagoon, its seasonal variability, and the importance of recognising these distributions before defining homogenous water body on the scope of Water Framework Directive.

Keywords: Coastal lagoon, Eutrophication, Nutrient dynamics, Sediment, Water Framework Directive

2.1 Introduction

Small rivers running towards the coast do not always reach the sea. Discharge to the ocean is often prevented by active beach-ridges forcing water and suspended particulate matter to accumulate in small coastal lagoons. The ecology of the lagoons is determined to a large extent by freshwater inputs and the mixing and circulation processes with the adjacent sea (Postma, 1981; Ittkoot et al., 2000). In lagoons with a permanent connection to the sea and strong tidal amplitudes, the circulation is highly influenced by semi-diurnal and fortnight tidal cycles, and only episodically freshwater discharges force the salinity gradient to move seaward near the inlet (Boynton et al., 1996). In densely populated regions, the discharge of nitrogen and phosphorous is augmented by domestic and industrial waste waters, urban drainage and agricultural effluents (Cabeçadas et al., 1999; Lillebø et al., 2005; Lopes et al., 2007). Additionally, most of the particulate organic matter that reaches the bottom is mineralised in top sediment layers, promoting a spatial variability in the nutrient exchange across the sediment-water interface (Lerat et al. 1990; Forja et al. 1994; Vidal and Morguí, 1995).

Coastal lagoons with these vulnerabilities, exhibit frequently a temporary and progressive decline of water quality. The increasing number of ecosystems with these symptoms led environmental managers to identify eutrophication as a major worldwide problem (Cloern, 2001; Hauxwell and Valiela, 2004; Lillebø et al., 2007). Besides the high nutrient loading, primary producers may be limited to growth due to alterations of ratios between DIN, P and Si (Newton and Mudge, 2005; Gikas et al., 2006; Lopes et al., 2007). Although the Redfield molar ratios for phytoplankton growth (Si:DIN:P=16:16:1) are merely used to define the resource availability (del Amo et al., 1997), a shift from these proportions may change the species dominance and composition, and results in loss of diversity (Tilman et al., 1982; Gikas et al., 2006; Lopes et al., 2007). Moreover, the limiting nutrient to primary production may vary seasonally (Falcão and Vale, 1998). Since regeneration of nutrients in upper sediments depends on the supply of particulate organic matter, temperature and oxygen availability (Forja et al. 1994; Chapelle 1995; Asmus et al. 2000), fluxes to the overlying water are influenced by these environmental factors (Kristensen 1993; Wilson and Brennan, 2004), and consequently also the succession of primary producers.

Within the framework of protecting the quality of surface water bodies, various ecological classification tools have been proposed to assess eutrophication (e.g. Bricker et al.,

1999; Crouzet et al., 1999; Wasmund et al., 2001). However, the indexes are based on annual or winter means of nutrient levels, which may be insufficient in coastal lagoons impacted by organic loads.

This work presents the nutritional status of Óbidos coastal lagoon, an impacted ecosystem in Portugal, and discusses its spatial and seasonal variations. This study emphasises the importance of recognising the variability of water quality parameters, in order to define homogeneous water body within the Water Framework Directive (WFD).

2.2 Material and Methods

2.2.1 Study area

The Óbidos lagoon is a shallow coastal lagoon with a mean depth of 1 m and a wet area of 7 km^2, located on the west coast of Portugal (Figure 2.1). The lagoon is permanently connected to the sea through a narrow inlet. The position of the inlet and the configuration of the channels in lower part of the lagoon have changed naturally during the last decades (Oliveira et al, 2006). Three areas with different morphological and sedimentary characteristics have been identified (Quintino, 1988; Oliveira et al., 2006): several sand banks, narrow channels and strong currents in the lower part, weaker velocity intensities in the broad middle lagoon, and muddy bottom sediments in the inner branches Barrosa and Bom-Sucesso. The freshwater tributaries, Cal, Vala do Ameal and Arnóia drain agricultural areas and enter the lagoon at Barrosa, Bom-Sucesso and between the two branches, respectively. The freshwater discharges are negligible in summer (<0.05 m^3 s^{-1}) (IST/IPIMAR, 2008) and annually amount to an average of 3 m^3 s^{-1} (Oliveira et al., 2006). During the past decades domestic effluents from the town Caldas da Rainha had been discharged to the Cal River that ends into the Barrosa branch. Although, at the present time, this nutrient load enters directly the coastal zone adjacent to the lagoon through a submersed outfall, Cal River continued to have deteriorated quality conditions according to the Portuguese classification of freshwater systems (IST/IPIMAR, 2008). In fact, monthly campaigns carried out in 2007 indicated flows varied from 0.1 and 0.5 m^3 s^{-1} with elevated levels of ammonium (2.9-9.4 mg L^{-1}), nitrite (0.17-1.2 mg L^{-1}) and phosphate (1.4-6.3 mg L^{-1}). Tide energy dissipates in the entire lagoon, and tides range between 1 and 2 m (Oliveira et al., 2006). The nutrient load and the longer

resident time of water in inner branches (24-26 and 4-10 days in Bom-Sucesso and Barrosa, respectively) in comparison to the middle/lower lagoon (1-4 days) (Santos, personal communication) favour the macroalgal cover (*Ulva* sp. and *Enteromorpha* sp.), as well as accumulation of organic matter in sediments (Loss of Ignition between 5.7 and 7.5%) (IST/IPIMAR, 2008). Opportunist macroinvertebrate species found in Barrosa corroborates the eutrophic condition in the branches (Carvalho et al., 2005, 2006).

2.2.2 Sampling and methodologies

Five sampling sites were selected to give an adequate representation of the various morphological conditions of the lagoon (Figure 2.1): site A located near the inlet; B and C in the middle of the lagoon; D and E in Bom-Sucesso and Barrosa branches, respectively. The sites were visited in October 2004, February, June and October 2005 and February, May, August and October 2006.

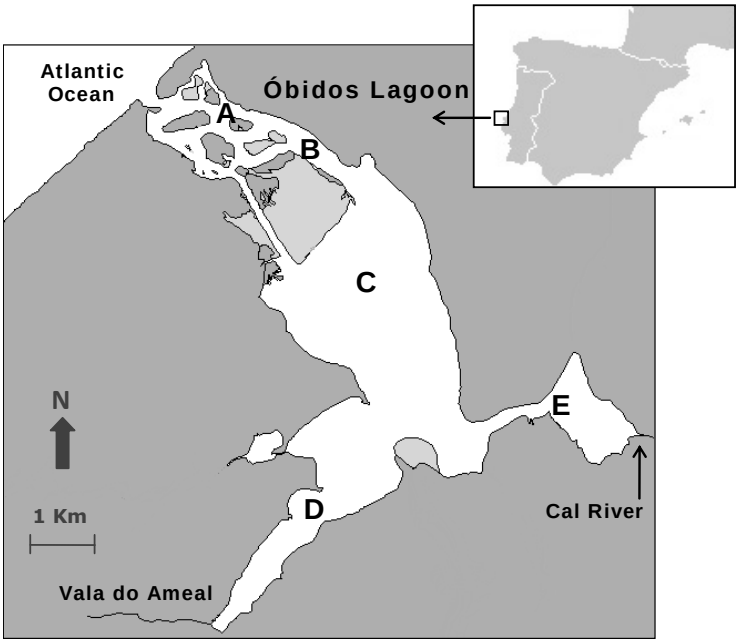

Figure 2.1. Location of the sampling sites at the Óbidos lagoon: A (inlet); B and C (middle lagoon); D and E (Bom Sucesso and Barrosa branches, respectively).

Surface water was sampled in high tide and low tide of the inlet channel. For logistic reasons, sampling started at the inlet and ended at inner branches. Each survey took less than two hours. Only temperature was measured *in situ* using an YSI, 650 meter. Water was sampled to various bottles according to the analytical specifications and transported to a field laboratory for filtration and preservation of the samples. Salinity was measured using an Autosal (Guildline Model 8400B Analyzer) analyzed against "IAPSO standard Sea Water" with accuracy of 0.003. Dissolved oxygen was determined by a modified Winkler method according to Carrit and Carpenter (1966). The coefficient of variation associated with this method was determined by analyzing replicates and was found to be less than 0.25%. Dissolved oxygen saturation was calculated according to OSPAR (2001). Suspended particulate matter (SPM) was obtained by filtering 250 mL of water through cellulose acetate membranes (0.45 µm) and determined gravimetrically (drying at 70 °C). Samples for the determination of dissolved inorganic nutrients (nitrate, NO_3^-+NO_2^-; ammonia, NH_4^+; phosphate, PO_4^{3-} and silicate, $Si(OH)_4$) determinations were filtered through MSI Acetate Plus filters, and analysis carried out using an autoanalyser TRAACS 2000 (Bran+Luebbe). Certified standards (WAKO, CSK Standard Solution) were used to ensure the accuracy of the procedures and precision was found to be ±1.0% for nitrate, ±2.0% for ammonia, ±1.9% for phosphate and ±1.1% for silicate. For chlorophyll *a* (Chl *a*) determinations, 250 mL of water was filtered through a Whatman GF/F (0.7 µm) filter that was immediately frozen at -20 °C and later extracted in 90% acetone, for analysis in a Perkin Elmer Fluorometer using the modified protocol by Lorenzen (1966). Commercial solutions (Sigma Chemical Co.) of Chl *a* were used to calibrate the fluorometer. The coefficient of variation associated with this method was less than 1.8%.

2.2.3 Data analysis

Statistical software (Statistica 6.1) was used for statistical analyses. ANOVA analysis was used to compare sampling sites and Tukey test was applied for post-hoc comparison (Zar, 1996). Differences between means were considered significant when $p<0.05$. A Pearson correlation was performed to evaluate the degree of relationship between the analysed parameters.

2.3 Results

2.3.1 Temperature, salinity, dissolved oxygen and suspended particulate matter

Water temperature at the five sampling sites ranged within narrower intervals in winter surveys (e.g. 10-12 °C in February 2005) than in summer (e.g. 18-25 °C in August 2006). Elevated values were registered in the afternoon (low tide) at the inner branches. The median, the percentile 25% and 75%, maximum and minimum of salinity in each site are shown in Figure 2.2. Outliers and extreme values marked in the figure were not taken into account for the median calculation. In most of the surveys salinity decreased landwards, from site A to E, reflecting the freshwater discharges to the inner branches. However, due to the small flows, the salinity gradient was located in the upper areas. Salinity in sites A and B ranged within narrow intervals centred at 36, the lower values being registered in low tide of February and October 2006 (extreme values in Figure 2.2). In sites D and E, values ranged within broader intervals (29.1-37.1 and 25.9-37.1, respectively): the lower ones were registered in periods of higher freshwater discharges (autumn and winter); and values exceeding seawater salinity were found in May 2005 and August 2006, which indicates that freshwater discharges did not compensate evaporation. Considering all salinity data, values in site E were significantly ($p<0.05$) different from those observed in sites A, B and C.

The dissolved oxygen concentrations in sites A, B and C varied from 85 to 135% saturation. The broader interval in sites D and E (60-185%) indicated supersaturation that is attributed to both macroalgae and phytoplankton. Undersaturation was usually recorded in the morning of warmer periods, reflecting higher consumption during the night by respiration and organic matter oxidation, while supersaturation was observed in the afternoon, as result of intense photosynthesis.

The suspended particulate matter (SPM) concentration in the sites A, B and C varied from 1.9 to 77 mg L^{-1}, reaching 141 and 170 mg L^{-1} in sites D and E, respectively. Values were lower in autumn/winter (October 2004, October 2005 and February and October 2006) than in spring/summer (e.g., May and August 2006), and variation between low and high tide was not consistent. The strong wind intensity occurred in the afternoon of several sampling dates, may have caused bottom resuspension that could be responsible for small differences of SPM between sites and sampling periods.

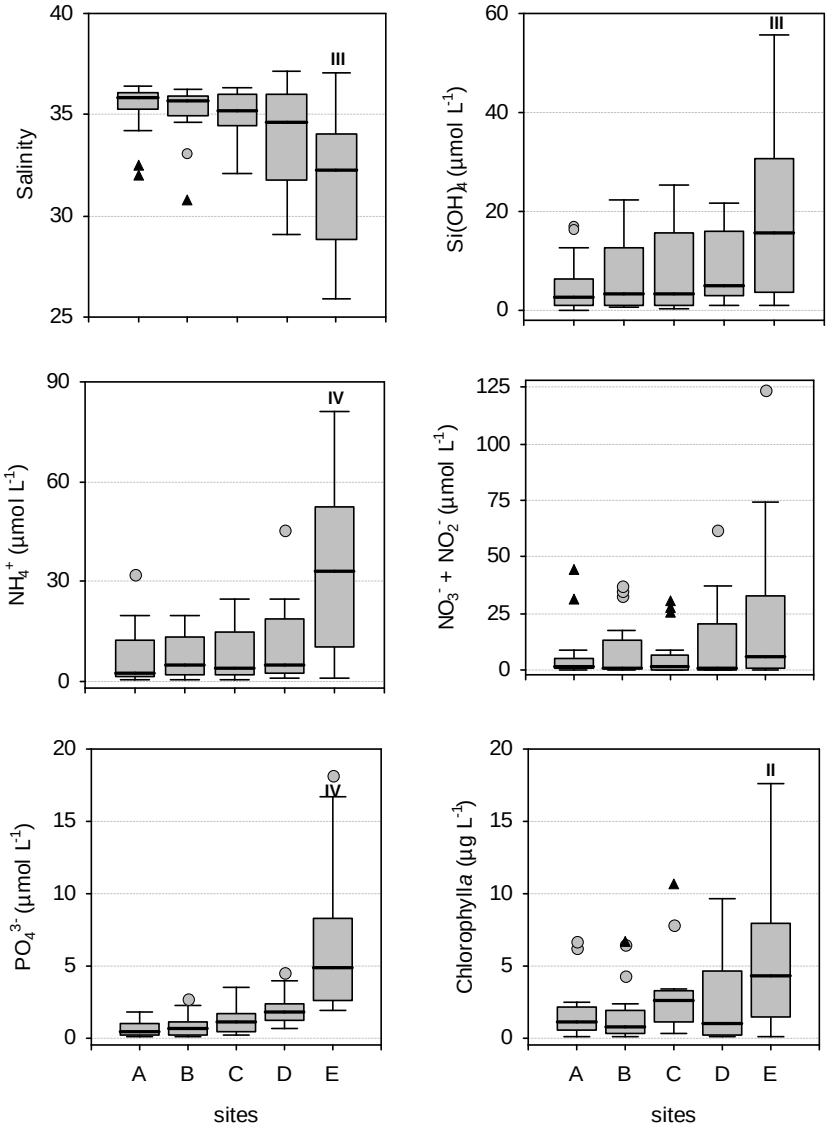

Figure 2.2. Salinity, $Si(OH)_4$, NH_4^+, NO_3^-+NO_2^-, PO_4^{3-} (µmol L^{-1}) and chlorophyll *a* (µg L^{-1}) in the sampling sites (A, B, C, D and E) for the surveyed period: median, percentile 25% and 75%, maximum and minimum, outliers (●) and extreme (▲) values. II- significant different from sites A and B; III - significant different from sites A, B and C; IV - significant different from sites A, B, C and D.

2.3.2 Nutrients and chlorophyll *a*

Figure 2.2 shows the median, the percentile 25% and 75%, maximum and minimum concentrations of ammonium, nitrate, silicate, phosphate and chlorophyll *a* in each sampling site. Site E exhibited broader concentration ranges of all determinations than the other sites. Silicate, ammonium and phosphate in site E (0.9-56 µmol L^{-1}, 1.2-81 µmol L^{-1}, 1.90-17 µmol L^{-}

^{1}, respectively) were significantly ($p<0.05$) higher than in sites A, B and C (0.5-25 µmol L^{-1}, 0.5-25 µmol L^{-1}, 0.10-3.9 µmol L^{-1}, respectively). Nitrate plus nitrite concentrations amounted to values comparable to ammonium (max. 74 µmol L^{-1}) and although sites D and E reached higher values, no significant ($p<0.05$) differences were obtained from sites A, B and C. Chlorophyll *a* was in general higher in site E (max. 18 µg L^{-1}) and significantly ($p<0.05$) higher than in sites A and B.

Figure 2.3 shows the concentrations of silicate, ammonium, nitrate+nitrite, phosphate and chlorophyll *a* registered, in low and high tides, in sites A and E that represent the marine- and the river-end members of the lagoon, respectively. All the values of the two sites were considered, including those identified as outliers and extremes for the calculation of the median. In site A, nutrient concentrations in high tide were lower than in low tide, indicating that the incoming of seawater diluted the nutrient concentrations of the lagoon. The dilution effect was more accentuated in periods of elevated nutrient concentrations, although a seasonal pattern could not be identified for all nutrients. In site E, maximum nutrient values correspond always to low tide.

Ammonium was elevated in October 2005, February and October 2006 (max. 32 and 81 µmol L^{-1} for sites A and E, respectively) and was low in June 2005 and August 2006 (min. 0.5 and 1.2 µmol L^{-1} for sites A and E, respectively). A similar trend was observed for silicates that had also a peak in the same sampling periods (max. 17 and 56 µmol L^{-1}) and minimum values in February 2005 (min. 0.9 µmol L^{-1} in both sites). The nitrate variation in the two sites was dominated by a pronounced peak in February 2006 (45 and 124 µmol L^{-1}). Phosphate in sites A and E showed elevated levels in June 2005 (1.5 and 18 µmol L^{-1}, respectively). In site A other maximum values were observed in February and October 2006, with considerable differences between low and high tide. The semi-diurnal differences of Chl *a*, superimposed the seasonal variation in site E particularly in October 2004, October 2005 and August 2006. In site A emerged the high values in low and high tide of October 2005.

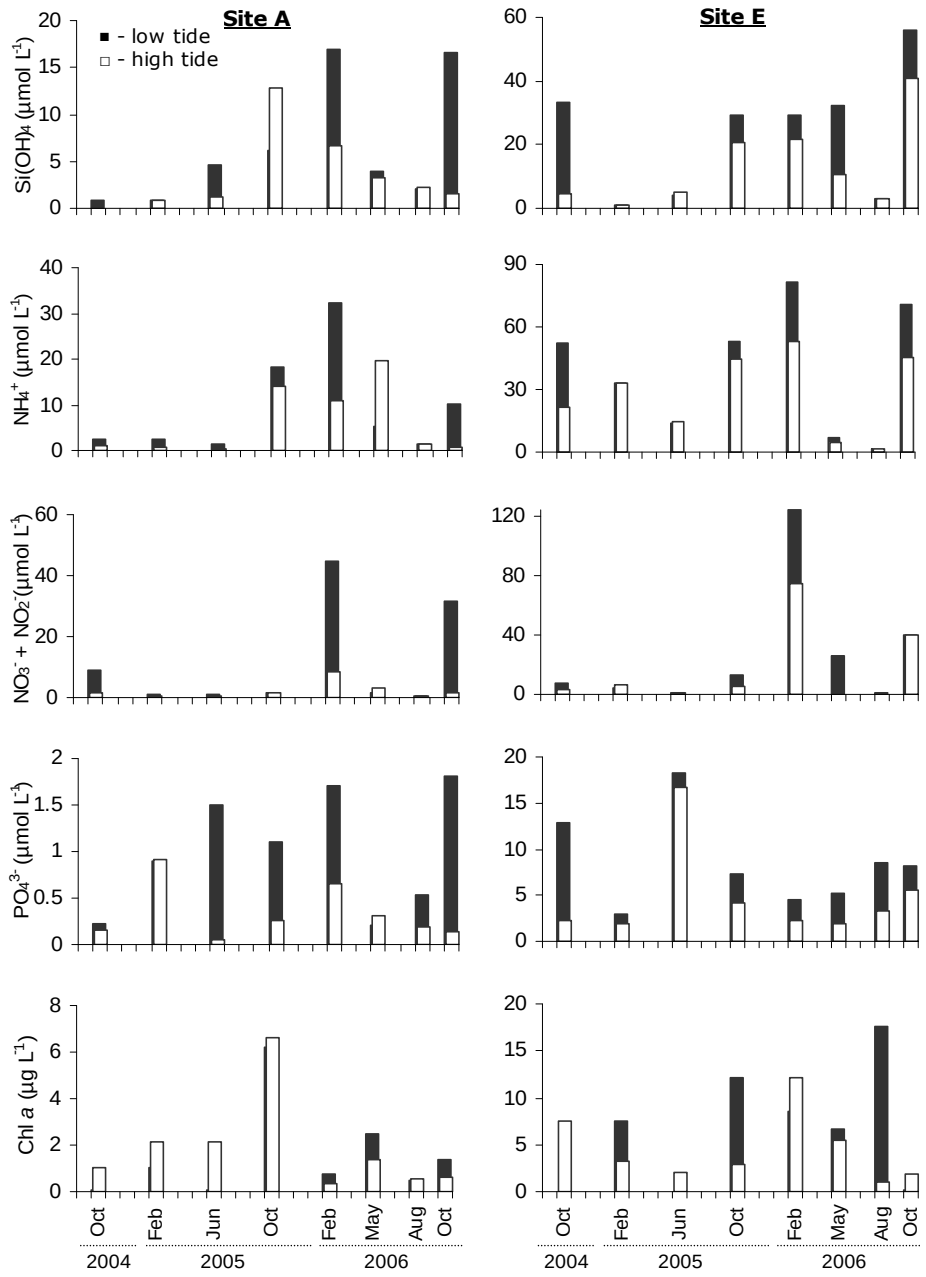

Figure 2.3. Seasonal variation of $Si(OH)_4$, NH_4^+, NO_3^-+NO_2^-, PO_4^{3-} (µmol L^{-1}) and chlorophyll *a* (µg L^{-1}) in sites A and E, in low and high tides.

2.3.3 Relationships between nutrients and salinity

Pearson correlations (r) between each nutrient and salinity were applied to all data (n=80). Silicate, ammonium and nitrate+nitrite exhibited significant ($p<0.05$) inverse linear relationships with salinity: -0.77, -0.62 and -0.60, respectively. Despite the narrow interval of salinity in most of the surveys, the obtained correlation coefficients suggest that nutrients were relatively well explained by conservative mixing between the nutrient-rich freshwater

and seawater. The decrease of silicate, ammonium, nitrate+nitrite and phosphate with salinity (Figure 2.4) was better observed in February 2006 (-0.95, -0.95, -0.96 and -0.93 respectively). The low chlorophyll *a* in the lagoon (except in site E) favours the observed conservative behaviour of nutrients during this rainy period (59 mm of monthly average rainfall) (SNIRH, 2008). Rainfall decreased in May and August 2006 (1.2 and 4.5 mm, respectively) and increased in October (189 mm). In August 2006, salinity ranged within the interval 36.0 to 37.1, increasing landwards. The higher nutrient levels in samples where salinity exceeded the seawater value and under negligible freshwater discharge conditions should result from evaporation of the lagoon water in summer. Phosphate presented a positive correlation with salinity (r= 0.98).

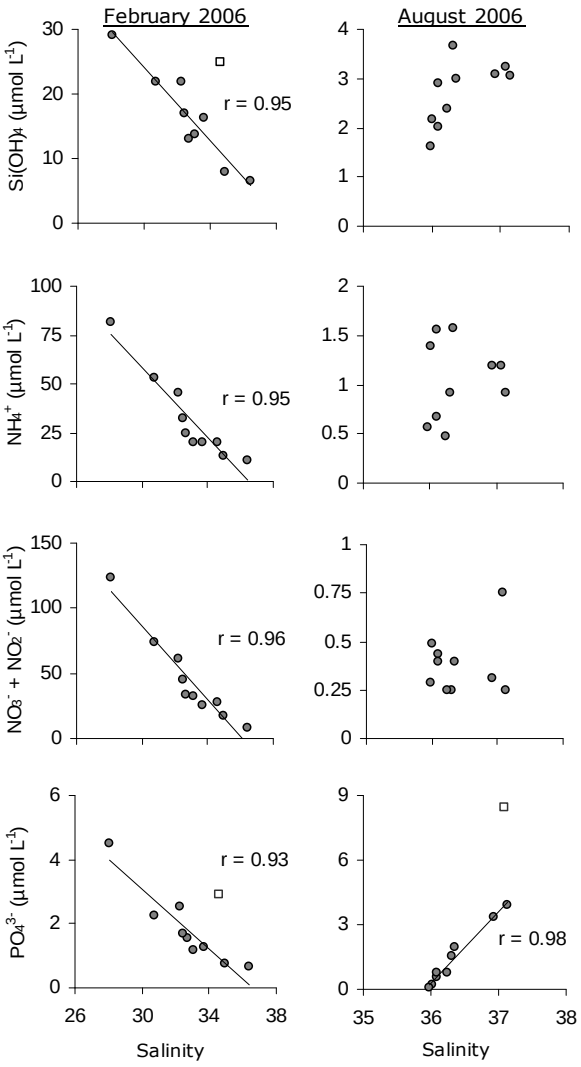

Figure 2.4. Relationships between salinity and nutrients (µmol L^{-1}) at all sites in February 2006 and August 2006. (□ - value not considered in the relationship).

2.3.4 Molar ratios

The molar ratios of dissolved inorganic nitrogen (DIN=NH_4^++NO_3^-+NO_2^-):P and DIN:Si were calculated for all data. Figure 2.5 shows the median of these ratios, the percentile 25% and 75%, maximum and minimum in each site. The identified outliers and one extreme value were not taken into account for the median calculation. The two plots present broader ranges of values in site A decreasing towards the site E. Ratios in this site were significantly ($p<0.05$) lower than in site A. Most of the DIN:P ratios in sites C, D and E were lower than the Redfield ratio (16), while the median in A and B were 19 and 18, respectively. Almost all the Si:P ratios in the five sites were below the respective Redfield ratio (16), and the median of DIN:Si ratios for the five sites were above the respective Redfield ratio (1).

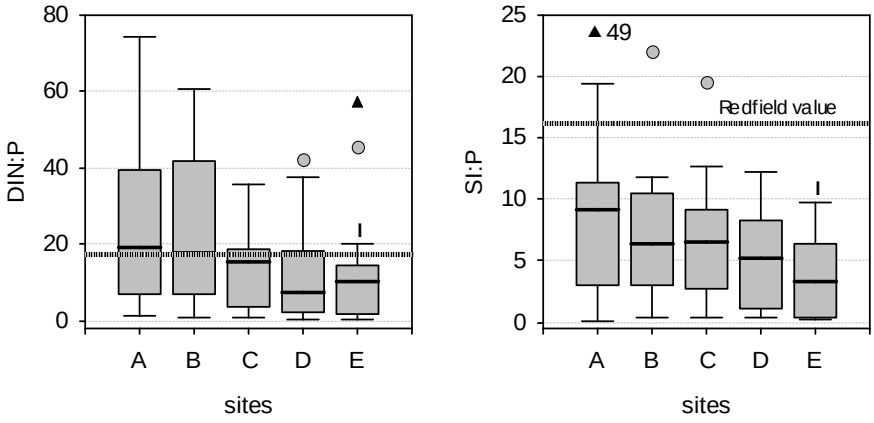

Figure 2.5. DIN:P and SI:P ratios in the sampling sites for the surveyed period; median, percentile 25% and 75%, maximum and minimum, outliers (●) and extreme (▲) values. I - significant different from site A.

Figure 2.6 shows DIN:P and Si:P ratios in sites A and E, in low and high tides, at each sampling periods. Site A exhibited broader differences of the ratios between low and high tide, resulting from the exchange with adjacent seawater that masked an eventual seasonal signal of the ratios. Otherwise, a seasonal variation was observed in site E. The ratios DIN:P were lower than 16 (Redfield ratio) in all surveys, except in February 2005 and February 2006. The ratios Si:P were always lower than 16, with lowest values recorded in February 2005 (0.3) and June 2005 (0.2) and August 2006 (0.4) and maximum ones in February 2006 (10).

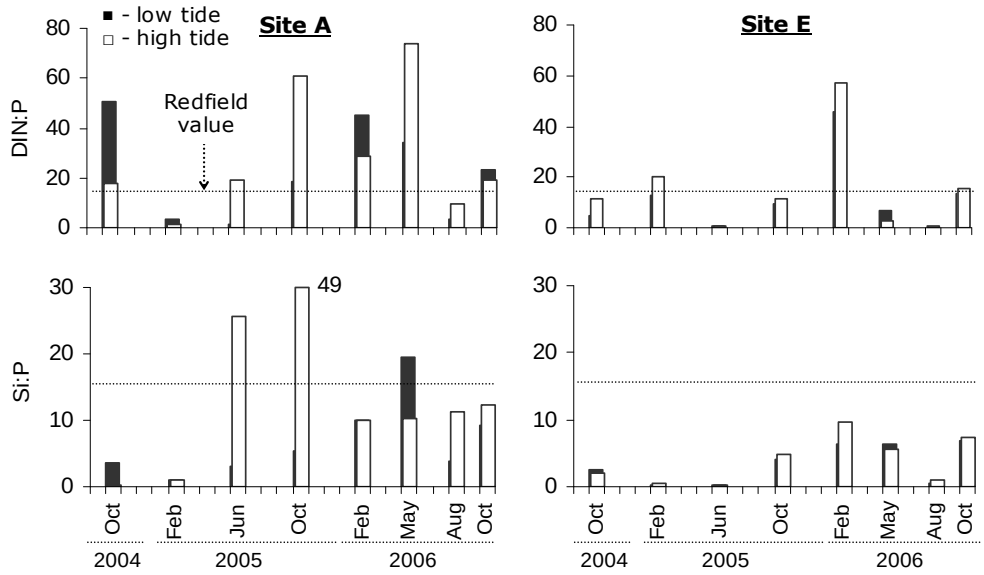

Figure 2.6. Seasonal variation of DIN:P and SI:P ratios in sites A and E, in low and high tides.

2.4 Discussion

The availability of nutrients in the water column of Óbidos lagoon varied between autumn/winter and spring/summer, although concentrations in northern inner branch (site E) exceeded always those found in other areas (except for nitrate). Compared to other temperate estuarine systems the mean concentrations of DIN, phosphate and silicate fell within the range of values described for the Mondego estuary (Lillebø et al., 2005) and Ria de Aveiro, located in western Atlantic coast of Portugal (Lopes et al., 2007). As in Óbidos lagoon, tributaries receiving domestic and agriculture effluents discharge into confined areas of those systems, which exchange water with the rest of the system through narrow channels. Otherwise, nutrients in northern inner branch of Óbidos lagoon are frequently more abundant than in Ria Formosa, a shallow coastal lagoon in south-western Iberia exchanging 70% of its water volume in spring tides (Falcão and Vale, 2003).

Despite the small quantity of freshwater discharged annually into the inner parts of the lagoon (Oliveira et al., 2006), a longitudinal trend of nutrients was observed, as in many other estuarine systems with an important freshwater input (Cabeçadas et al., 1999; Lopes et al., 2007). The inverse relationships between nutrients and salinity in autumn/winter indicate the input of silicate, ammonium, nitrate and phosphate derived from the drainage of the catchment area that consists of agriculture fields and small villages. In other surveyed

periods, with small salinity intervals, correlations were not obtained. However, the availability of nutrients appears to favour the high biological production, as evidenced by the high chlorophyll *a* and macroalgae (*Ulva* sp. and *Enteromorpha* sp.) that covers almost the entire sediment (Carvalho et al, 2006).

Table 2.1. Trophic status of Óbidos lagoon according to indexes proposed by Crouzet et al. (1999), Carlson (1977) and Wasmund et al. (2001). * Annual mean.

Parameters	Sites	Classification method				
		Annual mean	Crouzet et al., 1999	Carlson, 1977	Winter mean	Wasmund et al., 2001
$NO_3^- + NO_2^-$ ($\mu mol\ L^{-1}$)	A,B,C,D	6.7 – 12	fair - poor	-	-	-
	E	22	Bad			
PO_4^{3-} ($\mu mol\ L^{-1}$)	A,B	0.67 – 0.80	fair - poor	Mesotrophy	1.0 - 1.7	Eutrophic
	C,D	1.4 – 1.6	Bad	Eutrophy		
	E	6.4		Hypereutrophy	3.0	Polytrophic
DIN ($\mu mol\ L^{-1}$)	A,B,C,D	-	-	-	25 – 47	Eutrophic
	E				102	Polytrophic
Chorophyll *a* ($\mu g\ L^{-1}$)	A,B,C,D	1.7 – 2.6	-	Hypolimnia	1.7 – 2.6*	Mesotrophic
	E	5.6		Mesotrophy	5.6*	Eutrophic

The specificity of that branch was confirmed by the application of three methods for trophic classification (Table 2.1). On the basis of the EU-Crouzet et al. (1999) method, which considers the annual means of nitrate+nitrite and phosphate, sites E, D and C were classified as “bad” (based on phosphate), while only site E was “bad” using the nitrate+nitrite. The status of other sites was between “poor” to “fair”. The Carlson’s Trophic State Indices (TSI) (1977) uses annual means of phosphate and Chl *a* as metrics. The TSI values (Chl *a*) point site E as “mesotrophic”, while according to phosphate was “hypereutrophic”. The method proposed by Wasmund et al. (2001) is based on the annual means of Chl *a*, and winter means of phosphate and DIN concentrations. According to Chl *a* only site E was “eutrophic”, but using DIN and phosphate this site was “polytrophic”. The others sites were classified as “mesotrophic” (Chl *a*) to “eutrophic” (DIN and phosphate). All these tools pointed to an extreme deterioration conditions in the northern inner branch (site E) and better quality status in other areas of the Óbidos lagoon, although having a less coherent classification. These classifications agree with the results obtained from the use of the marine biotic index

AMBI in the assessment of the ecological status of the Óbidos lagoon (Carvalho et al., 2006). The increase in organic matter content of sediments from down- to upstream areas was associated with the dominance of opportunistic benthic species, while sensitive species to organic enrichment were mainly associated with the clean sandy area in the middle and lower lagoon. Regardless the low levels of chemical contaminants, the AMBI index allowed identifying the northern branch as an impacted area.

Although this study was not designed to investigate the role of sediments as internal source of nutrients to the water column, their influence in the regeneration of nutrients was apparent. The increase of phosphate in inner branches of Óbidos lagoon in summer surveys suggests its release from sediments as oxidation of organic matter increases under high temperatures. Salinity by exceeding the seawater value (Figure 2.2) rules out the hypothesis of the input of freshwater enriched in phosphate. It has been demonstrated the relevance of nutrient regeneration in sediments to the ecology of various shallow ecosystems. For example, studies developed in Ria Formosa showed that inter-tidal areas are apparently capable of supplying most of the daily N and P requirements of phytoplankton in the overlying water (Falcão and Vale, 1998).

Temperature is a key factor influencing the benthic fluxes of nutrients (Van Raaphorst et al. 1992; Kristensen 1993; Wilson and Brennan 2004) and promotes a temporal variability in sediment-water nutrient exchanges in coastal environments with marked seasons (Forja et al. 1994; Vidal and Morguí, 1995). Nevertheless, only phosphate concentrations were positively correlated with temperature in summer (August 2006) ($r=0.95$, $p<0.05$), indicating the release of phosphate from sediments of the inner areas of the lagoon where higher temperature was registered. Significant correlation was not obtained considering all data from June 2005, May and August 2006 (periods of negligible rainfall), suggesting influences of other variables besides temperature. No correlations were also found for ammonium and nitrate, reflecting complex processes associated with the cycle of nitrogen. Ammonium may differ from steady-state conditions reflecting the balance between production through organic matter mineralization (Bally et al. 2004), nitrification/denitrification (Vidal and Morguí, 1995) and consumption by the abundant primary producers living near the sediment-water interface (*Ulva* sp. and *Enteromorpha* sp.), corroborated by the increase in chlorophyll *a* in summer. The undersaturation levels of dissolved oxygen observed in the inner branches of Óbidos lagoon suggest insufficient oxygen diffusion across the water-sediment interfaces

during the night. The decrease of nitrification rates explains the high variation of nitrate concentrations. Nitrification/denitrification processes are also temperature-dependent. Thus, during the period of higher temperature (max. of 25 °C), denitrification of nitrate to the gaseous forms of nitrogen N_2 and N_2O (Cartaxana et al. 1999) may contribute to drop nitrate levels in sediment porewater of the extensive inter-tidal areas exposed to the atmosphere around low tide.

The positive linear relationship between phosphate and salinity emphasises two important aspects: sediment becomes a relevant internal source of P when temperature increases, and its consumption by the abundant primary producers does not cause a negative deviation on its correlation with salinity. These conclusions are supported by the positive relationship between phosphate and temperature in August 2006. It is well documented that phosphorus reacts with a wide variety of surfaces, being taken up and released from biogenic and abiogenic particles (Van Raaphorst and Kloosterhuis 1994). The retention/release of phosphate in marine systems is controlled by temperature and dissolved oxygen as referred in several studies (Slomp et al. 1998; Asmus et al. 2000). Phosphorus accumulated in solid phase during winter, partially due to P-sorption onto iron oxides, is released to sediment porewater and transferred to the overlying water in periods of elevated temperature due to reducing sediment conditions (Ohtake et al. 1984; Van Raaphorst and Kloosterhuis, 1994). Low dissolved oxygen in water column of the Óbidos inner branches registered during the morning, points to reducing conditions in the sediment during the night.

Presumably, the phosphate released from the sediments was not consumed in the inner branches as ammonium, since DIN:P ratio was low (median of 7 to 10). Although Redfield molar ratio (DIN:P=16) are merely indicative of resource availability (del Amo et al, 1997), the obtained values fall into a region in which P excess is most likely to occur. The excess was more noticed in warmer periods presumably due to the increase of phosphate released from the sediments. Furthermore, DIN:P ratios was also low in other sites of the lagoon, suggesting the dispersion of phosphate by the tide and supporting the hypothesis of phosphate being in excess in the entire lagoon during spring/summer. The two illustrative conditions presented in Figure 2.6 indicate that only when substantial amounts of freshwater are discharged into the lagoon (e.g. February 2006), the DIN:P ratios in the northern inner branch, became higher than the Redfield ratio (16:1), meaning that external input of fresh nitrogen compensates the excess of phosphate and inverses the nutrient limiting situation.

The low Si:N and Si:P ratios point to a limitation in silicate, more accentuated in the upper part of the lagoon. These conditions seem to favour the non-siliceous-based phytoplankton food webs and it has been compared to a loss of environmental quality status (Rocha et al., 2002; Domingues et al., 2005).

Given the implementation of the Water Framework Directive in a large number of impacted coastal lagoons in Europe, it is important to assess the seasonal fluctuation of quality status before the designing of the monitoring programmes. A particular relevant challenge is to establish scenarios, eventually supported by models, in order to predict changes and spatial variability on nutrient availability and its implications on phytoplankton, macroalgae and benthic macroinvertebrates, which are key biological elements to define the quality of coastal and transitional waters.

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CHAPTER III

Metal and nutrient dynamics in a eutrophic coastal lagoon (Óbidos, Portugal): the importance of observations at different time scales

3 Metal and nutrient dynamics in a eutrophic coastal lagoon (Óbidos, Portugal): the importance of observations at different time scales

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Abstract

Water and sediment quality was monitored at four sites of Óbidos coastal lagoon (Portugal) in February, May, July and October 2006, covering different hydrological conditions. Concentrations of nutrients and metals increased in autumn/winter, particularly in an inner branch with symptoms of eutrophication that receives a small tributary contaminated by agro-industrial activities. Moreover, concentrations of PO_4^{3-}, $Si(OH)_4$ and Mn (DGT-measured) varied inversely with salinity. Additionally, that branch was monitored over 26-hours in July 2006 to assess variations of water quality parameters, nutrients and metals on short time-scale. During the night, O_2 in water reached a minimum of 40% saturation followed by a pronounced increase of DGT-measured metals and nutrients in water column: Fe and Mn (10 times); Cr, Co, PO_4^{3-} and $Si(OH)_4$ (6 times). Enhancements were also registered for metal/Al ratios in suspended particulate matter: Mn, Cr and Cd (4-6 times); Fe, Ni and Co (1.5 times). The metal distribution coefficients calculated along the 26-hour survey showed a maximum at daylight suggesting a preferential association of metals with suspended particles. Data recorded under different hydrological conditions and over the 26-h survey allowed to address the influence of external and internal sources on water quality. The results of this study highlight the importance of day/night cycles on the availability of nutrients and metals in eutrophic environments.

Keywords: Coastal lagoon; Sediment; Water quality; Trace-elements; Nutrients; Day-night measurements

3.1 Introduction

Eutrophication became common in transitional and coastal waters, as a result of continued industrialization and growth of human populations. In shallow coastal lagoons with deficient connection to the sea, phytoplankton photosynthesis is often supplemented with photosynthesis by benthic macroalgae (Kennish, 2000). Under those conditions, a large fraction of total primary production occurs near the sediment surface (Serpa et al., 2007). High biogeochemical activity is found in the first centimetres of sediment where organic matter is mineralized by bacteria using oxygen, nitrate, metal oxides and sulphate as electron acceptors (Froelich et al., 1979). Oxygen is energetically the most favourable electron acceptor and is in general abundant in water above the sediment surface. Thus, oxygen is considered a key element of early diagenesis acting as a re-oxidizer of reduced compounds originated from anoxic mineralization by diffusing up to the oxic zone from deeper layers (Candfield et al., 1993).

The surface layer of coastal sediments suffers a complex set of chemical reactions that rarely achieves steady states (Sundby, 2006 and references herein), partially because it is frequently perturbed by the physical activities of organisms that inhabit sediments (Aller, 1994) and by changes in the fluxes of light and organic matter. In fact, the oscillation of the oxic-anoxic boundary, near the sediment-water interface, operates on variable time scales ranging from the momentary supply of oxygen to anoxic sediment during photosynthesis and the passage of a burrowing animal, to erosion-resedimentation of large volumes of sediment in a fluctuating bottom current regime (Sundby, 2006). One of the common characteristics of coastal lagoons undergoing eutrophication is the development of hypoxic or anoxic bottom waters on a temporarily basis or even permanently (Barnes, 1980). The decrease of oxygen availability near the sediment-water interface promotes alterations on biogeochemical processes that may have pronounced repercussions on cycling of nutrients, iron, manganese and trace elements (Point et al., 2007).

A number of studies have documented the daily variations of nutrient concentrations in shallow environments due to changes on the sediment-water fluxes (e.g. Escaravage, 1990; Lillebø et al., 2002). Broad daily variations in concentrations of Cd, Cu, Fe, Mn and Zn were found in metal-rich streams and examined relatively to: pH- and/or temperature-dependent adsorption onto secondary minerals and biofilms on the streambed (Gammons et al.,, 2005); redox reactions involving secondary Mn and Fe oxyhydroxide minerals (Gammons et al.,

2007); and biological uptake (Morris et al. 2006). Despite the fluctuation of those parameters in eutrophic environments, metal measurements on a daily scale are poorly documented. The present work reports the seasonal variation of metal and nutrient concentrations in four sites of Óbidos coastal lagoon and their variation over a 26-hour survey in an inner branch with symptoms of eutrophication.

3.2 Materials and Methods

3.2.1 Study area

The Óbidos lagoon is a shallow coastal lagoon with a mean depth of 1 m and a wet area of 7 km^2, located on the west coast of Portugal (Figure 3.1). The lagoon is permanently connected to the sea through a narrow inlet (Oliveira et al., 2006). The morphology and sediment characteristics contrast between the lower part with sand banks and narrow channels, and the inner branches (Barrosa and Bom-Sucesso) with muddy sediments and residence times longer than two weeks (Quintino, 1988; Oliveira et al., 2006). During the past decades, domestic effluents from the town Caldas da Rainha (50000 inhabitants) had been discharged to the Cal River that ends into the Barrosa branch. The nutrient load and the weak water renewal favour: high *Ulva* sp. growth, reaching 13±8 g AFDW m^{-2} in October 2006 (Carvalho, personal communication); opportunist macroinvertebrate species (Carvalho et al., 2005; 2006); and a broad variation of dissolved oxygen concentration (IST/IPIMAR, 2008). That branch was classified as eutrophic according to several trophic tools (Pereira et al., 2009).

3.2.2 Sampling and *in situ* measurements

Four sites were surveyed in 1st February, 4th May, 27th July and 30th October 2006 (Figure 3.1): sites A and B located in the lower part of the lagoon; and sites C and D in the two inner branches (Bom-Sucesso and Barrosa, respectively). Water temperature was measured *in situ* with an YSI, 650 meter and surface water (0.2 m depth) was sampled in high- and low-tide at daylight hours for determinations of salinity, dissolved oxygen, suspended particulate matter (SPM), dissolved inorganic nutrients, chlorophyll *a* (Chl *a*), DGT-measured metals and

particulate metals. Surface sediments were collected in the four sites with a Van-Veen grab and sub-samples of the first 2 cm depth were stored.

In addition, site D was surveyed every two hours between 10H00 of 19th July and 12H00 of 20th July 2006: solar radiation was measured by a LI-COR quantum sensor; water temperature, salinity, pH and dissolved oxygen concentration were measured *in situ* with an YSI, 650 meter; water was sampled at 0.2 m depth to polypropylene bottles for the determination of DGT-measured metal concentrations; water was collected at the same depth for SPM concentration and associated metal levels. An auto-analyser (Ecolab) was deployed *in situ* (at 0.2 m depth) for the quantification in shorter time intervals (20 to 30 minutes) of turbidity, dissolved inorganic nutrients [NO_3^-+ NO_2^-; PO_4^{3-} and $Si(OH)_4$] and Chl *a* (by fluorescence measurement).

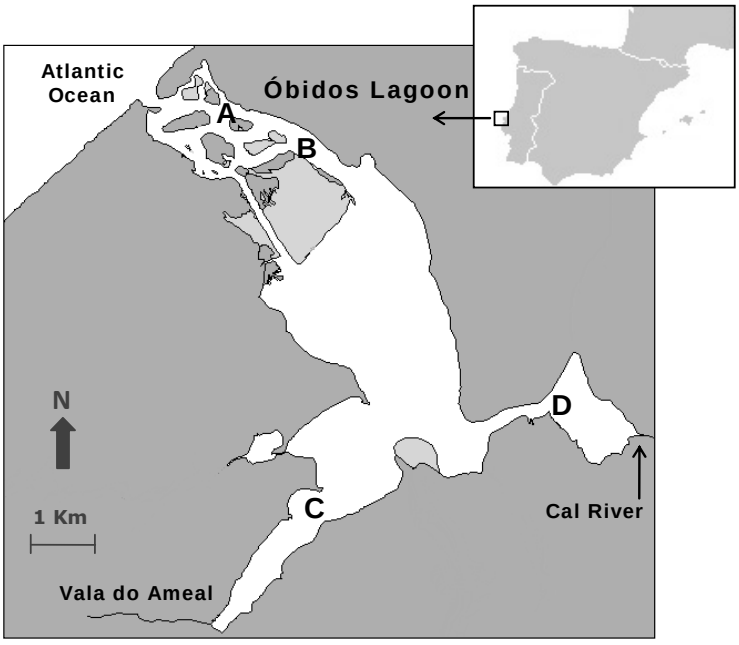

Figure 3.1. Location of the sampling sites A, B, C, and D at Óbidos lagoon.

3.3 Analytical determinations

3.3.1 Salinity, dissolved oxygen, SPM, nutrients and chlorophyll *a*

Salinity in seasonal campaigns was measured using an Autosal (Guildline Model 8400B Analyzer) analysed against "IAPSO standard Sea Water". Dissolved oxygen was determined by a modified Winkler method according to Carrit and Carpenter (1966). Dissolved oxygen

saturation was calculated according to OSPAR (2001). SPM both in seasonal campaigns and diel cycle was obtained by filtering 250 mL of water through polycarbonate membranes (0.45 µm) and determined gravimetrically. Water collected in the seasonal campaigns were filtered through MSI Acetate Plus filters and analysis of dissolved inorganic nutrients were carried out using an autoanalyser TRAACS 2000 (Bran+Luebbe). Detection limits were: 0.1 µM for NO_3^-, NO_2^- and $Si(OH)_4$; 0.01 µM for PO_4^{3-}. For Chl *a* determinations, 250 mL of water was filtered through a Whatman GF/F (0.7 µm) filter that was immediately frozen at -20 °C and later extracted in 90% acetone. The analysis of Chl *a* was carried out in a Perkin Elmer Fluorometer using the modified protocol by Lorenzen (1966) with a detection limit of 0.1 µg L^{-1}. Detection limits of the autoanalyser deployed *in situ* were 0.1 µM for nutrients and 0.255 µg L^{-1} for Chl *a*.

3.3.2 DGT-measured metals

Manganese, Fe, Cu, Cr, Ni, Co and Cd were measured in the collected waters using diffusive gradients of thin films (DGT). All DGT holders, Chelex-100 resins and diffusive gels (type APA, 0.8 mm thickness, open pore) (Zhang and Davison, 1999) were purchased from DGT Research (Lancaster, UK, http://www.dgtresearch.com). The DGT devices were deployed in 2 L polypropylene bottles with unfiltered sampled water and stirred at 24±1 °C for 48 hours. After devices retrieval, water pH was measured and resins were eluted by immersion in 5 mL of 1 M HNO_3 (prepared from suprapur nitric acid) for a minimum of 24 h. Eluates were analysed directly by a quadropole ICP-MS (Thermo Elemental, X-Series) equipped with a Peltier Impact bead spray chamber and a concentric Meinhard nebuliser for the quantification of Mn, Cu, Cr, Ni, Co and Cd. Iron was quantified in the eluate by graphite furnace atomic absorption spectrometry (AAS) (Perkin Elmer, Zeeman 4110ZL). The performance of DGT devices under those conditions was previously tested with spiked samples prepared in ultra-pure water with trace element concentrations between 10 and 100 ng L^{-1}. Added and obtained concentrations by DGT-measurements were not significantly different ($p<0.05$). All eluates were analysed with reagents blanks to control eventual contaminations during the analytical procedure, and with an international standard of river water (SLRS-4) to control the accuracy of the procedure. Detection limits varied between 0.01 nM for Cd and 1.7 nM for Ni.

The eluate concentration was converted into the mass of metal accumulated on the resin using an elution factor with a yield value of 0.8 (Zhang and Davison, 1995) and a resin gel volume of 0.15 mL. DGT-measured metal concentrations (C_{DGT}) were calculated according to the following equation (Davison and Zhang, 1994):

$$C_{DGT} = \frac{(M\Delta g)}{(tAD)} \qquad (1)$$

Where M is the mass of metal accumulated on the resin during the emersion time (t); Δg the diffusive gel thickness (0.08 cm); A the exposure area (3.14 cm^2); and D the diffusion coefficient of the metal in the gels as provided by DGT Research (Lancaster, UK, http://www.dgtresearch.com) ($5.69x10^{-6}$ cm^2 s^{-1} for Mn, $5.95x10^{-6}$ cm^2 s^{-1} for Fe, $4.91x10^{-6}$ cm^2s^{-1} for Cr, 6.06 $x10^{-6}$ cm^2 s^{-1} for Cu, $5.62x10^{-6}$ cm^2 s^{-1} for Ni, $5.78x10^{-6}$ cm^2 s^{-1} for Co, $5.93x10^{-6}$ cm^2 s^{-1} for Cd at 24 °C).

3.3.3 Metal concentrations in particulates

Suspended particulate matter retained on 0.45 µm membranes, as well as sediment samples (100 mg) were mineralized completely with HF (40%) and Aqua Regia (HCl-36% : HNO_3-60%; 3:1) in closed Teflon bombs (100 °C for 1 h), evaporated to near dryness (DigiPrep HotBlock – SCP Science), redissolved with 1 mL of doubled-distilled HNO_3 and 5 mL of ultra-pure water, heated for 20 min at 75 °C, and diluted to 50 mL with ultra-pure water (Caetano et al., 2007a). Reagents blanks and international certified standards of sediments from the National Research Council of Canada (1646a; BCSS-1; MESS-3; AGV-1; MAG-1) were prepared in a similar way of samples to control the accuracy of the procedure. The concentrations of Al, Fe and Mn were determined by flame atomic absorption spectrometry (AAS) (Perkin Elmer Analyst 100) with a nitrous oxide-acetylene flame (Al) and air-acetylene flame (Fe, Mn). The concentrations of Cu, Cr, Ni, Co and Cd were determined by ICP-MS. Levels of the analysed elements obtained in the reference materials were consistently within the ranges of certified values.

To avoid contamination, all bottles and labware used for metal determinations were cleaned with HNO_3 (25%) and HCl (25%), subsequently rinsed three times with ultra-pure water obtained from a Millipore-Q model (18.2 MΩ).

3.3.4 Carbon, nitrogen and phosphorus

Organic carbon (C_{org}) and total nitrogen (N) contents in sediments were determined using the method described by Verardo et al. (1990) in a CHN Fisons NA 1500 Analyser. Total phosphorus (P) concentrations in sediments were determined by the Anderson (1976) method using a spectrophotometer Spectronic Genesys 5.

3.4 Statistical analyses

Statistical software (Statistica 7.0) was used for statistical analyses. ANOVA analysis was used to compare sampling sites in seasonal campaigns and Tukey test was applied for Post-Hoc comparison (Zar, 1996). Differences between means were considered significant when $p<0.05$. A Pearson correlation analysis was performed to evaluate the degree of relationship between parameters.

3.5 Results

3.5.1 Sediment chemical composition

Table 3.1 presents the concentration ranges of Al, Fe, Mn, Cr, Cu, Ni, Co, Cd, N, P and C_{org} in surface sediments collected in the sites A, B, C and D in February, May, July and October 2006. The intervals obtained reflect differences on sediment properties inside each surveyed site. Nevertheless, element concentrations in sites C and D (inner branches) were one to two orders of magnitude higher than in sites A and B (lower lagoon). Differences between sampling sites are partially explained by the contrasting Al content, which is a proxy for the fine particles (Windom et al., 1989). Indeed, bottom of inner branches are dominated by fine particles (91±11%), while only a minor fraction (6±10%) is present in sediments of the lower lagoon (Carvalho et al., 2006).

Table 3.1. Minimum and maximum concentrations of metals, organic carbon (C org), nitrogen and phosphorus in surface sediments of sites A, B, C and D at Óbidos lagoon. Data from 1st February, 4th May, 27th July and 30th October 2006.

		Site A	Site B	Site C	Site D
Al	%	0.21 - 0.42	0.39 - 0.69	11 - 12	9.1 – 11
Fe		0.030 - 1.2	0.12 - 2.0	1.3 - 4.9	3.4 - 5.2
C org		0.016 - 0.30	0.35 - 1.0	1.3 - 2.9	1.2 - 2.6
N		0.009 - 0.026	0.010 - 0.15	0.14 - 0.25	0.10 - 0.21
P	$\mu g\ g^{-1}$	14 - 108	52 - 390	434 - 666	505 – 855
Mn		19 - 38	26 - 45	266 - 322	269 – 301
Cr		0.78 – 1.8	2.2 - 3.3	56 - 111	71 – 90
Cu		0.16 – 1.3	0.58 – 2.6	46 - 68	41 – 83
Ni		0.39 – 1.3	1.3 - 1.9	24 - 49	29 – 39
Co		0.31 - 0.84	0.38 - 0.78	10 - 20	12 – 16
Cd		0.019 - 0.066	0.021 - 0.042	0.13 - 0.23	0.15 - 0.29

3.5.2 Water quality at daylight hours

Table 3.2 presents the median and ranges of seasonal data for temperature, salinity, dissolved oxygen, SPM, nutrients, Chl *a*, DGT-measured metals and metal/Al ratios of particulate metals in the sites A, B, C and D.

3.5.2.1 Physicochemical parameters

Water temperature ranged from 8.5 to 25 °C, with elevated values recorded at the inner branches in July. Salinity in site D (25.9-37.1) was significantly ($p<0.05$) lower than in sites A and B. The minimum values of salinity were registered in February and May, whereas in July at sites C and D salinity exceeded the seawater value. This indicates that evaporation in the inner branches was not compensated by the freshwater inputs. The dissolved oxygen levels in sites C and D ranged within broader intervals (65-170% saturation) than in sites A and B (105-120%) but no significant differences were found between lower and upper lagoon. Undersaturation was usually recorded in the morning of warmer sampling periods (high-tide),

whereas supersaturation occurred in the afternoon (low-tide). Concentration of SPM was lower in February and significantly higher ($p<0.05$) in site D (56-169 mg L^{-1}) than in sites A and B (15-65 mg L^{-1}).

Table 3.2. Physicochemical parameters, nutrients, chlorophyll *a*, DGT-measured metals and metal/Al ratios in suspended particles in sites A, B, C and D at Óbidos lagoon. Median, minimum and maximum values in high tide and low tide of 1st February, 4th May, 27th July and 30th October 2006.

		Site A	Site B	Site C	Site D
Temperature	°C	19 (9.4-20)	19 (9.1-21)	20 (8.9-25)	21 (8.5-25)
Salinity		35.8 (32.0-36.4)	35.1 (30.8-36.1)	31.7 (29.1-37.1)	29.4 (25.9-37.1)
O_2	%	98 (105-115)	90 (110-120)	108 (85-170)	100 (65-145)
SPM	mg L^{-1}	29 (15-65)	33 (16-62)	92 (31-141)	105 (56-169)
$NO_3^-+NO_2^-$		2.3 (0.4-45)	9.1 (0.3-38)	16 (0.2-62)	27 (0.2-124)
PO_4^{3-}	µM	0.42 (0.1-1.8)	0.76 (0.1-2.7)	2.1 (0.7-3.9)	4.8 (1.9-8.5)
$Si(OH)_4$		3.6 (1.6-17)	6.5 (1.6-23)	9.3 (3.0-22)	25 (3.1-56)
Chl *a*	µg L^{-1}	0.68 (0.4-2.5)	0.72 (0.4-2.4)	0.95 (0.4-3.9)	6.0 (1.0-18)
Mn		60 (13-402)	104 (7.0-319)	279 (20-721)	488 (239-1579)
Cu		2.9 (0.08-5.2)	2.3 (0.51-4.1)	3.6 (1.9-4.1)	4.8 (1.9-6.8)
Cr	nM	4.3 (2.1-7.8)	2.0 (7.7-9.5)	5.6 (2.0-9.1)	5.3 (2.1-13)
Ni		5.3 (3.3-16)	5.0 (3.5-11)	7.0 (2.2-13)	8.3 (3.9-13)
Co		0.71 (0.33-1.5)	1.0 (0.52-3.8)	0.61 (0.34-2.9)	1.2 (0.46-3.9)
Cd		0.11 (0.06-0.37)	0.14 (0.09-0.22)	0.08 (0.05-0.19)	0.10 (0.07-0.29)
Fe/Al		0.45 (0.26-0.54)	0.50 (0.13-0.54)	0.52 (0.28-0.81)	0.49 (0.41-0.55)
Mn		70 (31-154)	59 (23-117)	105 (23-645)	86 (41-708)
Cu		5.8 (5.0-7.9)	6.5 (5.3-11)	5.9 (4.9-18)	6.8 (6.0-9.7)
Cr	$x(10^{-4})/Al$	9.1 (3.0-26)	9.6 (1.7-11)	9.8 (5.0-28)	8.2 (5.7-16)
Ni		4.4 (2.8-12)	4.0 (3.0-7.5)	4.3 (3.2-11)	3.8 (3.4-6.6)
Co		1.5 (0.82-2.6)	1.4 (0.44-2.6)	1.5 (0.82-3.5)	1.4 (0.81-2.5)
Cd		0.07 (0.02-0.35)	0.05 (0.02-0.24)	0.03 (0.01-0.20)	0.03 (0.01-0.10)

3.5.2.2 Nutrients

Site D exhibited the maximum levels of $NO_3^-+NO_2^-$ (124 µM) and $Si(OH)_4$ (56 µM) in wet periods (February and October, respectively), whereas PO_4^{3-} (8.5 µM) peaked in summer (July). Nevertheless, significantly higher levels were only registered for PO_4^{3-} (site D *versus* A and B) and for $Si(OH)_4$ (site D *versus* A). Chlorophyll *a* was significantly ($p<0.05$) higher in site D than in the other three sites and reached maximum values in summer (18 µg L^{-1}).

3.5.2.3 Metals

Among the DGT-measured metals, only Mn was significantly ($p<0.05$) higher in site D than in site A. Metal concentrations in SPM were normalised to Al in order to minimise possible grain-size effect (Windom et al., 1989). Metal/Al ratios showed no significant differences between sites but narrower ranges were recorded in site D.

3.5.3 Water quality in site D over a 26-hour period

Solar radiation varied between 2224 and 2420 W m^{-2} from 10H00 to 16H00, decreased abruptly to 78 W m^{-2} after 20H00, and remained in darkness until 6H00. The water temperature varied from 23 to 30 °C following approximately the variation of solar radiation.

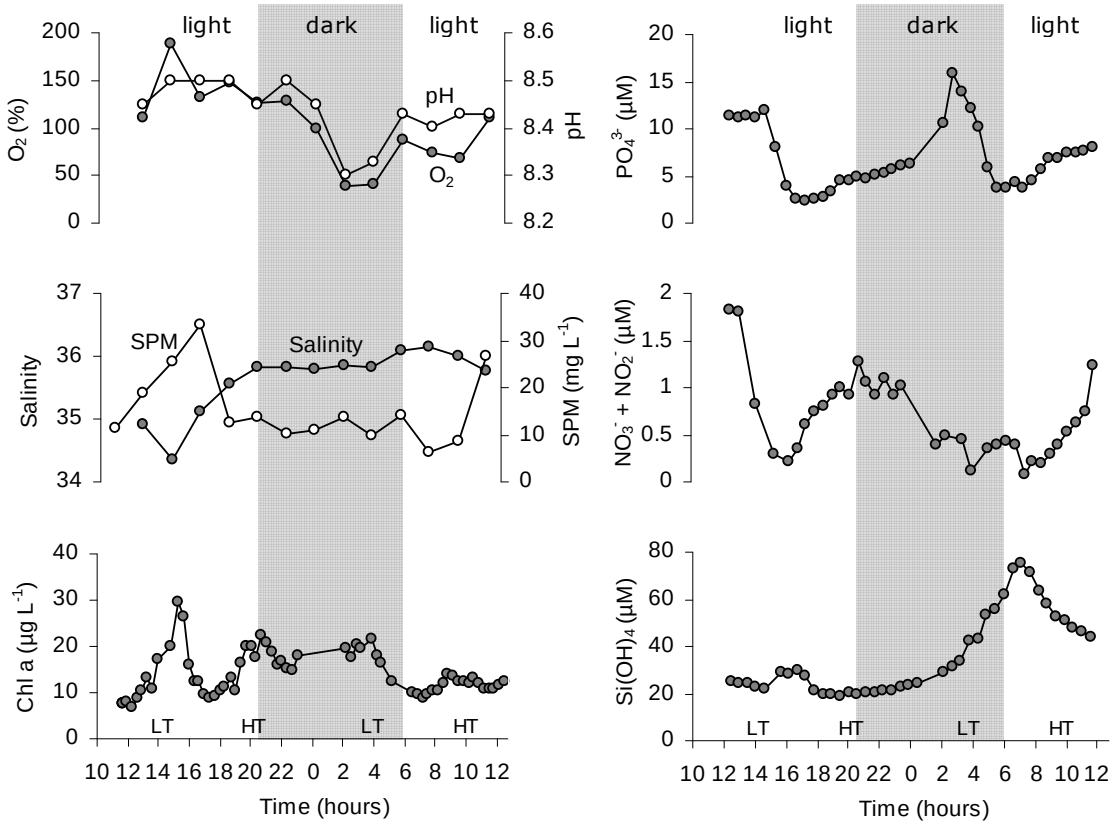

Figure 3.2. Variation of O_2 (%), pH, salinity, SPM (mg L^{-1}), Chl *a* (µg L^{-1}), PO_4^{3-}, NO_3^-+NO_2^- and $Si(OH)_4$ (µM) in site D, over a 26-h survey (19-20th July 2006). LT= low-tide; HT= high-tide.

3.5.3.1 Physicochemical parameters

Figure 3.2 shows the fluctuations of O_2, pH, salinity, SPM and Chl *a*. The O_2 concentrations in daylight hours exceeded 100% saturation (max. 190% at 14H00) but after sunset decreased gradually until a minimum of 40% at 2H00 and 4H00. The pH ranged from 8.3 to 8.5, showing a similar variation to that of O_2 (r^2=0.80, p<0.001). Salinity varied within a narrow interval (35.6–36.1) during most of the surveyed period, except between 12H00 and 16H00 when decreased to 34.4. Enhanced turbidity (max. 17 NTU) and SPM concentrations (33 mg L^{-1}) were registered in that period, and then decreased to 6±1.6 NTU and 11±2.6 mg L^{-1}, respectively. The Chl *a* showed a less marked pattern, although presented a peak (29 µg L^{-1}) around 16H00 and a slight decrease thereafter (6.9-22 µg L^{-1}).

3.5.3.2 Nutrients

Phosphate concentrations exhibited enhanced values (max. 11 µM) in the period of lower salinity and a pronounced peak (16 µM) during the night when dissolved oxygen was undersaturated (Figure 3.2). The NO_3^-+NO_2^- levels were higher around 12H00 (max. 1.8 µM), had a minimum between 14H00 and 17H00 when Chl *a* enhanced (0.21 µM), then increased until approximately 1 µM and then decreased again in the period of lower oxygen levels (min. 0.08 µM). The $Si(OH)_4$ concentrations started to increase also during the night, showing a peak of 75 µM when O_2 enhanced in the early morning.

3.5.3.3 Metals

The concentrations of Mn, Fe, Cu, Cr, Ni, Co and Cd measured by the DGT technique follow two distribution patterns (Figure 3.3). Levels of Mn, Cr and Co increased during the night until 8H00 followed by pronounced decreases. Iron varied identically but peaked at 6H00. The fluctuation of Cd and Ni levels was marked by a sharp peak when salinity lowered (at 14H00 for Cd; 16H00 for Ni) and minor enhancements during the night. The maximum values of Fe (104 µM) and Mn (240 µM) exceeded approximately 10 times the lowest ones. The increases were less pronounced for Co, Cr and Cu (5x), Cd (3x) and Ni (2x). The Al concentrations were relatively constant between 10H00 and 22H00 (7.8±0.98%) and decreased until 3.8±0.58% at 8H00 (Figure 3.4). An identical distribution pattern was observed for Fe, Mn, Cr, Co and Ni ratios to Al characterised by an increase during the night

and a maximum value in the early morning, which was followed by a pronounced decrease. Among the Me/Al ratios, only the Cu/Al increased considerably in the period of lower salinity. A peak was registered for Cu/Al and Cd/Al ratios at 4H00. The maximum ratios of Mn, Cd and Cu exceeded 3-5 times the lowest values registered during the survey, whereas of Fe, Co, Cr and Ni only 1.3-2 times.

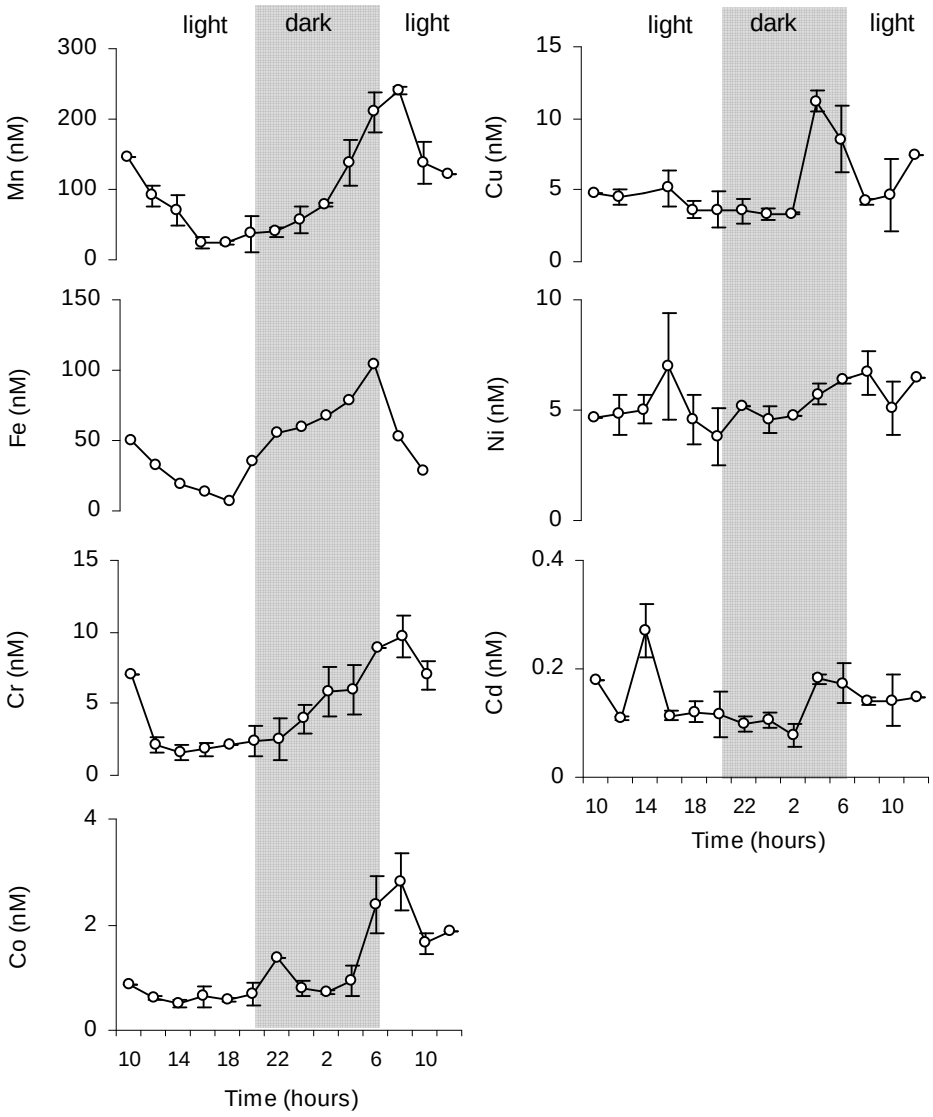

Figure 3.3. Variation of DGT-measured metals [Mn, Fe, Cr, Co, Cu, Ni, Cd (nM)] in site D over a 26-h survey (19-20th July 2006). Mean and standard deviation are presented.

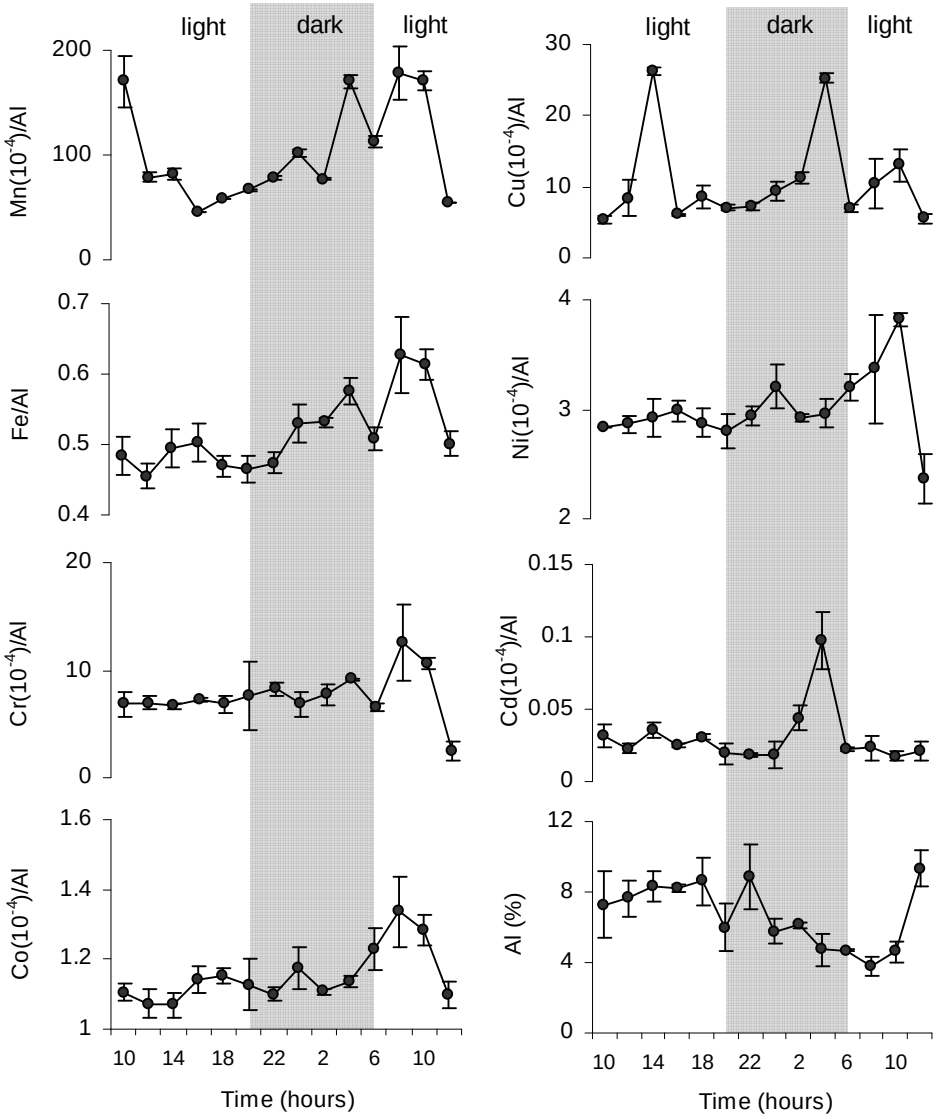

Figure 3.4. Variation of $Mn(10^{-4})/Al$, Fe/Al, $Cr(10^{-4})/Al$, $Co(10^{-4})/Al$, $Cu(10^{-4})/Al$, $Ni(10^{-4})/Al$ and $Cd(10^{-4})/Al$ ratios and Al (%) in suspended particulate matter from site D over a 26-h survey (19-20th July 2006). Mean and standard deviation are presented.

3.5.4 Metal and nutrient concentrations *versus* salinity

The levels of nutrients and DGT-measured metals were plotted against salinity considering data obtained in seasonal campaigns (black circles) and over the 26-h cycle (open squares). Figure 3.5 presents the plots for Mn, Cu, Ni, $NO_3^-+NO_2^-$, PO_4^{3-} and $Si(OH)_4$. Measurements obtained in seasonal campaigns showed a tendency to decrease along the salinity gradient with significant correlations found for Mn, $Si(OH)_4$ and PO_4^{3-} ($p<0.001$). Elevated levels of PO_4^{3-} (unfilled diamond) found in July in inner branches were not

considered in the correlation. The obtained relationships suggest that nutrients and metals in water column are relatively well explained by the conservative mixing between freshwater enriched in these compounds and seawater. Two situations were obtained for data of the 26-hour cycle: Mn, Ni, and $NO_3^-+NO_2^-$ are included in the respective relationships established with the seasonal values; whereas Cu, PO_4^{3-} and $Si(OH)_4$ are plotted above the linear relationships and surpassed the values corresponding to lower salinity conditions of the seasonal campaigns.

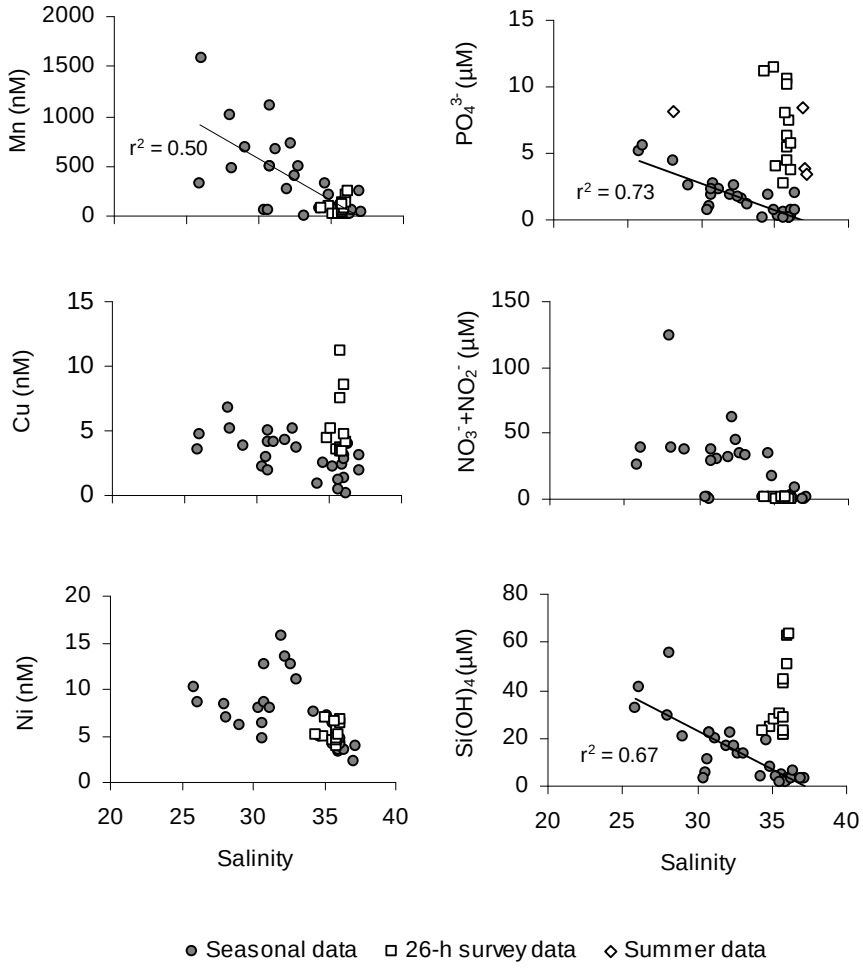

Figure 3.5. Concentrations of Mn, Cu, Ni, PO_4^{3-}, $NO_3^-+NO_2^-$ and $Si(OH)_4$ *versus* salinity. Data of seasonal campaigns and 26-h survey. Unfilled diamond corresponds to summer data, which was not considered in the relationship.

3.6 Discussion

The chemical composition of sediments from inner branches corroborates the elevated accumulation of organic matter in fine sediments observed in previous studies (Quintino, 1988; Carvalho et al., 2005; 2006). Organic loads enter in the inner branches by two tributaries (IST/IPIMAR, 2008) and the weak tidal currents facilitate the settling of organically enriched particles. The increase of nutrient concentrations in site D, during the period of lower salinity, reflects mainly the discharge of organic matter by the Cal River into the Barrosa inner branch. This input was also noted in the 26-h cycle (between 12H00 and 16H00) leading to a decrease of salinity. At the present time, domestic-effluent loads enter directly the coastal zone adjacent to the lagoon through a submersed outfall. Nevertheless, the Cal River continued to have deteriorated quality conditions according to the Portuguese classification of freshwater systems (IST/IPIMAR, 2008). Despite moderate metal concentrations in sediments, the increase of DGT-measured Ni and Cd, as well as of particulate Cu in the period of lower salinity in the 26-h cycle also indicates inputs associated with the freshwater discharge (Cal River). This evidence is also supported by the seasonal data obtained for Mn, Ni and Cu in water column that decreased along the salinity gradient.

The higher levels of nutrients and DGT-measured Mn in site D, as well as the broader ranges of other measured metals in seasonal campaigns may also result from internal regeneration of nutrients and release of metals from sediments. In fact, the observations over 26 hours in summer revealed pronounced increases of nutrient and metal concentrations during the night, following a substantial decrease of O_2. Moreover, these increases were not related with salinity as shown in Figure 3.5. The decrease of O_2 to 40% saturation during the night resulted from the superimposed consumption by benthic respiration and organic matter oxidation. Several works have invoked the photosynthesis-respiration cycle to explain daily variations of O_2 concentrations (Escaravage, 1990; Gammons et al., 2007; Serpa et al., 2007). Fluctuation of pH during the 26-h survey appears to reflect also the photosynthesis- and respiration-induced changes (Escravage, 1990).

At night, the release of PO_4^{3-}, $Si(OH)_4$ and Cu from sediments lead to concentrations in water column that exceed the highest values registered in periods of high flows of Cal River (Figure 3.5). This release has a pulse nature that is only observable in day/night surveys.

3.6.1 Release of nutrients and metals from sediments

The maximum levels of PO_4^{3-} and $Si(OH)_4$ registered in site D under low oxygen concentrations indicate the upward diffusion of these compounds regenerated near the sediment-water interface. That diffusion may have been facilitated by the displacement of the oxic-anoxic barrier from the topmost sediments to the near-bottom water. As this chemical barrier moved to the water column, dissolved constituents resulted from reactions in the topmost sediments diffuse towards the overlying water. Retention of the released compounds by sorption onto Fe- and Mn-oxides or by participation in reactions near the oxic-anoxic barrier was presumably minimised (Sundby, 2006 and references herein). Moreover, the rapid decline of PO_4^{3-} during the night (2H30-8H00) suggests that phosphorus was scavenged as Fe-oxides precipitated in the water column, which was corroborated by the increase of Fe/Al ratios. These results are in line with several works that proved the relationship between phosphorus availability in bottom water and the Fe(III)/Fe(II) cycle (Sundby et al., 1992; Slomp et al., 1998). The levels of $Si(OH)_4$ remained elevated until daylight and only then decreased progressively. The most plausible explanation is its daily biological consumption by the abundant photosynthetic organisms and minor inorganic removal. Observations performed over 24 hours in shallow waters of Mondego estuary pointed also to an increase of PO_4^{3-} efflux from sediments during the night under anoxic conditions (Lillebø et al., 2002).

The enhanced values of Mn, Fe, Cu, Cr, Ni, Co and Cd measured by the DGT technique and in suspended particulate matter during the night and early morning in site D indicates an additional input of metals to the water column. The increases may have resulted from diagenetic reactions in sediments and subsequent transport to the water column under low oxygenated conditions. During the night, photosynthesis ceased and respiration consumed O_2 resulting in low oxygenation and low pH values in the overlying water. These conditions promoted the reductive dissolution of Mn- and Fe-oxyhydroxides in the topmost sediments, with the concomitant release of Mn^{2+}, Fe^{2+}. Other adsorbed trace elements were probably released to the water column. Migration of oxic-anoxic chemical boundary facilitates the upward diffusion of those elements (Sundby, 2006 and references herein). Daily cycling of Fe, Mn and other divalent elements have been reported in metal-rich freshwater environments and related to redox changes of Fe(III)/Fe(II) and Mn(II)/Mn(IV) near the streambed (Nagorski et al 2003; Gammons et al., 2007).

3.6.2 Metal partitioning in the water column

The maximum of metal/Al ratios in suspended particles were observed with the increasing of O_2 concentrations. The ratios peaked concomitantly or after the peak of DGT-measured metals (Figure 3.3 and Figure 3.4), suggesting that elements were rapidly transferred to the suspended particulate fraction as photosynthesis promoted oxygenation of the water column. Fast precipitation of Fe- and Mn-oxyhydroxides (Thamdrup et al., 1994; Caetano et al., 1997) facilitates the rapid exchange of Fe and Mn between DGT-measurements and particulate fraction. The rapid increase of trace-element ratios to Al, and hence their removal from solution, may resulted from incorporation onto the freshly formed oxides. Indeed, it is generally recognized that cycling of trace elements near the oxic-anoxic boundary is closely linked to the diagenetic reactions and to the interaction with forms of Fe, Mn and S (Fones et al., 2004; Naylor et al., 2004). Furthermore, the interactions between trace elements and Fe- and Mn- oxides may occur in the minute scale (Caetano et al., 2007b).

It is well documented that the distribution coefficient (K_d) provides empirical information on the combined effect of heterogeneous reactions on the solid-solution distribution of an element (Hatje et al., 2003). The distribution coefficient was calculated as following:

$$K_d = \frac{[Me]_P}{[Me]_{DGT}} \times [SPM] \qquad (2)$$

Where [SPM] is the concentration of suspended particulate matter in g L^{-1}; $[Me]_p$ the metal concentration in SPM (µg g^{-1}); and $[Me]_{DGT}$ the DGT-measured metal (µg L^{-1}). The K_d values varied from <1 for Cd to 10^3 for Fe (Figure 3.6). Such a broad range of K_d reflects different affinities of the elements to be associated and transported with the solid phase (Hatje et al., 2003). The computed K_d were based on DGT values that may not correspond to the total dissolved metals. Nevertheless, the values obtained for Cu and Cd are in accordance with those reported for the Portuguese coastal waters adjoining the major estuaries and coastal lagoons (Caetano and Vale, 2003). These K_d , as well as K_d(Fe) and K_d (Mn), were lower than those documented for Seine and Scheldt due to elevated metal concentrations in suspended particulate matter in these contaminated estuaries (Zwolsman et al., 1997; Oodane et al., 1999).

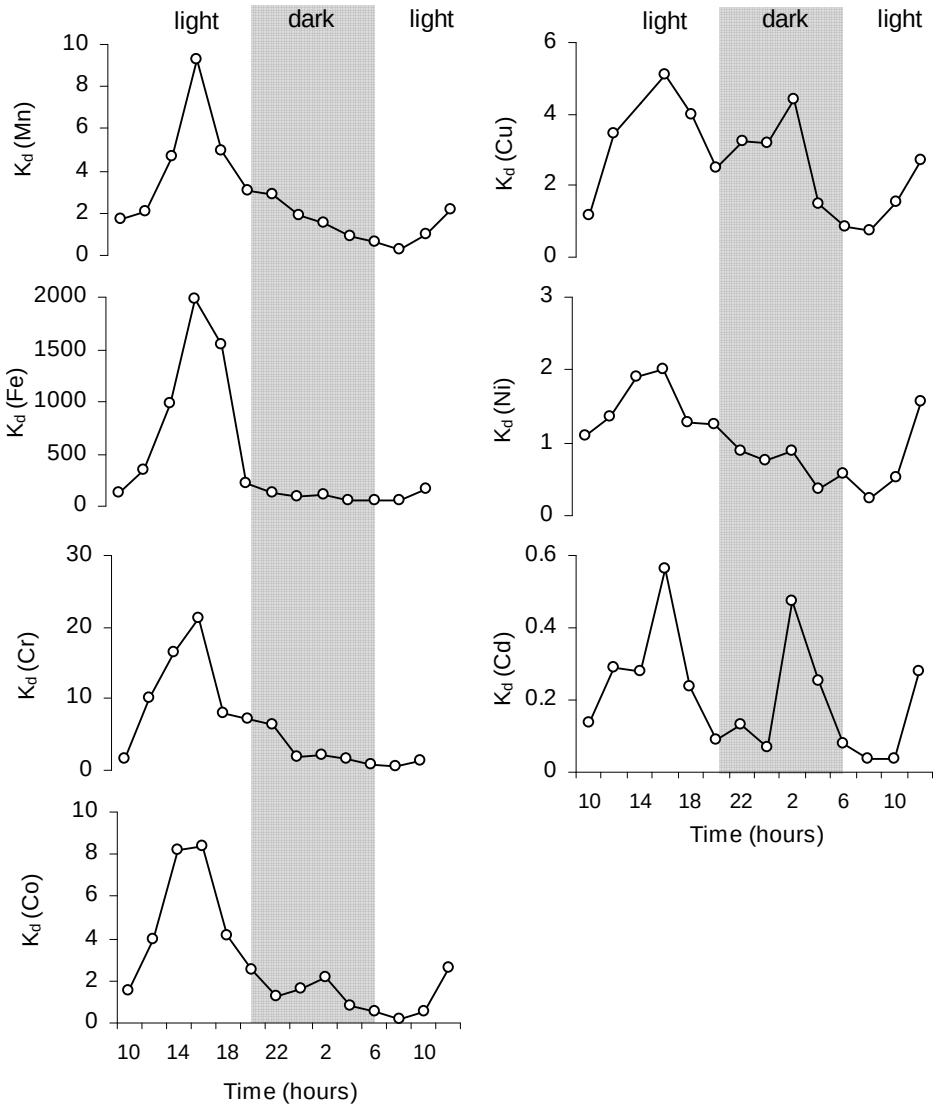

Figure 3.6. Variation of metal distribution coefficients (K_d) (Mn, Fe, Cr, Co, Cu, Ni, Cd) in site D over a 26-h survey (19-20th July 2006).

The diel fluctuations of K_d for all measured elements are marked by a pronounced maximum at daylight hours between 14H00 and 18H00 with a peak at 16H00 (Figure 3.6). Moreover, the K_d of Mn, Fe, Cr, Co and Ni were almost constant during the night. At daylight hours, metals were preferentially associated with particulate fraction, being maximum values of K_d registered in the period of higher oxygenation, SPM and Chl *a*. The significant ($p<0.05$) positive correlations between K_d and O_2 concentrations (r^2= 0.74, 0.74, 0.61 and 0.59 for Mn, Cr, Co and Ni, respectively) suggests that the transference of metals from dissolved fraction to particulates can be determined by processes dependent on oxygen availability. Although

the particles' nature may influence the metal sorption, the lack of significant correlations between K_d and Chl *a* indicates that changes on K_d were not solely influenced by alterations on the abundance of phytoplankton. The lower K_d recorded during the night suggests that the equilibrium was dislocated towards the DGT-measured fraction. Furthermore, the values remained relatively constant (except for Cu and Cd) at night, which indicates that rapid exchanges between DGT-measurements and particulate fraction may compensate the metal inputs from sediment to the dissolved fraction that occurs during lower oxygenation periods.

3.7 Summary

The results obtained in this eutrophic environment pointed to the occurrence of pulse releases of nutrients and metals from sediments during summer. These internal inputs are of great importance for water quality, since could lead to levels that exceed those recorded in periods under effect of diffuse sources. Moreover, the results emphasise the importance of addressing day-night variations in sampling designs performed in eutrophic environments. Automatic devices to register levels of water quality parameters over small-time scales are needed.

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CHAPTER IV

Metal accumulation and oxidative stress in *Ulva* sp. substantiated by response integration into a general stress index

4 Metal accumulation and oxidative stress in *Ulva* sp. substantiated by response integration into a general stress index

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Abstract

In an attempt to correlate external levels of exposure, bioaccumulation markers and oxidative stress responses in the green macroalgae *Ulva* sp., an investigative biomonitoring study was performed in a Portuguese coastal lagoon with a complex contamination scenario (eutrophication and moderate metal levels). *Ulva* sp. was collected seasonally in three stations: BB (Barrosa branch) and BS (Bom-Sucesso branch) located in confined areas of the upper lagoon; LL (lower lagoon) closer to the lagoon inlet and selected as reference station. Water and sediment were both monitored for metals (Mn, Cu, Cr, Ni, Cd). Additionally, nutrients and organic matter were measured in water and sediment, respectively. Catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GR), glutathione-S-transferase (GST), total glutathione (GSH_t) and lipid peroxidation (LPO), as well as Mn, Cu, Cr, Ni, Cd were measured in the algae. Metal levels in *Ulva* sp. consistently reflected their occurrence in water. *Ulva* sp. from BB exhibited higher CAT activity (autumn and spring) in agreement with Cu and Ni increases in water and algae and supported by significant Pearson correlations. The involvement of GPx on algae defences was considered since a positive correlation was observed with accumulated Mn. At BB, a direct implication of accumulated metals on increases of GR (autumn) and GSH_t (summer) was also assumed. The GST contribution to algae defences seemed to be less prominent. Despite the enzymatic antioxidants activation at BB, *Ulva* sp. collected in autumn and spring showed no efficiency to cope with ROS overproduction as LPO occurred. Globally, the adopted multi-biomarker approach coupled with environmental chemical characterization, pointed out BB as a major impacted area. The integration of oxidative stress responses into a general stress index (IBR) substantiated the data interpretation, providing a more definitive identification of the level of pollution, and therefore contributing to the understanding of cause-effect relationships.

Keywords: *Ulva* sp.; Oxidative stress; Metals; IBR; Eutrophication; ROS

4.1 Introduction

Chemical analyses of the water and sediment can reveal metal concentrations in the environment but are insufficient to assess toxicity on organisms and the impact on ecosystem (Amiard et al., 1998). Metals usually appear in the aquatic environment as a part of a complex mixture of contaminants that can cause interactive effects on the biota, which are difficult to predict by chemical analyses (Orbea et al., 2002). Hence, the use of biosentinel species in monitoring programs as a mean to assess the bioavailability of contaminants was proposed, in order to determine the impact of anthropic action on biocenosis vitality and its progression (Rainbow and Phillips, 1993).

A biosentinel organism should be sedentary, widespread, widely studied, easy to sample and to keep in the laboratory, as well as sensitive to environmental variations (Rainbow and Phillips, 1993). Macroalgae fulfil these requirements and have shown to be particularly promising in monitoring trace metal contamination (Vasconcelos and Leal, 2001; Villares et al., 2002) as a result of their metal-biding properties (Phillips, 1977). As primary producers, macroalgae allow the early detection of potential harmful effects in the ecosystems. *Ulva* sp. appears as a valuable biosentinel of water quality in eutrophic littoral lagoons or sheltered bays due to its massive development and wide distribution (Lazaridou et al., 1997). Despite some reported difficulties associated with seasonal variations of abiotic parameters and intrinsic factors such as age and growth rate, species of the genus *Ulva* have been used as biosentinel (Haritonidis and Malea, 1995; Villares et al., 2002). The high surface area:volume ratio of *Ulva* sp. provided by its laminar shape, together with a structurally uniform and physiologically active thallus (Villares et al., 2001) constitute additional favourable characteristics.

Beyond certain concentration thresholds, metals can induce the overproduction of reactive oxygen species (ROS), such as $O_2^{\bullet -}$, $HO^{\bullet}$ and H_2O_2, which are able to generate oxidative damage on lipids, proteins and nucleic acids (Collén et al., 2003). To counteract oxidative stress and protect the cells, an armoury of endogenous antioxidants can be mobilized. Therefore, changes in expression of antioxidant enzymes or in the content of non-enzymatic ROS scavengers have been used in several aquatic organisms as sensitive indicators of exposure to exogenous pro-oxidants, including metals (Orbea et al., 2002;

Gravato et al., 2006). Nonetheless, biochemical responses have been neglected in macroalgae, being the few existing studies performed under controlled laboratory conditions (Collén et al., 2003; Malea et al., 2006; Nimptsch and Pflugmacher, 2007) rather than in real field scenarios (Cairrão et al., 2004; Pawlik-Skowrońska et al., 2007). This is probably related to a dominant assumption that plants are less sensitive to chemicals than aquatic animals (Mohan and Hosetti, 1999). However, according to Nimptsch et al. (2005) the susceptibility to toxicants depends on the species used as well as on the chemicals tested, rather than on differences between plants and animals. Even so, the available data offer encouraging insights into the use of macroalgae (e.g. *Fucus* sp. and *Enteromorpha* sp.) for the assessment of environmental quality, particularly on the basis of their oxidative stress responses (Rijstenbil et al., 1998; Cairrão et al., 2004).

In view of that, this paper aimed to evaluate on a seasonal basis the oxidative stress responses and metal levels in *Ulva* sp. from a coastal system (Óbidos lagoon, Portugal) impacted by eutrophication and moderate metal contamination. Peroxidative damage and antioxidant responses were measured in *Ulva* sp. and their relationship with metal accumulation, as well as with environmental chemical data (metals and nutrients) were assessed. A general stress index termed "Integrated Biomarker Response" (IBR), combining all the oxidative stress endpoints, was used to summarize the biomarker responses. It was also intended to evaluate the suitability of the adopted combined strategy (external levels of exposure/bioaccumulation markers/oxidative stress responses) on monitoring coastal ecosystems in a multi-pollution context.

4.2 Materials and Methods

4.2.1 Study area

The Óbidos lagoon is a shallow coastal ecosystem, located on the west coast of Portugal with a wet area of 7 km^2, which is permanently connected to the sea through a narrow inlet (Figure 4.1). It comprises areas of different morphological and sedimentary characteristics: sand banks and narrow channels in the lower lagoon, and muddy bottom sediments in the two inner branches at Barrosa and Bom-Sucesso. The Barrosa branch is shallower (mean depth 0.5-1 m) and water circulation is mostly driven by tides and by freshwater inputs from a small tributary (Cal River) that drains agriculture fields. Urban effluents from a nearby town (Caldas da Rainha, 50,000 inhabitants) were discharged into

the Barrosa branch by the Cal River. Consequently, this area presents the highest nutrient concentrations of the lagoon, being classified as eutrophic (Pereira et al., 2009a). High nutrient concentrations are in line with abundant macroalgae (*Ulva* sp. and *Enteromorpha* sp.) and a broad daily variation of dissolved oxygen concentrations during the summer months (Pereira et al., 2009a). Previous water quality surveys of the lagoon showed maximum concentrations of Mn, Ni, Cu and Cd in Barrosa. Major metal sources were identified to be the Cal River in periods of higher discharges, and remobilization from sediments in summer (Pereira et al., 2009b). The Bom-Sucesso branch is also a confined area but receives a smaller freshwater flow (Vala do Ameal) with better water quality than the Cal River, according to the Portuguese categorization of freshwater systems. In this area, metal remobilization from sediments is less plausible due to the higher depths and stronger currents. The lower lagoon was previously characterised by coarser sediments with a low affinity for metals (Carvalho et al., 2006), as well as by a better water quality (Pereira et al., 2009b), giving support to its selection as a reference area.

4.2.2 Sampling

Sampling was carried out at three stations in the Óbidos lagoon in autumn (October 2005), winter (February 2006), spring (May 2006) and summer (August 2006) (Figure 4.1): LL is located in the lower lagoon and considered reference site, BS (Bom-Sucesso) and BB (Barrosa) are located in the branches. Surface water (0.2 m depth) was sampled in high tide (morning) and low tide (afternoon) for the determination of nutrients, chlorophyll *a* and metals. Surface sediments were also collected in the three stations with a Van-Veen grab.

Due to a complex taxonomy of the genus *Ulva*, sampled specimens were not identified to species level, which is an option previously assumed by other authors (Aveytua-Alcázar et al., 2008), including in metal accumulation studies (Villares et al., 2002; Pérez et al., 2007). Samples of *Ulva* sp. were collected by hand at the three stations in low tide (afternoon). To account for intra-station variability, five samples of algae were collected at each station for metal determinations (≈100 g each) and five samples for biochemical quantifications (≈10 g each). All samples were washed *in situ* with lagoon water to remove the majority of adherent particles. Samples for biochemical analyses were carefully rewashed with ultra-pure water [Millipore-Q model (18.2 MΩ)] in the field, before they were shock frozen and transported to the laboratory in liquid nitrogen, where

they were stored at -80 °C until analysis. In August 2006, *Ulva* sp. was not found in BS station.

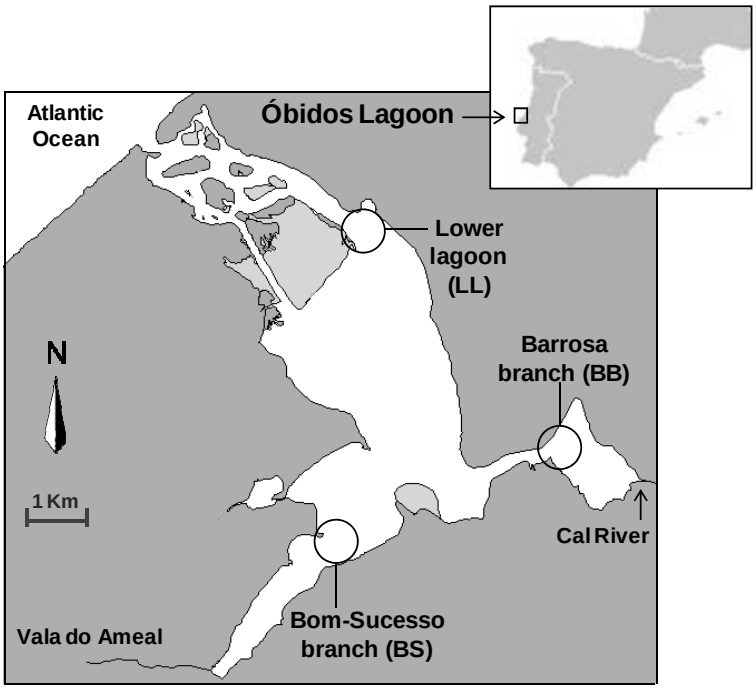

Figure 4.1. Location of the sampling stations at the Óbidos lagoon: lower lagoon (LL) assumed as reference station and inner branches (Bom-Sucesso – BS, Barrosa - BB).

4.2.3 Analytical procedures

4.2.3.1 Water and sediment physicochemical analyses

Water temperature, salinity and dissolved oxygen were measured *in situ* using an YSI, 650 meter. For the determination of nitrate+nitrite (NO_3^-+NO_2^-), ammonium (NH_4^+), phosphate (PO_4^{3-}) and silicate $Si(OH)_4$ water was filtered through MSI Acetate Plus filters, and analyses were carried out using an autoanalyser TRAACS 2000 (Bran+Luebbe). For chlorophyll *a* determinations, water was filtered through a Whatman GF/F (0.7 µm) filter that was immediately frozen at -20 °C and later extracted in 90% acetone, for analysis in a Perkin Elmer Fluorometer using the protocol modified by Lorenzen (1966). Organic matter content of sediments was determined by loss on ignition (4h at 500 °C).

4.2.3.2 Metal determinations in environmental matrices and Ulva sp.

Manganese, Cu, Ni, Cr and Cd were measured in water samples using diffusive gradients of thin films (DGT). DGT holders, Chelex-100 resins and diffusive gels (type APA, 0.8 mm thickness, open pore> 5 nm) (Zhang and Davison, 1999) were purchased from DGT

Research (Lancaster, UK). The DGT devices were deployed in 2 L polypropylene bottles with unfiltered sampled water and stirred at 21±1 °C for 48 hours. After the retrieval of the devices, resins were eluted by immersion in 5 mL of 1 M HNO_3 (prepared from suprapur nitric acid) for a minimum of 24 h. Eluates were analysed directly by a quadropole inductively coupled plasma mass spectrometer (Thermo Elemental, X-Series). All eluates were analysed with reagent blanks and an international standard of river water (SLRS-4) to control eventual contaminations during the analytical procedure and the procedure accuracy, respectively. The eluate concentration was converted into the mass of metal accumulated on the resin using an elution factor with a yield value of 0.8 (Zhang and Davison, 1995) and a resin gel volume of 0.15 mL. Metal concentrations in water were calculated according to the following equation: [Metal]=(MΔg)/(tAD); where M is the mass of metal accumulated on the resin during the emersion time (t); Δg the diffusive gel thickness (0.08 cm); A the exposure area (3.14 cm^2); and D the diffusion coefficient of the metal in the gels as provided by DGT Research.

Sediment samples (100 mg) were mineralized completely with HF (40%) and Aqua Regia (HCl-36%: HNO_3-60%; 3:1) in closed Teflon bombs (100 °C for 1 h), evaporated to near dryness (DigiPrep HotBlock – SCP Science), redissolved with 1 mL of doubled-distilled HNO_3 (prepared from 65% pro analysis) and 5 mL of ultra-pure water, heated for 20 min at 75 °C, and diluted to 50 mL with ultra-pure water (Caetano et al., 2007). At the laboratory, *Ulva* sp. samples were additionally washed with gentle rubbing (to remove the remaining particles and epiphytes), lyophilised and grounded to get a homogenous mixture. Approximately 100 mg of algae were digested by adding 2 mL of HNO_3/$HCLO_4$ (7:1, v/v) (HNO_3 doubled-distilled from 65% pro analysis; $HCLO_4$ 70%) in Teflon bombs at 100 °C for 3 hours according to the method described by Caçador et al. (1994). Reagent blanks were prepared in a similar way and international certified standards (1646a, BCSS-1, MESS-3 for sediments; CRM-281, BCR-60, BCR-61 for algae) were used to control the accuracy of the procedure. The concentrations of Mn were determined by flame atomic absorption spectrometry (Perkin Elmer Analyst 100), while levels of Cu, Cr, Ni and Cd were determined by a quadropole inductively coupled plasma mass spectrometer.

4.2.3.3 Biochemical analyses in Ulva sp.

Lipid peroxidation (LPO) was measured according to Collén et al. (2003). Briefly, approximately 100 mg of fresh algae samples were weighted, grounded in liquid nitrogen,

mixed with 1 mL of trichloroacetic acid (12%) and 1 mL of thiobarbituric acid (0.73%), incubated for 30 min in boiling water, diluted to 10 mL before centrifugation (15,000 g) and the absorbance measured in the supernatant at 532 nm. The rate of LPO was expressed as nmoles of thiobarbituric acid reactive substances (TBARS) formed per milligram of fresh tissue, using a molar extinction coefficient of 1.55×10^5 M^{-1} cm^{-1} (Rama Devi and Prasad, 1998).

Algae portions (100-200 mg) for determinations of soluble catalase (CAT), glutathione reductase (GR), glutathione S-transferase (GST) and glutathione peroxidase (GPx) were weighted, grounded in liquid nitrogen and extracted with 50 mM potassium phosphate buffer (pH 7.0) (1:5; fresh weight:volume) containing 0.25% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 1% isopropanol and 1% polyvinylpyrrolidone (Collén et al., 2003). The extracts were centrifuged (16,000 g) for 5 min at 4 °C before assaying. Protein concentrations were determined according to the Biuret method (Gornall et al., 1949) using bovine serum albumin (E. Merck-Darmstadt) as a standard. CAT activity was analysed according to Aebi (1984) following the decrease of absorbance at 240 nm during 1 min, after addition of 2 nM H_2O_2 and calculated in terms of nmol H_2O_2 consumed/min/mg protein. GR activity was determined according to Carlberg and Mannervik (1975) by measuring NADPH oxidation during 1 min at 340 nm and expressed as µmol NADPH oxidised/min/mg protein. GST activity was determined by Drotar et al. (1985) method in Cairrão et al. (2004) by following, during 3 min, the increase in absorbance at 340 nm due to the formation of the conjugate 1-chloro-2,4-dinitrobenzene (CDNB) (substratum)/GSH, catalysed by GST, and calculated as µmol CDNB conjugate formed/min/mg protein using a molar extinction coefficient of 9.6 mM^{-1} cm^{-1}. GPx activity was determined by measuring the oxidation of NADPH at 340 nm during 3 min using H_2O_2 as substrate (Mohandas et al., 1984).

For the determination of total glutathione (GSH_t), extracts were prepared by adding 7.5 mL of sulphosalicylic acid (5%) to 1 g of algae material (fresh weight) (Nimptsch and Pflugmacher, 2007). The mixture was centrifuged (18,000 g) at 4 °C for 12 min. In the supernatant, GSH_t was determined by adopting the enzymatic recycling method using GR excess, whereby the sulfhydryl group of GSH reacts with 5,5'-Dithiobis(2-nitrobenzoic acid) producing a yellow coloured 5-thio-2-nitrobenzoic acid (TNB). Formation of TNB was measured by spectrophotometry at 412 nm and the results were expressed as nmol TNB formed/min/mg protein.

4.2.4 Data analyses

A method for combining all the measured biochemical responses into one general index - Integrated Biomarker Response (IBR) (Beliaeff and Burgeot, 2002) - was applied in all surveys, except summer due to the absence of data in BS station. The basis of data processing is described here briefly according to Beliaeff and Burgeot (2002). First, the mean value (X_i) for each biomarker at each sampling area and season was calculated. In addition, the general mean (m_i) and standard deviations (s_i) of each biomarker were estimated for all sampling areas and seasons. The value of X_i was then standardized to obtain Y_i, where $Y_i = (X_i - m_i)/s_i$. Then, Z_i was computed via the equation $Z_i = -Y_i$ or $Z_i = Y_i$ in the case of a biological effect corresponding, respectively, to inhibition or activation. The minimum value (Min_i) of Z_i for each biomarker was calculated for all sampling areas and seasons, and then the score S_i was computed as $S_i = Z_i + |Min_i|$, where $|Min_i|$ is the absolute value. Finally, IBR for each sampling area and season was calculated via the following formula: $IBR = (S_1 x S_2)/2 + (S_2 x S_3)/2 + \ldots + (S_{n-1} x S_n)/2 + (S_n x S_1)/2$; in which the obtained score for each biomarker (S_i) is multiplied with the score of the next biomarker (S_{i+1}), arranged as a set, dividing each calculation by 2 and summing-up of all values. IBR values were compared with accumulated metals in *Ulva* sp. by star plots.

Statistical software (Statistica 7.0) was used for statistical analyses. The assumptions of normality and homogeneity of data were verified. ANOVA analysis was used to compare sampling stations and a Tukey test was applied for Post-hoc comparison (Zar, 1996). Differences between means (n=5) were considered significant when $p<0.05$. In order to detect relationships between the assessed parameters, a Pearson analysis ($p<0.05$) was performed between water variables (nutrients and metals) measured in the two sampling tides and *Ulva* biomarkers (metal levels and oxidative stress responses) thus considering the mean values of the four sampling seasons at each station. Identically, correlations were tested between metal levels in *Ulva* sp. and oxidative stress responses.

4.3 Results

4.3.1 Environmental data

Organic matter content, Mn, Cu, Cr, Ni and Cd in sediments from the branches (BS and BB) were considerably higher than those from the lower lagoon (LL) (Table 4.1). The value intervals for each station reflect differences on sediment properties rather than seasonal fluctuations.

Table 4.1. Ranges of organic matter and metals concentrations in surface sediments collected seasonally in the lower lagoon (LL) and in inner branches (Bom-Sucesso - BS, Barrosa - BB) at Óbidos lagoon.

Station	Organic matter (%)	Metals (μg g^{-1})				
		Mn	Cu	Cr	Ni	Cd
LL	0.61 - 3.9	26 – 39	0.58 - 2.6	1.1 - 2.6	0.8 - 1.9	0.02 - 0.04
BS	1.8 - 8.8	285 - 322	46 - 68	56 - 111	24 - 49	0.13 - 0.23
BB	5.7 - 10	269 - 300	41 - 83	72 - 90	30 - 39	0.15 - 0.29

In spring and summer, water temperature reached elevated values in the branches (Table 4.2). Salinity was lower in BB than in the other stations in autumn, winter and spring due to freshwater inputs. Dissolved oxygen in LL was around 100% in autumn and winter, while in spring it was approximately 100% but reached oversaturated values in BB and BS. In summer, undersaturated levels were registered in BB during daylight hours.

Concentrations of NH_4^+, NO_3^-+NO_2^- and $Si(OH)_4$ were higher in BB than BS and LL in autumn, winter and spring (Table 4.2). Phosphate and chlorophyll *a* were elevated in BB in all sampling situations. In summer, the three stations exhibited identical levels of NH_4^+, NO_3^-+NO_2^- and $Si(OH)_4$, whereas PO_4^{3-} and chlorophyll *a* increased considerably in BB in low tide. The maximum values of NH_4^+ and NO_3^-+NO_2^- were registered in winter at BB in low tide.

Manganese, Cu and Ni concentrations in water were generally higher in BB than in BS and LL at the four sampling periods (Table 4.3). Levels of Cr and Cd showed less variability in the lagoon and differences between stations were not consistent. Despite the fact that no seasonal pattern could be clearly discerned, maximum Mn levels were recorded at BB in winter, while Cu and Cd were highest in autumn at BB, and Ni and Cr at BS in winter and summer, respectively.

Table 4.2. Water temperature (T), salinity, dissolved oxygen (DO), ammonium (NH_4^+), nitrate+nitrite (NO_3^-+NO_2^-), phosphate (PO_4^{3-}), silicate ($Si(OH)_4$) and chlorophyll *a* (Chl *a*) determined seasonally on the lower lagoon (LL) and in inner branches (Bom-Sucesso - BS, Barrosa - BB) at Óbidos lagoon in high and low tide.

		High tide – Low tide							
Season	Station	T (°C)	Salinity	DO (%)	NH_4^+ (µM)	NO_3^-+NO_2^- (µM)	PO_4^{3-} (µM)	$Si(OH)_4$ (µM)	Chl *a* (µg L^{-1})
Autumn	LL	18-19	35.7-35.3	97-102	13-19	0.9-0.9	0.2-0.2	12-16	6.5-6.7
	BS	18-19	35.3-35.1	97-98	21-20	2.3-1.2	1.6-2.1	19-13	5.4-7.8
	BB	19-20	34.5-33.0	95-94	44-53	5.1-13	4.2-7.3	21-29	2.9-12
Winter	LL	12-9	34.9-33.1	103-108	13-20	17-33	0.7-1.2	7.8-14	0.8-0.7
	BS	11-9	32.7-32.3	107-102	25-46	34-62	1.6-2.5	13-22	0.8-1.1
	BB	9-11	30.8-28.1	103-124	53-81	74-124	2.2-4.5	22-29	12-8.5
Spring	LL	17-19	35.6-35.3	94-117	10-3.5	0.8-0.6	0.6-0.3	5.1-3.8	1.3-2.4
	BS	20-22	30.6-30.4	172-142	4.2-1.3	0.7-1.0	1.0-0.7	5.6-3.4	3.9-2.5
	BB	20-23	30.7-25.9	108-145	4.6-7.2	mv-26	1.9-5.1	11-33	5.5-6.6
Summer	LL	20-19	36.1-36.0	101-108	0.7-0.6	0.4-0.3	0.8-0.07	2.9-1.6	0.4-0.6
	BS	25-24	37.1-36.4	121-109	0.9-1.6	0.3-0.4	3.9-2.0	3.1-3.0	1.1-0.5
	BB	24-25	37.1-36.9	74-96	1.2-1.2	0.8-0.3	3.4-8.5	3.2-3.1	1.0-18

mv – missed value

Table 4.3. Metals in water determined seasonally on the lower lagoon (LL) and in inner branches (Bom-Sucesso - BS, Barrosa - BB) at Óbidos lagoon in high and low tide.

		High tide – Low tide				
Season	Station	Mn (µM)	Cu (nM)	Ni (nM)	Cr (nM)	Cd (nM)
Autumn	LL	0.02-0.18	1.8-2.7	5.7-4.3	8.6-7.2	0.08-0.05
	BS	0.23-0.44	2.6-3.5	5.5-5.0	8.5-6.5	0.08-0.05
	BB	0.30-0.18	9.6-4.5	12-5.9	6.5-8.7	0.15-0.17
Winter	LL	0.21-0.01	mv	mv-11	9.2-7.5	0.09-0.12
	BS	0.49-0.72	3.6-8.6	13-14	2.5-7.5	0.08-0.13
	BB	0.49-1.0	5.0-6.8	13-8.3	5.9-8.5	0.11-0.07
Spring	LL	0.11-0.07	0.51-2.1	4.0-7.2	2.0-3.3	0.13-0.11
	BS	0.07-0.05	2.9-2.2	4.6-7.9	2.1-2.5	0.06-0.05
	BB	0.49-0.32	1.9-3.5	6.3-10	2.4-3.3	0.08-0.07
Summer	LL	0.10-0.02	1.3-2.4	4.7-3.5	3.5-7.9	0.14-0.14
	BS	0.04-0.02	1.9-4.0	2.2-3.5	13-3.1	0.06-0.08
	BB	0.09-0.24	3.1-mv	3.9-mv	2.0-5.2	0.07-0.12

mv – missed value

4.3.2 Metals accumulated in *Ulva* sp.

Levels of Cu, Cr and Ni were significantly higher in *Ulva* sp. from BB than LL in spring, summer and autumn (Figure 4.2). *Ulva* sp. collected at BB also showed significantly higher accumulation of Mn in spring and summer, as well as Cd in autumn. *Ulva* sp. from BS displayed punctual elevations (comparing to LL) of Cu in spring and Cr in winter. The comparison of the two branches revealed significant increases in BB of Mn (autumn and spring), Cr (spring) and Ni (spring). The algae collected in BB have no seasonal differences for Cu, Cr and Ni, whereas Cd increased significantly in autumn (*versus* winter and summer) and Mn increased significantly in summer (*versus* winter). The metal levels in *Ulva* sp. from BS and LL varied also with the season: in BS, Cr and Ni were higher in winter than autumn and spring, whereas Mn and Cu were higher in spring than autumn; in LL, Cu and Cr were higher in winter than spring, whereas Mn increased in summer relatively to spring and Ni in winter relatively to autumn.

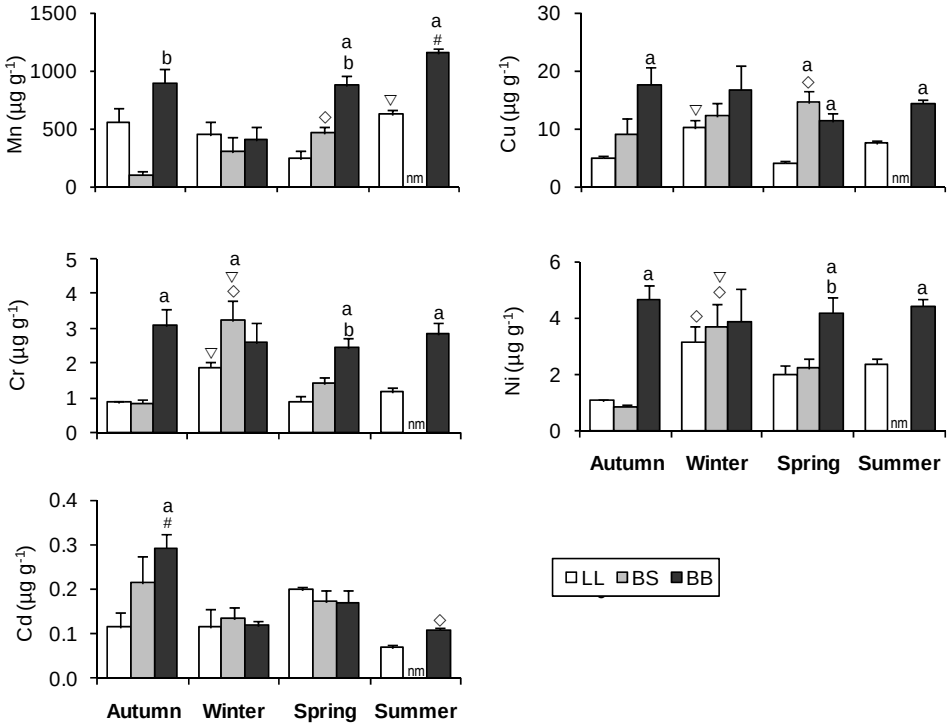

Figure 4.2. Metal levels in *Ulva* sp. collected seasonally in the lower lagoon (LL) and in the inner branches (Bom-Sucesso – BS, Barrosa - BB) at Óbidos lagoon. Letters and symbols denote statistically significant differences ($p<0.05$) between stations (in the same season) or seasons (for each station), respectively: (a) vs. LL; (b) vs. BS; (◊) vs. autumn; (#) vs. winter; (∇) vs. spring. Mean and associated standard errors are given. nm - Not measured.

4.3.3 Oxidative stress in *Ulva* sp.

Ulva sp. showed significant increases of CAT (autumn and spring) and GR (autumn) activities, as well as GSH_t content (summer) at BB in relation to LL (Figure 4.3). Moreover, higher levels of LPO were found at BB (*versus* LL) in autumn and spring. The comparison between both surveyed branches revealed increases at BB in autumn for all the parameters, excluding GSH_t content, as well as in spring for GPx, GSH_t and LPO. Seasonal differences were recorded at the three stations for all endpoints, except GPx. *Ulva* sp. from LL presented a decrease of GSH_t content in summer (compared to autumn) and enhanced LPO in winter (compared to spring and autumn). In BS, *Ulva* sp. presented higher GSH_t and LPO levels in winter (compared to spring and autumn, respectively). *Ulva* sp. collected in BB showed more seasonal variations than at the other two stations, since increased CAT, GST and GR activities in autumn (compared to summer) and LPO in spring (compared to winter) were observed.

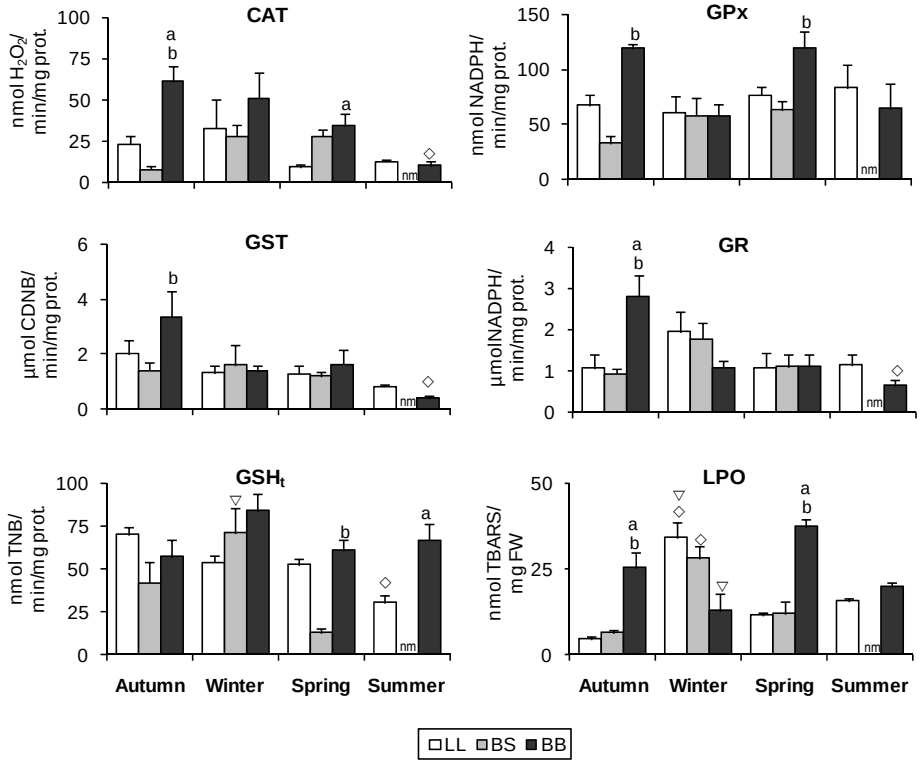

Figure 4.3. Oxidative stress in *Ulva* sp. collected seasonally in the lower lagoon (LL) and in the inner branches (Bom-Sucesso – BS, Barrosa - BB) at Óbidos lagoon: catalase (CAT), glutathione peroxidase (GPx), reduced glutathione (GR), glutathione S-transferase (GST) activities; content of total glutathione (GSH_t), and lipid peroxidation (LPO). Letters and symbols denote statistically significant differences ($p<0.05$) between stations (in the same season) or seasons (for each station), respectively: (a) vs. LL; (b) vs. BS; (◊) vs. autumn; (∇) vs. spring. Mean and associated standard errors are given. nm - Not measured.

4.3.4 Correlations between parameters and IBR calculation

The correlations obtained between water parameters and *Ulva* sp. biomarkers (metal levels and oxidative stress responses) were generally the same when variables were paired considering high- and low-tide data simultaneously or separately. Therefore, only correlations testing pooled tides data *versus Ulva* sp. biomarkers are shown in Figure 4.4. Non significant correlations were not presented. Dependence between nutrient levels and metals accumulation in *Ulva* sp. was apparent since Cu, Cr and Ni in algae were significantly correlated with PO_4^{3-} (Figure 4.4A). Moreover, Cu and Ni levels in *Ulva* sp. were correlated with those registered in water. CAT and GR activities were significantly correlated with NH_4^+, as well as with Ni and Cu in water (Figure 4.4 B).

The significant correlations obtained between metal levels in *Ulva* sp. and oxidative stress responses are presented in Figure 4.5. CAT increased linearly with accumulated Cu, Ni and Cr. Moreover, GPx, GST and GSH_t were positively correlated with Mn, Cd and Cr, respectively.

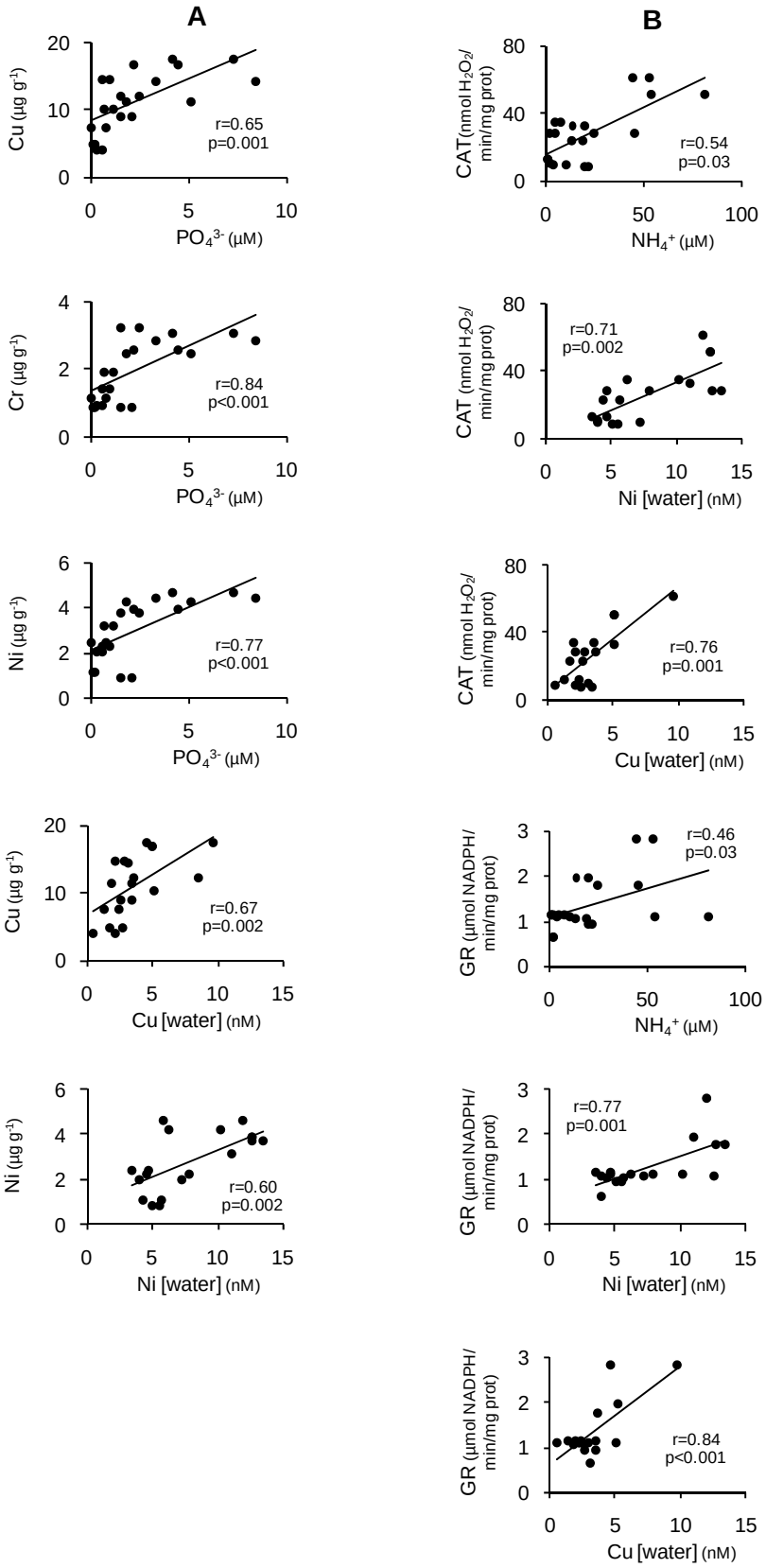

Figure 4.4. Significant correlations between water characteristics (nutrients and metals) and metal levels (A) or oxidative stress responses (B) in *Ulva* sp. Statistical significance and correlation coefficient are represented by p and r, respectively. Non significant correlations are not presented.

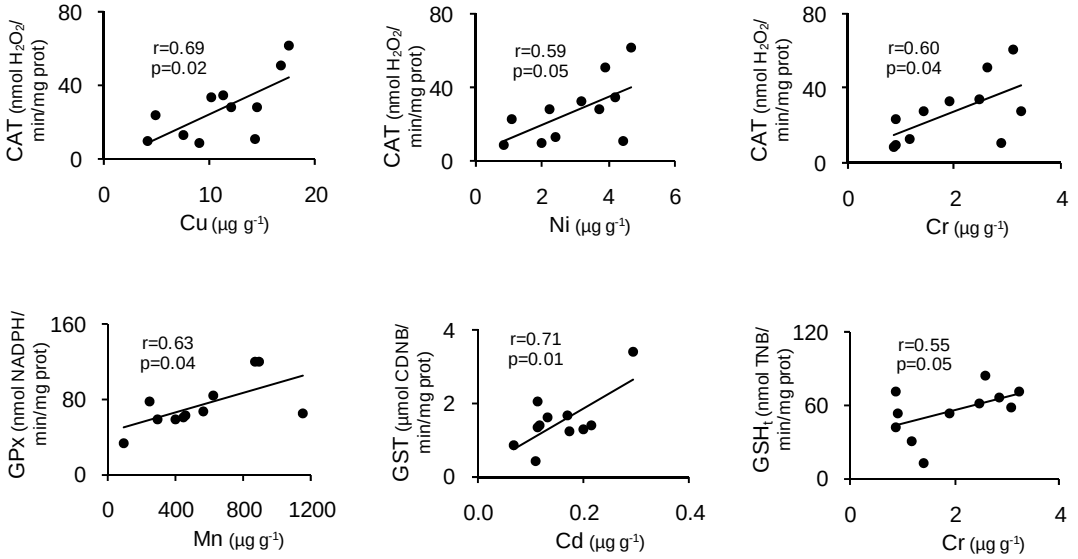

Figure 4.5. Significant correlations of oxidative stress responses with metal levels in *Ulva* sp. Statistical significance and correlation coefficient are represented by p and r, respectively. Non significant correlations are not presented.

In Figure 4.6, the radius coordinates in left star plots are integrated biomarker response (IBR) values and, adjacently, metal plot areas (measured in *Ulva* sp.) are also depicted. In general, IBR values showed different variation ranges along the year: in autumn they ranged from 0.1 at BS to 20 at BB, showing the highest index expression; in winter they ranged from 4.0 at BB to 6.6 at BS and in spring from 0.5 at BS to 10 at BB. The computed IBR values clearly indicate that *Ulva* sp. was more stressed at BB in autumn and spring, comparing with BS and LL. On the other hand, in winter no inter-station differences were discernible on the basis of IBR values. The visual comparison between the IBR black areas and the metal plot areas (grey) revealed an agreement in autumn for Mn, Cr, Cu and Ni. In winter, IBR matches to Cr area and to a lesser extent with Cd. In spring, this agreement is not so evident.

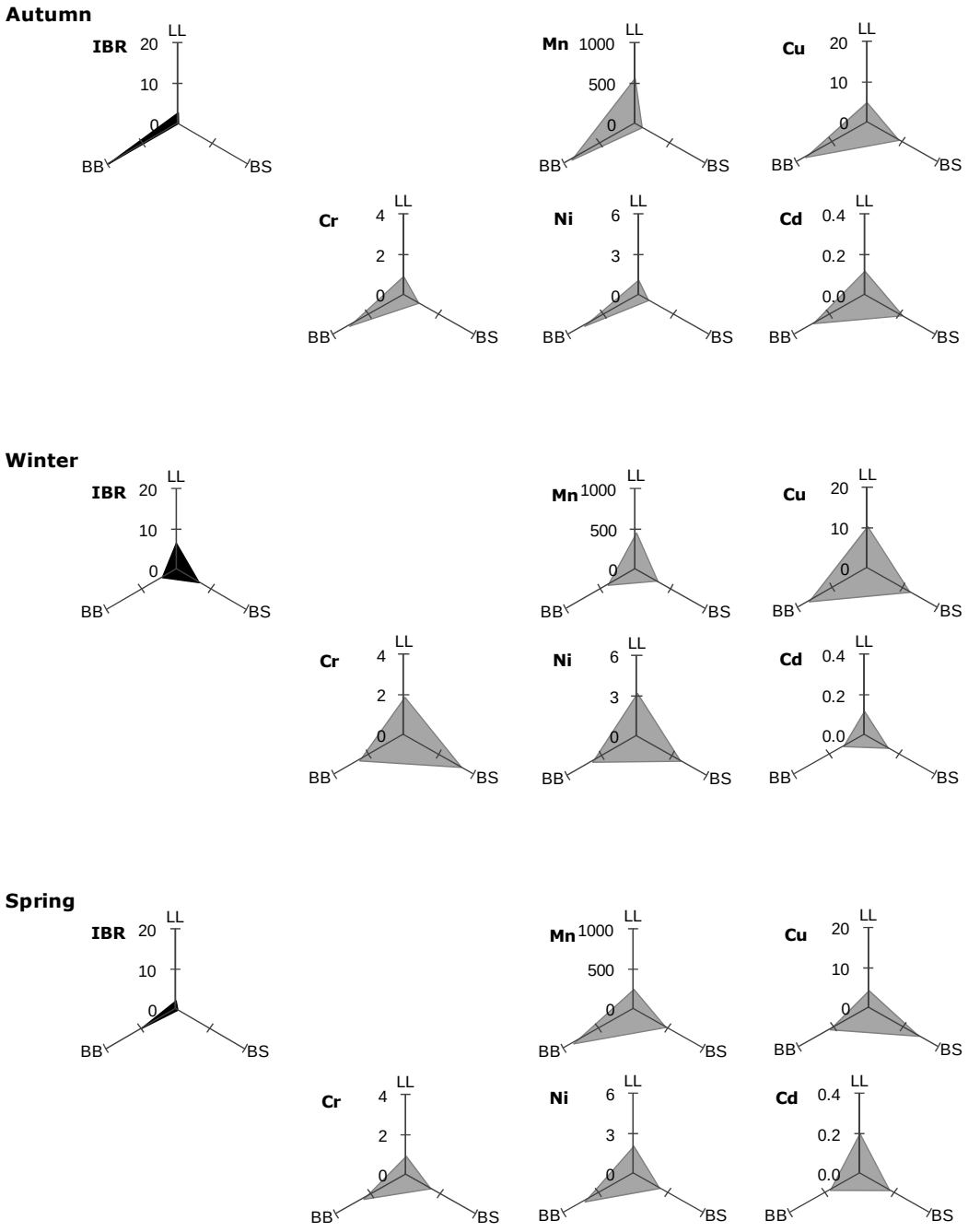

Figure 4.6. Integrated biomarker response (IBR) index and metals (Mn, Cu, Cr, Ni, Cd) star plots measured in *Ulva* sp. collected in autumn, winter and spring in the lower lagoon (LL) and in the inner branches (Bom-Sucesso – BS, Barrosa - BB) at Óbidos lagoon. Metals are expressed as µg g^{-1}.

4.4 Discussion

4.4.1 Spatial and temporal variation of environmental conditions

The accumulation of organic matter in fine sediments in the inner branches is related to urban effluents and agriculture activities, combined with weak tidal currents that promote the settling of organically enriched particles. Metal concentrations were higher in sediments of the branches due to their affinity to organic matter and fine particles, which is in line with a previous work (Carvalho et al., 2006). The comparison of sediment metal concentrations with values proposed by Long et al. (1995) for "Effects Range-Low" (ERL) (34, 81, 21, 1.2 μg g^{-1} for Cu, Cr, Ni, Cd, respectively) within the sediment quality guidelines indicates that the two branches (BS and BB) presented values above ERL for Cu and Ni but to a lesser extent for Cr. Metal concentrations in LL were largely below ERL.

The elevated levels of nutrients and chlorophyll *a* in BB are associated with eutrophication processes, as previously described (Pereira et al., 2009a). In summer, oxygen may decrease to less than 50% saturation during the night and, under these conditions, sediments act as an internal source of regenerated nutrients (Pereira et al., 2009b). Freshwater inputs enriched with nutrients (occurring in all seasons except summer) should also be considered for the elevated values recorded at that branch. Despite the fact that no significant differences were detected between metals in sediments from the two branches, Mn, Cu and Ni in water were generally higher in BB. The small tributary that enters this branch (Cal River) can contribute to metal inputs in periods of higher freshwater discharges, particularly in winter. However, higher values were also recorded in summer when freshwater flows seem to be negligible. Similar to the results found for nutrients, sediments can act as an internal source of metals to the water column during the night. The extension and intensity of these peaks were reported in a previous work (Pereira et al., 2009b). The enhanced values of Mn, Cu and Ni recorded in BB, particularly in the morning period (high tide) of autumn, may therefore be indicative of the existence of a high release of metals during the night. Taken as a whole, current chemical characterization of Óbidos lagoon pointed to a contamination scenario at BB marked by moderate metal levels and high nutrient availability.

4.4.2 Metal levels in *Ulva* sp. as biomarkers of exposure

Current work explores whether metal levels in *Ulva* sp. reflect environmental contamination, particularly in the water. Copper and Ni in *Ulva* sp. were higher at BB in autumn and spring in agreement with the corresponding measured water levels. These associations are statistically supported by Pearson analysis. Concerning Mn, Cd and Cr, no significant correlations were observed between water and algae levels when all seasons/stations/tides were jointly analysed. Nonetheless, coincidental patterns are perceptible for Mn and Cd. In fact, the significantly higher Mn levels found in *Ulva* sp. collected at BB in spring and summer match with Mn concentration increase in water. Similarly, Cd increased concomitantly in water and *Ulva* sp. at BB in autumn (compared to LL).

The significantly higher levels of Cr measured in algae from BB (compared to LL) in autumn, spring and summer are apparently in disagreement with the corresponding water values. A possible explanation for this discrepancy can be the occurrence of nocturne Cr releases from the sediment, as demonstrated by Pereira et al. (2009b), which are not detected in the current water samples collected at daylight hours.

Metal levels in *Ulva* sp. also accounted for the distinction between the surveyed branches since levels of Mn (autumn and spring), Cr and Ni (spring) were higher in BB than in BS. Overall, metal levels in *Ulva* sp. pointed BB as a major impacted area in the Óbidos lagoon reflecting distinct metal availabilities resulting from spatial and seasonal differences on metal concentrations in water. This is in conformity with previous studies that recorded metal levels in seaweeds from polluted sites to be 3- to 10-fold higher than in specimens sampled in less polluted coastal waters (Vasconcelos and Leal, 2001; Pawlik-Skowrońska et al., 2007).

The accumulation of metals in biota is a complex process that does not only depend on metal levels in the environment (Vasconcelos and Leal, 2001; Villares et al., 2002). In this direction, particular attention should be dedicated to endogenous as well as exogenous factors such as nutrient availability. In fact, significant correlations were obtained between Cu, Cr and Ni levels accumulated in *Ulva* sp. and PO_4^{3-} in water, suggesting that a higher uptake of metals occurs with the increasing availability of this nutrient. The dependence of metal accumulation on major nutrients was previously demonstrated in laboratory for *Ulva fasciata* (Lee and Wang, 2001).

The seasonality of metal accumulation in macroalgae is not a consensual matter. It was demonstrated that metal concentrations decrease during periods of growth and increase in the dormant winter period (Riget et al., 1995; Villares et al., 2001). Contrarily, Catsiki and Papathanassiou (1993) found elevated metal levels in the summer growth period of *Ulva* sp., which was explained by higher rates of photosynthesis and respiration, favouring the assimilation of metals. Other authors claimed that the accumulation of non-essential elements (Cr and Cd) is independent of physiological requirements (Rice and Lapointe, 1981). Current results revealed inter-season variations in LL and BS, showing increased Cu, Cr and Ni accumulation predominantly in winter. In relation to Cu and Ni, this increase cannot be dissociated from the slight increases of these metals in water recorded in that season; inversely, Cr accumulation seems to have a relation with seasonal factors, possibly the lower growth in winter as stated by Riget et al. (1995) and Villares et al. (2001). A divergent pattern was recorded in BB with Cd accumulation increasing in autumn and Mn enhancing in summer (both in relation to winter). Cadmium increase in *Ulva* sp. agrees with the Cd water levels, thereby showing no clear dependence from seasonal factors, while Mn increase can be attributed to seasonal variables as Mn availability clearly decreased in summer. Manganese, as an essential metal, plays a major role in photosynthesis, respiration and activation of several enzymes (Marschner, 1995). Therefore, the implicit regulation of Mn uptake and retention by the algae is probably on the basis of the absence of a significant correlation between environment (water) and algae levels.

4.4.3 Oxidative stress in *Ulva* sp. and causative factors

CAT activity displayed significantly higher levels at BB in autumn and spring, which is in agreement with Cu and Ni increases measured both in water and in *Ulva* sp. Furthermore, CAT is causally linked to Cu and Ni in water and in algae when the overall lagoon survey is considered as demonstrated by the correlations obtained. Additionally, CAT increased linearly with Cr accumulation in algae that was significantly higher at BB in autumn and spring, though the correlation with Cr in water was not apparent. CAT induction provided an indication of peroxide overproduction in *Ulva* sp. at BB in autumn and spring, suggesting the presence of redox-active compounds. Increased CAT activity has been described in several aquatic species from impacted areas both by metals and organic contaminants (Orbea et al., 2002; Nimptsch et al., 2005). Nevertheless, CAT induction in

plants was only reported under laboratory conditions (Roy et al., 1995; Rama Devi and Prasad, 1998; Collén et al., 2003). In this direction, the pro-oxidant action of accumulated Cu, Ni and Cr in autumn and spring at BB should be hypothesised. For Cu in particular, the potential to enhance intracellular ROS generation via Fenton-like reactions in the free floating macrophyte *Ceratophyllum demersum* was demonstrated (Rama Devi and Prasad, 1998). Globally, current data evidenced a linkage between external levels of exposure, bioaccumulation markers and oxidative stress responses (as CAT induction) when Cu and Ni (and, to a less extent, Cr) are considered.

The involvement of GPx on *Ulva* sp. defences against peroxides should also be considered (although there is an absence of significant differences to LL) since significant increases were observed at BB in relation to BS coinciding with CAT increases (autumn and spring). GPx also displayed a positive correlation with accumulated Mn. Notwithstanding that Mn is an essential metal for plants; this correlation suggests that high Mn concentrations also cause oxidative stress. This relation was previously demonstrated in *Populus cathayana* showing accumulation of hydrogen peroxide and malondialdehyde content following exposure to Mn (Lei et al., 2007).

GSTs are also capable of detoxifying ROS and their activity increases in cases of oxidative stress in plant cells (Pflugmacher et al., 2000). Taking into account a sole increase in GST activity at BB in autumn (compared to BS), its involvement on current algae defences seems to be less prominent. Nonetheless, a good correlation was detected with Cd accumulation.

GR was also significantly correlated with Cu and Ni in water but no significant correlation was found with accumulated metals. However, a direct implication of Cu and Ni accumulated in *Ulva* sp. on GR activity increase as observed at BB in autumn should be assumed due to the concomitance of this enzymatic activation with the highest concentrations of these metals measured in the algae. Elevated GR activity was previously observed in aquatic plants exposed to pro-oxidant stressors (Nimptsch et al., 2005). The higher GR activity at BB reflects increased glutathione recycling, as an adaptation to a GSSG/GSH ratio increase due to the presence of pro-oxidants and the subsequent attempt to inactivate them. Analysing the GR response profile, as well as GSH_t content (discussed later), it is perceptible that GPx and GST activities were not affected by limiting levels of GSH.

A GSH_t increase was observed in summer at BB (compared to LL), suggesting its involvement as a co-substrate in catalyzed or spontaneous conjugation reactions, as a consequence of a physiological adjustment involving the synthesis of new GSH. This response showed to be particularly associated with Cr toxicity, since a significant correlation was found with its accumulation in *Ulva* sp. Earlier, laboratory studies reported an increase in GSH content following metal exposure in plants (Tukendorf and Rauser, 1990), including *Ulva* sp. (Pawlik-Skowrońska et al., 2007), that can be associated to the activation of GSH synthesizing enzymes (Tukendorf and Rauser, 1990). However, under field conditions an opposite pattern was also observed in *Ulva* sp. with higher metal accumulation being accompanied by lower GSH levels (Pawlik-Skowrońska et al., 2007).

Besides the importance of metals in the macroalgae oxidative stress induction, the role of ammonium should also to be considered in the current study. In fact, significant positive correlations between CAT and GR activities and ammonium were found, which showed higher levels at BB water in autumn, winter and spring. Indeed, the toxicity of ammonium has been described in plants (Nimptsch and Pflugmacher, 2007), explained by the decrease of pH resulting from the excess of intracellular H^+, i.e. due to the high rate of proton extrusion from the plant into the surrounding medium (Taylor and Bloom, 1998). The involvement of ammonium on oxidative stress generation should be considered either through its direct action or, indirectly, trough a synergistic interaction with metals, as well as via an interference with metals accumulation and algae growth. On the other hand, it was previously observed that *Ulva* sp. in shallow environments could suffer from nutrient limitation in summer periods and consequently from oxidative stress (Malta et al., 2003). Current summer results displayed a significant GSH_t increase at BB in the presence of dissolved inorganic nitrogen levels in the same range of that found by Malta and co-authors in the Veerse Meer (Netherlands) and described as presenting oxidative stress potential (expressed as GSH_t increase and glutathione redox ratio decrease). However, the nitrogen deficiency per se cannot explain the present GSH_t response since dissolved inorganic nitrogen levels at LL were even lower than at BB. The same explanation is applicable to LPO increase observed in spring. As stated above for ammonium excess, nitrogen limitation cannot be excluded as an additional stressor, playing a synergistic role on the challenge posed to *Ulva* sp. by metals.

Despite the activation of enzymatic antioxidants in autumn and spring, *Ulva* sp. did not cope efficiently with ROS production and oxidation of polyunsaturated fatty acids

occurred at BB. In fact, LPO was significantly higher at BB than LL and BS, highlighting the great challenge posed to antioxidant defences of *Ulva* sp. and the corresponding state of environmental degradation in that area. This contaminant-induced membrane destabilization may lead to a significant loss in chlorophyll content and severe damage on cell structure and function as demonstrated by Rama Devi and Prasad (1998) and thus, diminishing algae fitness. In winter, no LPO was observed, which is consistent with the antioxidant parameters. In summer, a successful ROS neutralization occurred probably due to higher levels of GSH_t that is an indication of a less hazardous condition at BB in comparison with autumn and spring.

The absence of significant correlations between LPO and contaminant levels in water or in algae is not surprising in view of the multiplicity of factors preceding the occurrence of peroxidative damage, namely a complex antioxidant system and metal sequestering mechanisms (e.g. phytochelatins), which can impair the perception of such linear association.

4.4.4 Interpretation of integrated biomarker response (IBR)

By combining the different biomarker signals, the IBR index provides a simple mean for a general description of the health status of populations (Broeg and Lehtonen, 2006; Pytharopoulou et al., 2008). The IBR was previously used to integrate oxidative stress responses and other biomarkers measured in *Mytilus galloprovinciallis*, allowing for the discrimination of stations, independently of the sampling season (Pytharopoulou et al., 2008). In this present work, the IBR revealed spatial-temporal interactions, with algae showing a high “stress syndrome” at BB in autumn, followed by spring (comparing both with BS and LL). On the other hand, in winter no inter-station differences were discernible on the basis of IBR values. Despite the fact that some criticisms have been made to the application of IBR, mainly related with the choice of biomarkers and its position on the star plot, the main outcomes from its application here are in line with the data interpretation done without the help of this tool. Hence, by the comparison of IBR area contour and metal plots in autumn, algae stress at BB is apparently related with the higher accumulation of Mn, Cr, Cu and Ni. In winter, IBR values are in agreement with the absence of significant differences between stations concerning metal accumulation in *Ulva* sp. (with the sole exception of Cr). Beside this corroborative information, IBR brought three new perspectives to the present discussion: (i) *Ulva* sp. was more severely affected

at BB in autumn (IBR value = 20) in comparison to spring (IBR value = 10); the assertion that winter conditions at BB are not of concern must be carefully assumed since the increase in IBR values at BS and LL in relation to the other seasons can have a masking effect; (ii) In general, metal levels in *Ulva* sp. in spring were not substantially lower that in autumn; therefore, allowing for the statistical correlations obtained, we could be impelled to establish a causal relation between accumulated metals and oxidative stress responses in that season. However, the visual comparison of IBR and metal plot areas revealed no agreement in spring, indicating that other factors (instead of/beside metals) should be hypothesized as responsible by algae responses, namely the occurrence of high nutrient levels; (iii) In autumn and spring, BS displayed the lowest IBR levels, even lower than at the pre-defined reference station (LL); in view of that, the valuation of significant differences between BB and BS adopted in point 4.4.3. is legitimated.

Analyzing the effectiveness of the IBR index, it can be stated that it offered an "aerial picture" of the overall biomarker responses, diminishing the degree of uncertainty on the interpretation of results.

4.5 Conclusions

The adopted multiparametric approach applied to *Ulva* sp., coupled with environmental chemical characterization, clearly pointed out Barrosa branch (BB) as a critical area at the Óbidos lagoon, with potential impact on autochthonous populations. Obvious signs of toxicity were observed (lipid peroxidation) surmounting the antioxidant defences. These effects were clearly correlated with metals, though a contributory role of high ammonium levels (associated to the BB eutrophic status) can not be overlooked.

Despite the complexity and variety of processes affecting metal uptake and accumulation, metal load in *Ulva* sp. consistently reflected their occurrence in water within moderate contamination levels, namely in the case of Cu and Ni. In addition, the responsiveness of oxidative stress parameters in this green macroalgae showed to be comparable to other taxa, namely aquatic animals, demonstrating their ability as early alert signals.

The combination of exposure and effect biomarkers applied to *Ulva* sp. enabled the health assessment of a multi-impacted ecosystem. Moreover, the integration of oxidative stress responses into a general stress index (IBR) substantiated data interpretation, and

provided a more definitive identification of the extent of pollution, thus contributing to the understanding of cause-effect relationships.

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CHAPTER V

Biochemical responses of the shore crab (*Carcinus maenas*) in a eutrophic and metal-contaminated coastal system (Óbidos lagoon, Portugal)

5 Biochemical responses of the shore crab (*Carcinus maenas*) in a eutrophic and metal-contaminated coastal system (Óbidos lagoon, Portugal)

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Abstract

A eutrophic and metal-contaminated coastal system (Óbidos lagoon, Portugal) was monitored combining water/sediment quality parameters and *Carcinus maenas* biomarkers (accumulated metals, oxidative stress and biotransformation responses). Two confined branches (Barrosa and Bom-Sucesso) were surveyed and compared with a reference area. Both crab genders from Barrosa exhibited activation of hepatopancreas CAT, GPx and GST, pointing-out this area as the major impacted in the lagoon. Females captured at Barrosa were more vulnerable to peroxidative damage while only males showed decreased EROD activity, reinforcing genders specificities. In general, responses were not directly attributed to metals in hepatopancreas, as supported by principal component analysis (PCA). However, higher metals (Ni, Cu, Cd) and nutrients levels registered in Barrosa water were associated with the observed oxidative stress responses by PCA. Despite the difficulty to establish cause-effect relationships due to the co-occurrence of various stressors and their interactions, the adopted integrated monitoring strategy appears to be promising.

KEYWORDS: Oxidative stress; biotransformation; metals; eutrophication; *Carcinus maenas*; Óbidos lagoon

5.1 Introduction

A general decrease in the anthropogenic pressure on coastal ecosystems has been observed recently in developed countries, but coastal lagoons are still undergoing major human impact (Lotze et al., 2006). A main environmental concern is the enhancement of contaminant dispersion and algal production due to eutrophication, associated with an increase in the duration of intermittent periods of anoxia (Point et al., 2007). The low water exchange with seawater may slow down the dilution process and enhance the sediment accumulation and/or retention of toxic compounds, which are transferred to biota through the food chain (Mucha et al., 2004). Several studies demonstrated that metals, such as Fe, Mn, Cu and Pb are easily remobilized from sediments during organic matter decomposition under anaerobic conditions (Point et al., 2007). Aquatic organisms inhabiting those environments are exposed to multiple stress agents with different mechanisms of toxicity, each contributing to a final overall adverse effect. Therefore, the integration of chemical data and biological responses is strongly recommended in monitoring programmes of ecological status (Allan et al., 2006). Among the wide range of biological endpoints pointing to environmental contamination, biochemical markers had played a singular role, representing early-warning signals which detection can avoid adverse effects at higher hierarchical levels (Van der Oost et al., 2003). In this context, the cytochrome P450 1A (CYP1A) (Pacheco et al., 2005) and oxidative stress responses (Martín-Díaz et al., 2007, 2008; Lima et al., 2006) are among the best-studied biomarkers. The induction of ethoxyresorufin-*O*-deethylase (EROD) activity, a functional and specific assessment of CYP1A, has been widely used as an indicator for wildlife exposure to xenobiotics that bind to the aryl hydrocarbon (Ah) receptor like organic contaminants (Van der Oost et al., 2003). On the other hand, several reports have implicated metals as modifiers of CYP1A function (Vakharia et al., 2001), being EROD activity inhibited at relatively low concentrations of Cu, Pb and Cd (Viarengo et al., 1993; Oliveira et al., 2004). Enzymatic and non-enzymatic antioxidant systems are triggered to neutralize the impact of reactive oxygen species (ROS), allowing organisms to overcome oxidative stress (Winston and DiGiulio, 1991), thereby preventing DNA damage, enzymatic inactivation and peroxidative damage. The contaminant-induced ROS generation is ubiquitous in aquatic species, being antioxidants induction an important component of the adaptive response in organisms exposed to different classes of chemicals (Stohs and Bagghi, 1995). The modulation of antioxidant enzymes and alterations on glutathione content demonstrated

to serve as biomarkers of exposure to metals, PAH, nitroaromatic compounds, dioxins and halogenated hydrocarbons (Van der Oost et al., 2003). On the other hand, the oxidative stress potential of nitrogen compounds in aquatic organisms has been less investigated. Nonetheless, recent works demonstrated that antioxidant defences were induced by ammonia, nitrites and nitrates under field and laboratory conditions (Nimptsch et al., 2005; Nimptsch and Pflugmacher, 2007), highlighting the relevance of eutrophic conditions on the overall responses of the organisms.

The shore crab *Carcinus maenas* has a wide geographical distribution and abundance in coastal and estuarine waters, presents relatively low mobility (Stentiford and Feist, 2005), and is an aggressive and voracious predator living in close contact with the sediments (Baeta et al., 2005). *C. maenas* is a burrowing and opportunistic feeder, consuming a large variety of prey items, including organisms from animal (mainly Crustacea, Mollusca and Annelida), plant and protest phyla (Cohen et al., 1995). Its sexual dimorphism represents an advantage in order to assess eventual gender-specific responses. This species has been successfully used in the biomonitoring of contaminated sites, showing its sensitivity to contaminants both in water and sediment (Martín-Díaz et al., 2007, 2008). However, a search in specialized literature revealed that field studies with this epibenthic crustacean combining bioaccumulation, biotransformation and oxidative stress endpoints are scarce.

This paper reports biochemical effects in a wild population of *C. maenas* from a coastal lagoon (Óbidos lagoon, Portugal) impacted by eutrophication and moderate metals contamination, and examines the implications of the co-occurrence of these environmental stressors on the ecosystem health. Peroxidative damage, antioxidants and biotransformation responses were measured and its relationship with metal accumulation in crabs' hepatopancreas as well as with environmental chemical data (metals and nutrients) was assessed. In addition, it was intended to evaluate the suitability of the adopted strategy, using *C. maenas*, on monitoring coastal ecosystems in a multi-pollution context.

5.2 Materials and Methods

5.2.1 Study area characterization

The Óbidos lagoon is a shallow coastal ecosystem, located on the west coast of Portugal with wet area of 7 km^2, permanently connected to the sea through a narrow inlet (Figure 5.1). It comprises areas of different morphological and sedimentary characteristics: several sand banks and narrow channels in the lower/middle lagoon; muddy bottom sediments of the two inner branches Barrosa and Bom-Sucesso. Tidal energy dissipates in the entire lagoon with tidal amplitudes ranging between 1 and 2 m (Oliveira et al., 2006). The semi-diurnal tidal cycle in the upper part of the lagoon is asymmetrical, being dominated by the ebb period with approximately 8 hours (Pereira et al., 2009b).

Barrosa branch is shallower (mean depth 0.5-1 m) and water circulation is mostly driven by tides and by a small tributary (Cal River) that drains agriculture fields. Urban effluents from a nearby town (Caldas da Rainha, 50 000 inhabitants) have been discharged to Barrosa branch by Cal River. Consequently, this area presents the highest nutrient availability of the lagoon, being classified as eutrophic (Pereira et al., 2009a). High nutrient concentrations are in line with abundant macroalgae (*Ulva* sp. and *Enteromorpha* sp.) and a broad daily variation of dissolved oxygen concentration in summer (Pereira et al., 2009a). Water quality surveys of the lagoon also showed maximum concentrations of Mn, Ni, Cu and Cd in Barrosa area. Major metal sources were identified to be the Cal River in periods of higher discharges, and remobilization from sediments in summer (Pereira et al., 2009b). Bom-Sucesso branch is also a confined area but receives a smaller freshwater flow (Vala do Ameal) with better water quality than Cal River, according to the Portuguese categorization of freshwater systems. In this area, metal remobilization from sediments is less plausible due to the higher depth and stronger currents. Middle lagoon was previously characterised by a better water quality than the two confined branches (Pereira et al., 2009a), giving support to the reference area selection.

5.2.2 Sampling

Water, sediments and crabs were collected in October 2005 at three sites (Figure 5.1): BB, located in the impacted Barrosa branch, BS in the Bom-Sucesso branch, and ML in the middle part of the lagoon. Due to the distance from the impacted area and the influence of seawater influx, ML was assumed as reference site. Surface water was sampled in high tide (10-11am) and low tide (4-5pm) to various bottles according to the

analytical specifications and transported to a field laboratory for filtration and preservation. Surface sediments were collected at each site with a Van-Veen grab, oven-dried to constant weight at 40 °C and homogenised.

Twelve specimens of *Carcinus maenas* L. (six females and six males) were captured at each site during the morning using baited circular drop nets. Males and females were considered separately based on previous works, which claimed that background concentrations of metals in tissues of *C. maenas* can be affected by gender (Bjerregaard et al., 2005; Martín-Díaz et al., 2005) and consequently biochemical responses. Following collection, crabs were placed in thermally insulated 10 L boxes filled with water from the sampling site, transported to the laboratory where they were weighted (total fresh weight) and measured (cephalothorax width). Crabs were sacrificed and their hepatopancreas were excised, divided in two parts, immediately frozen in liquid nitrogen and stored at -80 °C until further metal analysis and biochemical assays. Hepatopancreas was selected based on previous works that demonstrated its responsiveness to environmental contaminants in the field (Martín-Díaz et al., 2008) and under laboratory conditions (Morales-Caselles et al., 2008). Due to its key role on food and xenobiotics processing, hepatopancreas presents high levels of biotransformation and antioxidant enzymes (Livingston, 1998).

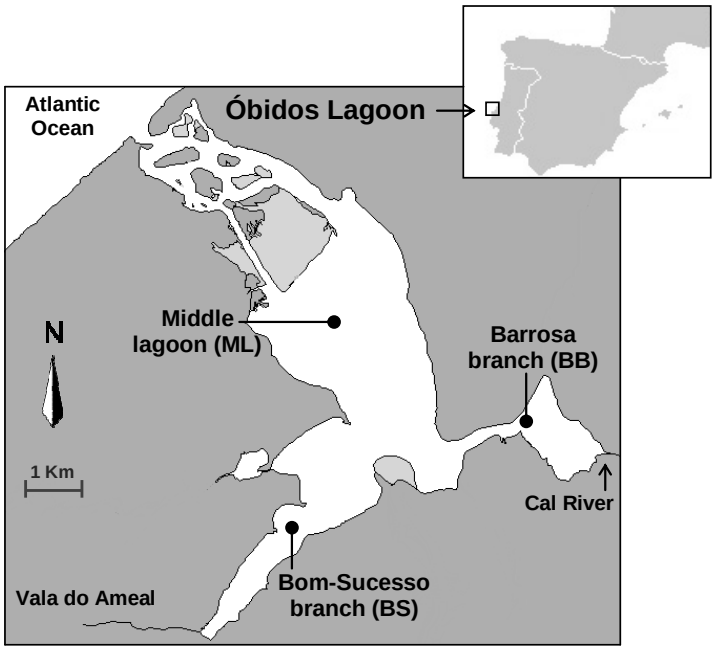

Figure 5.1. Map of Óbidos lagoon (Portugal) with location of the sampling sites: ML in the middle lagoon (Reference; 39º24'50.48''N, 9º12'42.82''W); BS (39º23'44.88''N, 9º13'01.75''W) and BB (39º26'16.84''N, 9º11'33.18''W) in the inner branches (Bom-Sucesso and Barrosa, respectively).

5.2.3 Analytical procedures

5.2.3.1 Water physicochemical parameters

Temperature, salinity and pH were measured *in situ* using an YSI, 650 meter. Dissolved oxygen was determined in the laboratory by a modified Winkler method (Carrit and Carpenter, 1966) after *in situ* fixation. The coefficient of variation associated with this method was determined by analysing replicates and was found to be less than 0.25%. Dissolved oxygen saturation was calculated according to OSPAR (2001). Suspended particulate matter (SPM) was obtained by filtering 250 mL of water through cellulose acetate membranes (0.45 µm) and determined gravimetrically. SPM quantifications were performed in triplicates. Samples for dissolved inorganic nutrients (nitrate+nitrite, NO_3^- +NO_2^-; ammonium, NH_4^+, phosphate PO_4^{3-}) determinations were filtered through MSI Acetate Plus filters, and analysed in triplicate using an autoanalyser TRAACS 2000 (Bran+Luebbe). The precision was found to be ±1.0% for NO_3^-+NO_2^-, ±2.0% for NH_4^+ and ±1.9% for PO_4^{3-}.

5.2.3.2 Analyses of metals, carbon and nitrogen

The measurement of dissolved Mn, Cu, Cr, Ni, Pb and Cd was performed by diffusive gradients of thin films (DGT) samplers deployed in surface water (2 L) collected in polypropylene bottles, during 48 hours (Pereira et al., 2009b). DGT holders, Chelex-100 resins and diffusive gels (type APA, 0.8 mm thickness, open pore> 5 nm) (Zhang and Davison, 1995) were purchased from DGT Research (Lancaster, UK). After the retrieval of the devices, resins were eluted by immersion in 5 mL of 1 M HNO_3 (prepared from suprapur nitric acid) at a minimum of 24 h. Eluates were analysed directly by a quadropole ICP-MS (Thermo Elemental, X-Series). All eluates were analysed with reagent blanks and an international standard of river water (SLRS-4) to control eventual contaminations during the analytical procedure and the procedure accuracy, respectively. The eluate concentration was converted into the mass of metal accumulated on the resin using an elution factor with a yield value of 0.8 (Zhang and Davison, 1995) and a resin gel volume of 0.15 mL. Dissolved metal concentration were calculated according to the following equation: [Metal]=(MΔg)/(tAD); where M is the mass of metal accumulated on the resin during the emersion time (t); Δg the diffusive gel thickness (0.08 cm); A the exposure area (3.14 cm^2); and D the diffusion coefficient of the metal in the gels as provided by DGT Research. Detection limits varied between 0.01 nM for Cd and 1.7 nM for Ni.

Sediment samples (≈100 mg) were mineralized completely with HF (40%) and Aqua Regia (HCl-36%:HNO_3-60%; 3:1) in closed Teflon bombs (100 °C for 1 h) evaporated to near dryness (DigiPrep HotBlock – SCP Science), redissolved with 1 mL of HNO_3 (65%) and 5 mL of ultra-pure water, heated for 20 min at 75 °C and diluted to 50 mL with ultra-pure water (Caetano et al., 2007). Freeze-dried and homogenized hepatopancreas samples (≈50 mg) were digested with a mixture of HNO_3 and H_2O_2 at 60 °C for 12 hours, at 100 °C for 1 hour and at 80 °C for 1 hour, according to the method described in Ferreira et al. (1990). The accuracy of the analytical procedures was assessed by the analysis of certified reference materials (MESS-3, AGV-1, BCSS-1, 1646a for sediments; TORT-1, TORT-2, DOLT-1, DOLT-3 for crabs' hepatopancreas). In order to examine for any possible contamination during the analytical procedure, blanks were analysed. The concentrations of Al, Fe and Zn were determined by flame atomic absorption spectrometry (Perkin Elmer Analyst 100). The concentrations of Mn, Cu, Cr, Ni, Pb, and Cd were determined by ICP-MS. Levels of the analysed elements obtained in the reference materials were consistently within the ranges of certified values.

Organic carbon and nitrogen were determined in sediment samples using the method described by Byers et al. (1978) in a CHN Fissons NA 1500 Analyser.

All labware for metal determinations was soaked for two days with HNO_3 (20%), then for two days with HCl (20%) and rinsed with ultra-pure water in order to avoid contamination.

5.2.3.3 Biochemical analyses

Hepatopancreas was homogenized in a 1:10 w/v ratio (hepatopancreas weight:buffer volume) of 0.1 mol L^{-1} Tris–HCl (pH 7.4) containing 0.15 mol L^{-1} KCl and 20% glycerol, using a Potter–Elvehjem glass–Teflon homogenizer at 2500 rpm. An aliquot of homogenate (150 µL) was taken for thiobarbituric acid reactive substances (TBARS) measurement and stored after adding 10 µL BHT (1-1 butylated hydroxytoluene) to prevent oxidation, and the rest was centrifuged. Microsomes were obtained by differential centrifugation at 4 °C in a Beckman Optima TL Ultracentrifuge (TLA-100.4 fixed angle rotor), according to the method of Lange et al. (1993): the homogenate was first centrifuged at 12,000 g for 20 min to remove cell debris, nuclei and mitochondria; the supernatant was recentrifuged at 135,000 g for 75 min. The resulting microsomal pellet was resuspended in 200 µL of the previous buffer. Both cytosolic and microsomal fractions

were frozen in liquid nitrogen, and stored at −80 °C until use. EROD activity was measured in the microsomal fraction (at 25 °C), whereas all the other measurements were carried out in the cytosolic fraction (at 25 °C).

Total glutathione (GSH_t) content was determined adopting the enzymatic recycling method using glutathione reductase (GR) excess, whereby the sulfhydryl group of GSH reacts with DTNB (Ellman's reagent) producing a yellow coloured 5-thio-2-nitrobenzoic acid (TNB). Formation of TNB was measured by spectrophotometry at 412 nm and the results were expressed as nmol TNB formed/min/mg protein. Glutathione-S-transferase (GST) activity was determined using CDNB (1-chloro-2,4-dinitrobenzene) as substrate according to the method of Habig et al. (1974), the increase in absorbance was recorded at 340 nm during 3 min and enzyme activity calculated as nmol CDNB conjugate formed/min/mg protein (ε=9.6 mM^{-1} cm^{-1}). Catalase (CAT) activity was assayed by Claiborne (1985) method. Change in absorbance was recorded at 240 nm and CAT activity was calculated in terms of nmol H_2O_2 consumed/min/mg protein (ε=43.5 M^{-1} cm^{-1}). Glutathione peroxidase (GPx) activity was assayed by Mohandas et al. (1984) method. Oxidized glutathione (GSSG), produced upon reduction of H_2O_2 by GPx is recycled to its reduced state by glutathione reductase (GR) and NADPH. The decrease in absorbance at 340 nm resulting from NADPH oxidation is directly proportional to the GPx activity, and results were expressed as nmol NADPH oxidized/min/mg protein (ε=6.22×10^3 M^{-1} cm^{-1}). The determination of LPO was carried out based in the TBARS measurement (535 nm), as adapted by Filho et al. (2001). The rate of LPO was expressed as nmol of TBARS formed per milligram of fresh tissue (ε=1.56×10^5 $M^{-1}cm^{-1}$). EROD activity was measured as described by Burke and Mayer (1974). The reaction was carried out in the fluorometer cuvette containing 1 mL 0.5 µmol L^{-1} ethoxyresorufin (in homogenization buffer, pH 7.4) and 50 µL of microsomal suspension. The reaction was initiated by adding 10 µL of NADPH (10 mmol L^{-1}) and the progressive increase in fluorescence, resulting from the resorufin formation, was measured for 3 min (excitation wavelength 530 nm, emission wavelength 585 nm). EROD activity was expressed as pmol/min/mg protein. Microsomal and cytosolic protein concentrations were determined according to the Biuret method (Gornall et al., 1949) using bovine serum albumin (E. Merck-Darmstadt) as a standard.

5.2.4 Statistical analyses

Statistical software (Statistica 7.0) was used for univariate statistical analyses. All the data were first tested for normality and homogeneity of variance to meet statistical demands. ANOVA analysis was used to compare results between crabs groups, followed by Tukey test (Zar, 1996). Differences between means were considered significant when $p<0.05$. A Pearson correlation was performed to evaluate the degree of relationship between the accumulated metal levels in crabs and biochemical responses.

The link between accumulated metals and biochemical responses (biomarkers) in *C. maenas*, as well as between environmental data and biomarkers was assessed by means of a Principal Component Analysis (PCA) performed on the log transformed and standardized variables (Legendre and Legendre, 1998; Clarke and Warwick, 2001). In the second approach, two sets of PCAs were performed: one linking biomarkers to water data (nutrients and metals); and other linking biomarkers to sediment data (metals, organic carbon and organic nitrogen). A total of 12 cases were analyzed. The significance of the correlation between these two data sets was investigated by applying the RELATE procedure to the Euclidean-distance dissimilarity matrices. All multivariate analyses were carried out with the statistical package PRIMER 6.0+ (PRIMER-E, Plymouth).

5.3 Results

5.3.1 Water and sediment characteristics

Temperature ranged between 18 °C and 20 °C (Table 5.1). Salinity was 2-6% lower in BB than in BS and ML reflecting the freshwater inputs in the former. Suspended particulate matter concentration was higher in BB, particularly in low tide. Dissolved oxygen concentration was approximately 100% saturation in all sites, for the two sampling periods, and pH ranged within 7.9-8.4. NH_4^+ accounted to 80-94% of the total inorganic nitrogen concentrations and values in BB were twice higher than in BS and ML. Higher levels of NO_3^-+ NO_2^- and PO_4^{3-} were also registered in BB, and doubled in low tide. Dissolved Mn, Cu, Cr, Ni, Pb and Cd levels pointed to a low/moderate contamination of the Óbidos lagoon (Table 5.1), but values of Cu, Ni and Cd in BB exceeded always those registered in BS and ML. In addition, metal concentrations in BB were higher in the morning (high tide) than in the afternoon.

The metal concentrations in surface sediments were slightly higher in BB (Table 5.2). Sediments from inner branches (BB and BS) contained higher organic carbon and organic nitrogen than from ML.

Table 5.1. Concentration ranges of physicochemical parameters, nutrients (NH_4^+, NO_3^-+NO_2^-, PO_4^{3-}) and dissolved metals (Mn, Cu, Cr, Ni, Pb and Cd) in water collected in the middle lagoon (ML - reference) and in the inner branches (Bom-Sucesso – BS, Barrosa - BB) at Óbidos lagoon. Intervals represent concentrations in high- and low-tide. SPM- suspended particulate matter.

		Sampling sites		
		ML	BS	BB
Temperature	(°C)	18 - 19	18 - 19	19 - 20
Salinity		35.1- 35.4	35.3 - 35.1	34.5 - 33.0
SPM	(mg L^{-1})	31 - 35	31 - 30	56 - 62
pH		8.0 - 8.2	7.9 - 8.3	7.9 - 8.4
O_2	(%)	97 - 102	97 - 98	94 - 95
NH_4^+		25 - 16	21 - 20	44 - 53
NO_3^-+NO_2^-	(µM)	1.8 - 2.0	2.3 - 1.2	5.1 - 13
PO_4^{3-}		1.7 - 1.1	1.6 - 2.1	4.2 - 7.3
Mn	(µM)	0.21 - 0.17	0.23 - 0.44	0.31 - 0.18
Cu		1.6 - 2.2	2.6 - 3.5	9.6 - 4.5
Cr		7.5 - 6.9	6.5 - 8.7	8.5 - 6.8
Ni	(nM)	6.3 - 4.4	5.5 - 5.0	12 - 6.0
Pb		0.55 - 0.79	0.38 - 0.55	0.56 - 0.42
Cd		0.089 - 0.069	0.080 - 0.054	0.15 - 0.17

Table 5.2. Concentrations of organic carbon (C org), organic nitrogen (N org), Al, Fe, Mn, Zn, Cu, Cr, Ni, Pb and Cd in surface sediments collected in the middle lagoon (ML - reference) and in the inner branches (Bom-Sucesso – BS, Barrosa - BB) at Óbidos lagoon.

		Sampling sites		
		ML	BS	BB
C org	(%)	0.5	1.5	1.1
N org		0.036	0.16	0.14
Al	(%)	11	10	12
Fe		4.6	4.3	5.0
Mn		298	286	300
Zn		125	121	144
Cu		40	56	52
Cr	($\mu g\ g^{-1}$)	83	87	89
Ni		33	35	35
Pb		35	35	39
Cd		0.15	0.17	0.19

5.3.2 Metal levels in hepatopancreas of *C. maenas*

Copper, Zn, Mn, Ni and Cr concentrations in crab's hepatopancreas did not vary significantly among sites, both in males and females (Table 5.3). On the contrary, Cd levels in females from BB were significantly higher than those from ML. Moreover, females from BS showed Fe concentrations two times higher than those from BB and ML; males from BS presented higher Pb and Cd levels than those from ML. Identical spatial trends were observed when data from both genders were pooled (Table 5.3). In fact, crabs from BB presented higher Cd levels than ML, whereas those from BS accumulated higher Pb (than ML) and Fe (than BB and ML). Nonetheless, gender differences were perceptible as males exhibited higher accumulation of Cu (BB and BS), Zn (BS), Mn (BB and ML), Ni (BB) and Cd (BS) comparing with females within the same site. Females from BS showed higher levels of Fe than males.

Table 5.3. Mean and associated standard errors of weight, cephalothorax width and metal concentrations in hepatopancreas of *C. maenas* captured in the middle lagoon (ML - reference) and in the inner branches (Bom-Sucesso – BS, Barrosa - BB) at Óbidos lagoon. Data concern genders separately and jointly. Letters denote statistically significant differences ($p<0.05$): (a) vs. ML; (b) vs. BS; (s) between genders.

		Sampling sites		
	Gender	ML	BS	BB
Width (cm)	♀	5.0 ± 0.04	5.4 ± 0.10	5.1 ± 0.10
	♂	6.0 ± 0.05	4.7 ± 0.12	5.9 ± 0.30
	Both	5.6 ± 0.17	5.1 ± 0.13	5.4 ± 0.19
Weight (g)	♀	23 ± 0.49	29 ± 0.94	25 ± 0.07
	♂	44 ± 2.2	21 ± 1.8	45 ± 7.4
	Both	35 ± 3.8	25 ± 1.6	33 ± 4.5
Fe ($\mu g\ g^{-1}$)	♀	551 ± 120	1208 ± 43 [a]	518 ± 66 [b]
	♂	494 ± 96	777 ± 157 [s]	616 ± 68
	Both	523 ± 73	992 ± 111 [a]	573 ± 48 [b]
Cu ($\mu g\ g^{-1}$)	♀	357 ± 115	237 ± 76	239 ± 69
	♂	475 ± 94	779 ± 82 [s]	637 ± 136 [s]
	Both	410 ± 75	478 ± 109	416 ± 96
Zn ($\mu g\ g^{-1}$)	♀	94 ± 8.6	85 ± 3.7	101 ± 14
	♂	118 ± 20	152 ± 20 [s]	113 ± 11
	Both	106 ± 11	119 ± 15	107 ± 8.9
Mn ($\mu g\ g^{-1}$)	♀	14 ± 1.5	22 ± 5.2	15 ± 1.7
	♂	30 ± 2.9 [s]	30 ± 4.1	29 ± 4.5 [s]
	Both	21 ± 3.2	26 ± 3.8	22 ± 2.9
Ni ($\mu g\ g^{-1}$)	♀	3.7 ± 0.46	5.6 ± 1.2	2.5 ± 0.22
	♂	5.3 ± 2.3	4.2 ± 1.0	11 ± 4.3 [s]
	Both	4.6 ± 1.2	4.9 ± 0.78	7.6 ± 2.6
Cr ($\mu g\ g^{-1}$)	♀	0.69 ± 0.19	1.0 ± 0.33	0.88 ± 0.19
	♂	0.59 ± 0.16	1.1 ± 0.29	0.89 ± 0.12
	Both	0.64 ± 0.12	1.1 ± 0.21	0.88 ± 0.11
Pb ($\mu g\ g^{-1}$)	♀	0.11 ± 0.05	0.31 ± 0.10	0.25 ± 0.07
	♂	0.10 ± 0.04	0.35 ± 0.08 [a]	0.23 ± 0.02
	Both	0.10 ± 0.03	0.33 ± 0.06 [a]	0.24 ± 0.04
Cd ($\mu g\ g^{-1}$)	♀	0.12 ± 0.03	0.08 ± 0.02	0.20 ± 0.04 [a]
	♂	0.11 ± 0.02	0.18 ± 0.01 [a, s]	0.14 ± 0.01
	Both	0.11 ± 0.02	0.13 ± 0.02	0.17 ± 0.02 [a]

5.3.3 Oxidative stress and biotransformation responses

The activities of CAT, GPx and GST recorded in females from BB were significantly higher than those from ML (Figure 5.2). In addition, females from BB presented significantly higher GPx and GST activities than those from BS. The males from BB showed elevated GPx and GST activities in relation to specimens from ML. GSH_t content in males from BS was also significantly lower than in males from ML. Only females from BB exhibited significant increases of LPO relatively to ML. EROD activity was significantly lower in males from BB than from ML (Figure 5.2). The same EROD spatial pattern was observed in females, but with no statistical support. Considering data from pooled genders, CAT, GPx and GST were higher in BB than ML, and GPx and GST were also significantly increased in BB in relation to BS. No spatial differences were found for GSH_t, EROD and LPO. Considering significant differences between sexes captured at the same site, it was found that females from BB presented higher CAT activity, while males from BS displayed higher GST activity.

The correlations between the accumulated metals and biochemical responses in crabs were statistically tested considering genders either separately or jointly. Since no significant correlations were obtained, test results were not represented.

In the PCA, the criterion for consideration of a variable as being associated with a particular component was defined as its having a loading of 0.25 or higher. Three principal components were defined for explaining the major amount of total variance (63%) when accumulated metals and biomarkers were considered (Table 5.4). The first principal component (PC1) accounts for 28% of the variance and shows a positive association between CAT, GPx, GST and LPO with accumulated Cd. PC1 has a positive score (averaged by sampling site) only at BB (Figure 5.3A). The PC2 and PC3 account for 20% and 15%, respectively. PC2 did not establish any association between biochemical responses and metal levels (Table 5.4). According to PC3, GPx and EROD induction, as well as higher GSH_t content are positively related with accumulated Cr (Table 5.4) being this association more relevant in ML (Figure 5.3A).

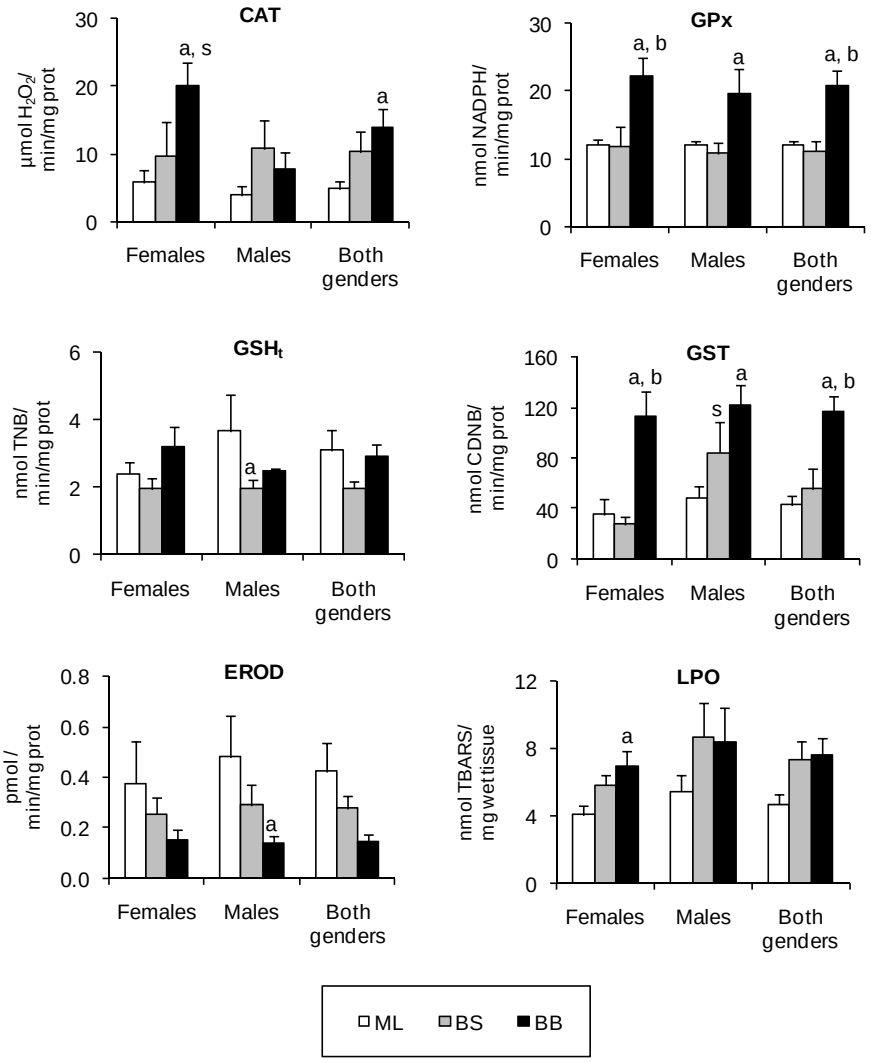

Figure 5.2. Oxidative stress and biotransformation parameters in *C. maenas* captured in the middle lagoon (ML - reference) and in the inner branches (Bom-Sucesso – BS, Barrosa - BB) at Óbidos lagoon. Data concern genders separately and jointly. Hepatopancreas activity of catalase (CAT), glutathione peroxidase (GPx), glutathione S-transferase (GST) and ethoxyresorufin-*O*-deethylase (EROD); total glutathione (GSH_t) content and lipid peroxidation (LPO). Letters denote statistically significant differences ($p<0.05$): (a) vs. ML; (b) vs. BS; and (s) between genders. Mean and associated standard errors are given.

Table 5.4. Results of the Principal Component Analysis (PCA) on the data set with biomarkers (metals, oxidative stress and biotransformation) in *C. maenas*: coefficients of the eigenvectors (loadings of the original variables) in the linear combination of variates from which the PCs are computed (loadings <0.25 were omitted). The percentage of total variance explained by each PC is indicated in brackets.

		Principal components		
		PC1 (28%)	PC2 (20%)	PC3 (15%)
Biochemical responses	CAT	0.44	-	-
	GPx	0.38	-	0.26
	GSH_t	-	-	0.49
	GST	0.44	-	-
	EROD	-0.25	-	0.51
	LPO	0.30	-	-
Accumulated metals	Fe	-	-	-0.39
	Cu	-	-	-
	Zn	-	-	-
	Mn	-	-0.53	-
	Ni	-	-0.46	-
	Cr	-	-0.46	0.29
	Pb	-	-	-
	Cd	0.34	-	-

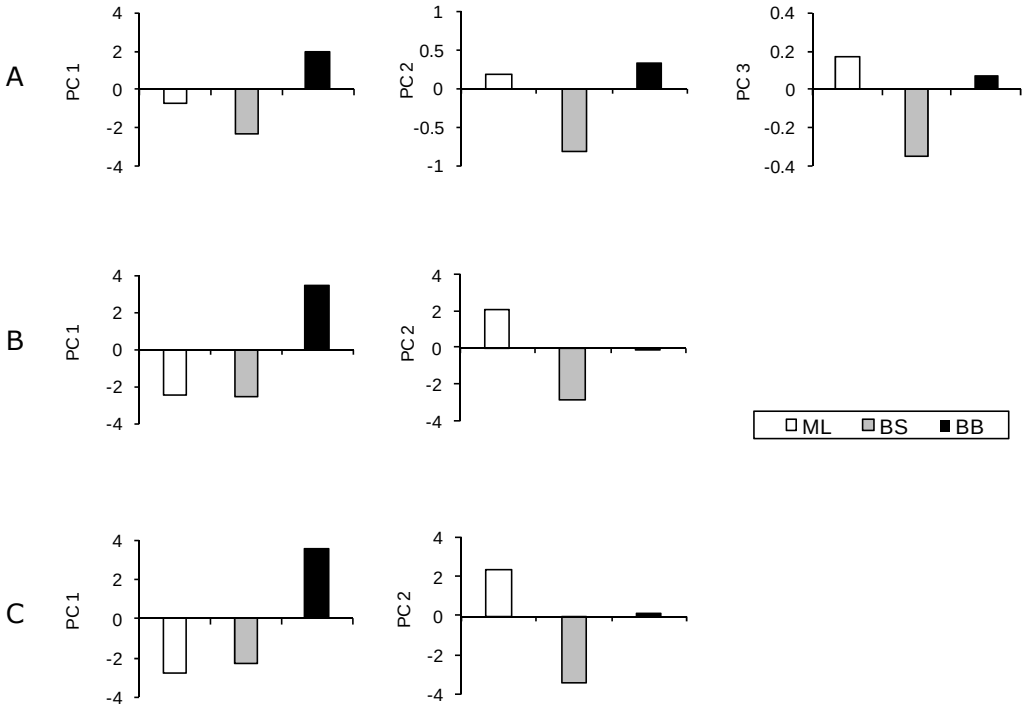

Figure 5.3. Results of the Principal Component Analysis (PCA) on the following data sets: (A) accumulated metals and biochemical responses (biomarkers) in *C. maenas*; (B) water parameters and biomarkers; (C) sediment parameters and biomarkers. Average scores for the principal components (PCs) obtained from the ordination of sites ML, BS, and BB are presented.

5.3.4 Linking environmental data and crabs biomarkers

The PRIMER software Relate routine allowed to find a significant correlation (ρ = 0.96, p = 0.001) between sediment and water data sets, further supporting the separated ordination analysis carried out for each set. The criterion for consideration of a variable was defined as its having a loading of 0.25 or higher.

5.3.4.1 Water quality versus crab biomarkers

Two principal components were defined as explaining the major amount of total variance (59%) (Table 5.5). The first principal component (PC1) accounts for 42% of the variance and shows a positive association between GST, CAT and GPx induction with levels of nutrients (NH_4^+, NO_3^-+NO_2^-, PO_4^{3-}) and Cu, Ni and Cd in water. PC1 has a positive score only in BB branch (Figure 5.3B). The PC2 accounts for 17% and links positively GST induction with Pb in water (Table 5.5), being this association only relevant at ML site (Figure 5.3B).

5.3.4.2 Sediment quality versus crab biomarkers

Two principal components were defined as explaining the major amount of total variance (62 %) (Table 5.6). PC1 accounts for 41% of the variance and indicates an association at BB between the induction of CAT activity and the presence of higher metal levels (Zn, Cr, Ni, Pb, Cd and Fe) in sediments (Table 5.6, Figure 5.3C). The PC2 accounts for 21% linking the induction of GST at ML with higher Mn levels in sediment (Table 5.6, Figure 5.3C).

Table 5.5. Results of the Principal Component Analysis (PCA) on the data set with water parameters (nutrients and metals) and biomarkers (metals, oxidative stress and biotransformation) in *C. maenas*: coefficients of the eigenvectors (loadings of the original variables) in the linear combination of variates from which the PCs are computed (loadings <0.25 were omitted). The percentage of total variance explained by each PC is indicated in brackets.

		Principal components	
		PC1 (42%)	PC2 (17%)
Crab	CAT	0.29	-
	GPx	0.25	-
	GSH_t	-	-
	GST	0.26	0.26
	EROD	-	-
	LPO	-	-
	Fe	-	-0.36
	Cu	-	-
	Zn	-	0.27
	Mn	-	-
	Ni	-	-
	Cr	-	-
	Pb	-	-
	Cd	-	-
Water	NH_4^+	0.32	-
	NO_3^- + NO_2^-	0.32	-
	PO_4^{3-}	0.32	-
	Mn	-	-0.48
	Cu	0.31	-
	Cr	-	-0.44
	Ni	0.32	-
	Pb	-	0.42
	Cd	0.32	-

Table 5.6. Results of the Principal Component Analysis (PCA) on the data set with sediment data (metals, organic carbon and nitrogen) and biomarkers (metals, oxidative stress and biotransformation) in *C. maenas*: coefficients of the eigenvectors (loadings of the original variables) in the linear combination of variates from which the PCs are computed (loadings <0.25 were omitted). The percentage of total variance explained by each PC is indicated in brackets.

		Principal components	
		PC1 (41%)	PC2 (21%)
Crab	CAT	0.27	-
	GPx	-	-
	GSH_t	-	-
	GST	-	0.25
	EROD	-	-
	LPO	-	-
	Fe	-	-0.32
	Cu	-	-
	Zn	-	-
	Mn	-	-
	Ni	-	-
	Cr	-	-
	Pb	-	-
	Cd	-	-
Sediment	Mn	-	0.35
	Zn	0.30	-0.25
	Cu	-	-0.28
	Cr	0.27	-
	Ni	0.25	-
	Pb	0.31	-
	Cd	0.29	-
	Fe	0.28	-
	Al	-	-
	C org	-	-0.40
	N org	-	-0.36

5.4 Discussion

5.4.1 Spatial variation of environmental conditions

The elevated levels of nutrients registered in BB branch are in line with previous studies on water quality (Pereira et al., 2009a; Pereira et al., 2009b), as well as benthic

macro-invertebrates and sediment quality (Carvalho et al., 2006). Those studies demonstrated that this confined branch is a major impacted area of the lagoon, being classified as a eutrophic zone. The abundant biomass of *Ulva sp*. and *Enteromorpha sp*. (Carvalho et al., unpublished data) as well as the high levels of suspended particles found in this study reinforce that classification. In the current work, observations were done in high and low tides, since in transitional waters major differences in concentrations are related with salinity (Lopes et al., 2007). Higher nutrient concentrations in low tide suggest the influence of freshwater discharges enriched in organic loads. Moreover, regeneration in organically-rich sediments and upwards diffusion during periods of low oxygenation should be considered as an internal input of NH_4^+ and PO_4^{3-} to the water column. This internal source of nutrients was previously detected at BB in Óbidos lagoon (Pereira et al., 2009b) and in other eutrophic lagoons (Point et al., 2007). Nutrients in ML and BS were lower and ranged within similar concentration intervals.

Metal concentrations in sediments of the three sites differed less than 30% among them. The comparison of sediment metal concentrations with values proposed by Long et al. (1995) for "Effects Range-Low" (ERL) (150, 34, 81, 21, 47, 1.2 μg g^{-1} respectively for Zn, Cu, Cr, Ni, Pb, Cd) within the sediment quality guidelines indicates that the three sites presented values above ERL for Cu, Ni and, to a lesser extent, for Cr. Nonetheless, for the large majority of the measured metals BB displayed levels slightly higher than the other sites. The enrichment in organic carbon and organic nitrogen of sediments from inner branches (BS and BB) reflects the discharge of domestic and agro-industrial effluents via Cal River (Pereira et al., 2009a).

Enhanced concentrations of dissolved metals were consistently found in BB. Since the differences were more accentuated in the morning period, corresponding to high tide, increases are hardly attributed to freshwater inputs. The most plausible explanation is the pulse input from sediments occurring during the night, as previously hypothesised in Óbidos lagoon (Pereira et al., 2009b). Decomposition of organic matter in sediments under low oxygenated conditions may lead to a more effective diffusion of metals from pore-water to the water column (Metzger et al., 2007). The importance of benthic mineralization to the trophic chain increases as the water column gets shallower (Lillebø et al., 2002) and could represent an additional source of metals to benthic organisms with possible transfer to pelagic food web (Mucha et al., 2004). The influence of that input of metals may be more prominent than what is depicted in the current data (concerning high

tide), when pulse input conjugates with low tide (minimum water volume). Narrower ranges of metal concentrations between low and high tide in the other two sites suggest that the hypothesised punctual release from sediment is confined to the BB branch.

5.4.2 Crab responses

A common pathway of toxicity induced by several stressful conditions is the imbalance between ROS generation and the efficiency of antioxidant defences (Frenzilli et al., 2001). Therefore, the susceptibility to oxidative stress was investigated in crab's hepatopancreas from three areas of the Óbidos lagoon. The results pointed to alterations on the assessed parameters in females and males from BB, revealing compensatory mechanisms to cope with the environmental changes. The general activation of enzymatic antioxidant defences, expressed through the increased activity of CAT, GPx and GST in females as well as GPx and GST in males, indicate that crabs suffered pro-oxidant pressure in BB branch. Moreover, when males and females were analysed jointly identical conclusions could be extracted concerning environmental quality of the Óbidos lagoon, since specimens from BB showed significantly higher activities of CAT, GPx and GST.

Catalase and GPx induction provides an indication of peroxides overproduction in hepatopancreas, suggesting the presence of redox-active compounds. Hydrogen peroxide is the main cellular precursor of the hydroxyl radical, the most toxic ROS, and it is well known that CAT directly catalyses its decomposition to O_2 and water, while GPx transforms organohydroperoxide to alcohol and water at the expense of GSH. In *C. maenas* it was previously observed GPx induction in *C. maenas* under field exposure linked by PCA to Zn sediment contamination (Morales-Caselles et al., 2008). Moreover, GPx induction in specimens from Spanish ports was also associated with high PCB and PAH concentrations in sediments (Martín-Díaz et al., 2007; Martín-Díaz et al., 2008). Increased activities of CAT and GPx have been described in several other aquatic species, particularly fishes from impacted sites both by metals and organic contaminants (Di Giulio et al., 1993; Regoli et al., 2002). Nevertheless, more research is required in invertebrate species as concluded by Martín-Díaz et al. (2008). In this study, the pro-oxidant action of Cd in crabs' hepatopancreas from BB should be hypothesised as pointed out by PC analysis, in agreement with the Cd potential to enhance intracellular ROS generation via Fenton-like reactions demonstrated by Winston and Di Giulio (1991). However, this association must be carefully assumed considering the relatively low percentage of total variance explained

by respective PC (28%) and the fact that Cd accumulated levels were low in comparison with crabs from other polluted environments (Pedersen et al., 1997; Bjerregaard et al., 2005).

The definition of which enzyme, CAT or GPx, is more efficient and has the main H_2O_2-detoxifying action is a controversial issue and both positions can be found in the literature (Bouzyk et al., 1997; Dorval et al., 2003). In spite of that, the simultaneous CAT and GPx activity increase observed in females from BB represents a reinforcement of the defence mechanisms, providing an additional evidence of a serious of environmental heath degradation in that area.

GSTs are a multigenic superfamily of multifunctional enzymes less studied in marine invertebrates in comparison to mammals and insects. GSTs may play a dual protective role associated to their activity on conjugation of electrophilic compounds (or phase I metabolites) with GSH (Van der Oost et al., 2003), and as a result of a direct antioxidant action, carried out by GST α-class, catalyzing the reduction of organic hydroperoxides by GSH (Wang and Ballatori, 1998). Taking into account that the adopted methodology (using CDNB, which is conjugated by all GST isoforms with the exception of the q-class) determines total GST activity, it is not easily perceptible the meaning of the observed GST activity increase at BB branch. However, considering the concomitance of this response with an EROD activity reduction (discussed below), the GST involvement on an incremented phase II conjugation seems to be less plausible.

The increase on GPx and GST activities constitutes a challenge to the glutathione recycling system in the cell, which efficacy is determinant to support antioxidant defences. Nevertheless, no significant alterations were detected on GSH_t contents in crabs from BB branch, being symptomatic of a balanced metabolism in terms of glutathione consumption, regeneration and synthesis. Moreover, as no alterations were detected on GSH_t, it can be stated that enzymatic antioxidants are the first line of defence against contaminants in BB branch. The few biomonitoring studies using *C. maenas* didn't measured GSH_t content and thus no comparison could be made with previous data.

The punctual depletion on GSH_t observed in males at BS branch indicates an increased use without a compensatory synthesis. This depletion coincided with an elevation of Pb levels in hepatopancreas. This association, though not supported by Pearson or PCA analyses, should not be ignored since previous findings showing a GSH decrease in rats exposed to Pb (Gurer and Ercal, 2000). Additionally, the present

glutathione depletion cannot be related with its use on GPx and GST activities, and thus indicating spontaneous conjugation with xenobiotics.

Oxidative damage can occur when antioxidant and detoxifying systems are deficient and not able to neutralise the active intermediates produced by toxics and their metabolites (Frenzilli et al., 2001). Despite the described activation of antioxidant enzymes (CAT, GPx and GST), female crabs did not cope efficiently with ROS production and oxidation of polyunsaturated fatty acids occurred, leading to lipid peroxidation (LPO). This highlights the environment state degradation at BB. Despite the evidences of ROS production in males, no LPO was observed, indicating a successful ROS neutralization by hepatopancreas antioxidants.

A tendency to EROD activity reduction was noticed at BB, with higher evidence in males. The involvement of metals in EROD inhibition was reported by different authors (Viarengo et al., 1997; Sanchez et al., 2005), which described that they can affect CYP1A by direct biding, leading to a conformational change or altering protein turnover. Taking into consideration the levels of metals measured in crabs' hepatopancreas, only Cd could be linked with that inhibition. Though it was demonstrated that Cd strongly inhibits EROD activity in aquatic organisms (Henczová et al., 2006), neither Pearson nor PC analyses corroborate that association. On the other hand, oxidative stress has been shown to repress the expression of the CYP1A1 gene at the transcriptional level (Morel and Barouki, 1998). Hence, the involvement of ROS on the observed EROD inhibition cannot be overlooked. This damaging action can be understood as an alternative toxicity pathway or in combination with a Cd-induced suppression.

Despite the suggested association between Cd accumulated in hepatopancreas and biochemical responses, it is important to highlight that in general no direct cause-effect relationships could be established between metal levels in hepatopancreas and oxidative stress or biotransformation responses.

An overall comparison of the results in males and females reveals identical response patterns. Nonetheless, females displayed higher vulnerability towards peroxidative damage, despite the general activation of enzymatic antioxidants (including CAT induction only observed in this gender). The higher susceptibility of males to EROD inhibition should not be cogitated since a clear reduction was also discernible in females from BB despite the lack of statistical significance. Moreover, it is important to notice that differences between genders were more recurrent for accumulated metals than oxidative stress

parameters. In general, current data reinforced the righteousness of the decision to analyse genders separately, as the jointly analysis could increase the intra-group variability masking significant effects (e.g. current GSH_t, LPO and EROD responses) and diminishing the assessment efficacy.

Prolonged oxidative stress may be responsible for profound alterations to cell physiology impairing the individual fitness, leading to the disappearance of less tolerant species and, consequently, to the biodiversity reduction (Frenzilli et al., 2001). In this perspective, the results obtained in BB reflect a serious environmental risk to populations inhabiting that impacted area.

5.4.3 Searching for cause-effect relationships between environmental conditions and crab responses

A parallel analysis of metal concentrations in water or sediment and accumulated in crabs' hepatopancreas revealed no direct associations, which was supported by PCA results. Presumably, enhanced availability of metals in water at BB was insufficient to record increases in the corresponding hepatopancreas levels. In view of that and also considering the lack of statistically supported links between metals in hepatopancreas (with the sole exception of Cd) and biochemical responses, the association between environmental metals contamination (water and sediment) and crab responses (CAT, GPx and GST) pointed out by PCA results at BB must be regarded with some scepticism.

Furthermore, higher levels of ammonium, nitrate+nitrite and phosphate were registered at BB suggesting an association with the observed oxidative stress responses. In fact, the PCA linked CAT, GPx and GST with those nutrients in water at BB. It has been proved that ammonia and nitrite can be important sources of pro-oxidants in fish, mussels and aquatic plants, leading to nitric radicals' production (Lima et al., 2006; Nimptsch and Pflugmacher, 2007). To the best of our knowledge no other work correlated oxidative stress responses with water phosphate levels. For a more definitive causal linkage between increased nutrients and oxidative stress responses it would be decisive the quantifications of those compounds in the crab tissues.

Coastal lagoons typically present a combination of human impact and wide variability of natural environmental factors. Among these factors, alterations on dissolved oxygen assume a particular importance in the context of the assessed biomarkers. It was demonstrated that hyperoxia/anoxia cycles increase the production of ROS, leading to

oxidation of cellular components after the turn back of oxygen. Therefore, besides the euryoxic nature of *C. maenas*, these phenomena should be also considered as contributory to the increased risk towards oxidative stress at BB due to drastic nocturne reductions of dissolved oxygen described in that area. A similar consideration can be presented for the EROD activity reduction, since it was also found that acute hypoxia reduces the rate of biotransformation by CYP1A (Fradette et al., 2007).

In light of the above discussion, additive effects of metals, nutrients, and other abiotic variables, as well as their interactions (synergism/potentiation) can play a crucial role on the induction of the biochemical effects measured in crabs.

In general, currents results demonstrate the rational of the biomarker selection, giving support to the idea presented by Monserrat et al. (2007) that when aquatic species are facing complex environmental changes the determination of non-specific biomarker is recommended.

5.5 Conclusions

Current results clearly pointed out Barrosa branch (BB) as a critically impacted area in Óbidos lagoon and the consequent ecological risk for autochthonous populations. Overall data suggest that the biochemical changes measured in crabs from BB are causally linked to variety of insults where interactions (synergism/potentiation) can play a crucial role.

In general, crab biochemical responses could not be directly attributed to metal uptake and accumulation (despite some evidences towards Cd involvement), making weaker the association pointed out by PCA between the increased metals environmental availability and the effects detected in the crabs. In addition, the increment on crab antioxidants matched with the nutrients levels in water, bringing into the light this ubiquitous class of compounds as potential pro-oxidants to aquatic fauna.

Though it was reinforced the difficulty to establish cause-effect relationships under field conditions, particularly with a co-occurrence of different stressors, the implemented strategy integrating environmental chemical data and crab biomarkers showed to be promising.

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CHAPTER VI

Bioaccumulation and biochemical markers in feral crab (*Carcinus maenas*) exposed to moderate environmental contamination - the impact of non-contamination-related variables

6 Bioaccumulation and biochemical markers in feral crab (*Carcinus maenas*) exposed to moderate environmental contamination - the impact of non-contamination-related variables

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Abstract

Moderate contamination state is a challenging scenario for ecotoxicologists due to the occurrence of subtle biomarker responses and the increased relevance of non-contamination-related variables. The current investigative biomonitoring study was performed in a moderately contaminated coastal system (Óbidos lagoon, Portugal) to examine winter-summer variations on biochemical responses and accumulated metals in *Carcinus maenas*, searching for associations with environmental and biological factors. Males and females were collected in three sites: BB and BS in confined areas of the upper lagoon; ML closer to the lagoon inlet. Water and sediment were both monitored for metals (Cu, Mn, Ni, Cr, Cd). CAT, GPx, GST, GSH_t, LPO and EROD, as well as Cu, Mn, Ni, Cr and Cd were measured in crabs' hepatopancreas. The few spatial differences suggested that BB site presents stressors for crabs. This was particularly obvious in summer when higher GST as well as lower GSH_t and EROD were found in females and accompanied by higher Ni accumulation. Seasonal differences of biochemical responses superimposed spatial variations in line with the contrasting winter-summer conditions regarding water quality and, in lesser extent, with metal bioaccumulation. CAT, GSH_t and LPO were higher in summer whereas enhancements of GPx and GST were recorded in winter. Winter increases were in agreement with the higher availability of metals in water and enhancement of accumulated levels, particularly in females as emphasized by a bioaccumulation index. On the other hand, increases in summer were mainly driven by non-contamination-related factors. Males and females exhibited different patterns of metals accumulation and biochemical responses, with females being more responsive as

confirmed by a general stress index (IBR). Results recommend genders separation in biomonitoring programs using crabs. The integration of biochemical responses into IBR substantiated data interpretation. This is particularly relevant under moderate contamination allowing a better site-distinction than biochemical responses considered individually.

Keywords: Oxidative stress; biotransformation; metals; *Carcinus maenas*; seasonal variability; gender-specific responses

6.1 Introduction

Costal lagoons have been progressively degraded due to increasing human activities such as agriculture, industry and urbanisation of the drainage basin, as well as unsustainable aquaculture practices. Degradation caused by the input of nutrients and toxic compounds has been particularly pronounced in cases of little exchange of lagoon water with seawater, being reported transference of toxics to the biota. Organisms may be therefore exposed to multiple stress agents causing synergistic/antagonistic effects. Complementarily to the measurement of contaminant body burdens, the use of biochemical responses can offer more complete and biologically relevant information on the potential impact on the organism's health (Van der Oost et al., 2003).

The cytochrome P450 1A (CYP1A) and oxidative stress responses (Guilherme et al., 2008; Martín-Díaz et al., 2008) are among the best-studied biomarkers in aquatic organisms. The induction of ethoxyresorufin-*O*-deethylase (EROD) activity, a functional measure of CYP1A, has been widely used as an indicator of exposure to xenobiotics that bind to the aryl hydrocarbon (Ah) receptor like organic contaminants (Van der Oost et al., 2003). On the other hand, several reports have implicated metals as modifiers of CYP1A function, being EROD activity inhibited at relatively low concentrations of Cu, Pb and Cd (Viarengo et al., 1993; Oliveira et al., 2004). Many pollutants are capable of inducing oxidative stress in aquatic animals by disturbing the antioxidants efficiency and enhancing the intracellular reactive oxygen species (ROS), which often prelude in DNA damage, lipid peroxidation (LPO) and enzyme inhibition (Van der Oost et al., 2003). A number of studies confirmed the successful employment of the modulation of antioxidant enzymes (e.g. catalase - CAT, glutathione peroxidase - GPx, glutathione S-transferase - GST) and of non-

enzymatic antioxidants (e.g. glutathione) in identifying environmental stress (Van der Oost et al., 2003; Martín-Díaz et al., 2008). LPO estimation has been also found to have a high predictive importance as biomarker of effect (Guilherme et al., 2008).

One key question in ecotoxicology is the integration of individual biomarker responses into a set of tools and indices capable of detecting and monitoring the degradation of the organisms' condition (Broeg and Lehtonen, 2006). In this direction, Beliaeff and Burgeot (2002) developed the "Integrated Biomarker Response" (IBR) method based on data obtained in the Seine estuary and in the Baltic Sea, thereafter employed in several investigative biomonitoring studies (Broeg and Lehtonen, 2006; Pereira et al., 2009a). IBR emerged in these studies as a feasible tool to examine differences in responses between sampling areas and surveys, giving an "aerial picture" of the overall biomarker responses. In general, IBR values were in agreement with pollution levels measured at the different areas regardless of the considerable variability in the biomarkers sets used for the index calculations.

The selection of a suitable sentinel species among the myriad of species existent in any aquatic ecosystem appears as a critical issue in biomonitoring programs. Previous studies adopted the shore crab (*Carcinus maenas*) as a source of environmental information in impacted shallow estuaries (Pereira et al., 2006; Martín-Diaz et al., 2008) and coastal lagoons (Pereira et al., 2009b). In addition, this crustacean also showed its usefulness in laboratory experiments addressing sub-lethal effects of contaminated sediments (Morales-Caselles et al., 2008). In fact, *C. maenas* has favourable features as sentinel in shallow sediment-contaminated systems. On one hand, is opportunist species that feeds on a large variety of prey items, from animal phyla (mainly Crustacea, Mollusca and Annelida) to plant and protest (Cohen et al., 1995); on the other, its sexual dimorphism represents an advantage in order to assess eventual gender-specific responses. Furthermore, biochemical changes in *C. maenas* demonstrated its sensitivity to contaminants both in water and sediment (Martín-Díaz et al., 2008; Morales-Caselles et al., 2008). Nevertheless, field studies combining bioaccumulation, biotransformation and oxidative stress endpoints on *C. maenas* are still scarce.

The Óbidos lagoon, in the western Portuguese coast, is impacted by eutrophication and moderate metal contamination due to the longstanding discharges of agricultural and domestic sewage. In that way, this coastal lagoon could represent an ecosystem-prototype of those environmental problems, as pointed in a previous ecotoxicological study using a

primary producer (*Ulva* sp.) as sentinel (Pereira et al., 2009a). In that work, the biochemical responses recorded seasonally were correlated with metals bioaccumulation. *C. maenas* was also used as sentinel in a preliminary study carried out at Óbidos lagoon (Pereira et al., 2009b). However, important questions associated with the influence of season- and gender-related variables on biological endpoints had arisen in that study and remained unanswered. These potential modifying factors (Van der Oost et al., 2003) assume an additional relevance considering the moderate contamination profile of the ecosystem under evaluation, and should be considered when interpreting the biological effects.

In view of that, the current study was designed to provide information on *C. maenas* biomarkers variability associated with the season and gender. The paper reports biochemical effects and metals accumulation in a feral population of *C. maenas* (males and females) sampled in two contrasting seasons (winter and summer) in the Óbidos lagoon. Peroxidative damage, antioxidant (enzymatic and non-enzymatic) and biotransformation responses were measured in the hepatopancreas. In order to summarize the biomarker responses, a general ‘‘stress index’’ (IBR) was applied.

6.2 Materials and Methods

6.2.1 Study area

The Óbidos lagoon is a shallow coastal ecosystem, located on the west coast of Portugal with a wet area of 7 km^2, permanently connected to the sea through a narrow inlet (Figure 6.1). It comprises areas of different morphological and sedimentary characteristics: sand banks and narrow channels in the lower/middle lagoon; muddy bottom sediments in the two inner branches (Barrosa and Bom-Sucesso). The Barrosa branch is shallower (mean depth 0.5-1 m) and water circulation is mostly driven by tides and by a small tributary (Cal River) that drains agriculture fields. Urban effluents from a nearby town (Caldas da Rainha, 50,000 inhabitants) also have been discharged to the Barrosa branch by the Cal River. Consequently, this area presents the highest nutrient concentrations of the lagoon, being classified as eutrophic (Pereira et al., 2009c). High nutrient availability was in line with abundant macroalgae (*Ulva* sp. and *Enteromorpha* sp.) and a broad daily variation of dissolved oxygen concentration during the summer months (Pereira et al., 2009d). Previous water quality surveys of the lagoon showed maximum

concentrations of Mn, Ni, Cu and Cd in Barrosa branch. Major metal sources were identified to be the Cal River in periods of higher inflow and remobilization from sediments in summer months (Pereira et al., 2009d). The Bom-Sucesso branch is also a confined area but receives a smaller freshwater flow (Vala do Ameal) with better water quality than the Cal River, according to the Portuguese categorization of freshwater systems. In this area, metal remobilization from sediments is less plausible due to the higher depths and stronger currents. The middle lagoon was previously characterised by coarser sediments with a low affinity for metals (Carvalho et al., 2006), as well as by a better water quality (Pereira et al., 2009c). Moreover, previous ecotoxicological studies with *Ulva* sp. and *C. maenas* pointed out Barrosa branch as a critical area at the lagoon with potential impact on autochthonous populations, whereas the lower/middle lagoon was considered the reference area (Pereira et al., 2009a; 2009b).

6.2.2 Sampling

Sampling was carried out in the Óbidos lagoon, in winter (February 2006) and summer (August 2006), at three sites (Figure 6.1): ML located in the middle part of the lagoon; BS and BB in the two inner branches, Bom-Sucesso and Barrosa, respectively. Surface water (0.2 m depth) was sampled in high tide (morning) and low tide (afternoon) for determinations of nutrients, chlorophyll *a* and metals. Surface sediments were collected with a Van-Veen grab. Twelve specimens of *Carcinus maenas* L. (six females and six males) were captured at each site during the morning using baited circular drop nets. Following collection, crabs were placed in thermally insulated 10 L boxes filled with water from the sampling site, transported to the laboratory where specimens were weighted and measured (Table 6.1). Crabs were sacrificed and their hepatopancreas were excised, divided in two parts, snap-frozen in liquid nitrogen and stored at -80 °C until further metal analysis and biochemical assays.

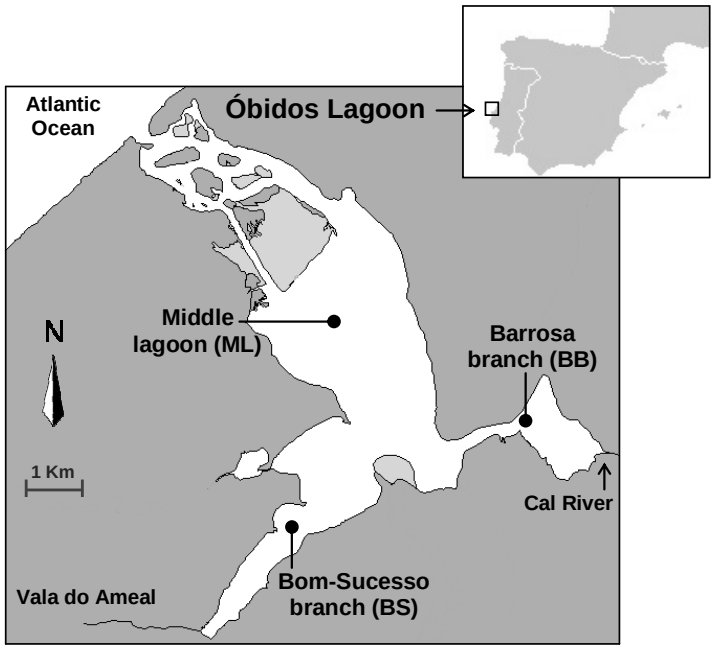

Figure 6.1. Location of the sampling sites at the Óbidos lagoon (Portugal): middle lagoon (ML - 39º24'50.48''N, 9º12'42.82''W); inner branches Bom-Sucesso (BS - 39º23'44.88''N, 9º13'01.75''W) and Barrosa (BB - 39º26'16.84''N, 9º11'33.18''W).

6.2.3 Analytical procedures

6.2.3.1 Water and sediment physicochemical parameters

Water temperature, salinity and dissolved oxygen were measured *in situ* using an YSI, 650 meter. Water samples for determinations of nitrate+nitrite (NO_3^-+NO_2^-), ammonium (NH_4^+), phosphate (PO_4^{3-}) and silicate $Si(OH)_4$) were filtered through MSI Acetate Plus filters, and analyses carried out using an autoanalyser TRAACS 2000 (Bran+Luebbe). For chlorophyll *a* determinations, water was filtered through a Whatman GF/F (0.7 µm) filter that was immediately frozen at -20 °C and later extracted in 90% acetone, for analysis in a Perkin Elmer Fluorometer using the protocol modified by Lorenzen (1966). Organic matter content in sediments was determined by loss on ignition (4h at 500 °C).

6.2.3.2 Metal determinations in environmental matrices and C. maenas hepatopancreas

Manganese, Cu, Ni, Cr and Cd in the collected waters were measured using diffusive gradients of thin films (DGT). DGT holders, Chelex-100 resins and diffusive gels (type APA, 0.8 mm thickness, open pore> 5 nm) (Zhang and Davison, 1999) were purchased from DGT

Research (Lancaster, UK). The DGT devices were deployed in 2 L polypropylene bottles with unfiltered sampled water and stirred at 21±1 °C for 48 hours. After devices retrieval, resins were eluted by immersion in 5 mL of 1 M HNO_3 (prepared from suprapur nitric acid) at a minimum of 24 h. Eluates were analysed directly by a quadropole ICP-MS (Thermo Elemental, X-Series). All eluates were analysed with reagents blanks and an international standard of river water (SLRS-4) used to control eventual contaminations during the analytical procedure and the procedure accuracy, respectively. The eluate concentration was converted into the mass of metal accumulated on the resin using an elution factor with a yield value of 0.8 and a resin gel volume of 0.15 mL. Metals concentrations in water were calculated according to the following equation: [Metal]=(MΔg)/(tAD); where M is the mass of metal accumulated on the resin during the emersion time (t); Δg the diffusive gel thickness (0.08 cm); A the exposure area (3.14 cm^2); and D the diffusion coefficient of the metal in the gels as provided by DGT Research.

Sediment samples (100 mg) were mineralized completely with HF (40%) and Aqua Regia (HCl-36%: HNO_3-60%; 3:1) in closed Teflon bombs (100 °C for 1 h), evaporated to near dryness (DigiPrep HotBlock – SCP Science), redissolved with 1 mL of doubled-distilled HNO_3 and 5 mL of ultra-pure water, heated for 20 min at 75 °C, and diluted to 50 mL with ultra-pure water (Pereira et al., 2009a). Freeze-dried and grounded hepatopancreas samples (≈50 mg) were digested with a mixture of HNO_3 and H_2O_2 at 60 °C for 12 hours, 100 °C for 1 hour and at 80 °C for 1 hour, according to the method described in Pereira et al. (2009b). The accuracy of the analytical procedures was assessed by the analysis of Certified Reference Materials (MESS-3, BCSS-1, 1646a for sediments; TORT-1, TORT-2, DOLT-3 for crabs' hepatopancreas). The concentrations of Mn, Cu, Cr, Ni and Cd were determined by ICP-MS. In order to examine for any possible contamination during the analytical procedure blanks were analysed. Metal levels obtained in the reference materials were consistently within the ranges of certified values.

6.2.3.3 Biochemical analyses in C. maenas hepatopancreas

Tissue was homogenized in a 1:10 w/v ratio (hepatopancreas weight:buffer volume) of 0.1 mol L^{-1} Tris–HCl (pH 7.4) containing 0.15 mol L^{-1} KCl and 20% glycerol, using a Potter–Elvehjem glass–Teflon homogenizer at 2500 rpm. An aliquot of homogenate (150 µL) was taken for thiobarbituric acid reactive substances (TBARS) measurement and stored at −80 °C after adding 10 µL BHT (1-1 butylated hydroxytoluene) to prevent oxidation, and

the rest was centrifuged. Microsomes were obtained by differential centrifugation at 4 °C in a Beckman Optima TL Ultracentrifuge (TLA-100.4 fixed angle rotor), according to the method of Lange et al. (1993): the homogenate was first centrifuged at 12,000 g for 20 min to remove cell debris, nuclei and mitochondria; the supernatant was recentrifuged at 135,000 g for 75 min. The resulting microsomal pellet was resuspended in 200 µL of the previous buffer. Both cytosolic and microsomal fractions were frozen in liquid nitrogen and stored at −80 °C until use. EROD activity was measured in the microsomal fraction (at 25 °C), whereas all the other measurements were carried out in the cytosolic fraction (at 25 °C).

Protein content in the tissue homogenate was precipitated with trichloroacetic acid (5%) for 1 h and then centrifuged at 13,400 g for 20 min at 4 °C, and total glutathione content (GSH_t) was determined in the resulting supernatant adopting the enzymatic recycling method using glutathione reductase (GR) excess, whereby the sulfhydryl group of reduced gluthathione reacts with DTNB (Ellman's reagent) producing a yellow coloured 5-thio-2-nitrobenzoic acid (TNB). Formation of TNB was measured by spectrophotometry at 412 nm and the results were expressed as nmol TNB formed/min/mg protein using a molar extinction coefficient (ε) of 14.1×10^3 M^{-1} cm^{-1}. Glutathione-S-transferase (GST) activity was determined using CDNB (1-chloro-2,4-dinitrobenzene) as substrate according to the method of Habig et al. (1974). The increase in absorbance was recorded at 340 nm during 3 min and enzyme activity calculated as nmol CDNB conjugate formed/min/mg protein (ε=9.6 mM^{-1} cm^{-1}). Catalase (CAT) activity was assayed by Claiborne (1985) method. Change in absorbance was recorded at 240 nm and CAT activity was calculated in terms of µmol H_2O_2 consumed/min/mg protein (ε=43.5 M^{-1} cm^{-1}). Glutathione peroxidase (GPx) activity was assayed by Mohandas et al. (1984) method. Oxidized glutathione (GSSG), produced upon reduction of H_2O_2 by GPx is recycled to its reduced state by GR and NADPH. The decrease in absorbance at 340 nm resulting from NADPH oxidation is directly proportional to the GPx activity, and results were expressed as nmol NADPH oxidized/min/mg protein (ε=6.22×10^3 M^{-1} cm^{-1}). The determination of LPO was carried out based in the TBARS measurement (535 nm), as adapted by Filho et al. (2001). The rate of LPO was expressed as nmol of TBARS formed per milligram of fresh tissue (ε=1.56×10^5 M^{-1} cm^{-1}). EROD activity was measured as described by Burke and Mayer (1974). The reaction was carried out in the fluorometer cuvette containing 1 mL 0.5 µmol L^{-1} ethoxyresorufin (in homogenization buffer, pH 7.4) and 50 µL of microsomal suspension. The reaction was

initiated by adding 10 µL of NADPH (10 mmol L^{-1}) and the progressive increase in fluorescence, resulting from the resorufin formation, was measured for 3 min (excitation wavelength 530 nm, emission wavelength 585 nm). EROD activity was expressed as pmol/min/mg protein. Microsomal and cytosolic protein concentrations were determined according to the Biuret method (Gornall et al., 1949) using bovine serum albumin (E. Merck-Darmstadt) as standard.

6.2.4 Data analyses

A method for combining all the measured biochemical responses into a general index - Integrated Biomarker Response (IBR) (Beliaeff and Burgeot, 2002) - was applied for males and females captured in both surveys, as well as for polled genders. The basis of data processing is described here briefly. First, the mean value (X_i) for each biomarker at each sampling area and season was calculated. In addition, the general mean (m_i) and standard deviations (s_i) of each biomarker were estimated for all sampling areas and seasons. The value of X_i was then standardized to obtain Y_i, where $Y_i = (X_i - m_i)/s_i$. Then Z_i was computed via the equation $Z_i = -Y_i$ or $Z_i = Y_i$ in the case of a biological effect corresponding, respectively, to inhibition or activation. The minimum value (Min_i) of Z_i for each biomarker was calculated for all sampling areas and seasons, and then the score S_i was computed as $S_i = Z_i + |Min_i|$, where $|Min_i|$ is the absolute value. Finally, IBR for each sampling site and season was calculated via de following formula: $IBR = (S_1 x S_2)/2 + (S_2 x S_3)/2 + \ldots + (S_{n-1} x S_n)/2 + (S_n x S_1)/2$; in which the obtained score for each biomarker (S_i) is multiplied with the score of the next biomarker (S_{i+1}), arranged as a set, dividing each calculation by 2 and summing-up of all values. Since the IBR value is directly dependent on the number of biomarkers used in the set, the value estimated as described previously was divided by the number of biomarkers, as proposed by Broeg and Lehtonen (2006).

Statistical software (Statistica 7.0) was used for statistical analyses. The assumptions of normality and homogeneity of data were verified. ANOVA analysis was used to compare sampling sites and Tukey test was applied for Post-hoc comparison (Zar, 1996). Differences between means were considered significant when $p<0.05$.

The individual mean bioaccumulation index (IMBI) was calculated according to Maes et al. (2005) by dividing the individual concentration of metal i (C_i) by the maximum observed concentration (C_{imax}) and averaging all metals (n), as:

$$IMBI = \frac{\sum_{i=1}^{n} Ci / Ci\max}{n}$$

The link between biomarkers (accumulated metals including IMBI, oxidative stress and biotransformation) in *C. maenas* was assessed by means of a Principal Component Analysis (PCA) performed on the log-transformed and standardized variables (Clarke and Warwick, 2001). In addition, two sets of PCAs were performed in order to associate biomarkers and environmental data: one linking biomarkers to water data (temperature, salinity, chlorophyll *a*, nutrients and metals) and the other linking biomarkers to sediment data (metals, organic matter content). The significance of the correlation between these two data sets was investigated by applying the RELATE procedure to the Euclidean-distance dissimilarity matrices. The importance of the associations (within biomarkers and between biomarkers and environmental data) evidenced by the PCA's scores was further compared separating sampling sites and seasons. The factor gender could not be taken into account in the multivariate approach owing to analysis constraints occurred mainly in winter. All multivariate analyses were carried out with the statistical package PRIMER 6.0+. The influence of crab's weight on the ordination results provided by the PCA was assessed by calculating the Pearson correlations between body weight and the first two PCA axes obtained. Thereafter, Pearson correlations between weight and each biomarker (accumulation of metals and biochemical responses) were also investigated.

6.3 Results

6.3.1 Biometric parameters

In general, width and weight did not differ significantly between sites (within each season) either for females and males. A sole exception was recorded for width of both genders from BS being significantly lower than those from ML. However, males in both seasons were always significantly wider and heavier than females. Moreover, specimens captured in summer had significantly higher width and weight than those from winter, with the exception of males at ML.

Table 6.1. Width and weight of *C. maenas* captured in winter and summer in the middle lagoon (ML) and in inner branches (Bom-Sucesso – BS, Barrosa – BB) at Óbidos lagoon. Letters denote statistically significant differences ($p<0.05$) between sites (within the same survey and crab's gender), between surveys (within the same site and crab's gender) or between genders (within the same site and survey): (a) vs. ML; (s) between genders; (t) between surveys. Mean and associated standard errors are given.

Season	Gender	Site	Width (cm)	Weight (g)
Winter	Females	ML	4.7 ± 0.05	20 ± 0.7
		BS	4.4 ± 0.20 [a]	18 ± 1.7
		BB	5.0 ± 0.11	24 ± 0.5
	Males	ML	5.9 ± 0.18 [s]	43 ± 2.8 [s]
		BS	5.1 ± 0.11 [a, s]	32 ± 4.2 [s]
		BB	6.1 ± 0.38 [s]	54 ± 9.6 s
Summer	Females	ML	4.9 ± 0.02 [t]	23 ± 1.1 [t]
		BS	5.3 ± 0.09 [t]	25 ± 1.3 [t]
		BB	5.8 ± 0.05 [t]	31 ± 0.9 [t]
	Males	ML	5.6 ± 0.26 [s]	40 ± 4.5 [s]
		BS	7.1 ± 0.57 [s, t]	60 ± 4.3 [s, t]
		BB	6.8 ± 0.21 [s, t]	63 ± 3.1 [s, t]

6.3.2 Sediment and water characteristics

Organic matter, Cu and Cd in surface sediments reached slightly higher levels at BB, while Mn was higher at BS (Table 6.2). Chromium and Ni in sediment showed no clear inter-site differences. The intervals obtained reflect differences on sediment properties within each surveyed site rather than seasonal fluctuations.

Table 6.2. Ranges of organic matter content and metal concentrations in surface sediments collected in winter and summer in the middle lagoon (ML) and in inner branches (Bom-Sucesso - BS, Barrosa - BB) at Óbidos lagoon.

Site	Organic Matter (%)	Metals (μg g^{-1}) Mn	Cu	Cr	Ni	Cd
ML	6.9 - 7.5	252 - 297	43 - 52	69 - 91	29 - 37	0.21 - 0.22
BS	6.9 - 7.3	285 - 322	46 - 63	56 - 91	24 - 37	0.20 - 0.23
BB	7.0 – 8.6	269 - 284	58 - 83	72 - 90	30 - 35	0.28 - 0.29

Concerning water, temperature in summer was higher at BB and BS and salinity exceeded the seawater values in the three sites, suggesting that evaporation was not compensated by the freshwater inputs (Table 6.3). In winter, water temperature ranged within identical intervals in the three sites but salinity was lower at BB due to freshwater

discharges in this area of the lagoon. Dissolved oxygen was around 100% at ML in both surveys, while in BS was in the order of 100% in winter but reached oversaturation in summer. In BB undersaturated levels were registered in summer, reaching oversaturation in winter. Concentrations of all nutrients and chlorophyll *a* were higher at BB than BS and ML in winter. In summer, the three sites exhibited identical levels of NH_4^+, NO_3^-+NO_2^- and $Si(OH)_4$, whereas PO_4^{3-} and chlorophyll *a* increased considerably at BB in low tide. The maximum values of NH_4^+ and NO_3^-+NO_2^- were registered in winter at BB in low tide. Manganese was higher at BB in both tides and surveys (

Table **6.4**). Moreover, Cu and Ni were higher at BB in high tide of winter and summer. For Cr and Cd a spatial pattern couldn't be clearly discerned. Generally, levels of all measured metals in water were higher in winter than in summer.

Table 6.3. Water temperature (T), salinity, dissolved oxygen (DO), ammonium (NH_4^+), nitrate+nitrite (NO_3^-+NO_2^-), phosphate (PO_4^{3-}), silicate ($Si(OH)_4$) and chlorophyll *a* (Chl *a*) in winter and summer in middle lagoon (ML) and in inner branches (Bom-Sucesso - BS, Barrosa - BB) at Óbidos lagoon in high tide and low tide.

		High tide – Low tide							
Season	Site	T (°C)	Salinity	DO (%)	NH_4^+ (µM)	NO_3^-+NO_2^- (µM)	PO_4^{3-} (µM)	$Si(OH)_4$ (µM)	Chl *a* (µg L^{-1})
Winter	ML	11-11	34.6-33.7	108-103	20-20	28-26	2.9-1.3	mv-16	2.6-2.6
	BS	11-9	32.7-32.3	107-102	25-46	34-62	1.6-2.5	13-22	0.8-1.1
	BB	9-11	30.8-28.1	103-124	53-81	74-124	2.2-4.5	22-29	12-8.5
Summer	ML	21-21	36.6-36.2	101-108	0.9-0.5	0.3-0.2	1.6-0.8	3.7-2.4	0.6-0.7
	BS	25-24	37.1-36.4	121-109	0.9-1.6	0.3-0.4	3.9-2.0	3.1-3.0	1.1-0.5
	BB	24-25	37.1-36.9	74-96	1.2-1.2	0.8-0.3	3.4-8.5	3.2-3.1	1.0-18

mv – missed value

Table 6.4. Levels of metals in water in winter and summer in the middle lagoon (ML) and in inner branches (Bom-Sucesso - BS, Barrosa - BB) at Óbidos lagoon in high tide and low tide.

		High tide – Low tide				
Season	Site	Mn (µM)	Cu (nM)	Ni (nM)	Cr (nM)	Cd (nM)
Winter	ML	0.43-0.41	4.7-3.5	11-11	9.5-6.1	0.11-0.07
	BS	0.49-0.72	3.6-8.6	13-14	2.5-7.5	0.08-0.13
	BB	0.49-1.0	5.0-6.8	13-8.3	5.9-8.5	0.11-0.07
Summer	ML	0.04-0.05	1.8-3.0	2.7-3.9	2.2-2.9	0.07-0.12
	BS	0.04-0.02	1.9-4.0	2.2-3.5	13-3.1	0.06-0.08
	BB	0.09-0.24	3.1-mv	3.9-mv	2.0-5.2	0.07-0.12

mv – missed value

6.3.3 Metal levels in hepatopancreas of *C. maenas*

Manganese and Cr in crab's hepatopancreas did not varied significantly among sites, in males, females and pooled genders for the two surveyed seasons (Figure 6.2). Contrarily, Cu in winter was significantly higher in pooled genders at BB than ML, as well as Cd in crabs (males, females and pooled genders) from BB than BS. In summer, Ni increased significantly at BS in males and pooled genders, as well as at BB in females in comparison with ML.

Concerning seasonal differences, females from BS accumulated significantly higher levels of Cu, Mn and Cr in winter than summer. An identical temporal difference was observed at ML for Ni in males, while Cr in males from ML increased in summer (Figure 6.2). Temporal trends were also recorded when data from both genders were pooled. In fact, crabs from BB exhibited significantly higher Cu and Cd in winter than summer and the same temporal variation was recorded for accumulated Cu and Mn in crabs from BS.

Comparing genders within the same site and season, in winter females showed higher levels of Cu at BS and Cr at ML, while in summer it was observed that females accumulated higher levels of Ni (ML and BB) and Cr (BB).

In general, the individual mean bioaccumulation index (IMBI) did not vary significantly among sampling sites for males, females or pooled genders. However, IMBI estimated for females and pooled genders were significantly higher in winter than summer at all sites. Gender differences were registered only in winter with females exhibiting higher IMBI values than males at all sites.

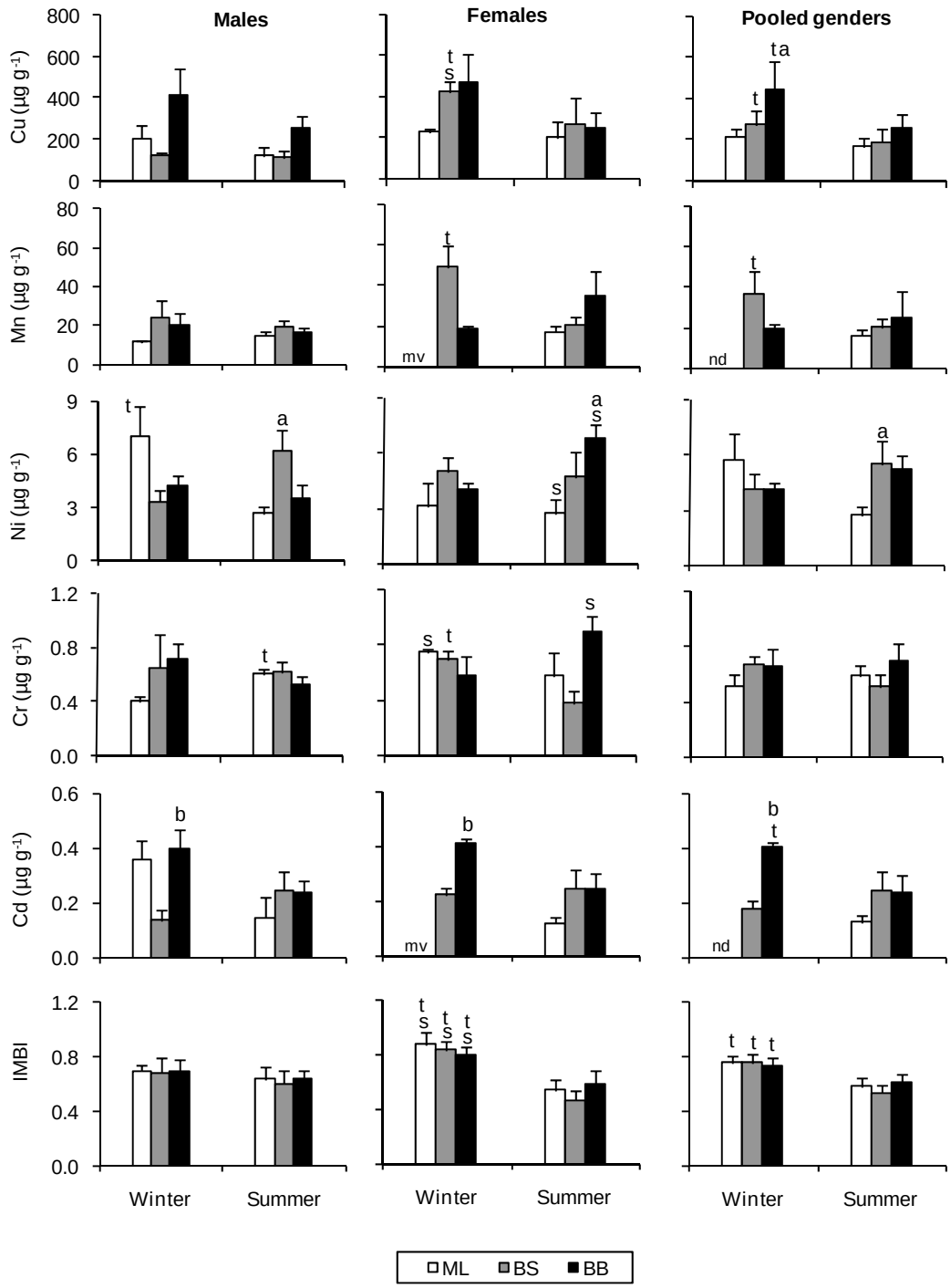

Figure 6.2. Metal levels and individual mean bioaccumulation index (IMBI) in *C. maenas* captured in winter and summer in the middle lagoon (ML) and in the inner branches (Bom-Sucesso – BS, Barrosa - BB) at Óbidos lagoon. Data concern genders separately and pooled. Letters denote statistically significant differences ($p<0.05$) between sites (in the same survey and crab's gender), between surveys (for each site and crab's gender) or between genders (in the same site and survey): (a) vs. ML; (b) vs. BS; (s) between genders; (t) between surveys. Mean and associated standard errors are given. mv – missed value. nd – not determined.

6.3.4 Oxidative stress and biotransformation responses in hepatopancreas of *C. maenas*

Significant differences between sites were registered only in summer (Figure 6.3), namely: increased activity of GST in females from BB than those from BS; lower GSH_t content in males, females and pooled genders from BB (comparing with BS, with ML and BS, and with BS, respectively); higher GSH_t content in males and pooled genders from BS than ML; decrease of EROD activity in males from BB and BS, both compared with ML, as well as in pooled genders from BB in relation to ML.

Differences between the two sampling surveys were found for all the quantified parameters. Males, females and pooled genders presented higher CAT activity (all sites; ML and BS; ML and BS, respectively), GSH_t content (all sites) and LPO levels (ML and BS; BS; all sites, respectively) in summer. Contrarily, males, females and pooled genders revealed higher GPx (ML and BB; all sites; ML and BB, respectively) and GST (BS and BB; ML and BS; BS and BB, respectively) activities in winter than summer. Moreover, males from BB presented significantly higher EROD activities in winter than summer.

Significant differences between genders captured within the same site in winter were only found for GST at BS, with females presenting higher activities than males. Moreover, in summer EROD at BB was also higher in females.

The antioxidants, LPO and EROD values measured in crabs were processed in order to calculate the IBR index (Figure 6.4). IBR values showed different variation ranges in winter and summer, being higher in winter (for males, females and pooled genders). In winter, IBR values did not differed substantially between sites for males and pooled gender, while for females were clearly higher at BB. In summer, the computed IBR values were always higher at BB than other sites. Moreover, IBR values did not differ considerably between genders in summer at all sites. However, in winter females exhibited higher IBR than males at BB but an opposite pattern was recorded at ML.

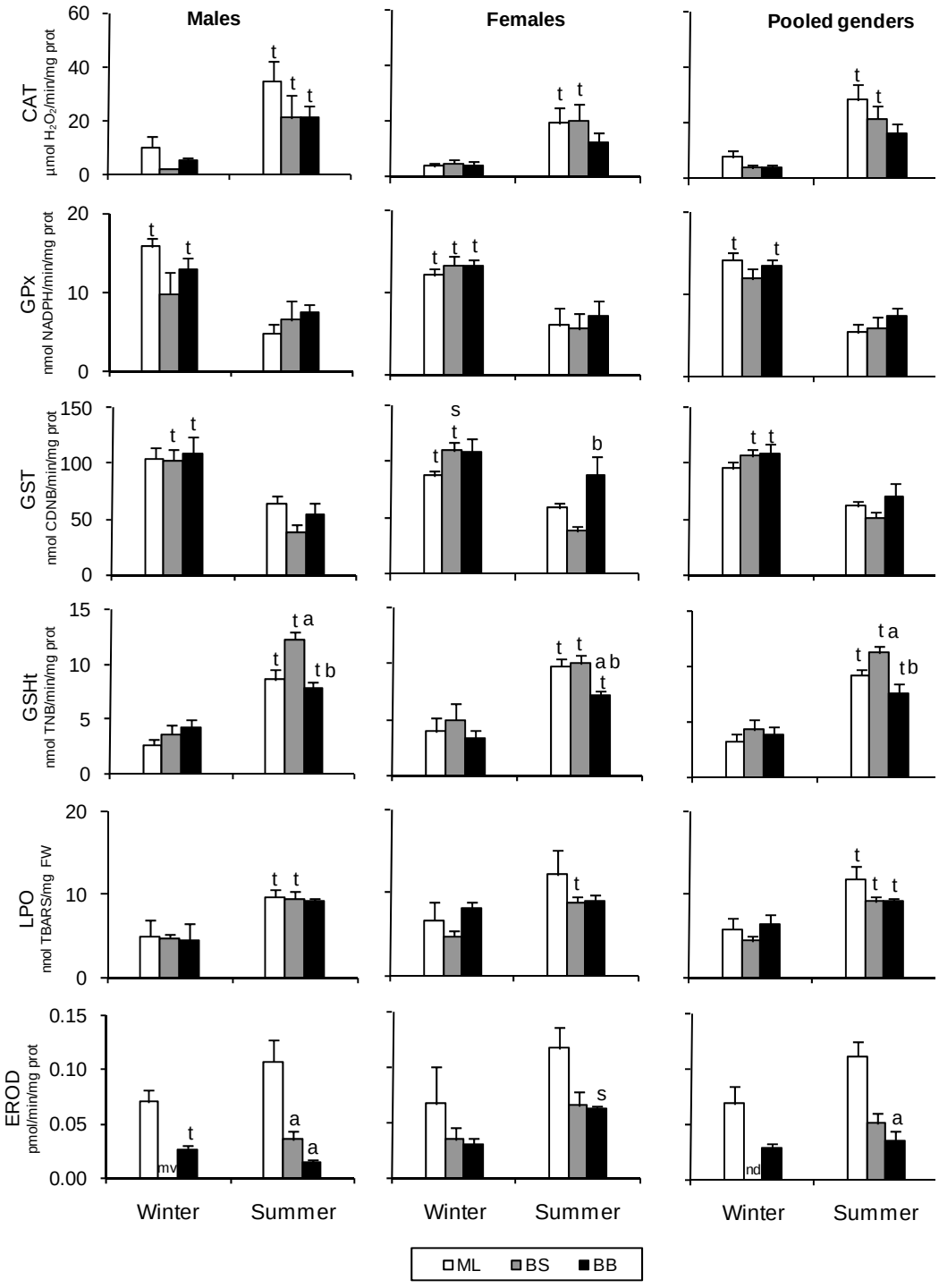

Figure 6.3. Oxidative stress and biotransformation responses in *C. maenas* captured in winter and summer in the middle lagoon (ML) and in the inner branches (Bom-Sucesso – BS, Barrosa - BB) at Óbidos lagoon. Data concern genders separately and pooled. Letters denote statistically significant differences ($p<0.05$) between sites (in the same survey and crab's gender), between surveys (for each site and crab's gender) or between genders (in the same site and survey): (a) vs. ML; (b) vs. BS; (s) between genders; (t) between surveys. Mean and associated standard errors are given. mv – missed value; nd – not determined.

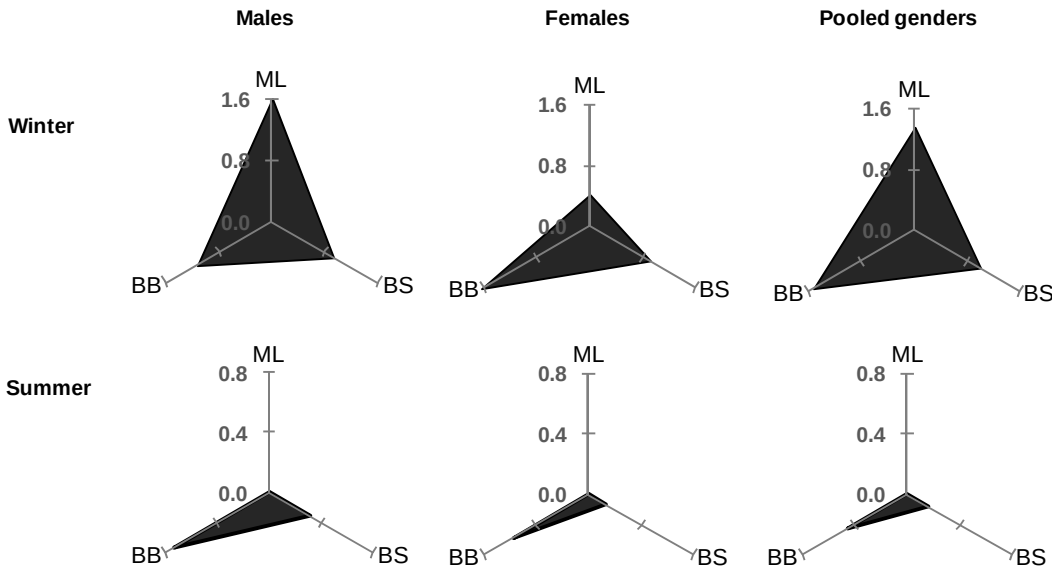

Figure 6.4. Integrated biomarker response (IBR) index in *C. maenas* (males, females and pooled genders) captured in winter and summer in the middle lagoon (ML) and in the inner branches (Bom-Sucesso – BS, Barrosa - BB) at Óbidos lagoon.

6.3.5 Linking accumulated metals and biochemical endpoints in hepatopancreas of *C. maenas*

In the PCA, the criterion for consideration of a variable as being associated with a particular component was defined as its having a loading of 0.25 or higher. Two principal components were established for explaining the major amount of total variance (56%) when accumulated metals and biochemical endpoints were considered (Table 6.5). The first principal component (PC1) accounted for 37% of the variance and showed a positive association between GPx and GST with accumulated Cu, Ni and Cd, as well as IMBI. PC1 has a positive score (averaged by sampling site and season) at all sites in winter, being slightly higher at BB (Figure 6.5A). According to PC2 (19% of the total variance), CAT, GSH_t and LPO were linked to accumulated Cu, Mn and Cr, as well as to IMBI, and this association was more relevant at BS (winter and summer) and BB (summer).

The influence of crab's weight on the ordination results provided by the PCA was assessed by calculating the correlations between body weight and the first two PCA axes obtained. No significant correlations were found pointing to a minor influence of weight on the overall PCA results. This conclusion was corroborated by the absence of significant

Pearson correlations between weight and each biomarker (accumulation of metals and biochemical responses).

Table 6.5. Results of the Principal Component Analysis (PCA) on the data set with biomarkers (accumulated metals, oxidative stress and biotransformation) in *C. maenas*: coefficients of the eigenvectors (loadings of the original variables) in the linear combination of variates from which the principal components (PC) are computed (loadings <0.25 were omitted). The percentage of total variance explained by each PC is indicated in brackets.

		PC1 (37%)	PC2 (19%)
Biochemical responses	CAT	-0.29	0.33
	GPx	0.35	-0.27
	GST	0.35	-
	GSH_t	-0.31	0.28
	LPO	-0.32	0.29
	EROD	-0.26	-
Accumulated metals	Cu	0.33	0.29
	Mn	-	0.38
	Ni	0.25	-
	Cr	-	0.48
	Cd	0.31	-
	IMBI	0.32	0.36

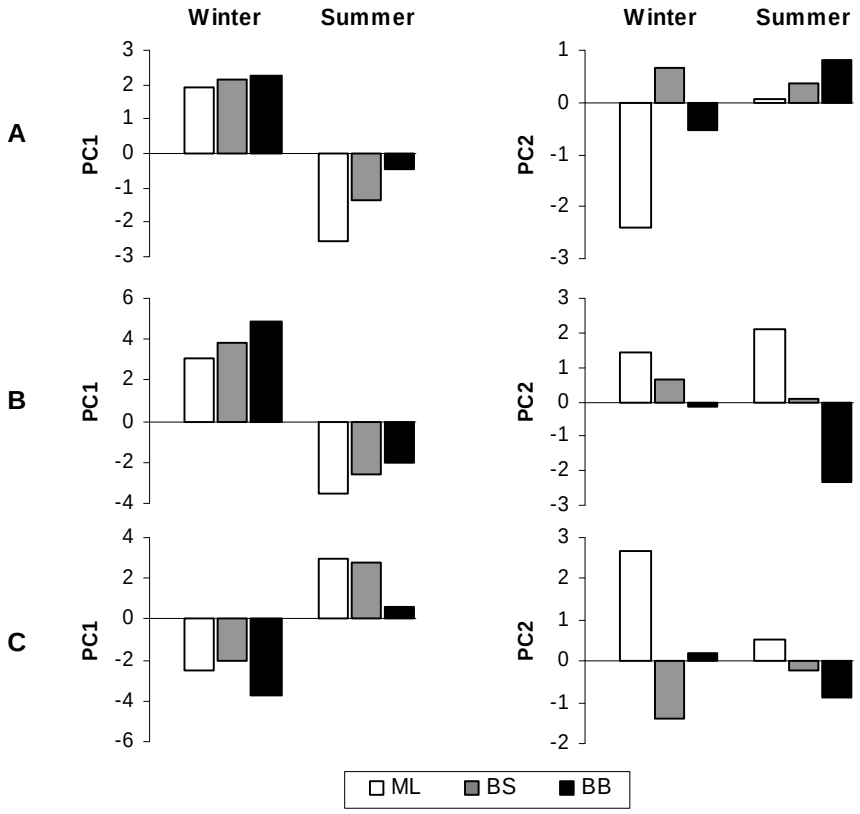

Figure 6.5. Results of the Principal Component Analysis (PCA) on the following sets: (A) accumulated metals and biochemical responses (biomarkers) in *C. maenas*; (B) water parameters and biomarkers; (C) sediment parameters and biomarkers. Average scores for the principal components (PCs) obtained from the ordination of sites ML, BS and BB are presented.

6.3.6 Linking environmental data and *C. maenas* biomarkers

The Primer Software Relate routine established a significant correlation (ρ=1.0, p=0.001) between water and sediment data sets, further supporting the separated ordination analysis for each set. The criterion for consideration of a variable as being associated with a particular PC was defined as it having a loading of 0.25 or higher.

6.3.6.1 Water quality versus C. maenas biomarkers

Two principal components were defined as explaining the major amount of total variance (62%) (Table 6.6). The first principal component (PC1) accounted for 48% of the total variance but did not establish any association between biomarkers and water data. The PC2 accounted for 14% of the total variance and linked, with positive coefficients, EROD activity with dissolved oxygen (Table 6.6), being this association more relevant at ML

in summer (Figure 6.5B). PC2 also evidenced a link between Cu and Cr accumulated in the hepatopancreas and PO_4^{3-} and chlorophyll *a*, which was associated with BB in summer (Figure 6.5B).

Table 6.6. Results of the Principal Component Analysis (PCA) on the data set with water parameters (temperature, salinity, dissolved oxygen - DO, nutrients, chlorophyll *a* - Chl *a*, and metals) and biomarkers (accumulated metals, oxidative stress and biotransformation) in *C. maenas*: coefficients of the eigenvectors (loadings of the original variables) in the linear combination of variates from which the principal components (PC) are computed (loadings <0.25 were omitted). The percentage of total variance explained by each PC is indicated in brackets.

		PC1 (48%)	PC2 (14%)
Crab	CAT	-	-
	GPx	-	-
	GST	-	-
	GSH_t	-	-
	LPO	-	-
	EROD	-	0.36
	Cu	-	-0.28
	Mn	-	-
	Ni	-	-
	Cr	-	-0.28
	Cd	-	-
	IMBI	-	-
Water	Temperature	-0.27	-
	Salinity	-0.27	-
	DO	-	0.32
	NH_4^+	0.29	-
	NO_3^-+ NO_2^-	0.29	-
	PO_4^{3-}	-	-0.48
	$Si(OH)_4$	0.28	-
	Chl *a*	-	-0.37
	Mn	0.28	-
	Cu	0.28	-
	Ni	0.28	-
	Cr	-	-
	Cd	-	-

6.3.6.2 Sediment quality versus C. maenas biomarkers

Two principal components were defined as explaining the major amount of total variance (56%) (Table 6.7). PC1 accounted for 41% of the total variance and revealed an association at BB in winter between the induction of GPx and GST and the presence of higher metal levels (Cu, Cr, Ni) and organic matter content in sediments (Table 6.7, Figure 6.5C). The PC2 accounted for 15% of the total variance and linked accumulated Mn and levels in sediments at BS (winter) and BB (summer) (Table 6.7, Figure 6.5C).

Table 6.7. Results of the Principal Component Analysis (PCA) on the data set with sediment data (metals, organic matter content - OM) and biomarkers (accumulated metals, oxidative stress and biotransformation) in *C. maenas*: coefficients of the eigenvectors (loadings of the original variables) in the linear combination of variates from which the principal components (PC) are computed (loadings <0.25 were omitted). The percentage of total variance explained by each PC is indicated in brackets.

		PC1 (41%)	PC2 (15%)
Crab	CAT	0.25	-
	GPx	-0.29	-
	GST	-0.28	-
	GSH_t	0.29	-
	LPO	0.26	-
	EROD	-	-
	Cu	-	-0.35
	Mn	-	-0.40
	Ni	-	-
	Cr	-	-0.46
	Cd	-	-
	IMBI	-	-0.38
Sediment	Mn	-	-0.28
	Cu	-0.32	-
	Cr	-0.33	-
	Ni	-0.32	-
	Cd	-	-
	OM	-0.32	-

6.4 Discussion

6.4.1 Spatial and seasonal variations of environmental conditions

The comparison of sediment metal concentrations with values proposed by Long et al. (1995) for "Effects Range-Low" (ERL) (34, 81, 21 and 1.2 μg g^{-1} respectively for Cu, Cr, Ni and Cd) within the sediment quality guidelines indicates that the three sites presented values above ERL for Cu, Ni and, to a lesser extent, Cr. Metals in sediment varied only slightly between sites in accordance with the similar organic matter contents. Nevertheless, in water Mn (both tides) and, to a lesser extent, Cu and Ni (high tide) exhibited increases at BB site, in both seasons. The tributary that enters at BB branch may contribute to metal inputs in periods of higher freshwater discharges (Pereira et al., 2009d). In fact, metal levels registered in winter at the three sites were generally higher than those found in summer. The higher concentrations recorded in winter may be also related with the enhancement of sediment resuspension in that period as a consequence of rainfall and strong currents (Guilherme et al., 2008). These factors could be particularly relevant in the Óbidos lagoon due to it shallowness. However, enhanced values of Mn were also recorded in summer at BB, when freshwater flows are almost insignificant. These increments are probably related with the pulse release of metals from sediments during periods of low oxygenation, as undersaturation was simultaneously recorded at BB. This internal source was already identified in other eutrophic coastal lagoons (Point et al., 2007) and hypothesized to occur in BB branch (Pereira et al., 2009d). At BS, the prominence of these sediment-related processes on the water quality is probably lower due to the better trophic state (Pereira et al., 2009c). Interestingly, sediments from BS exhibited the highest levels of Mn, while in water this metal increased at BB. According with the previous hypothesis, the importance of those processes at ML are certainly even lower than at BS.

BB branch exhibited elevated levels of nitrogenous compounds and silicates in winter, as well as phosphate and chlorophyll *a* in both seasons, which is in line with a previous study on Óbidos lagoon water quality (Pereira et al., 2009c). In fact, that confined branch was already proposed as the major impacted area in the lagoon, being classified as eutrophic (Pereira et al., 2009c). Higher concentrations of NH_4^+ and NO_3^-+NO_2^- in low tide of the winter survey highlight the influence of freshwater discharges enriched in nutrients for the water quality of BB branch. Nutrients in the other two sites were lower and ranged within similar concentrations intervals. Moreover, regeneration could occur in organically-

rich sediments, and upwards diffusion during periods of low oxygenation should be considered as an additional input of NH_4^+ and PO_4^{3-} to the water column at BB site, as previously noticed (Pereira et al., 2009d).

6.4.2 Metal levels in *C. maenas* and associations with environmental and biological factors

6.4.2.1 Spatial variations

Pooled genders data revealed higher Cu accumulation at BB than ML in winter, being in accordance with differences found in water (both tides). Spatial variation of Cu in crabs can also be the result of sediment contamination. In fact, a Cu gradient can be clearly established (BB>BS>ML) in the sediment. In previous studies, Cu concentrations in crabs demonstrated to reflect the sediment gradient (Pedersen et al., 1997; Martín-Díaz et al., 2008). Nevertheless, PCA in the current work did not provide any association between accumulated Cu in crabs and the corresponding water or sediment levels. The absence of associations, particularly with levels in water, can be related to the fact that metals in this environmental compartment can reflect transitory circumstances, while accumulated levels (particularly in hepatopancreas) reflect continuous exposures, influenced by several variables (biotic and abiotic).

Conversely, Mn accumulation in crabs was associated with sediment contamination by PCA at BS (winter) and BB (summer). In spite of that, accumulation in crabs' hepatopancreas did not reflect the notorious Mn increase found in BB water (in both seasons and tides).

The enhancement of Ni in females captured in summer at BB (in comparison with those from ML) is apparently in line with levels measured in water (high tide) at BB. In that season, Ni levels in males and pooled genders also enhanced at BS (in comparison with ML) contrarily to the levels in water that were lower than at ML in both tides. Increases at BS cannot be related with Ni contamination of sediments since very similar values were found in the two sites (ML and BS). In fact, PCA did not link accumulated Ni with levels in the environment (water or sediment).

Cadmium was higher at BB (than BS) in males, females and pooled genders captured in winter but values found in water do not allow the discrimination of the sampling sites. In fact, Cd levels in high tide were higher at BB than BS, while in low tide was the opposite. That spatial difference is much more in line with levels found in sediment, which were

consistently higher at BB than BS. This association was also observed in other field studies using *C. maenas* as sentinel (Pedersen et al., 1997).

Overall, spatial differences on metals accumulation were only recorded punctually, leading to the absence of significant variations when computed in a bioaccumulation index (IMBI). *Ulva* sp. reflected much better than *C. maenas* metal contamination of water in the Óbidos lagoon, since accumulated metals were correlated to their occurrence in water (Pereira et al., 2009a). It is well described that macroalgae, as primary producers, allow the early detection of contamination in ecosystems. Moreover, the uptake of metals occurred almost exclusively from the water phase, while in crabs the diet represents also an important source of contamination, namely metals, explaining the difficulty in obtaining correlations with the water and sediment loads. Nevertheless, PCA associated accumulation of Mn and levels of that element in the sediment, which is in accordance with the benthic lifestyle of *C. maenas*, feeding on epifauna and shallow infauna.

6.4.2.2 Seasonal variations

Seasonal variations of metals accumulated in crabs were more pronounced than spatial differences being observed for all metals individually, as well as for IMBI. In fact, PC1 provided by analysis of water and sediment data both *versus* accumulated metals established a separation between winter and summer for all studied sites. Despite PCA did not establish associations between any specific accumulated metal and the respective level in water, the recorded seasonal variations in crabs were generally in line with levels found in water, both showing increases in winter. Nesto et al. (2007) also observed metals increase in winter in mussels from the Venice lagoon, as well as Orbea et al. (2005) for organic contaminants in *C. maenas* from Basque estuaries.

There is evidence of regulation of Cu body burdens in *C. maenas* (Rainbow, 1985). Estimations suggest that decapods need between 31.3 and 38.1 μg g^{-1} to meet the requirements of Cu-associated enzymes and haemocyanin (Abdennour, 1997). Thus, the Cu regulation will eventually minimize seasonal differences. In spite of that, crabs caught at BB and BS (pooled genders), as well as females from BS, presented higher levels in winter than in summer. These enhancements are probably a consequence of the higher Cu levels found in water during winter. An increased metal availability in water during winter can also explain the higher levels of Mn and Cr in females captured at BS, as well as Mn (BS) and Cd (BB) in pooled genders. In general, at ML no significant winter increases were

observed for the different accumulated metals when analysed individually (only IMBI reflected winter increases), in spite of the higher water levels recurrently found in winter. This finding points out the involvement of other factors on the bioaccumulation dynamics besides the metals availability. Since those temporal differences were predominantly registered in females, the estimated IMBI for females was higher in winter (all sites) and consequently in pooled genders but not in males.

Seasonal variations in the abundance and diversity of crab's diet components could produce temporal variations in the metals availability via dietary uptake. In this direction, macrobenthic communities are known to oscillate seasonally at the Óbidos lagoon, with higher density and biomass in winter (Carvalho et al., unpublished data). Additionally, other factors besides metal availability may be on the basis of the higher metal accumulation in winter, such as the influence of abiotic parameters on crab's physiology including metals uptake. In winter, a salinity gradient was observed at the Óbidos lagoon, with lower values in upstream areas (BB and BS) and increasing with the proximity to the sea. This can play a relevant role since it has been previously reported that metals accumulation from water in crabs is higher for lower salinity (Pereira et al., 2006). However, in the current study the influence of salinity on metals accumulation was not corroborated by PCA, since no associations were obtained between those variables. On the other hand, the accumulation of Cu and Cr was related by PCA with PO_4^{3-} levels in water at BB (summer). Associations between metals uptake and nutrients were previously reported in *Ulva* sp. at Óbidos lagoon (Pereira et al., 2009a).

The body weight has been extensively reported in invertebrates, especially in bivalves, as an important factor affecting metal accumulation (Mouneyrac et al., 1998). Larger individuals accumulated more metals (Noel-Lambot et al., 1980) but the opposite was also observed (Mouneyrac et al., 1998). In the current study, crabs captured in summer were generally wider and heavier than those from winter, thereby contradicting the findings of Noel-Lambot et al. (1980). Moreover, the opposite hypothesis should be also refuted as body weight and width had a minor effect on accumulated metals, as evidenced by the absence of statistical correlations. A negligible effect was also recorded in the PCA.

It is well known that metals are associated with the exoskeleton and thus, moulting may be a way for arthropods to depurate metals. In isopods, moulting has been shown to reduce metal body burdens (Raessler et al., 2005). Concerning crustaceans, increased

accumulation of Cd was recorded in several tissues of crabs (males and females) during the postmoult stages compared to other stages (Bondgaard et al., 2000). The moulting periodicity of *C. maenas* at the Óbidos lagoon was not accurately investigated but, according to our field observations, males moulting has no a seasonal pattern (occurs repeatedly throughout the year), whereas females moulting starts on May prolonging until September/October (Pereira et al., unpublished data). Therefore, for females the moulting process can also be on the basis of the observed seasonal variations on hepatopancreas metals load.

Metals accumulation in females could be also dependent on the ovarian stage since it was previous observed in *C. maenas* a decrease on Cd accumulation during the ovarian maturation (Bondgaard et al., 2000). This was related with a lower branchial net Cd influx. At the Óbidos lagoon the breeding period of *C. maenas* occurs from December to April (unpublished data). Though egg-bearing females were not found in the two sampling periods, the influence of this process on metals accumulation dynamics of winter females (captured in February) cannot be ignored. Nevertheless, the mechanism proposed by Bondgaard et al. (2000) does not appear to be applicable to the current circumstances as females showed higher metal levels (including IMBI) in winter comparing to summer.

6.4.2.3 Gender related variations

Gender differences of accumulated metals are not commonly investigated in crabs. However, in this study, females presented higher metal levels than males. Identically, females of the rock crab *Thalamita crenata* presented higher Cd, Cu, Mn and Zn than males (Chen et al., 2005). Divergent results were also found as higher levels of Cd were recorded in males of *C. maenas* in Danish waters which was associated with the diet (Bjerregaard et al., 2005). It has been proved that food is a major source of Cd accumulation in *C. maenas*. Consequently, female crabs which are generally smaller than males and with smaller chelae, handled with preys of small size (Bjerregaard et al., 2005). This explanation is not valid for the current work since an opposite pattern was observed.

Based on the higher width and weight of males currently observed at Óbidos lagoon, it is expectable a larger number of moults comparing to females. By this process, males have the opportunity to excrete higher quantities of metals than females and thus, contributing to explain the lower metals load in males. However, the involvement of body weight on the observed gender differences (e.g. through the interference on the diet

profile or through the associated variation on moulting frequency) was not statistically demonstrated by the analysis of correlations and by the influence on PCA. Anyhow, when males and females were analysed jointly some spatial differences were unseen (e.g. accumulated Ni) due to a distinct responsiveness of genders.

6.4.3 Biochemical endpoints in *C. maenas* and associations with environmental and biological factors

6.4.3.1 Spatial variations

Increased activities of antioxidant enzymes have been described in several aquatic species from impacted sites, being considered as an adaptation enabling the biological systems to partially or totally overcome stress resulting from exposure to an unsafe environment (Guilherme et al., 2008; Martín-Díaz et al., 2008). On the other hand, impairment in the defence system by the chemical reactive species, which can act as antioxidant enzymes inhibitors, reduces the cell protection and the organism fitness becomes precarious (Escobar et al., 1996). Apparently, this is occurring in crabs of the Óbidos lagoon since, in general, a tendency for lower levels of CAT (though no statistically significant) was observed at BB in winter and summer. This distinction was supported by PCA for BB in summer, linking CAT activity with accumulated levels of Cu, Mn, Cr and IMBI. Conversely, in a previous work carried out in the same ecosystem during autumn, CAT exhibited increased activities in *C. maenas* from BB (Pereira et al., 2009b). This discrepancy could be related to the influence of temporal factors on enzymatic activities as discussed in the next section. Moreover, the activity of antioxidant enzymes in environmentally exposed organisms reflects a balance between the action of inducers and inhibitors. Accordingly, the current findings seem to reflect an ascendancy of the inhibitors contrarily to the previous work. The overproduction of ROS (Ahmad et al., 2000), such as superoxide anion (Kono and Fridovich, 1982), was previously associated to CAT activity decrease. Other dissimilarity between the two studies occurs for GPx, which was induced previously at BB (Pereira et al., 2009b) but herein did not discriminate sites in the two surveys. Thus, associations provided by PCA between GPx and metals in hepatopancreas (Cu, Ni, Cd and IMBI) or in sediments (Cu, Cr and Ni) should be regarded in the context of the overall data.

In response to environmental changes GSTs (a multigenic superfamily of multifunctional enzymes) may play a dual protective role associated to their activity on

conjugation of electrophilic compounds (or phase I metabolites) with GSH (Van der Oost et al., 2003) and to a direct antioxidant action carried out by GST α-class catalyzing the reduction of organic hydroperoxides by GSH (Wang and Ballatori, 1998). Taking into account that the adopted methodology (using CDNB, which is conjugated by all GST isoforms with the exception of the q-class) determines total GST activity, it is not easily perceptible the meaning of the observed GST activity increase in summer at BB for females in comparison with BS. When considering the overall data, the induction of GST was associated by PCA with higher accumulation of Cu, Ni, Cd and IMBI, with more relevance at BB in winter. This sampling condition (site/season) was also put in evidence by PCA concerning the link of GST response with levels of Cu, Cr and Ni in sediments. GST induction was also previously observed in *C. maenas* exposed *in situ* to metal contaminated areas (Martín-Díaz et al., 2008), as well as at BB site in the Óbidos lagoon (Pereira et al., 2009b).

The depletion on GSH_t observed in females at BB in summer indicates an increased use without a compensatory synthesis, which can be related with its involvement on conjugation activities catalyzed by GST, as this enzymatic activity revealed a concomitant increment. The PCA linked GSH_t variation with accumulated levels of Cu, Mn, Cr and IMBI. It is well known that GSH_t may play a role in inducing resistance to metals by protecting macromolecules against attack by free radicals.

The absence of LPO in *C. maenas* from BB points to the effectiveness of the overall defense mechanisms. Thus, the absence of enzymatic antioxidants induction and the reduction on GSH_t levels at BB had no repercussions on cellular damage. Conversely, in a previous work carried out at the Óbidos lagoon LPO increased significantly in crabs from BB site (Pereira et al., 2009b).

The involvement of metals in EROD inhibition was reported by different authors (Oliveira et al., 2004; Pereira et al., 2009b), who described that metals can affect CYP1A by direct biding, leading to a conformational change or altering protein turnover. Herein, EROD activity decreased significantly in males and pooled genders at BB in summer, being the same spatial tendency recorded in winter for males and in both seasons for females. Taking into consideration the levels of metals measured in crabs' hepatopancreas, Ni is the one potentially linked with that inhibition in females but this was not confirmed by PCA, which did not establish any association between accumulated metals and EROD. This is in accordance with the absence of significant inter-site differences for IMBI, pointing to a

minor influence of metals in the EROD inhibition. Moreover, PCA did not show any association between EROD inhibition and contamination of water or sediment by metals. Besides metals, oxidative stress has been shown to repress the expression of the CYP1A1 gene at the transcriptional level (Morel and Barouki, 1998). Hence, the involvement of ROS on the observed EROD inhibition cannot be overlooked. This damaging action can be understood as an alternative toxicity pathway or in combination with a Ni-induced suppression. Furthermore, EROD was not associated by PCA with nutrients levels in water as observed in a previous work at the Óbidos lagoon (Pereira et al., 2009b).

The spatial differences recorded in biochemical responses when computed in a general stress index (IBR) revealed "stressful" conditions for crabs at BB. The distinction of BB site was particular evident in summer, while in winter only females discriminated BB from the other sites.

6.4.3.2 Seasonal variations

In this study, a marked difference between winter and summer was observed for all oxidative stress endpoints. For instance, CAT activity, GSH_t content and LPO levels showed a summer increase.

The few field studies investigating seasonal variations of biochemical responses are mainly focused on bivalves and fish, being absent for *C. maenas*. Because water temperature rose 10 to 15 °C in summer, its interference on oxidative stress must be hypothesised. Low temperature reduces the metabolic rates in ectothermal organisms, and hence lower enzymatic activities are in general observed in colder seasons. In the bivalve *Corbicula fluminea* lower CAT activity was recorded in winter in comparison to summer (Vidal et al., 2002). Comparable results were reported for CAT in the digestive gland of mussels (Regoli, 1998). The current GSH_t increase detected in summer is in agreement with previous studies with ectothermal marine animals (Heise et al., 2006). This variation was related to spontaneous, nonenzymatic GSH oxidation by emerging ROS under hyperthermia. Elevated temperatures accelerate mitochondrial respiration and increase mitochondrial ROS formation (Heise et al., 2003). As the current summer GSH_t increase was measured in all the sites, it appears as an indication that glutathione pool responds to stressful conditions enhancing the baseline levels through a synthesis de novo.

It was reported for fish that increase in environmental temperature modifies the chemical and physical state of membranes (Cossins, 1981). This aspect, together with the enhancement of oxygen consumption and the previously mentioned increased mitochondrial ROS generation, could lead to the higher lipid peroxidation recorded in crabs of the Óbidos lagoon in summer.

Metal levels in tissues are likely to change with the season, reflecting variability in the environmental inputs, but also changes in the metabolism (Regoli, 1998). However, the hypothesis of the observed seasonality in CAT, GSH_t and LPO being partially driven by changes in the accumulated metals in unlikely. In general, metals in water and accumulated in crabs had minimum values when those biochemical responses were maxima (summer). Orbea et al. (2002) investigated the variability of peroxisomal enzymes and their relation to changes in the bioavailability of organic contaminants in field conditions, coming up with a conclusion that under moderate pollution conditions seasonal factors may affect biomarker responses to a greater extent than pollutant stress. This is likely to be the case also in the present study carried out at the Óbidos lagoon namely for CAT, GSH_t and LPO.

An opposite temporal pattern was recorded for GPx and GST, showing maximum activities in winter, which is in agreement with the higher availability of metals in that season. In fact, GPx and GST were linked by PCA with metals in sediments (Cu, Cr, Ni) and accumulated in hepatopancreas (Cu, Ni, Cd and IMBI) in winter (preferential at BB), pointing to a major importance of metals, rather than temperature or other abiotic factors for these particular enzymes.

The current data suggest that summer conditions potentiate the occurrence of inhibitory actions, pointing to higher crab´s susceptibility in this period. This can be related with the tendency for EROD elevation in summer in the less impacted site (ML) where contaminant-associated variables have minor importance. Seasonal changes in temperature showed to modulate the response of cytochrome P4501A in wild fish populations, as observed in *Anguilla anguilla* by the enhancement of EROD levels in warmer periods (Gorbi et al., 2005).

The influence of season on biochemical parameters is also evidenced by IBR values, which reached higher values in winter than summer. Nevertheless, inter-site differences based on this index are more obvious in summer.

6.4.3.3 Gender related variations

Females from BS exhibited higher GST activities than males (winter). Another interesting point is that only females signalled inter-site differences for GST. No mechanism is known to explain this male-female variation in crabs. However, gender differences could be related to the higher levels of accumulated metals found in females. Winzer et al. (2002) reported a higher vulnerability of hepatocytes of female fish to oxidative stress associated with a lower metabolic capacity (indicated by induction of CYP450) than males when exposed to xenobiotics. This higher susceptibility of females to toxicants could explain the higher incidences of tumours in marine environments in relation to males (Winzer et al., 2002). Sanchez et al. (2008) also reported lower EROD activity in female fish and claimed that its suppression could be due to protein dilution generated by vitellogenesis. However, female crabs in this work had higher EROD activities than males and thus that phenomenon seems to be negligible. Moreover, females showed to be less vulnerable to EROD inhibition.

Males and females varied appreciably on accumulation of metals but this was not accompanied by significant gender differences on biochemical responses (except punctually for EROD and GST). Nevertheless, when males and females were analysed jointly some spatial differences were unseen for GST, GSH_t and EROD pointing to the relevance of gender separation in biomonitoring programs using crabs. Gender differences were also evidenced by IBR with females being more responsive than males to site differences in winter. Differently, in summer both genders point to BB as the major hazard area of the lagoon.

6.5 Conclusions

The inter-site differences signalled by bioaccumulation and biochemical markers, though scarce, pointed BB branch as the main impacted area of the lagoon. The occurrence of subtle responses should be regarded as a reflex of a moderate contamination state and slight inter-site differences on the contamination pattern/extent, rather than as a lack of sensitivity of the adopted biomonitoring approach.

Seasonal impact on crab responses was more pronounced than spatial variations. This is in accordance with the contrasting winter-summer conditions regarding environmental conditions. Though in winter biochemical responses could be associated to the higher availability of metals in water and enhancement of accumulated levels, in

summer were driven mainly by non-contamination-related factors (biotic and abiotic). In addition, the impact of seasonal variables on metals bioaccumulation showed to be site- and gender-specific.

Males and females exhibited different patterns of metals accumulation and biochemical responses, with females being more responsive as suggested by IMBI and IBR. This highlights the importance of working with separated genders in order to avoid misinterpretations and improve the efficacy of biomonitoring programs.

Under moderate contamination, biomarkers (accumulation of metals and biochemical responses) could vary only slightly between sites. Beneath those conditions, the application of biomarkers integration tools like IBR could be particular relevant, allowing a better site-discrimination than biomarkers individually.

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CHAPTER VII

Combined use of environmental data and biomarkers in *Liza aurata* inhabiting a eutrophic and metal-contaminated coastal system – gills as a mirror door for environmental contamination

7 Combined use of environmental data and biomarkers in *Liza aurata* inhabiting a eutrophic and metal-contaminated coastal system – gills as a mirror door for environmental contamination

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Abstract

An investigative biomonitoring study was carried out in a eutrophic coastal system with a moderate contamination by metals (Óbidos lagoon, Portugal), combining the evaluation of exposure levels with metals accumulation and oxidative stress responses in gills of *Liza aurata*. Two contrasting seasons (winter and summer) were considered at three sites: Barrosa (BB) and Bom-Sucesso (BS) branches; Middle lagoon (ML). Data on water column pointed to a higher metals and nutrients availability at BB that was reflected in the higher metal contents in gills, particularly in winter. Similarly, oxidative stress responses demonstrated a pro-oxidant challenge at BB (winter and summer), being substantiated by a general stress index (IBR). Gills' metals load was higher in summer than winter, explained by the increased environmental concentrations in combination with elevated metabolic rates. CAT, GST, GSH_t and LPO increases observed in winter at BB were related with metal accumulation, while summer enhancement of GPx, GR, GST and GSH_t was associated with other stressors. Inter-site differences on the basis of IBR were more accentuated in winter. Based on its confirmation as an important route of entry for contaminants and the currently demonstrated ability to reflect environmental status, gills can be considered as a "mirror door" in the context of water contamination assessment.

KEYWORDS: Oxidative stress; fish; metals; coastal lagoon; temporal variability.

7.1 Introduction

The suitability of fish gills for environmental biomonitoring was recommended by several recent works (Fernandes et al., 2008; Dautremepuits et al., 2009; Oliveira et al., 2009). In fact, gills have a wide surface area that is continuously in contact with the external medium, being, thus, the main uptake route of contaminants from aqueous phase (Playle, 1998). On the other hand, fish gills are able to accumulate chemicals that were taken up by other exposure routes, due to their position between the venous and arterial circulation, thus receiving nearly all of the cardiac output (Levine and Oris, 1999). Moreover, gills play a key role on fish physiology, such as gaseous exchange, osmotic and ionic regulation, acid-base balance and nitrogenous waste (Ahmad et al., 2008). It is documented that fish gills responded earlier than kidney and liver under field contamination exposure (Ahmad et al., 2004; Santos et al., 2004). Its applicability is also incremented by the fact that gills' epithelium suffers an intense cell division to counteract its exfoliation and erosion and by this feature gills usually reflect current exposures, while more quiescent tissues/organs with high storage propensity can reflect past exposures and, thus, increasing the risk of misinterpretations (Mieiro et al., 2009). This seems to be particularly relevant when fish are used as bioindicators due to their non-sessile behaviour.

Monitoring the state of coastal systems has traditionally been almost entirely based on measurements of contaminants concentrations in sediment, water column and organisms. However, this chemical monitoring alone is no longer considered meaningful or cost-effective. Additionally, it has become evident that measuring concentrations in the tissues does not provide the information needed to assess biological effects that contaminants may cause in organisms (Lehtonen and Schiedek, 2006). In fact, contaminants usually appear in the environment as very complex mixtures that can cause interactive effects on the biota impossible to evaluate only by means of chemical analyses. Thus, recent investigative monitoring studies combined environmental levels and body burdens with the evaluation of biomarkers that represent early signs of biological effects (Van der Oost et al., 2003). In this context, many pollutants are capable of inducing oxidative stress in aquatic animals by disturbing the antioxidants efficiency and enhancing the intracellular reactive oxygen species (ROS), which often prelude in DNA damage, lipid peroxidation (LPO) and enzyme inhibition (Van der Oost et al., 2003). Antioxidant defence systems include antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT),

glutathione peroxidase (GPx) and glutathione reductase (GR), as well as non-enzymatic antioxidants (e.g. glutathione – GSH) which have been extensively used as biomarkers of oxidative stress (Ahmad et al., 2006; 2008; Guilherme et al., 2008). LPO estimation has been also found to have a high predictive importance as biomarker of effect (Guilherme et al., 2008). To our knowledge, only few studies combined the measurement of oxidative stress in fish gills with levels of accumulated contaminants (Hansen et al., 2006; Fernandes et al., 2008). Additionally, an investigative approach with the previous information and environmental contamination levels is more uncommon (Hansen et al., 2006).

Previous studies employed the golden grey mullet (*Liza aurata*) as a model on field studies (Oliveira et al., 2009; Guilherme et al., 2008) and under laboratory conditions (Cionna et al., 2006; Oliveira et al., 2007). In fact, *L. aurata* has favourable features as sentinel of contaminated systems. This is a pelagic species that is in frequent contact with sediments, filtering its superficial layer and particles in the water column. Moreover, mullets play an important role in the estuarine trophic web (Almeida, 2003).

In order to access the environmental health of a coastal lagoon impacted by eutrophication and moderate metal contamination (Óbidos lagoon, Portugal), gills of *Liza aurata* were monitored for metal accumulation and oxidative stress in two contrasting seasons: winter and summer. Peroxidative damage and antioxidant (enzymatic and non-enzymatic) responses were measured in *L. aurata*' gills and their relationship with metal accumulation as well as with environmental chemical data (metals and nutrients) was assessed. It was also intended to evaluate the suitability of the adopted combined strategy (external levels of exposure/bioaccumulation markers/oxidative stress responses) applied to fish gills on monitoring coastal ecosystems in a multi-contamination context.

7.2 Material and Methods

7.2.1 Study area

The Óbidos lagoon is a shallow coastal ecosystem, located on the west coast of Portugal with a wet area of 7 km^2, permanently connected to the sea through a narrow inlet (Figure 7.1). It comprises areas of different morphological and sedimentary characteristics: sand banks and narrow channels in the lower/middle lagoon; muddy bottom sediments in the two inner branches (Barrosa and Bom-Sucesso). The Barrosa branch is shallower (mean depth 0.5-1 m) and water circulation is mostly driven by tides

and by a small tributary (Cal River) that drains agriculture fields. Urban effluents from a nearby town (Caldas da Rainha, 50 000 inhabitants) also have been discharged to the Barrosa branch by the Cal River. Consequently, this area presents the highest nutrient availability of the lagoon, being classified as eutrophic (Pereira et al., 2009a). High nutrient concentrations were in line with abundant macroalgae (*Ulva* sp. and *Enteromorpha* sp.) and a broad daily variation of dissolved oxygen concentration during the summer months (Pereira et al., 2009b). Previous water quality surveys of the lagoon showed maximum concentrations of Mn, Ni, Cu and Cd in Barrosa branch. Major metal sources were identified to be the Cal River in periods of higher inflow and remobilization from sediments in summer months (Pereira et al., 2009b). The Bom-Sucesso branch is also a confined area but receives a smaller freshwater flow (Vala do Ameal) with better water quality than the Cal River, according to the Portuguese categorization of freshwater systems. In this area, metal remobilization from sediments is less plausible due to the higher depths and stronger currents. The middle lagoon was previously characterised by coarser sediments with a low affinity for metals (Carvalho et al., 2006), as well as by a better water quality (Pereira et al., 2009a). Moreover, previous ecotoxicological studies with *Ulva* sp. and *Carcinus maenas* pointed out Barrosa branch as a critical area at the lagoon with potential impact on autochthonous populations, whereas the lower/middle lagoon was considered the reference area (Pereira et al., 2009c; 2009d).

7.2.2 Sampling

Two surveys were carried out at Óbidos lagoon in winter (December 2006) and summer (July 2007) during low-tide and juveniles of the golden grey mullet (*L. aurata*) were collected (n=10) using a traditional beach-seine net named “chincha”. Three sampling areas were selected taking into account previous ecotoxicological studies (Pereira et al., 2009c, 2009d) (Figure 7.1): Middle lagoon (ML); Bom-Sucesso (BS) and Barrosa (BB) located in the inner branches of the lagoon. Fish biometrical parameters, such as weight and length ranged from 20-40 g and 13-17 cm. Juveniles were selected in order to aside the gender interference and diminish the background of previous exposures to contaminants (Ahmad et al., 2008). Immediately after catching, fish were sacrificed and gills were removed and frozen in liquid nitrogen in two sets of samples: one for oxidative stress quantifications and other corresponding to the rest of the organ for metal

determinations. In the laboratory, samples were preserved until further processing at -80 and −20 °C for oxidative stress and metal determinations, respectively.

Surface water (0.2 m depth) was sampled in the three areas for determinations of nutrients, chlorophyll *a* and metals.

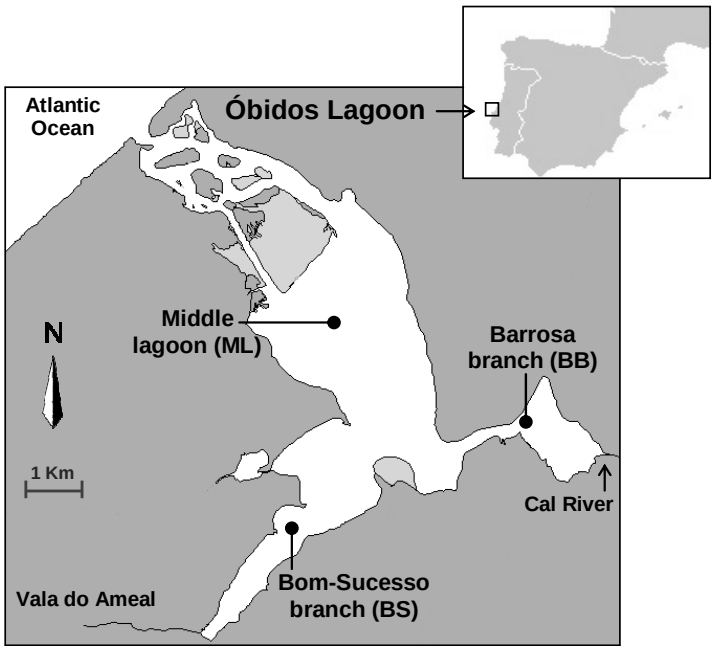

Figure 7.1. Location of the sampling sites at the Óbidos lagoon: middle lagoon (ML) (39º24'50.48''N, 9º12'42.82''W); inner branches Bom-Sucesso (BS) (39º23'44.88''N, 9º13'01.75''W) and Barrosa (BB) (39º26'16.84''N, 9º11'33.18''W).

7.2.3 Determination of physicochemical parameters and metals in water

Water temperature, salinity and dissolved oxygen were measured *in situ* using an YSI, 650 meter. Water samples for determinations of nitrate+nitrite (NO_3^-+NO_2^-), ammonium (NH_4^+), phosphate (PO_4^{3-}) and silicate $Si(OH)_4$) were filtered through MSI Acetate Plus filters and analyses carried out using an autoanalyser TRAACS 2000 (Bran+Luebbe). For chlorophyll *a* determinations, water was filtered through a Whatman GF/F (0.7 µm) filter that was immediately frozen at -20 °C and later extracted in 90% acetone, for analysis in a Perkin Elmer Fluorometer using the protocol modified by Lorenzen (1966).

Manganese, Cu, Ni, Cr, Pb and Cd in the collected waters (triplicate samples) were measured using diffusive gradients of thin films (DGT). All DGT holders, Chelex-100 resins and diffusive gels (type APA, 0.8 mm thickness, open pore> 5 nm) (Zhang and Davison, 1999) were purchased from DGT Research (Lancaster, UK). The DGT devices were

deployed in 2 L polypropylene bottles with unfiltered sampled water and stirred at constant temperature for 48 hours. After devices retrieval, resins were eluted by immersion in 5 mL of 1 M HNO_3 (prepared from suprapur nitric acid) at a minimum of 24 h. Eluates were analysed directly by a quadropole ICP-MS (Thermo Elemental, X-Series). All eluates were analysed with reagents blanks and an international standard of river water (SLRS-4) used to control eventual contaminations during the analytical procedure and the procedure accuracy, respectively. The eluate concentration was converted into the mass of metal accumulated on the resin using an elution factor with a yield value of 0.8 (Zhang and Davison, 1995) and a resin gel volume of 0.15 mL. DGT-metal concentrations were calculated according to the following equation: [Metal]=(MΔg)/(tAD); where M is the mass of metal accumulated on the resin during the emersion time (t); Δg the diffusive gel thickness (0.08 cm); A the exposure area (3.14 cm^2); and D the diffusion coefficient of the metal in the gels as provided by DGT Research.

Water was collected in triplicates for separation of suspended particulate matter (SPM), which was retained on 0.45 µm membranes that were mineralized completely with HF (40%) and Aqua Regia (HCl-36%:HNO_3-60%; 3:1) in closed Teflon bombs (100 °C for 1 h), evaporated to near dryness (DigiPrep HotBlock – SCP Science), redissolved with 1 mL of doubled-distilled HNO_3 and 5 mL of ultra-pure water, heated for 20 min at 75 °C, and diluted to 50 mL with ultra-pure water. Reagents blanks and international certified standards of sediments from the National Research Council of Canada (1646a; BCSS-1; MESS-3) were prepared in a similar way of samples to control the accuracy of the procedure. The concentrations of Mn and Zn were determined by flame atomic absorption spectrometry (Perkin Elmer Analyst 100) whereas Cu, Ni, Cr, Pb and Cd were determined by ICP-MS. Levels of the analysed elements obtained in the reference materials were consistently within the ranges of certified values.

7.2.4 Metal analyses in gills

Approximately 50 mg of freeze dried tissue was digested with a mixture of HNO_3 (doubled distilled from 65%) and H_2O_2 (suprapure, 30%) at 60 °C for 12 h, at 100 °C for 1 h and at 80 °C for 1 h according to the method described in Pereira et al. (2009e). Procedural blanks were prepared using the same analytical procedure and reagents. Concentrations of Mn, Zn, Cu, Ni, Cr, Pb and Cd were determined by ICP-MS. International certified

standards (TORT-2, DOLT-2 and DOLT-3) were use to control the accuracy of the analytical procedure.

7.2.5 Biochemical analyses in gills

Tissue handling and preparation of post-mitochondrial supernatant (PMS) fraction - Tissue samples were homogenized, using a Potter-Elvehjem homogenizer, in chilled phosphate buffer (0.1 M, pH 7.4) (1 g of tissue/10 ml buffer). This homogenate was then divided in two aliquots for LPO and PMS preparation. The PMS preparation was obtained by centrifugation in a refrigerated centrifuge (Optima TL, Beckman) at 13,400 g for 20 min at 4 °C. Aliquots of PMS were divided in microtubes and stored at -80 °C until analyses.

Estimation of lipid peroxidation (LPO) - LPO was determined in the previously prepared homogenate as adapted by Filho et al. (2001). Briefly, to 150 µl of homogenate, 10 µl of BHT (4% in methanol) was added and mixed well to prevent oxidation. The absorbance of each sample was measured at 535 nm. The rate of LPO was expressed in nmol of TBARS formed per milligram of fresh tissue using a molar extinction coefficient of $1.56x10^5$ $M^{-1}cm^{-1}$.

Catalase (CAT) activity measurement - CAT activity was assayed in PMS by Claiborne (1985) method (at 25 °C). Briefly, the assay mixture consisted of 1.95 ml phosphate buffer (0.05 M, pH 7.0), 1 ml hydrogen peroxide (0.019 M) and 50 µl of sample in final volume of 3 ml. Change in absorbance was recorded at 240 nm and CAT activity was calculated in terms of µmol H_2O_2 consumed $min^{-1}mg^{-1}$ protein using a molar extinction coefficient of 43.5 $M^{-1}cm^{-1}$.

Glutathione peroxidase (GPx) activity measurement - GPx activity was determined in PMS according to the method described by Mohandas et al. (1984) with some modifications (at 25 °C). The assay mixture consisted of 0.72 ml phosphate buffer (0.05 M, pH 7.0), 0.05 ml EDTA (1 mM), 0.05 ml sodium azide (1 mM), 0.025 ml GR (1 IU/ml), 0.05 ml GSH (4 mM), 0.05 ml NADPH (0.8 mM), 0.005 ml H_2O_2 (1.0 mM) and 0.05 ml of PMS in a total volume of 2 ml. Oxidation of NADPH was recorded at 340 nm, and GPx activity was calculated in terms of nmol NADPH oxidized $min^{-1}mg^{-1}$ protein using a molar extinction coefficient of $6.22x10^3$ $M^{-1}cm^{-1}$.

Glutathione reductase (GR) activity measurement - GR activity was assayed by the method of Cribb et al. (1989) with some modifications (at 25 °C). Briefly, the assay mixture contained 0.025 ml of PMS fraction and 0.925 ml of NADPH (0.2 mM), GSSG (1 mM) and DTPA (0.5 mM). The enzyme activity was quantified by measuring the disappearance of NADPH at 340 nm during 3 min. The enzyme activity was calculated as nmol NADPH oxidized $min^{-1}mg^{-1}$ protein using a molar extinction coefficient of 6.22×10^3 $M^{-1}cm^{-1}$.

Glutathione-S-transferase (GST) activity measurement - GST activity was determined using CDNB (1-chloro-2,4-dinitrobenzene) as substrate according to the method of Habig et al. (1974) (at 25 °C). The assay was carried out in a quartz cuvette with a 2 ml mixture of phosphate buffer (0.2 M, pH 7.4), CDNB (0.2 mM) and 0.2 M GSH. The reaction was initiated by addition of 10 µl of PMS and the increase in absorbance was recorded at 340 nm during 3 min. The enzyme activity was calculated as nmol CDNB conjugate formed $min^{-1}mg^{-1}$ protein using a molar extinction coefficient of 9.6 $mM^{-1}cm^{-1}$.

Total glutathione (GSH_t) content measurement - Protein content in the tissue homogenate was precipitated with TCA (5%) for 1 h and then centrifuged at 13,400 g for 20 min at 4 °C. GSH_t content was determined in the resulting supernatant (deproteinated PMS) (at 25 °C) adopting the enzymatic recycling method using glutathione reductase (GR) excess, whereby the sulfhydryl group of GSH reacts with DTNB (Ellman's reagent) producing a yellow coloured 5-thio-2-nitrobenzoic acid (TNB). The rate of TNB production is directly proportional to this recycling reaction, which is in turn directly proportional to the concentration of GSH in the sample. Formation of TNB was measured by spectrophotometry at 412 nm. It should be noted that GSSG is converted to GSH by GR in this system, which consequently measures total GSH. The results were expressed as nmol TNB formed min^{-1} mg^{-1} protein using a molar extinction coefficient of 14.1×10^3 $M^{-1}cm^{-1}$.

Protein measurement - Total protein contents were determined according to the Biuret method (Gornall et al., 1949), using bovine serum albumin (E. Merck-Darmstadt, Germany) as a standard.

7.2.6 Statistical analyses

A method for combining all the measured oxidative stress endpoints into a general index - Integrated Biomarker Response (IBR) (Beliaeff and Burgeot, 2002) - was applied for data of both surveys. The basis of data processing is described here briefly. First, the mean value (X_i) for each biomarker at each sampling area and season was calculated. In addition, the general mean (m_i) and standard deviations (s_i) of each biomarker were estimated for all sampling areas and seasons. The value of X_i was then standardized to obtain Y_i, where $Y_i = (X_i - m_i)/s_i$. Then Z_i was computed via the equation $Z_i = -Y_i$ or $Z_i = Y_i$ in the case of a biological effect corresponding, respectively, to inhibition or activation. The minimum value (Min_i) of Z_i for each biomarker was calculated for all sampling areas and seasons, and then the score S_i was computed as $S_i = Z_i + |Min_i|$, where $|Min_i|$ is the absolute value. Finally, IBR for each sampling site and season was calculated via de following formula: $IBR = (S_1 x S_2)/2 + (S_2 x S_3)/2 + \ldots + (S_{n-1} x S_n)/2 + (S_n x S_1)/2$; in which the obtained score for each biomarker (S_i) is multiplied with the score of the next biomarker (S_{i+1}), arranged as a set, dividing each calculation by 2 and summing-up of all values.

Statistical software (Statistica 7.0) was used for statistical analyses. The assumptions of normality and homogeneity of data were verified. ANOVA analysis was used to compare sampling sites and Tukey test was applied for Post-hoc comparison (Zar, 1996). Differences between means were considered significant when $p<0.05$. The correlation between metals in the water column (DGT-measured and SPM fractions) and accumulated in gills, as well as between accumulated metals and oxidative stress responses in gills was tested (considering the mean values of the two surveys at each site) by Pearson analysis ($p<0.05$).

7.3 Results

7.3.1 Water characteristics

Water temperature was slightly higher at BB and BS in summer and winter, while salinity was lower at BB than at the other sites, particularly in winter (Table 7.1). In general, dissolved oxygen was around 100% but undersaturation was recorded at BB in summer and BS in winter. In general, the maximum values of nitrogenous compounds, $Si(OH)_4$, PO_4^{3-} and chlorophyll *a* were recorded at BB in both surveys. NH_4^+, NO_3^-+NO_2^- and $Si(OH)_4$ exhibited a typical temporal fluctuation in the lagoon with lower values in summer.

Table 7.2 presents the levels of metals in water (DGT-measured fraction) at the three sites in winter and summer. Generally, BB presented higher levels of metals in winter than the other sites. In summer, a similar pattern was observed for Cu, Pb and Cd. Moreover, Cr was higher at BB and BS than at ML. Concerning temporal differences, higher levels of Mn and Cd were found in winter, while Cu, Cr and Pb enhanced in summer.

Manganese, Cu, Ni, Cr and Cd in SPM collected in winter were higher at BB (Table 7.3). Moreover, Zn increased at ML in winter and Pb at BS. In summer, Cu, Ni, Cr and Pb in SPM peaked at BB, while SPM from ML contained higher levels of Mn, Zn and Cd than other sites. Moreover, Ni increased in summer in relation to winter (BB), as well as Mn (all sites), whereas Pb (all sites) and Cd (BB) were higher in winter than in summer. A temporal pattern was difficult to discern for Zn, Cu and Cr.

Table 7.1. Water temperature (T), salinity, dissolved oxygen (DO), ammonium (NH_4^+), nitrate+nitrite (NO_3^-+NO_2^-), phosphate (PO_4^{3-}), silicate ($Si(OH)_4$) and chlorophyll *a* (Chl *a*), in winter and summer of low-tide, in middle lagoon (ML) and in inner branches (Bom-Sucesso - BS, Barrosa - BB) at Óbidos lagoon.

Season	Site	T (°C)	Salinity	DO (%)	NH_4^+ (µM)	NO_3^-+NO_2^- (µM)	PO_4^{3-} (µM)	$Si(OH)_4$ (µM)	Chl *a* (µg L^{-1})
Winter	ML	9	34.6	101	2.0	9.2	0.5	6.0	1.7
	BS	11	31.2	87	18	31	2.3	20	0.7
	BB	11	28.1	96	45	40	5.6	41	1.8
Summer	ML	20	34.6	95	1.0	1.0	2.9	5.1	4.9
	BS	23	35.2	108	1.0	1.2	2.1	4.6	3.7
	BB	24	33.3	83	0.9	1.3	6.5	12	10

Table 7.2. Levels of metals in water of low-tide, in winter and summer, in the middle lagoon (ML) and in inner branches (Bom-Sucesso - BS, Barrosa - BB) at Óbidos lagoon. Means and associated standard errors are presented.

Season	Site	Mn (µM)	Cu (nM)	Ni (nM)	Cr (nM)	Pb (nM)	Cd (nM)
Winter	ML	0.22±0.06	2.3±0.14	7.5±2.0	7.5±1.4	0.22±0.06	0.16±0.01
	BS	0.68±0.01	3.9±0.17	7.0±0.85	8.3±0.17	0.13±0.005	0.18±0.01
	BB	1.0±0.55	4.9±0.17	7.7±0.87	8.5±0.17	0.26±0.08	0.24±0.06
Summer	ML	0.13±0.01	3.3±0.21	8.6±2.3	6.4±1.2	0.47±0.12	0.07±0.006
	BS	0.12±0.01	4.9±0.18	6.3±0.81	14±1.9	0.72±0.02	0.09±0.007
	BB	0.04±0.02	7.9±1.0	8.0±0.38	14±1.3	0.99±0.21	0.14±0.03

Table 7.3. Levels of metals (μg g^{-1}) in suspended particles of low-tide, in winter and summer, in the middle lagoon (ML) and in inner branches (Bom-Sucesso - BS, Barrosa - BB) at Óbidos lagoon. Means and associated standard errors are presented.

Season	Site	Mn	Zn	Cu	Ni	Cr	Pb	Cd
Winter	ML	505±30	161±32	29±4.2	22±3.1	55±6.7	40±0.31	0.19±0.08
	BS	458±48	113±9.1	35±12	23±6.7	56±14	50±10	0.18±0.08
	BB	749±18	142±4.1	52±0.35	28±2.0	68±2.6	44±3.4	0.36±0.11
Summer	ML	1515±92	153±3.1	24±3.0	28±3.6	48±0.32	24±1.2	0.20±0.05
	BS	623±9.1	128±17	24±3.5	28±4.1	23±3.9	21±1.5	0.18±0.05
	BB	812±13	134±8.2	52±2.3	61±2.7	51±3.3	31±1.7	0.18±0.01

7.3.2 Metals in *L. aurata* gills

In winter, gills of fish from BB showed significantly higher accumulation of Zn, Cu, Cr and Cd than ML (Figure 7.2). Moreover, levels of Cr were significantly higher in gills of fish from BB than BS in winter, whereas at BS were recorded Zn and Pb increases (in comparison with ML). In summer, gills at BB showed lower levels of Cu (than BS) and Cr (than BS and ML).

Differences between the two surveys were found for all metals, except Mn and Pb. Gills in winter presented higher contents of Cu and Ni (BB) than in summer. Differently, in summer gills presented higher levels of Zn (all sites), Cu (BS), as well as Cr and Cd (ML, BS).

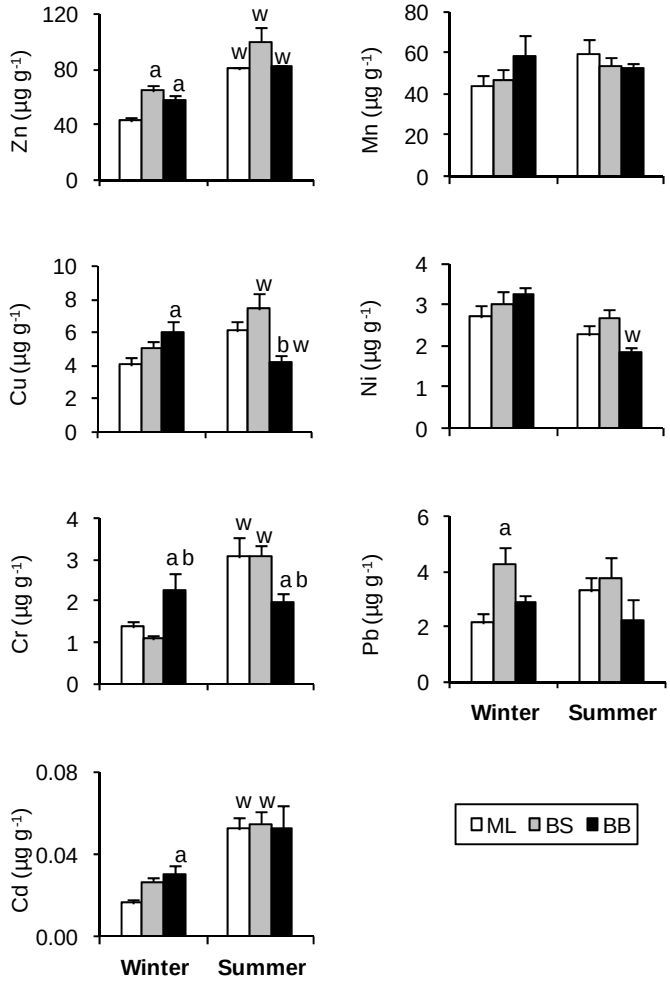

Figure 7.2. Metal levels in gills of *L. aurata* captured in winter and summer in the middle lagoon (ML) and in the inner branches (Bom-Sucesso – BS, Barrosa - BB) at Óbidos lagoon. Letters denote statistically significant differences ($p<0.05$) between sites (in the same survey) and between surveys (for each site): (a) vs. ML; (b) vs. BS; (w) vs. winter. Means and associated standard errors are given.

7.3.3 Oxidative stress in *L. aurata* gills

A spatial analysis of gills antioxidant defences revealed significant increases of CAT (winter), GPx and GR (summer) activities, as well as GSH_t content (winter and summer) at BB relatively to ML and BS (Figure 7.3). Moreover, GST activity in winter and summer enhanced significantly at BB in comparison to ML. In terms of peroxidative damage, significantly higher levels were also found at BB (*versus* ML and BS) in winter. Concerning temporal differences, significantly higher levels were found in summer for: CAT, GPx and GR at all sites; GST at ML and BS; and GSH_t at BB. In addition, LPO was higher in winter than summer at BB.

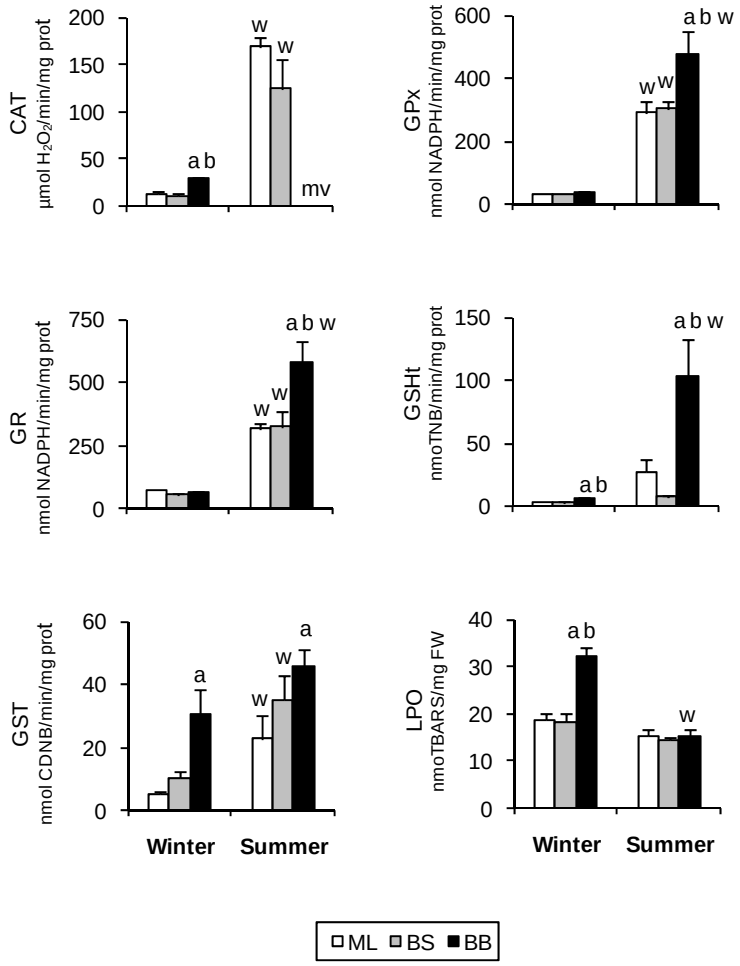

Figure 7.3. Oxidative stress responses in gills of *L. aurata* captured in winter and summer in the middle lagoon (ML) and in the inner branches (Bom-Sucesso – BS, Barrosa - BB) at Óbidos lagoon. Letters denote statistically significant differences ($p<0.05$) between sites (in the same survey) and between surveys (for each site): (a) vs. ML; (b) vs. BS; (w) vs. winter. Means and associated standard errors are given. mv – missed value.

7.3.4 Correlations between parameters and IBR calculation

The significant correlations obtained between metal levels in gills *versus* oxidative stress endpoints are shown in Figure 7.4 (non-significant correlations were not presented). Positive correlations were found between activities of GPx, GR and GST and accumulated levels of Zn, Cd and Cu, respectively. Significant correlations were not found between metals in the water column (DGT-measured fraction and SPM) and accumulated in *L. aurata* gills.

The antioxidants and LPO values measured in gills were processed in order to calculate the IBR index (Figure 7.5). IBR values showed different variation ranges in winter

and summer, being higher in summer. Even so, in both surveys the computed IBR values were always higher at BB than other sites. In particular, the estimated IBR value for BB was 100 times higher than ML in winter, whereas in summer the difference between the two sites was approximately 3 times.

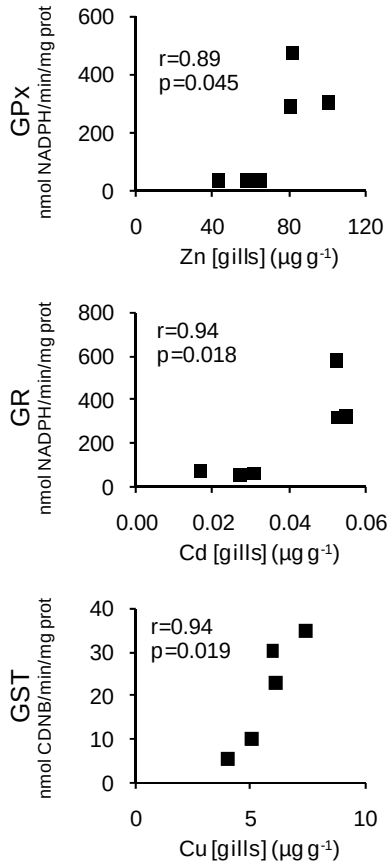

Figure 7.4. Significant correlations between metal levels and oxidative stress responses in gills of *L. aurata*. Statistical significance and correlation coefficient are represented by p and r, respectively. Non-significant correlations are not presented.

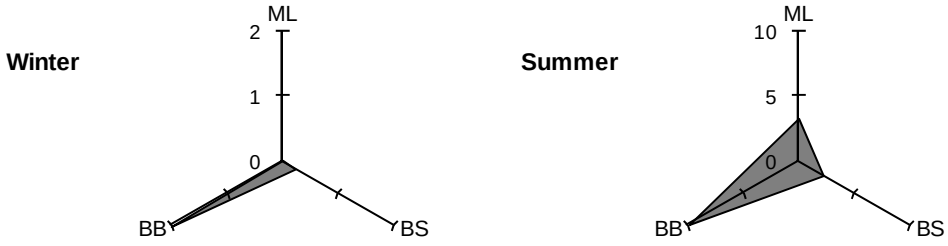

Figure 7.5. Integrated biomarker response (IBR) index in gills of *L. aurata* captured in winter and summer in the middle lagoon (ML) and in the inner branches (Bom-Sucesso – BS, Barrosa - BB) at Óbidos lagoon.

7.4 Discussion

7.4.1 Water quality and metal availability

The accurate consideration of site-specific water quality is of crucial importance to evaluate accumulation and effects of metals in fish (Reynders et al., 2008). Thus, a number of water quality parameters were currently measured at the three sites. In winter and summer BB exhibited a higher metal availability than ML and, in a lower extent, than BS. This was particular evident for Cu in water (DGT-measured fraction) and SPM, Cr in water, as well as Mn, Ni and Cd in SPM. The tributary that discharges at BB branch probably contributed to metal inputs, mainly in periods of higher freshwater flows (Pereira et al., 2009b). In fact, levels of water Cd and Mn (all sites) as well as particulate Pb (all sites) and Cd (BB) were higher in winter than in summer. Conversely, values of Cu (all sites) and Cr (BS, BB) in water were higher in summer than winter, when freshwater flows into the lagoon are almost insignificant. Nickel in particles also increased in summer at BB. These summer increments are probably related with the pulse release of metals from sediments during periods of low oxygenation as indicated by the simultaneous undersaturation of oxygen recorded mainly at BB. This internal source was already identified in other eutrophic coastal lagoons (Point et al., 2007) and hypothesized to occur in BB branch (Pereira et al., 2009b). The prominence of these sediment-related processes for the water quality is probably lower at BS and ML than at BB due to a better trophic state of these two areas (Pereira et al., 2009b). In fact, BB was already proposed as a major impacted area in the lagoon being classified as eutrophic (Pereira et al., 2009a). Accordingly, BB exhibited the highest levels of nutrients and chlorophyll *a* in both seasons. In wet seasons, the tributary that discharges at BB branch could introduce large amounts of nutrients into that area of the lagoon (Pereira et al., 2009a). Since PO_4^{3-} regeneration could occur in organically-rich sediments with upwards diffusion during periods of low oxygenation (mainly in summer), this should be considered as an additional input to the water column at BB as previously recorded (Pereira et al, 2009b).

7.4.2 Metal levels in *L. aurata* gills and associations with environmental factors

Gills are the first organ to be in contact with water and resuspended sediment particles, so they can be relevant sites of interaction with metal ions (Fernandes et al., 2007). Increased Cu, Cr and Cd concentrations in gills from BB in winter reflected higher metals uptake from water column, as previously observed in gills of other fish species

(Reynders et al., 2008). In fact, Cu, Cr and Cd in gills were higher at BB than ML in winter, which is in line with differences recorded in SPM and water (DGT-measured fraction). The Pb enhancement at BS in winter (in comparison with ML) is also probably a response to a higher availability as suggested by levels in SPM. Nevertheless, Pearson analysis did not provide any association between accumulated metals in gills and the corresponding levels in water or particles. Since data of the two seasons were considered jointly in Pearson analysis and the associations above discussed did not occur in summer, non-significant correlations were obtained.

Gills from BB and BS showed higher contents of Zn in winter but this was not accompanied by increases in water or SPM, which points to other explaining hypothesis for accumulation rather than Zn availability. This discrepancy was already observed by other authors for essential elements in fish under field exposure (Demirak et al., 2006; Reynders et al., 2008). Reynders et al. (2008) hypothesised that dissolved Cd could have disturbed metabolism of an essential element (Cu in that case), leading to its redistribution among organs. Accordingly, an identical process could be occurring for Zn (also essential metal) in *L. aurata* from the Óbidos lagoon in winter. An inconsistency between environmental data and accumulation was also found in summer for Cu and Cr. Despite the higher availability of those elements at BB their uptake and/or accumulation was lower than other sites. In the particular case of Cu, it was found that uptake by fish gills is strongly influenced by competing ions (like Ca^{2+} and H^{+}) presenting in the surrounding medium, as well as by dissolved organic matter and water pH (Playle, 1998). Moreover, it was demonstrated that Ca^{2+} and dissolved organic matter could prevent the binding of Cu to the gills (Playle, 1998).

Manganese accumulation in gills did not reflect the spatial differences recorded in water or particles in summer. These results suggested that *L. aurata* was able to regulate tissue Mn concentrations not evidencing the differences recorded in the environment. This was previously reported in the Óbidos lagoon for crabs (Pereira et al., submitted). Indeed, the concentrations of essential metals (as Mn) in organisms tend to be highly regulated compared to nonessential. Fish, in particular, can use distinct strategies of metal homeostasis to achieve a steady-state balance: reducing metal accumulation and toxicity including uptake inhibition, increased elimination and detoxification, and storage (Fernandes et al., 2007).

In general, gills exhibited higher metal contents in summer. For instance, Cr was higher in summer (ML and BS) in line with the enhancement recorded in water (DGT-measured fraction). Identically, a higher availability could also be on the basis of Cu enhancement in gills of summer at BS. However, gills levels of Zn and Cd increased in summer in opposition to levels relatively constant in the environment or enhancements in winter (respectively). Considering the essentiality of Zn (Fernandes et al., 2007), the enhancement of its uptake and subsequent accumulation in gills could represent an adaptation strategy in order to compensate a higher requirement during the summer period. Nonetheless, it is well known that the rates of metal uptake and accumulation increase with increasing temperature in ectothermal organisms (Sokolova and Lannig, 2008). An increase in metabolic rates at elevated temperatures may contribute to metal accumulation in ectotherms due to a higher energy demand, which results in elevated ventilation (Pörtner, 2002). Under a moderate contamination as in the Óbidos lagoon, the seasonal difference of metabolism had probably superimposed environmental availability of metals, explaining the scarcity of inter-site differences in gills metal load observed in summer.

7.4.3 Oxidative stress in *L. aurata* gills and causative factors

7.4.3.1 Spatial variations

Enzymatic antioxidant responses showed that fish are suffering from pro-oxidant challenges at BB in winter and summer. This site presents compounds that stimulate the production of H_2O_2 as depicted by the increase of CAT (winter) and GPx activities (summer). Hydrogen peroxide is the main cellular precursor of the hydroxyl radical that is the most toxic ROS. Those enzymes are known to protect the cell by reducing H_2O_2 to H_2O. The induction of both enzymes has been described previously in gills of *L. aurata* exposed to contaminants under field conditions (Oliveira et al., 2009). In particular, metals were previously associated with CAT induction in fish gills (Fernandes et al., 2008). In the Óbidos lagoon, CAT enhancement at BB in winter may be associated with higher accumulation of metals (particularly Cu, Cr and Cd) but this was not confirmed by Pearson analysis. On the other hand, it is improbable that GPx induction at BB in summer could be due to accumulated metals since substantial increases were not recorded in gills. In this direction, the significant correlation found between GPx and Zn accumulated in gills (provided by data of the two seasons jointly) has a limited value in establishing causal relationships

strictly in summer. It is more plausible that other stressors rather than metals could be on the basis of GPx induction in summer. The broad fluctuations of dissolved oxygen between day (180%) and night (30%) that were recorded in summer (Pereira et al., 2009a) could lead to an activation of oxidative stress defences. In fact, it is well known that organisms exposed to fluctuating levels of oxygen are subjected to marked oxyradical burst, which can be counteracted by elevated levels of antioxidants to prevent oxidative damage (Storey, 1996).

GR plays a major role in GPx and GST reactions as an adjunct in the control of peroxides and free radicals by maintaining the GSH redox status (Bompart et al., 1990). Thus, exposure to pro-oxidant stressors leads to elevated activities of GR in aquatic organisms (Regoli et al., 2002) as in *L. aurata* gills (Oliveira et al., 2009). This is also occurring at BB site of the Óbidos lagoon in summer but was not associated with concomitant elevation of metals in the gills. The high GR activity at BB reflects increased glutathione recycling (replenishing GSH in order to avoid its depletion), suggesting that the ratio GSSG/GSH has been increased as consequence of pro-oxidants. As stated for GPx induction at BB in summer, other stressors rather than metals are probably on the basis of GR response. Nevertheless, GR was correlated with gills accumulated Cd. As previously mentioned for GPx, this correlation has limited interest in explaining summer responses.

In response to environmental changes, GSTs (a multigenic superfamily of multifunctional enzymes) may play a dual protective role associated to their activity on conjugation of electrophilic compounds (or phase I metabolites) with GSH (Van der Oost et al., 2003) and to a direct antioxidant action carried out by GST α-class catalyzing the reduction of organic hydroperoxides by GSH (Wang and Ballatori, 1998). Taking into account that the adopted methodology (using CDNB, which is conjugated by all GST isoforms with the exception of the q-class) determines total GST activity, it is not easily perceptible the meaning of the observed GST activity increase at BB than other sites in summer. When searching correlations by Pearson analysis, the GST induction was correlated with levels of Cu in the gills. In fact, several studies showed that exposure to metals can lead to an increase of GST in liver (Amado et al., 2006; Guilherme et al., 2008; Oliveira et al., 2008) and, in a lower extent, in gills (Dautremepuits et al., 2009).

GSH act against ROS protecting the cells from the pernicious effects of metals and other electrophilic compounds (Wang and Ballatori, 1998). In this study, GSH_t was higher at BB in winter and summer, which indicates the presence of contaminants challenging cell

defences depending on this thiol. Indeed, the variation of the cellular glutathione status in fish is considered an indicator of the degree and duration of exposure to oxidant pollutants (Dautremepuits et al., 2009). The protective and adaptive role of GSH was previously observed in *L. aurata* gills from a contaminated ecosystem (Oliveira et al., 2009). Despite Pearson analysis did not provided any relationship between accumulated metals and GSH_t, it should be hypothesised the interference of metals (mainly Cu, Cr and Cd) in winter. In fact, metal metabolism involves the formation of GSH-metal complexes (Atli and Canli, 2008). However, in summer other compounds are probably challenging cell defences depending on this thiol. Elevated GSH_t levels represent a great ability to destroy ROS and are a result of a transfer from other organs (Oliveira et al., 2008). Indeed, most of the GSH is synthesized in liver and then exported to other tissues (Deneke and Fanburg, 1989). In addition, the concomitant increase of GR and GSH_t (summer) indicates that the turnover of reduced glutathione pools was not enough to maintain the redox cycling and cell homeostasis, forcing to a translocation from other organs to the gills. Moreover, this evidence of high GSH requirements is in agreement with the elevated GPx and GST activities concomitantly observed.

Free radical reactions in biological membranes could form lipid hydroperoxides that decompose double bonds of unsaturated fatty acids, destructing lipid membranes (Van der Oost et al., 2003). This complex process is known by lipid peroxidation. Our study revealed higher LPO at BB in winter, which is an indication of oxidative stress. This suggests that the observed antioxidant defences mobilization was not able to prevent gill LPO induced probably by metal exposure in winter. Nevertheless, no associations were established between LPO and accumulated metals.

The higher levels of NH_4^+, NO_3^-+ NO_2^- and PO_4^{3-} registered at BB in winter could be associated with the observed oxidative stress responses. In fact, it has been suggested that ammonia and nitrite can be important pro-oxidants in fish, mussels and aquatic plants, leading to nitric radicals' production (Lima et al., 2006; Nimptsch and Pflugmacher, 2007). At the Óbidos lagoon, the importance of those compounds on oxidative stress induction in crabs was previously invoked (Pereira et al., 2009d).

By combining the different biomarker signals, the IBR index provides a simple mean for a general description of populations' health status (Broeg and Lehtonen, 2006). In the present work, IBR substantiated the evidence that BB is the most impacted site of the lagoon.

7.4.3.2 Seasonal variations

In general, oxidative stress responses were enhanced in summer, namely activities of CAT, GPx, GR and GST and GSH_t content. The sole exception to this temporal pattern occurred for LPO at BB. It should be also emphasise that, despite the higher values in summer, GST and GSH_t were the only endpoints exhibiting and identical spatial patterns in both seasons (characterised by increases at BB).

Metal levels in tissues are likely to change with the season, reflecting variability in the environmental inputs, but also changes in the metabolism (Regoli, 1998). Herein, seasonal variations of metals in gills were mainly associated with the effect of temperature on metals uptake, which enhanced in summer. Maximum enzymatic activities and GSH_t content were recorded in summer when higher levels of Zn and Cd were found (all sites; ML and BS, respectively). These results point to a major importance of metals for the recorded temporal variations.

Nonetheless, since water temperature rose 11 to 13 °C in summer in relation to winter, its interference on oxidative stress responses must be hypothesised. A previous work carried out at the Óbidos lagoon, with crabs, also demonstrated activation of oxidative stress responses in summer (Pereira et al., submitted). Low temperature reduces the metabolic rates in ectothermal organisms, and hence lower enzymatic activities are in general observed in colder seasons. This was reported for CAT and GST in fish (Gorbi et al., 2005; Amado et al., 2006). Moreover, higher temperature lead to an increase in oxygen consumption and consequently to an enhancement of ROS generation (Amado et al., 2006). Most aquatic organisms are able to adjust antioxidant defences as a mechanism to temperature adaptation (Filho et al., 2001). The current GSH_t increase in summer is in agreement with previous studies with ectothermal marine animals (Heise et al., 2006). This variation was related to spontaneous, nonenzymatic GSH oxidation by emerging ROS under hyperthermia. Elevated temperatures accelerate mitochondrial respiration and increase mitochondrial ROS formation (Heise et al., 2003). As the current summer GSH_t increase was only measured at BB, it appears as an indication that glutathione pool responds to stressful conditions particularly in that area of the lagoon.

Conversely to other oxidative stress endpoints, LPO was enhanced in winter reinforcing the hypothesis of metals is inducing responses in this season.

The computed IBR values were higher in summer than winter at all sites, which is in agreement with the conclusions provided by the analysis of individual oxidative stress

endpoints. Nevertheless, an identical configuration of star plots representing IBR was found in winter and summer, highlighting BB as a major impacted area in the Óbidos lagoon. Despite this clear conclusion for both seasons, IBR seemed to have a higher capacity to discern sites in winter than in summer. In fact, the estimated IBR value for BB was 100 times higher than ML in winter, whereas in summer the difference between the two sites was approximately 3 times. Interestingly, it was in winter that spatial differences in individual oxidative stress endpoints were associated with a higher metal accumulation. In general, IBR represented a potential measure of *L. aurata* health at the Óbidos lagoon, providing a better evidence of the existing impact, as previously reported in that (Pereira et al., 2009c) and other systems (Beliaeff and Burgeot, 2002).

7.5 Conclusions

Results of this work provided these main findings:

- Oxidative stress responses of *L. aurata* gills were able to detect inter-site differences pointing BB as a critical area in the Óbidos lagoon, which was confirmed by a general stress index (IBR). Higher levels of accumulated Cu, Cr and Cd in winter could be on the basis of the pro-oxidant challenge at BB.

- Winter-summer variations were prevalent in gills of *L. aurata*, which exhibited a higher accumulation of metals in summer as well as increases of oxidative stress responses. IBR values corroborated a higher stress syndrome in summer at all sites than in winter. Nevertheless, peroxidative damage occurred at BB only in winter despite the activation of antioxidant defences. In terms of metals accumulation, gills were more responsive in winter than summer, while oxidative stress endpoints provided an evidence of existing impact at BB in both seasons.

- The applied approach combining biomarkers in gills (metal levels and oxidative stress) and environmental data (mainly metals and nutrients) demonstrated to be useful in the environmental health assessment, particularly under a moderate contamination scenario Moreover, based on its confirmation as an important route of entry for contaminants and the currently demonstrated ability to reflect environmental status (through metal bioaccumulation and oxidative stress responses), gills can be considered as a “mirror door” in the context of water contamination assessment.

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CHAPTER VIII

The relevance of temporal and organ specific factors on metals accumulation and biochemical effects in native fish (*Liza aurata*) under a moderate contamination scenario

8 The relevance of temporal and organ specific factors on metals accumulation and biochemical effects in native fish (*Liza aurata*) under a moderate contamination scenario

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ABSTRACT

Moderate contamination scenarios are nowadays challenging ecotoxicologists. An investigative biomonitoring study was performed under those environmental conditions (Óbidos lagoon, Portugal) focused on oxidative stress and accumulated metals in *Liza aurata* liver and kidney, also examining winter-summer variations and organ-specificities. Three sites were surveyed: Barrosa; Bom-Sucesso; Middle lagoon. The higher metal availability at Barrosa (in water and sediment) was reflected in both organ burdens, though liver was more responsive. Oxidative stress (both organs) pointed to a pro-oxidant challenge at Barrosa (both seasons), reinforced by a general stress index. In winter, oxidative stress at Barrosa was related with accumulated metals in both organs, while in summer this cause-effect relationship was only established for kidney since changes in liver were linked with non-contaminant related variables. Winter-summer variations were outstanding in liver and kidney oxidative stress endpoints, pointing to the relevance of considering distinct seasons and organs in the assessment of moderately contaminated systems.

KEYWORDS: Oxidative stress; Fish; Coastal lagoon; Temporal variability; Organ specificities

8.1 Introduction

Most of the investigative biomonitoring studies devoted to aquatic environment were carried out in ecosystems suffering from severe contamination (Gorbi et al., 2005; Kraemer et al., 2005; Amado et al., 2006; Guilherme et al., 2008; Reynders et al., 2008) or after spill accidents (Martínez-Gómez et al., 2006). These conditions represent an opportunity to test organisms' sensitivity when exposed to high levels of hazardous compounds under natural conditions. Regardless their ubiquity worldwide, systems with low to moderate levels of contamination were much less studied (Orbea et al., 2005; Sanchez et al., 2008). This emerging concern represents an adjustment on the society exigency related to environmental protection and a challenging scenario for ecotoxicologists due to the occurrence of subtle responses. Additionally, non-contamination-related variables like those associated with the season, assume high relevance under moderate levels of contamination (Orbea et al., 2005) and should be considered when interpreting the biological effects (Van der Oost et al., 2003).

Contaminants occur in the environment as complex mixtures with interactive effects, making the use of biomarkers particularly relevant for environmental health assessment (Orbea et al., 2005). In order to properly evaluate the effects of contaminants in organisms, the complementation of bioaccumulation with effect biomarkers is strongly recommend (Fernandes et al., 2008). Among the wide range of effect biomarkers pointing to environmental contamination, oxidative stress responses have been widely studied. In fact, contaminants can trigger oxidative stress by generation of reactive oxygen species (ROS) (Lesser, 2006). ROS can be detoxified by an enzymatic defence system, which includes superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and glutathione reductase (GR) (Van der Oost et al., 2003). Glutathione-S-transferases (GST) constitute a family of multifunctional phase II biotransformation enzymes, which occurs in the cytosol of most cells and catalyze the conjunction of glutathione to a variety of compounds. Moreover, lipid peroxidation (LPO) is an expression of oxidative damage, indicating that ROS production exceeded antioxidant defences (Ahmad et al., 2006; 2008). Changes in the levels of these oxidative stress endpoints are sensitive indicators of environmental disturbances (Orbea et al., 2005; Fernandes et al., 2008; Guilherme et al., 2008; Oliveira et al., 2009), which can be particularly useful when searching biological effects under a moderate contamination scenario.

Fish are generally considered to be suitable sentinel organisms for environmental health assessment (Van der Oost et al., 2003), being also successfully adopted under moderate contamination scenarios (Orbea et al., 2005; Sanchez et al., 2008). Liver is the most studied organ concerning both contaminants accumulation and oxidative stress endpoints in fish (Orbea et al., 2005; Amado et al., 2006; Guilherme et al., 2008; Reynders et al., 2008). The higher incidence of this choice is related with its multi-functionality and primary role in the metabolism of xenobiotics, essential for inactivation and detoxification of absorbed contaminants (Triebskorn et al., 1997). Particularly, it is well known that a large amount of metal sequestration occurs in fish liver associated with metallothioneins production (Olsvik et al., 2001). Fish kidney can present toxicant metabolizing rates (Ortiz-Delgado et al., 2008) and metal accumulation potential comparable to those of liver (Mieiro et al., 2009). Moreover, kidney plays a key role in the maintenance of homeostasis being a major route for elimination and rapid clearance of xenobiotics. Additionally, kidney has haematopoietic, endocrine and immune functions. Despite its relevance on fish physiology, kidney has been underemployed in environmental health assessment and, to our knowledge, none ecotoxicology study had considered liver and kidney simultaneously. Moreover, the application of an approach using biomarkers (i.e. tissue burdens and biochemical responses) combined with environmental data is still scarce.

The Óbidos lagoon (Portugal) is impacted by eutrophication and moderate metal levels due to the longstanding discharges of agricultural, livestock and domestic sewage. In that way, this coastal lagoon could represent an ecosystem-prototype of those environmental problems, as pointed previously by studies with *Ulva* sp. and *Carcinus maenas* (Pereira et al., 2009a; 2009b). Despite the high ecological relevance of fish, their adoption on an ecotoxicological point of view was never considered before at the Óbidos lagoon. Hence, the present work aimed to study metal accumulation and biochemical effects in native golden grey mullet (*Liza aurata*), coupled with water/sediment quality assessment. It was also intended to evaluate the influence of seasonal variations of metal availability as well as organ specific biomarkers responses. To accomplish these goals, three sites of the lagoon were surveyed in two contrasting seasons (winter and summer) and metal concentrations, enzymatic and non-enzymatic antioxidants, as well as LPO were quantified in liver and kidney of fish. Finally, it was used a biomarker integration index (IBR) in order to have a better evidence of the existing impact of contamination in fish liver and kidney.

8.2 Material and Methods

8.2.1 Study area

The Óbidos lagoon is a shallow coastal ecosystem, located on the west coast of Portugal with a wet area of 7 km^2, permanently connected to the sea through a narrow inlet (Figure 8.1). It comprises areas of different morphological and sedimentary characteristics: sand banks and narrow channels in the lower/middle lagoon; muddy bottom sediments in the two inner branches (Barrosa and Bom-Sucesso). The Barrosa branch is shallower (mean depth 0.5-1 m) and water circulation is mostly driven by tides and by a small tributary (Cal River) that drains agriculture fields. Urban effluents from a nearby town (Caldas da Rainha, 50,000 inhabitants) also have been discharged to the Barrosa branch by the Cal River. Consequently, this area presents the highest nutrient availability of the lagoon, being classified as eutrophic (Pereira et al., 2009c). High nutrient concentrations were in line with abundant macroalgae (*Ulva* sp. and *Enteromorpha* sp.) and a broad daily variation of dissolved oxygen concentration during the summer months (Pereira et al., 2009d). Previous water quality surveys of the lagoon showed maximum concentrations of Mn, Ni, Cu and Cd in Barrosa branch. Major metal sources were identified to be the Cal River in periods of higher inflow and remobilization from sediments in summer months (Pereira et al., 2009d). The Bom-Sucesso branch is also a confined area but receives a smaller freshwater flow (Vala do Ameal) with better water quality than the Cal River, according to the Portuguese categorization of freshwater systems. In this area, metal remobilization from sediments is less plausible due to the higher depths and stronger currents. The middle lagoon was previously characterised by coarser sediments with a low affinity for metals (Carvalho et al., 2006), as well as by a better water quality (Pereira et al., 2009c). Moreover, previous ecotoxicological studies with *Ulva* sp. and *Carcinus maenas* pointed out Barrosa branch as a critical area at the lagoon with potential impact on autochthonous populations, whereas the lower/middle lagoon was considered the reference area (Pereira et al., 2009a; 2009b).

8.2.2 Sampling

Two surveys were carried out at Óbidos lagoon in winter (December 2006) and summer (July 2007) and 10 juvenile specimens of the golden grey mullet (*Liza aurata*) were collected during low-tide using a traditional beach-seine net named “chincha”. Previous studies used successfully *L. aurata* as a model on field studies (Guilherme et al.,

2008; Oliveira et al., 2009) and under laboratory conditions (Cionna et al., 2006; Oliveira et al., 2007). This species shows potential as a sentinel because is abundant, adaptable to different environmental conditions, easy to catch and relatively sedentary among fishes. As a benthopelagic species, is in frequent contact with sediments, feeding on small benthic organisms and detritus. In addition, mullets play an important role in the estuarine trophic web (Almeida, 2003).

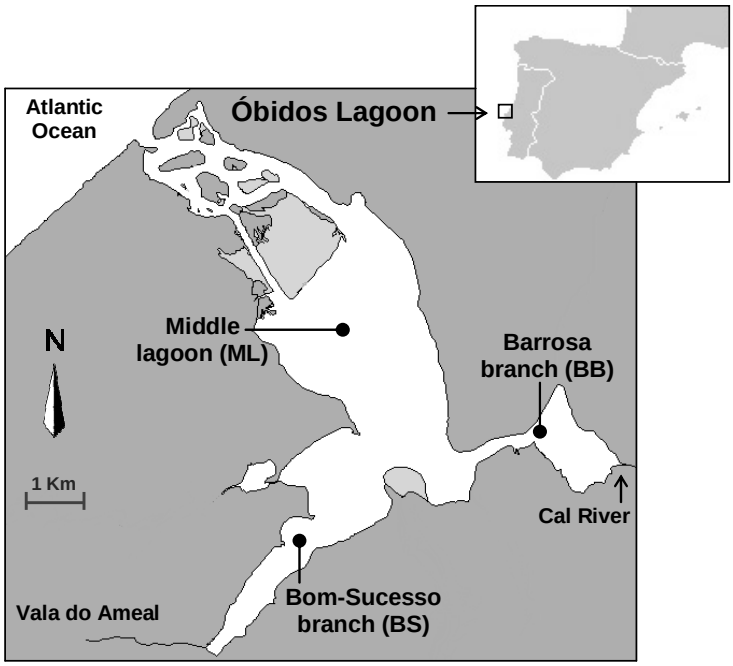

Figure 8.1. Location of the sampling sites at the Óbidos lagoon: middle lagoon (ML) (39º24'50.48''N, 9º12'42.82''W); inner branches Bom-Sucesso (BS) (39º23'44.88''N, 9º13'01.75''W) and Barrosa (BB) (39º26'16.84''N, 9º11'33.18''W).

Three sampling areas were selected taking into account previous ecotoxicological studies (Pereira et al., 2009a, 2009b) (Figure 8.1): Middle lagoon (ML) assumed as reference area; Bom-Sucesso (BS) and Barrosa (BB) located in the inner branches of the lagoon. Fish biometrical parameters, such as weight and length ranged from 20-40 g and 13-17 cm. Juveniles were selected in order to aside the gender interference and diminish the background of previous exposures to contaminants (Ahmad et al., 2008). Immediately after catching, fish were sacrificed and liver was removed and divided in two sets of samples: one for oxidative stress quantifications (n=10) and other for metal determinations (n=10). Kidney was also removed but, due to its lower mass, was handled differently; kidney of five individuals was used for oxidative stress quantifications (n=5),

while those of the remaining five fish allowed metal determinations (n=5). All tissue samples were immediately frozen in liquid nitrogen. In the laboratory, samples were preserved until further processing at -80 and -20 °C for oxidative stress and metal determinations, respectively.

Surface water (0.2 m depth) and surface sediments were sampled in the three areas for determinations of metals. Moreover, water was also surveyed for nutrients and chlorophyll *a*.

8.2.3 Determination of physicochemical parameters and metals in water

Water temperature, salinity and dissolved oxygen were measured *in situ* using an YSI, 650 meter. Water samples for determinations of nitrate+nitrite (NO_3^-+NO_2^-), ammonium (NH_4^+), phosphate (PO_4^{3-}) and silicate $Si(OH)_4$ were filtered through MSI Acetate Plus filters and analyses carried out using an autoanalyser TRAACS 2000 (Bran+Luebbe). For chlorophyll *a* determinations, water was filtered through a Whatman GF/F (0.7 µm) filter that was immediately frozen at -20 °C and later extracted in 90% acetone, for analysis in a Perkin Elmer Fluorometer using the protocol modified by Lorenzen (1966).

Manganese, Cu, Ni, Cr, Pb and Cd in the collected waters (triplicate samples) were measured using diffusive gradients of thin films (DGT). All DGT holders, Chelex-100 resins and diffusive gels (type APA, 0.8 mm thickness, open pore> 5 nm) (Zhang and Davison, 1999) were purchased from DGT Research (Lancaster, UK). The DGT devices were deployed in 2 L polypropylene bottles with unfiltered sampled water and stirred at constant temperature for 48 hours. After devices retrieval, resins were eluted by immersion in 5 mL of 1 M HNO_3 (prepared from suprapur nitric acid) at a minimum of 24 h. Eluates were analysed directly by a quadropole ICP-MS (Thermo Elemental, X-Series). All eluates were analysed with reagents blanks and an international standard of river water (SLRS-4) used to control eventual contaminations during the analytical procedure and the procedure accuracy, respectively. The eluate concentration was converted into the mass of metal accumulated on the resin using an elution factor with a yield value of 0.8 (Zhang and Davison, 1995) and a resin gel volume of 0.15 mL. DGT-metal concentrations were calculated according to the following equation: $[Metal]=(M\Delta g)/(tAD)$; where M is the mass of metal accumulated on the resin during the emersion time (t); Δg the diffusive gel

thickness (0.08 cm); A the exposure area (3.14 cm^2); and D the diffusion coefficient of the metal in the gels as provided by DGT Research.

Water was collected in triplicates for separation of suspended particulate matter (SPM) that was retained on 0.45 µm membranes. SPM and sediment samples (100 mg) were mineralized completely with HF (40%) and Aqua Regia (HCl-36%:HNO_3-60%; 3:1) in closed Teflon bombs (100 °C for 1 h), evaporated to near dryness (DigiPrep HotBlock – SCP Science), redissolved with 1 mL of doubled-distilled HNO_3 and 5 mL of ultra-pure water, heated for 20 min at 75 °C, and diluted to 50 mL with ultra-pure water. Reagents blanks and international certified standards of sediments from the National Research Council of Canada (1646a; BCSS-1; MESS-3) were prepared in a similar way of samples to control the accuracy of the procedure. The concentrations of Mn and Zn were determined by flame atomic absorption spectrometry (Perkin Elmer Analyst 100) whereas Cu, Ni, Cr, Pb and Cd were determined by ICP-MS. Levels of the analysed elements obtained in the reference materials were consistently within the ranges of certified values.

8.2.4 Metal analyses in liver and kidney

Freeze dried tissue was digested with a mixture of HNO_3 (doubled distilled from 65%) and H_2O_2 (suprapure, 30%) at 60 °C for 12 h, at 100 °C for 1 h and at 80 °C for 1 h according to the method described in Pereira et al. (2009e). Procedural blanks were prepared using the same analytical procedure and reagents. Concentrations of Mn, Zn, Cu, Ni, Cr, Pb and Cd were determined by ICP-MS. International certified standards (TORT-2, DOLT-2 and DOLT-3) were use to control the accuracy of the analytical procedure.

8.2.5 Biochemical analyses in liver and kidney

Tissue handling and preparation of post-mitochondrial supernatant (PMS) fraction - Tissue samples were homogenized, using a Potter-Elvehjem homogenizer, in chilled phosphate buffer (0.1 M, pH 7.4) (0.1 g of liver/1 ml buffer and 0.1 g of kidney/2 ml buffer). This homogenate was then divided in two aliquots for LPO and PMS preparation. The PMS preparation was obtained by centrifugation in a refrigerated centrifuge (Optima TL, Beckman) at 13,400 g for 20 min at 4 °C. Aliquots of PMS were divided in microtubes and stored at -80 °C until analyses.

Estimation of lipid peroxidation (LPO) - LPO was determined in the previously prepared homogenate as adapted by Filho et al. (2001). Briefly, to 150 µl of homogenate, 10 µl of BHT (4% in methanol) was added and mixed well to prevent oxidation. The absorbance of each sample was measured at 535 nm. The rate of LPO was expressed in nmol of TBARS formed per milligram of fresh tissue using a molar extinction coefficient of $1.56x10^5$ $M^{-1}cm^{-1}$.

Catalase (CAT) activity measurement - CAT activity was assayed in PMS by Claiborne (1985) method (at 25 °C). Briefly, the assay mixture consisted of 1.95 ml phosphate buffer (0.05 M, pH 7.0), 1 ml hydrogen peroxide (0.019 M) and 50 µl of sample in final volume of 3 ml. Change in absorbance was recorded at 240 nm and CAT activity was calculated in terms of µmol H_2O_2 consumed $min^{-1}mg^{-1}$ protein using a molar extinction coefficient of 43.5 $M^{-1}cm^{-1}$.

Glutathione peroxidase (GPx) activity measurement - GPx activity was determined in PMS according to the method described by Mohandas et al. (1984) with some modifications (at 25 °C). The assay mixture consisted of 0.72 ml phosphate buffer (0.05 M, pH 7.0), 0.05 ml EDTA (1 mM), 0.05 ml sodium azide (1 mM), 0.025 ml GR (1 IU/ml), 0.05 ml GSH (4 mM), 0.05 ml NADPH (0.8 mM), 0.005 ml H_2O_2 (1.0 mM) and 0.05 ml of PMS in a total volume of 2 ml. Oxidation of NADPH was recorded at 340 nm, and GPx activity was calculated in terms of nmol NADPH oxidized $min^{-1}mg^{-1}$ protein using a molar extinction coefficient of $6.22x10^3$ $M^{-1}cm^{-1}$.

Glutathione reductase (GR) activity measurement - GR activity was assayed by the method of Cribb et al. (1989) with some modifications (at 25 °C). Briefly, the assay mixture contained 0.025 ml of PMS fraction and 0.925 ml of NADPH (0.2 mM), GSSG (1 mM) and DTPA (0.5 mM). The enzyme activity was quantified by measuring the disappearance of NADPH at 340 nm during 3 min. The enzyme activity was calculated as nmol NADPH oxidized min-1mg-1 protein using a molar extinction coefficient of 6.22×10^3 $M^{-1}cm^{-1}$.

Glutathione-S-transferase (GST) activity measurement - GST activity was determined using CDNB (1-chloro-2,4-dinitrobenzene) as substrate according to the method of Habig et al. (1974) (at 25 °C). The assay was carried out in a quartz cuvette with a 2 ml mixture of

phosphate buffer (0.2 M, pH 7.4), CDNB (0.2 mM) and 0.2 M GSH. The reaction was initiated by addition of 10 µl of PMS and the increase in absorbance was recorded at 340 nm during 3 min. The enzyme activity was calculated as nmol CDNB conjugate formed $min^{-1}mg^{-1}$ protein using a molar extinction coefficient of 9.6 $mM^{-1}cm^{-1}$.

Total glutathione (GSH_t) content measurement - Protein content in the tissue homogenate was precipitated with TCA (5%) for 1 h and then centrifuged at 13,400 g for 20 min at 4 °C. GSH_t content was determined in the resulting supernatant (deproteinated PMS) (at 25 °C) adopting the enzymatic recycling method using glutathione reductase (GR) excess, whereby the sulfhydryl group of GSH reacts with DTNB (Ellman's reagent) producing a yellow coloured 5-thio-2-nitrobenzoic acid (TNB). The rate of TNB production is directly proportional to this recycling reaction, which is in turn directly proportional to the concentration of GSH in the sample. Formation of TNB was measured by spectrophotometry at 412 nm. It should be noted that GSSG is converted to GSH by GR in this system, which consequently measures total GSH. The results were expressed as nmol TNB formed min^{-1} mg^{-1} protein using a molar extinction coefficient of 14.1×10^3 $M^{-1}cm^{-1}$.

Protein measurement - Total protein contents were determined according to the Biuret method (Gornall et al., 1949), using bovine serum albumin (E. Merck-Darmstadt, Germany) as a standard.

8.2.6 Statistical analyses

A method for combining all the measured oxidative stress endpoints into a general index - Integrated Biomarker Response (IBR) (Beliaeff and Burgeot, 2002) - was applied for data of both surveys. The basis of data processing is described here briefly. First, the mean value (X_i) for each biomarker at each sampling area and season was calculated. In addition, the general mean (m_i) and standard deviations (s_i) of each biomarker were estimated for all sampling areas and seasons. The value of X_i was then standardized to obtain Y_i, where $Y_i= (X_i-m_i)/s_i$. Then Z_i was computed via the equation $Z_i= -Y_i$ or $Z_i= Y_i$ in the case of a biological effect corresponding, respectively, to inhibition or activation. The minimum value (Min_i) of Z_i for each biomarker was calculated for all sampling areas and seasons, and then the score S_i was computed as $S_i = Z_i+|Min_i|$, where $|Min_i|$ is the absolute value. Finally, IBR for each sampling site and season was calculated via de following formula: IBR=

$(S_1xS_2)/2 + (S_2xS_3)/2 +…+ (S_{n-1}xS_n)/2 + (S_nxS_1)/2$; in which the obtained score for each biomarker (S_i) is multiplied with the score of the next biomarker (S_{i+1}), arranged as a set, dividing each calculation by 2 and summing-up of all values.

Statistical software (Statistica 7.0) was used for statistical analyses. The assumptions of normality and homogeneity of data were verified. ANOVA analysis was used to compare sampling sites and Tukey test was applied for Post-hoc comparison (Zar, 1996). Differences between means were considered significant when $p<0.05$. The correlation between metals in the water column (DGT-measured and SPM fractions) and accumulated in liver and kidney, as well as between accumulated metals and oxidative stress responses in liver and kidney was tested (considering the mean values of the two surveys at each site) by Pearson analysis ($p<0.05$).

8.3 Results

8.3.1 Water and sediment characteristics

Surface sediments from BB presented slightly higher levels of Mn, Zn, Cu, Pb and Cd than other sites (Table 8.1). Chromium and Ni in sediment showed minor inter-site differences. The intervals obtained reflected differences on sediment properties within each surveyed site rather than seasonal fluctuations.

Water temperature was slightly higher at BB and BS in summer and winter, while salinity was lower at BB than at the other sites, particularly in winter (Table 8.2). In general, dissolved oxygen was around 100% but undersaturation was recorded at BB in summer and BS in winter. In general, the maximum values of nitrogenous compounds, $Si(OH)_4$, PO_4^{3-} and chlorophyll *a* were recorded at BB in both surveys. NH_4^+, NO_3^-+NO_2^- and $Si(OH)_4$ exhibited a typical temporal fluctuation in the lagoon with lower values in summer.

Table 8.3 presents the levels of metals in water (DGT-measured fraction). Generally, BB presented higher levels of metals in winter than the other sites. In summer, a similar pattern was observed for Cu, Pb and Cd. Moreover, Cr was higher at BB and BS than at ML. Concerning temporal differences, higher levels of Mn and Cd were found in winter, while Cu, Cr and Pb enhanced in summer.

Manganese, Cu, Ni, Cr and Cd in SPM collected in winter were higher at BB, whereas Zn increased at ML and Pb at BS (Table 8.4). In summer, Cu, Ni, Cr and Pb in SPM peaked at BB, while SPM from ML contained higher levels of Mn, Zn and Cd than other sites. Moreover, Ni increased in summer in relation to winter (BB), as well as Mn (all sites),

whereas Pb (all sites) and Cd (BB) were higher in winter than in summer. A temporal pattern was difficult to discern for Zn, Cu and Cr.

Table 8.1. Ranges of metal concentrations (μg g^{-1}) in surface sediments collected in winter and summer in the middle lagoon (ML) and in inner branches (Bom-Sucesso - BS, Barrosa - BB) at Óbidos lagoon.

Site	Mn	Zn	Cu	Cr	Ni	Pb	Cd
ML	273 - 297	101 - 110	43 - 43	69 - 72	29 - 30	37 - 39	0.17 - 0.22
BS	266 - 285	102 - 104	46 - 54	56 - 69	24 - 28	33 - 38	0.21 - 0.23
BB	284 - 301	112 - 130	57 - 58	71 - 72	29 - 30	44 - 48	0.23 - 0.29

Table 8.2. Water temperature (T), salinity, dissolved oxygen (DO), ammonium (NH_4^+), nitrate+nitrite (NO_3^-+NO_2^-), phosphate (PO_4^{3-}), silicate ($Si(OH)_4$) and chlorophyll *a* (Chl *a*), in winter and summer of low-tide, in middle lagoon (ML) and in inner branches (Bom-Sucesso - BS, Barrosa - BB) at Óbidos lagoon.

Season	Site	T (°C)	Salinity	DO (%)	NH_4^+ (μM)	NO_3^-+NO_2^- (μM)	PO_4^{3-} (μM)	$Si(OH)_4$ (μM)	Chl *a* (μg L^{-1})
Winter	ML	9	34.6	101	2.0	9.2	0.5	6.0	1.7
	BS	11	31.2	87	18	31	2.3	20	0.7
	BB	11	28.1	96	45	40	5.6	41	1.8
Summer	ML	20	34.6	95	1.0	1.0	2.9	5.1	4.9
	BS	23	35.2	108	1.0	1.2	2.1	4.6	3.7
	BB	24	33.3	83	0.9	1.3	6.5	12	10

Table 8.3. Levels of metals in water of low-tide, in winter and summer, in the middle lagoon (ML) and in inner branches (Bom-Sucesso - BS, Barrosa - BB) at Óbidos lagoon. Means and associated standard errors are presented.

Season	Site	Mn (μM)	Cu (nM)	Ni (nM)	Cr (nM)	Pb (nM)	Cd (nM)
Winter	ML	0.22±0.06	2.3±0.14	7.5±2.0	7.5±1.4	0.22±0.06	0.16±0.01
	BS	0.68±0.01	3.9±0.17	7.0±0.85	8.3±0.17	0.13±0.005	0.18±0.01
	BB	1.0±0.55	4.9±0.17	7.7±0.87	8.5±0.17	0.26±0.08	0.24±0.06
Summer	ML	0.13±0.01	3.3±0.21	8.6±2.3	6.4±1.2	0.47±0.12	0.07±0.006
	BS	0.12±0.01	4.9±0.18	6.3±0.81	14±1.9	0.72±0.02	0.09±0.007
	BB	0.04±0.02	7.9±1.0	8.0±0.38	14±1.3	0.99±0.21	0.14±0.03

Table 8.4. Levels of metals (μg g^{-1}) in suspended particles of low-tide, in winter and summer, in the middle lagoon (ML) and in inner branches (Bom-Sucesso - BS, Barrosa - BB) at Óbidos lagoon. Means and associated standard errors are presented.

Season	Site	Mn	Zn	Cu	Ni	Cr	Pb	Cd
Winter	ML	505±30	161±32	29±4.2	22±3.1	55±6.7	40±0.31	0.19±0.08
	BS	458±48	113±9.1	35±12	23±6.7	56±14	50±10	0.18±0.08
	BB	749±18	142±4.1	52±0.35	28±2.0	68±2.6	44±3.4	0.36±0.11
Summer	ML	1515±92	153±3.1	24±3.0	28±3.6	48±0.32	24±1.2	0.20±0.05
	BS	623±9.1	128±17	24±3.5	28±4.1	23±3.9	21±1.5	0.18±0.05
	BB	812±13	134±8.2	52±2.3	61±2.7	51±3.3	31±1.7	0.18±0.01

8.3.2 Metals accumulation in liver and kidney of *L. aurata*

In winter, liver of fish from BB showed significantly higher accumulation of Mn and Cr than ML and BS (Figure 8.2). Moreover, levels of Ni were significantly higher in liver of fish from BB than BS in winter, whereas at BS were recorded Pb and Cd increases (in comparison with ML, and ML and BB, respectively). In summer, liver at BB showed lower levels of Cu and Cd than ML and BS, whereas no inter-site differences were found for the other quantified metals. Less spatial differences were signalised by kidney. Nevertheless, in winter, Mn was also significantly higher at BB (than ML and BS). In summer, levels of Zn and Pb increased at BB (than BS) whereas Cd was lower at BB (than ML).

Differences between the two surveys were found for all metals measured in liver, which presented higher contents of Mn (at BB), Cr (at ML and BB), Pb (at BS) and Cd (at BB) in winter than in summer. Differently, Cu (at ML and BS), Zn (at ML and BB), Ni (at BS) and Cd (at ML) presented higher hepatic accumulation in summer. Seasonal differences were less pronounced in kidney, but the few recorded were identical to those of liver, i.e., Zn was higher in summer (at ML) and Mn was higher in winter (at BS).

Levels of the essential metals Cu and Zn were considerably higher in the liver than in kidney (around 30 and 2 times, respectively), while Mn, Ni, Cr and Pb ranged within similar concentration intervals. Though non-essential, Cd was higher in liver (2 - 8 times) than in kidney.

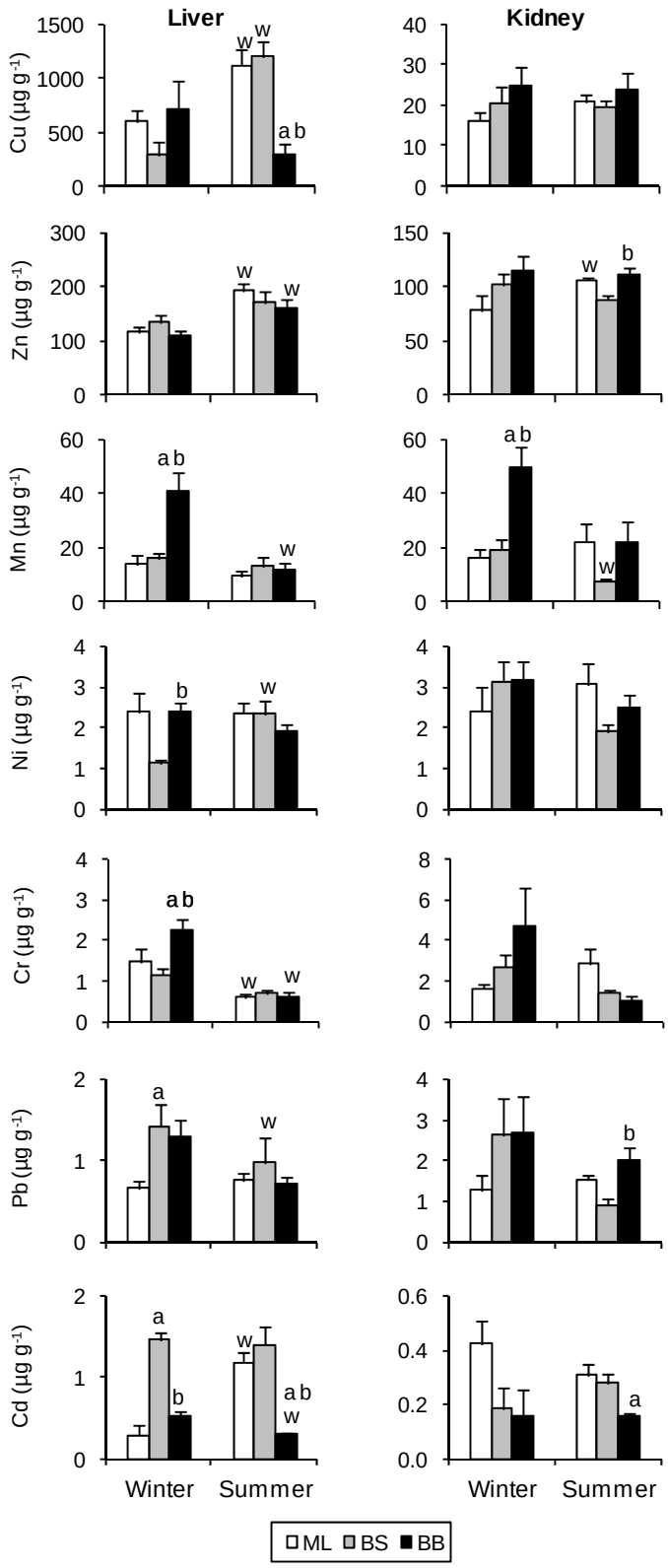

Figure 8.2. Metal levels in liver and kidney of *L. aurata* captured in winter and summer in the middle lagoon (ML) and in the inner branches (Bom-Sucesso – BS, Barrosa - BB) at Óbidos lagoon. Letters denote statistically significant differences ($p<0.05$) between sites (in the same survey) and between surveys (for each site): (a) vs. ML; (b) vs. BS; (w) vs. winter. Means and associated standard errors are given.

8.3.3 Oxidative stress in liver and kidney of *L. aurata*

A spatial analysis of liver antioxidant defences revealed significant increases of CAT and GPx (both in summer) at BB relatively to ML, while no differences were recorded for GR and GST (Figure 8.3). Moreover, in winter GSH_t content decreased at BB in comparison to ML. In terms of peroxidative damage, significantly higher levels were also found at BB (*versus* ML and BS) in winter. Similarly to liver, kidney exhibited increases of CAT at BB in summer (in relation to ML and BS), while GR and GST enhanced at BB in winter (comparing to ML and BS). However, GPx, GSH_t and LPO in kidney did not vary among sites.

Concerning temporal differences, liver GPx and LPO enhanced in summer (all sites, BS and BB, respectively), while GST and GSH_t were higher in winter (BS and all sites, respectively). Oxidative stress endpoints measured in kidney enhanced always in summer (except GR and GST that did not changed seasonally): CAT at ML and BB; GPx at ML and BS; GSH_t at all sites; LPO at BS.

Levels of CAT, GR, GST and LPO ranged within identical intervals in both organs but GSH_t doubled in liver in comparison to kidney and GPx doubled in kidney relatively to liver.

8.3.4 IBR results and correlations between *L. aurata* parameters

The correlations obtained between metals tissue load (in liver and kidney) and oxidative stress endpoints are shown in Figure 8.4 (non-significant correlations were not presented). Positive correlations were found between liver GST and LPO and accumulated levels of Pb and Zn, respectively. In kidney, activities of GPx and GST were correlated positively with accumulated Cu. Significant correlations were not found between metals in the water column (DGT-measured fraction and SPM) and accumulated in *L. aurata* organs.

The antioxidants and LPO values measured in liver and kidney were processed in order to calculate the IBR index (Figure 8.5). IBR values showed different variation ranges in winter and summer for both organs, being higher in summer (though in lower extent for kidney). Even so, in both surveys and organs the computed IBR values were always higher at BB than other sites. In particular, the estimated IBR values for liver at BB were 200 times higher than ML in winter, whereas in summer the difference between the two sites was approximately 3 times. Concerning kidney, the IBR in winter at BB exceeded 15 times ML, while in summer this difference was around 6 times. In winter, IBR values were higher in kidney than liver, while the opposite pattern was found in summer.

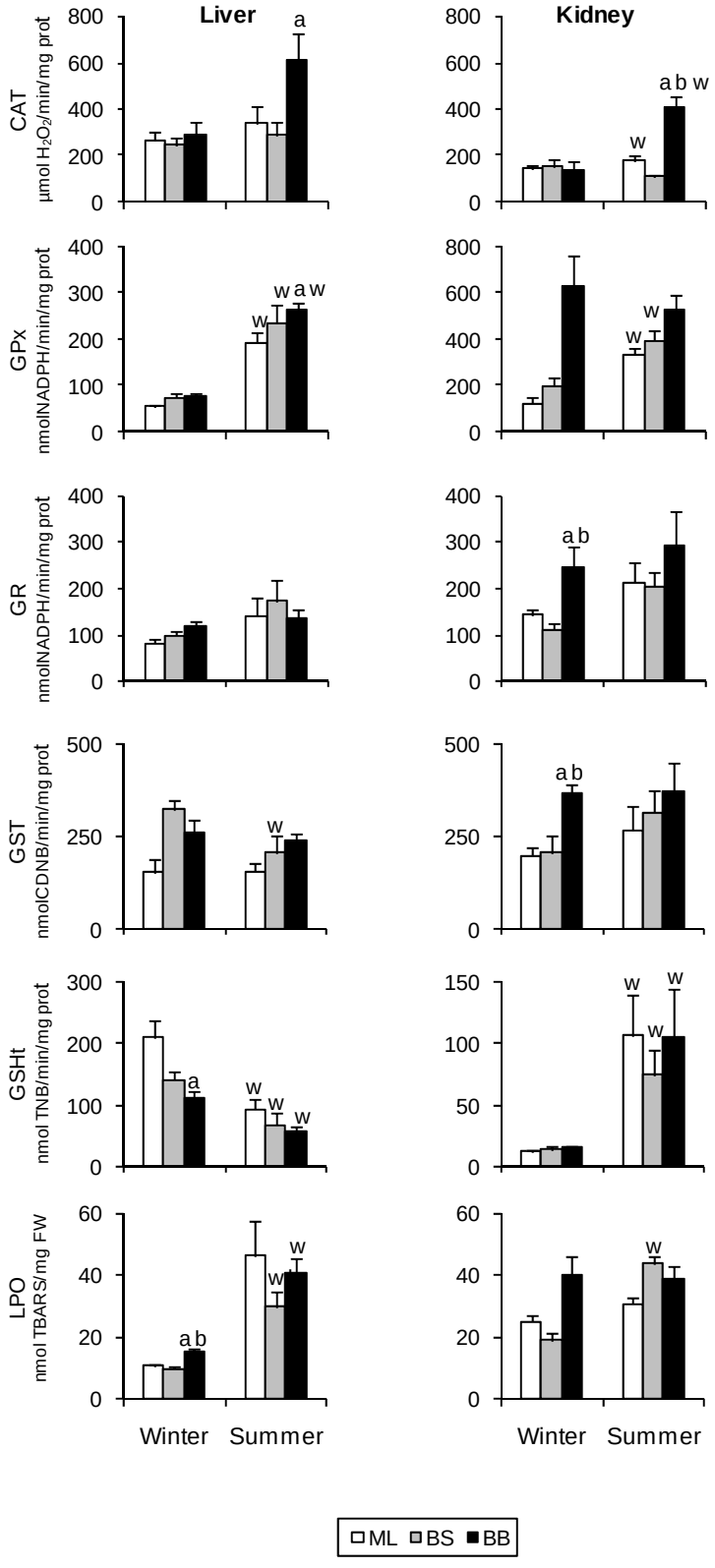

Figure 8.3. Oxidative stress responses in liver and kidney of *L. aurata* captured in winter and summer in the middle lagoon (ML) and in the inner branches (Bom-Sucesso – BS, Barrosa - BB) at Óbidos lagoon. Letters denote statistically significant differences ($p<0.05$) between sites (in the same survey) and between surveys (for each site): (a) vs. ML; (b) vs. BS; (w) vs. winter. Means and associated standard errors are given.

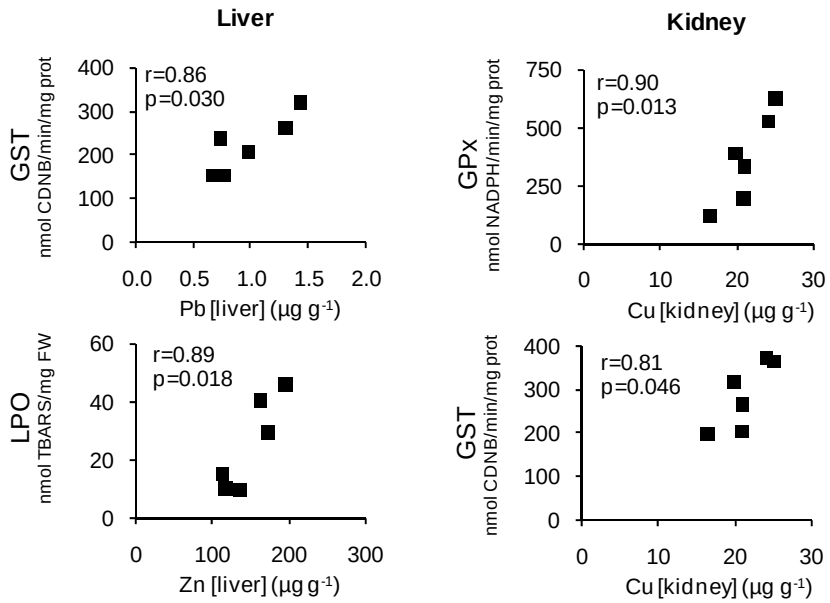

Figure 8.4. Significant correlations between metal levels and oxidative stress responses in liver and kidney of *L. aurata*. Statistical significance and correlation coefficient are represented by p and r, respectively. Non-significant correlations are not presented.

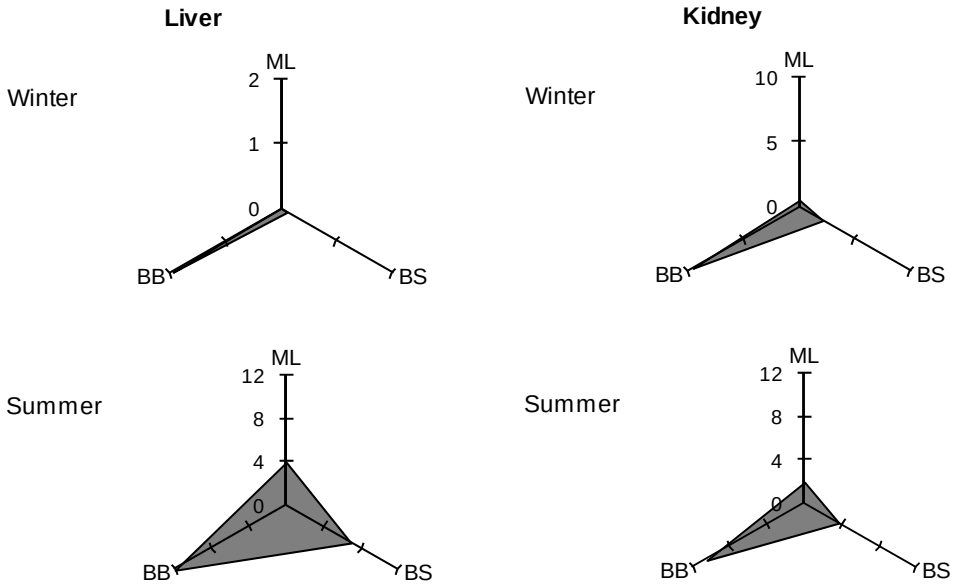

Figure 8.5. Integrated biomarker response (IBR) index in liver and kidney of *L. aurata* captured in winter and summer in the middle lagoon (ML) and in the inner branches (Bom-Sucesso – BS, Barrosa - BB) at Óbidos lagoon.

8.4 Discussion

8.4.1 Sediment and water quality

The comparison of sediment metal concentrations with values proposed by Long et al. (1995) for "Effects Range-Low" (ERL) (150, 34, 81, 21, 47, 1.2 μg g^{-1} respectively for Zn, Cu, Cr, Ni, Pb, Cd) within the sediment quality guidelines indicates that the three sites presented values above ERL for Cu and Ni. Nonetheless, metal concentrations were slightly higher at BB than other sites.

BB exhibited a higher metal availability than ML and, in a lower extent, than BS in both seasons. This was particular evident for Cu in water (DGT-measured fraction) and SPM, Cr in water, as well as Mn, Ni and Cd in SPM. The tributary that discharges at BB branch probably contributed to metal inputs, mainly in periods of higher freshwater flows (Pereira et al., 2009d). In fact, water levels of Cd and Mn (all sites) as well as particulate Pb (all sites) and Cd (BB) were higher in winter than in summer. In opposition, values of Cu (all sites) and Cr (BS, BB) in water as well as particulate Ni (BB) were higher in summer than winter, when freshwater flows into the lagoon are almost insignificant. These summer increments are probably related with the pulse release of metals from sediments during periods of low oxygenation as indicated by the simultaneous undersaturation of oxygen recorded mainly at BB. This internal source was already identified in other eutrophic lagoons (Point et al., 2007) and hypothesized to occur in BB branch (Pereira et al., 2009d). The prominence of these sediment-related processes for the water quality is probably lower at BS and ML than at BB due to the better trophic state in these two areas (Pereira et al., 2009c). In fact, BB was already proposed as a major impacted area in the lagoon being classified as eutrophic (Pereira et al., 2009c). Accordingly, BB exhibited the highest levels of nutrients and chlorophyll *a* in both seasons. In wet periods, the tributary that discharges at BB branch could introduce large amounts of nutrients into that area (Pereira et al., 2009c). Since PO_4^{3-} regeneration could occur in organically-rich sediments with upwards diffusion during periods of low oxygenation (mainly in summer), this should be considered as an additional input to the water column at BB as previously recorded (Pereira et al, 2009d).

Globally, sediment and water surveys pointed to a moderate contamination by metals in winter and summer. Moreover, the lagoon presented eutrophication symptoms with a higher nutrient availability in winter.

8.4.2 Metals accumulation and oxidative stress in *L. aurata* and their relationship with environmental data

Toxicity mechanisms could be better discussed when information on external levels of exposure, bioaccumulation and biological responses is available ensemble. Since environmental parameters and organism's physiology could vary considerably on a seasonal basis, ecotoxicological studies should be performed in contrasting periods, which is a particularly pertinent strategy under moderate contamination. Moreover, the few works concerning fish antioxidant capacities have shown organ specificity, associated with the anatomic location determining the exposure route of toxicants and their distribution (Ahmad et al., 2006). Hence, the current discussion will focus first on each organ individually, analysing inter-site differences and relating winter-summer changes both with seasonal alterations on metals availability and non-contamination related variables. Thereafter, a comparison of liver and kidney responses will be made.

8.4.2.1 Metal levels in liver and associations with environmental factors

Fish liver has a central role in basic metabolism and is the major site for accumulation, biotransformation and excretion of contaminants (Triebskorn et al., 1997). Particularly for liver, the diet is the main exposure route to contaminants, and thus could influence metals load recorded currently in mullet's liver. Despite that, the present work focused on searching relationships between accumulated metals and environmental availability (namely in water column and sediment). Accumulated Mn, Ni and Cr in liver increased at BB in winter, which is in line with the higher availability pointed by current measures in water (DGT-measured fraction) and SPM. Since metal concentrations in sediment varied only slightly among sites, metals accumulation in liver will be mainly related with water column levels. In fact, it is well documented that fish liver could accumulate metals in response to water exposure (Kraemer et al., 2005; Palaniappan and Karthikeyan, 2009). The Pb enhancement at BS in winter (in comparison with ML) is also probably the reflex of a higher availability as suggested by levels in SPM. Nevertheless, Pearson analysis did not provide any association between accumulated metals in liver and the corresponding levels in water or particles. Since data of the two seasons were considered jointly in Pearson analysis, the absence of associations in summer justifies the non-significant correlations obtained. Accordingly, an inconsistency between environmental data and liver accumulation was observed in summer. In fact, in that survey

liver Cu and Cd decreased at BB (in relation to ML and BS) despite the higher environmental availability. Moreover, hepatic metals load did not signalised differences on metal availability as pointed by water and SPM data, which was generally higher at BB than other sites in summer. It is well known that metal accumulation by fish in natural systems depends on many factors including water chemistry and temperature (Playle, 1998; Greenfield et al., 2001). Unpublished data from the Óbidos lagoon in summer indicated higher levels of particulate organic carbon in water column at BB than in other parts of the system. These data on particulate organic carbon allow inferring a higher content of dissolved organic matter that could eventually reduce Cu and Cd uptake via gills, as previously described (Playle, 1998).

The winter-summer variation pattern of liver accumulated metals showed to depend on the element. Thus, Cu and Ni exhibited higher values in summer (ML and BS; and BS, respectively), while Mn, Cr and Pb in liver peaked in winter (BB; ML and BB; BS, respectively) being both patterns in line with increases in water and/or SPM. Differently, Zn accumulation enhanced in summer comparing to winter (at ML and BB) regardless of lower values recorded in SPM. Bearing in mind the essentiality of Zn, the enhancement of accumulation in liver can represent an adaptation strategy in order to compensate a higher requirement during the summer period. Moreover, the higher temperatures of summer could be on the basis of the elevated accumulation of Zn. In fact, the rates of metal uptake and accumulation in ectothermal organisms increase with increasing temperature (Sokolova and Lannig, 2008). This hypothesis should also be considered in the interpretation of Cu and Ni levels previously discussed where temperature is probably having an additive action on their accumulation. A distinct pattern was found for Cd with increases recorded either in winter (BB) or summer (ML), while enhancements in the environment were regularly found in winter.

Under a moderate contamination status, the influence of seasonal changes had probably superimposed to environmental availability of metals, explaining the scarcity of inter-site differences in liver metals load recorded in summer.

8.4.2.2 Oxidative stress in liver and causative factors

The BB site of the Óbidos lagoon demonstrated to have pro-oxidant agents affecting the liver of *L. aurata*. In fact, CAT and GPx enhanced at BB in summer, as well as LPO in winter. Moreover, the decrease of GSH_t at BB in winter pointed to the same evidence.

Both CAT and GPx induction provides an indication of the higher H_2O_2 production in liver of BB fish in summer, suggesting the presence of redox active compounds in the environment and consequently accumulated in fish. The induction of both enzymes has been described in fish liver exposed to contaminants under field conditions (Orbea et al., 2005). In particular, metals were previously related with liver CAT induction in *L. aurata* (Fernandes et al., 2008). However, at the Óbidos lagoon it is improbable that CAT and GPx induction at BB in summer could be due to accumulated metals since substantial increases were not recorded in the liver. Thus, other stressors rather than metals could be on the basis of summer increases of these enzymatic antioxidants. In this context, the broad fluctuations of dissolved oxygen between day (180%) and night (30%) that were recorded in summer at the same area (Pereira et al., 2009d) may have represented a challenge for antioxidant defences, as previously proposed in a eutrophic lagoon (Gorbi et al., 2005). The concomitance of CAT and GPx increase in liver could be an additional indication of high levels of H_2O_2 and/or lipid hydroperoxides, as previously suggested for *L. aurata* gills (Oliveira et al., 2009).

GR plays a key role in GPx and GST reactions as an adjunct in the control of peroxides and free radicals by maintaining the proper GSH redox status (Bompart et al., 1990). Despite elevated hepatic GR activities have been previously recorded in *L. aurata* exposed to pro-oxidant compounds (Oliveira et al., 2008), as well as in other aquatic organisms (Regoli et al., 2002), in the current work no spatial differences were found for GR. Other studies have reported no alterations on liver GR under field exposures (Larsson et al., 2002). Moreover, detoxification enzymes like GST work on elimination of reactive compounds by forming conjugates with glutathione and subsequently eliminating them as mercapturic acid, thereby protecting the cells against ROS damage. In addition, it is recognized its role as antioxidant, participating in the reduction of organic hydroperoxides by GSH (Wang and Ballatori, 1998). Nevertheless, fish exposure to contaminants of the Óbidos lagoon did not alter liver GST activities significantly. Despite no statistical spatial differences were found for GST in liver, there was a tendency for higher values at BS and BB. Thus, when searching correlations by Pearson analysis, the GST induction was

correlated with levels of Pb in liver. In fact, several studies showed that exposure to metals can lead to an increase of GST in liver (Amado et al., 2006; Guilherme et al., 2008; Oliveira et al., 2008).

Variation of the cellular glutathione content is considered an indicator of the degree and duration of exposure to oxidant compounds in fish (Dautremepuits et al., 2009). Herein, it was recorded a GSH_t decrease in the liver of mullets from BB in winter. Identical results were found in crabs (*C. maenas*) from the Óbidos lagoon (Pereira et al., 2009b), as well as in perch (*Perca flavescens*) (Dautremepuits et al., 2009) and mussels (*Mytillus galloprovincialis*) (Lima et al., 2006) from other contaminated systems. The GSH_t depletion indicates an increased use without a compensatory synthesis, which can be attributed to the unbalance between GSSG production and GSH regeneration leading to GSSG accumulation and excretion from the cell to avoid NADPH exhaustion (Lima et al., 2006). In parallel with GSH_t decrease it was observed the enhancement of accumulated Mn, Ni and Cr at BB. In fact, it is well known that GSH_t may play a role in inducing resistance to metals by protecting macromolecules against attack by free radicals. Hence, the significantly higher LPO found in liver of fish captured at BB in winter can be causally linked with the GSH_t depletion. Moreover, since no alterations on enzymatic activities were recorded in winter, it can be suggested that low levels of contaminant-induced ROS can have a significant toxic effect if the threshold level for activation of antioxidant defences is not reached. The significant correlation found between LPO and Zn in liver (provided by data of the two seasons jointly) has a limited value in establishing causal relationships in winter strictly. In fact, it is more plausible that other stressors namely Mn, Ni and Cr (found to be elevated in liver), rather than Zn, are on the basis of LPO enhancement in winter. As a contributory part of water pollutants induced stress, the occurrence of high ammonium and nitrite levels at BB in winter can be an important source of pro-oxidants for fish (Ahmad et al., 2006), leading to nitric radicals production as demonstrated in mammals by Iijima et al. (2003). Thus, oxidative stress responses observed at BB in winter (namely lower GSH_t and higher LPO) could be also related with the presence of those compounds in water.

By combining the different biomarker signals, the IBR offers a potential measure of the organism's condition, which can be used in environmental health assessment as an additional aid to provide evidence of the existing impact (Broeg and Lehtonen, 2006). In

the present work, liver values of IBR substantiated the evidence that BB is the most impacted area of the lagoon both in winter and summer.

In this study, a marked difference between winter and summer was observed for oxidative stress endpoints. For instance, GPx activity (at all sites) and LPO levels (at BS and BB) showed higher levels in summer. However, this summer elevation at BB coincided with lower accumulated metals (i.e. Mn, Cr and Cd). This evidence points to a higher importance of seasonal non-contaminant related factors (e.g. temperature rise) on those oxidative stress responses in comparison with metal levels. A previous work carried out at the Óbidos lagoon, with crabs, also demonstrated increased LPO levels in summer (Pereira et al., submitted) and it was reported for fish that increase in environmental temperature modifies the chemical and physical state of membranes (Cossins, 1981). This aspect, together with the enhancement of oxygen consumption and the increase of mitochondrial ROS generation, could lead to the GSH_t reduction and higher hepatic LPO recorded in summer comparing to winter. In view of that, it would be plausible to record the induction of other antioxidants in summer relatively to winter. However, that was not observed (excluding for GPx) and an opposite temporal pattern was found for GST showing maximum levels in winter (at BS). Crabs from the Óbidos lagoon exhibited also higher activities of GST in winter at the same site, which were related with a higher availability of metals in that period (Pereira et al., submitted).

In winter IBR differences between sites were more accentuated than in summer. In fact, the estimated IBR values for liver at BB were 200 times higher than ML in winter, whereas in summer the difference between the two sites was approximately 3 times. Interestingly, it was in winter that spatial differences in individual biomarkers were more associated with metal contamination.

8.4.2.3 Metal levels in kidney and associations with environmental factors

Manganese enhanced at BB in winter (*versus* ML and BS) like occurred in liver, being probably related with a higher environmental availability as suggested by water (DGT-measured fraction) and SPM measurements. An identical explanation should be assumed for Pb increase at BB (than BS) in summer. Previous works showed that metals could increase in kidney as a response to water exposure (Palaniappan and Karthikeyan, 2009). Contrarily, the decreased Cd accumulation at BB in summer cannot be explained by the

environmental availability. This incongruence between water and/or SPM data and accumulation on summer was previously observed for liver, and thus an identical explanation should be considered in this section. Kidney from BS fish in summer showed lower contents of Zn than BB and this was not accompanied by water (DGT-measured fraction) or SPM levels, pointing to other explaining hypothesis rather than availability. Zinc has probably its uptake regulated in *L. aurata* since it is an essential element, which is supported by previous fish studies (Demirak et al., 2006; Reynders et al., 2008).

In general, no seasonal differences were found for accumulated metals in kidney. Only punctually Zn was higher in summer (ML) and Mn in winter (BS). Manganese increase could be the reflex of higher availability as suggested by water (DGT-measured fraction) levels. However, Zn peaked in summer without increases in the environment suggesting a higher physiological requirement in that season.

8.4.2.4 Oxidative stress in kidney and causative factors

The assessment of antioxidant defences in kidney was closely related with environmental contamination in previous studies (Oliveira et al., 2008; Dautremepuits et al., 2009). In the current work, increases of CAT (summer), as well as GR and GST (both in winter) occurred at BB suggesting the presence of redox active compounds. In fact, CAT has been reported as the first line of antioxidant defences, being very responsive to increasing levels of contaminant stimulated ROS production (Lima et al., 2006). Kidney CAT in summer was paralleled by enhanced values of accumulated Zn and Pb suggesting the involvement of these elements. Despite non-significant spatial differences were found for GPx or Cu in kidney, Pearson analysis provided a significant positive correlation between the two parameters.

In winter, GR was higher at BB than ML and BS. The elevated GR activity is related to the thiol status of cells because it regenerates GSH from GSSG. In this regard, the current GR induction indicates the enhancement of GSH oxidation resultant from the higher activities of GST and GPx (though not significant). The inductions of GR and GST recorded at BB in winter occurred concomitantly to higher Mn accumulation suggesting a causal relationship. Additionally, GST increase in kidney could be associated with accumulated Cu since a significant Pearson correlation was obtained and both parameters were increased at BB (though Cu without statistical support). As discussed for liver, the effect of

ammonium and nitrite levels at BB in winter should not be overlooked as an important source of pro-oxidants for fish.

The unchanged GSH_t levels indicate that this potent free radical scavenger was not a limiting factor for GST and GPx activities. Moreover, kidney LPO measurement revealed that fish were able to cope with the pollution stress (by the induction of CAT in summer and GR and GST in winter), avoiding damage.

The IBR values for kidney corroborated that BB is a critical area of the Óbidos lagoon with eventual risks for autochthonous populations.

In general, oxidative stress responses in kidney were enhanced in summer, namely activities of CAT and GPx, as well as levels of GSH_t and LPO. The most probable explanation is the influence of non-contamination related factors, such as temperature increase, on metabolism since no substantial augments of accumulated metals were found in that survey.

As discussed for liver, IBR seemed to have a higher discriminatory capacity in winter than summer, probably related with metal contamination. Indeed, IBR values in winter at BB exceeded 15 times ML, while in summer this difference was around 6 times.

8.4.2.5 Organ specificity

Two patterns were found concerning the hierarchy of liver and kidney for metal accumulation: Cu, Zn and Cd were higher in the liver, whereas Mn, Ni, Cr and Pb were identical in the two organs. Copper levels were around 30 times higher in liver than kidney, whereas Zn doubled in liver in comparison to kidney. These differences reveal a higher requirement of liver in those two essential elements, probably associated with its higher metabolic activity in relation to kidney. The partitioning recorded is also related with organs anatomic location that determines the exposure route and toxicants distribution. Thus, the higher Cu, Zn and Cd levels in liver point a preferential dietary uptake, since the liver is the main target organ of contaminants taken up via the intestine. On the other hand, it is known that kidney is preferentially targeted by chemicals when taken up through the gills (Pritchard and Bend, 1984). In general, the current partitioning of accumulated metals between liver and kidney is in agreement with other works (Olsvik et al., 2001; Papagiannis et al., 2004; Kraemer et al., 2005).

Comparing metal levels in liver and kidney between the three sampling sites, it is noteworthy that liver signalized better than kidney the spatial differences. The prevalence of liver as the most responsive organ is probably also related with its buffering role regulating the metals redistribution for the other organs.

Current results demonstrated an organ-specific antioxidant modulation as documented previously (Ahmad et al., 2006). This is resultant from organs differences concerning their propensity for toxicants accumulation, as well as distinct cellular metabolic rates regarding ROS production *versus* antioxidant potential. Concerning the hierarchy of liver and kidney for oxidative stress adaptability, it is interesting to note that GPx and GR basal activity doubled in the kidney, probably contributing determinately to avoid the occurrence of LPO in this organ. Conversely, liver was more vulnerable to LPO which cannot be dissociated from the higher retention of metals. Moreover, liver exhibited higher levels of GSH_t in comparison to kidney (expectable considering that liver is the main organ on GSH synthesis) which were, nevertheless, insufficient to protect it against peroxidative damage. Contrarily to liver, CAT activation in kidney was not accompanied by GPx induction, suggesting a higher H_2O_2 generation in the liver in agreement with its higher metabolic rate.

In general, identical spatial and seasonal trends were observed at the Óbidos lagoon for CAT, GPx and LPO in both organs, while different patterns were recorded for GR, GST and GSH_t, Indeed, only kidney GR and GST signalised spatial differences and thus this organ presents a higher adaptive capacity. Moreover, GSH_t of liver and kidney presented opposite temporal patterns with liver levels increasing in winter and kidney in summer.

In winter, IBR values were higher in kidney than liver, while the opposite pattern was found in summer. Nevertheless, IBR values of liver in winter had a higher capacity to discern BB site than kidney.

8.5 Conclusions

Results of this work provided these main findings:

- The applied approach combining biomarkers in liver and kidney (metal levels and oxidative stress) and environmental data (metals and nutrients) demonstrated to be useful in the environmental health assessment, particularly under such a moderate contamination scenario. In fact, the inter-site differences signalled by metals accumulation and biochemical markers in both organs pointed BB branch as a critical area in the Óbidos

lagoon, which was later substantiated by a general stress index (IBR). In winter, oxidative stress at BB was related with the enhancement of accumulated metals in both organs, while in summer this cause-effect relationship was only established for kidney since changes in liver were mostly linked with non-contaminant related variables.

- Organ-specific patterns were found. Hence, liver presented higher levels of Cu, Zn and Cd, being more responsive than kidney regarding metals inter-site and seasonal changes. Despite oxidative stress endpoints also clearly differed between organs (e.g. LPO occurring only at liver), within this context it was not possible to elect a more responsive organ.

- Winter-summer variations were prominent in both *L. aurata* organs. In terms of metals accumulation, both liver and kidney were more responsive in winter than summer, though the seasonal patterns were found to depend on the element. Oxidative stress endpoints provided evidence of impact at BB in both seasons, though exhibiting increased oxidative stress responses in summer (corroborated by IBR).

- Current data highlight the hazard of moderate contamination conditions to the icthiofauna populations, as pointed by the occurrence of oxidative damage without the previous prominent activation of antioxidant defences (as found in winter for liver from BB fish). In addition, under such contamination scenario, non-contamination related variables could be on the basis of biomarkers changes surpassing the influence of metals environmental availability.

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8.6 References

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CHAPTER IX

General Discussion

9 General Discussion

This general discussion section aims to provide an overview and integration of the main findings of each chapter (II – VIII), contributing with new interpretative perspectives. The current discussion was achieved taking into account the key objectives of the thesis and it's throughout progression. This work used the Óbidos lagoon as a prototype coastal system of eutrophication and moderate contamination by metals. A key-issue of this study was to evaluate if metals increase during the night of warm periods, as a consequence of dissolved oxygen depletion that is a common feature to eutrophic conditions. Other challenging aspect was to perform an investigative monitoring study under moderate contamination conditions and with the eventual intermittent increase of metals. Thus, it was searched in what extent those intermittent increases could be reflected by organisms both in terms of metals accumulation and biological effects. Moreover, a central aim of the current work was to associate biological effects with metals accumulation in target species. The selected organisms (*Ulva* sp., *Carcinus maenas* and *Liza aurata*) belong to distinct taxa and have different roles in the trophic chains.

Hence, it was adopted a combined approach with environmental chemical data, as well as bioaccumulation and biological effects in those target species. Water quality parameters, including dissolved oxygen, nutrients and metals were surveyed in the water column over a day-night scale and on a seasonal basis at different sites. Surface sediments were analysed to assess the pool of metals in several areas of the lagoon. Meanwhile, *Ulva* sp. (sea lettuce), *C. maenas* (shore crab) and *L. aurata* (golden grey mullet) were monitored for accumulated metal levels and biochemical responses in different seasons. Peroxidative damage and antioxidant (enzymatic and non-enzymatic) responses were measured in *Ulva* sp., crabs' hepatopancreas and mullets' organs (gills, liver and kidney). The current strategy was chosen in order to avoid the gaps of sole chemical monitoring (water, sediment or organisms) that does not provide any insights of toxicity. Thus, biological effects were related with external levels of exposure and accumulation concentrations to a toxicokinetic understanding. Until now, only few studies carried out in aquatic systems used such combined strategy for assessment of environmental health.

9.1 Environmental chemical data

At first, nutrients and metals were investigated for spatial and temporal dynamics (Chapters II and III) providing data that were on the basis of the following studies (Chapters IV to VIII). Thus, the ecotoxicological monitoring strategy comprised always sampling at both lagoon inner branches (Barrosa and Bom-Sucesso) and at a reference area (middle/lower lagoon), being performed seasonally (*Ulva* sp.; *Carcinus maenas*) or in winter and summer (*Liza aurata*) in order to cover periods of contrasting conditions, mainly in which concerns to metal sources and nutrients availability. The Barrosa branch presented the highest nutrient levels (particularly in winter) and was classified as eutrophic (Chapter II). Identically, also metals enhanced in water of Barrosa branch in wet periods due to freshwater inputs (Chapter III). Despite that, water metal concentrations recorded during summer nights at Barrosa branch could surpass values found in other sampling periods (always at daytime), pointing to the main importance of remobilization processes for metals availability in warm conditions. This is in line with previous studies that reported the importance of sediment-water exchanges for water quality parameters namely metals (e.g. Point et al., 2007). Accordingly, aquatic organisms at Barrosa branch would be exposed to metals both in winter and summer, which might be provided respectively from freshwater inputs and sediment remobilization. Besides metals, organisms living at Barrosa would be subjected to other stressors associated with the eutrophication process. Particularly in winter, the high levels of ammonium and nitrite (Chapter II) could represent an eventual hazard to aquatic organisms. On the other hand, in summer the broad fluctuations of dissolved oxygen between day (180%) and night (30%) (Chapter III) may challenge the organisms' physiology.

9.2 Metal levels in target species as biomarkers of exposure

Current work aimed to investigate whether metal levels in *Ulva* sp., *Carcinus maenas* (hepatopancreas) and *Liza aurata* (gills, liver and kidney) reflect environmental contamination in the lagoon, particularly in the water column and sediments (Chapters IV to VIII). Thus, those target species were selected considering their different roles in the trophic chains, which in turn leads to distinct biological characteristics, regarding for instance the major toxicant exposure routes and lifestyle (extent of dependence from the sediment or water), as well as metabolic and defence capabilities. In general, *Ulva* sp. (autumn, spring and summer) presented higher metal levels at Barrosa branch, as well as

gills and liver of *L. aurata* (only in winter). Spatial differences concerning accumulated metals were less pronounced for hepatopancreas of *C. maenas* (autumn, winter and summer) and kidney of *L. aurata* (winter and summer). The higher sensitivity of *Ulva* sp. in relation with other surveyed organisms must be related with its lower physiological complexity and due to the fact that metals are mainly absorbed from the surrounding water. Moreover, some metals could be taken up by macroalgae within few hours as previously recorded by Lee and Wang (2001). These features make *Ulva* sp. a privileged target species to detect intermittent increases of metals that occur during summer nights at the Barrosa branch. In fact, macroalgae collected in summer during daytime also exhibited higher levels of metals at Barrosa than other sites. Considering that in summer freshwater inputs into the lagoon are negligible, metals incorporated by *Ulva* sp. at Barrosa could only be provided from the sediment as described in Chapter III. Conversely, the repercussions of sediment-related processes on metals accumulation by *C. maenas* and *L. aurata* were not so clear. Particularly, accumulated metals in gills and liver of *L. aurata* decreased at Barrosa (in comparison with other sites) in summer.

Metals accumulation demonstrated to be more complex in internal organs (liver and kidney of fish, as well as in crabs' hepatopancreas) comparing with gills (an interface organ). This is partially related with the morphological complexity of liver and kidney and their anatomic location, which in turn lead to a higher relevance of the diet in comparison with the gills. Thus, gills were the organ that showed a capacity to reflect metals in the water column most comparable to the macroalgae *Ulva* sp. In fact, significant Pearson correlations between accumulated metals and water concentrations were searched for the three species but only found for *Ulva* sp. Nevertheless, gills and liver of fish exhibited increases of metals at Barrosa branch in winter in line with a higher environmental availability as suggested by measurements in the water column. This common pattern is an interesting point, bearing in mind that gills and liver are in general associated to different metal uptake routes (via water versus via diet, respectively). The kidney of *L. aurata* provided lower evidence that Barrosa branch presents a higher availability of metals than other sites. Similarly, the hepatopancreas of *C. maenas* was also less sensitive to metal contamination at the Barrosa branch. Anyhow, accumulated metals were mainly related with sediment contamination, in agreement with crabs' benthic lifestyle.

Accumulated metals in *Ulva* sp., *C. maenas* and *L. aurata* varied between winter and summer, though the seasonal pattern recorded showed to depend on the element and

sampling site. In general, seasonal differences of *Ulva* sp, *C. maenas* hepatopancreas and *L. aurata* organs were more prominent at other lagoon sites rather than Barrosa branch. At this site, only manganese levels in *Ulva* sp. peaked in summer in comparison to winter. It was hypothesised that those maxima could be related with alterations on algae physiology associated with the season, namely increase of photosynthesis and respiration. Although water measurements at daytime did not detected manganese increases at Barrosa, the higher accumulation by *Ulva* sp. could be resultant from an enhanced incorporation during the night due to the remobilization of that element from the sediment. Conversely, hepatopancreas of crabs (pooled genders) from Barrosa branch presented maxima of copper and cadmium in winter, associated with a higher environmental availability in that period. Identically, liver of *L. aurata* presented increases of manganese, chromium and cadmium in winter (in comparison with summer) at Barrosa in line with higher levels in water column. Differently from macroalgae at Barrosa, metal levels in organs of crabs and fish increased in winter (in comparison with summer) and thus, external metal levels (freshwater inputs) superimposed intermittent release of summer nights. An interesting point is that increases of accumulated levels in *Ulva* sp. from Barrosa branch (in comparison with other lagoon areas) occurred in several seasons, including summer. In this period, freshwater inputs into Barrosa are almost inexistent but sediment could represent a source of metals to the water column during the night (Chapter III). The prominence of these sediment-related processes for the water quality is probably lower both in Bom-Sucesso branch and in the middle/lower lagoon due to their better trophic state (Chapter II). Thus, it is most probable that those increments in the algae from Barrosa could be related with the release of metals from sediments during periods of low oxygenation that occur in the summer nights.

9.3 Biochemical responses in target species and causative factors

A common pathway of toxicity induced by several stressful conditions is the imbalance between generation of reactive oxygen species (ROS) and the efficiency of antioxidant defences. Therefore, the susceptibility to oxidative stress was investigated in *Ulva* sp., *C. maenas* (hepatopancreas) and *L. aurata* (gills, liver and kidney) from three areas of the Óbidos lagoon in several seasons. In general, the results pointed to alterations on the assessed parameters in the three target species from Barrosa branch, revealing compensatory mechanisms to cope with environmental changes. Indeed, this site presents

compounds that stimulate the production of H_2O_2 as depicted by the increase of CAT and GPx in *Ulva* sp. (autumn and spring), hepatopancreas of crabs (autumn), as well as gills, liver and kidney of *L. aurata* (more prevalent in summer). Moreover, the exposure to pro-oxidant stressors at Barrosa branch led to an elevation of GST in *Ulva* sp. (autumn), crabs (autumn and summer) and fish' kidney (winter). Additionally, GSH_t decreased in animals (crabs' hepatopancreas in summer and fish' liver in winter), whereas a punctual increase was recorded in *Ulva* sp. (summer). Despite the general activation of antioxidant defences, in several sampling periods target species did not cope efficiently with ROS production and oxidation of polyunsaturated fatty acids occurred, leading to LPO. This was recorded for *Ulva* sp. (autumn and spring), *C. maenas* (autumn) and *L. aurata* gills and liver (winter). Results on LPO highlight the environment state degradation at Barrosa branch relatively to other areas of the lagoon.

The seasonal comparison of biochemical responses in the three target species revealed that only crabs and fish exhibited differences between winter and summer on oxidative stress responses. In general, CAT, GSH_t and LPO peaked in summer both for crab (hepatopancreas) and fish (gills and kidney). This seasonal pattern was also found for LPO in fish liver. However, maxima in liver were recorded in winter for GSH_t and GST, whereas no differences were found for CAT. Moreover, seasonal variations of GPx differed between crab and fish, since higher enzymatic activities were found in winter for crab and in summer for fish (all the organs). This discrepancy was found partially for GST with crab presenting higher activities in winter and gills of fish in summer.

In the present work, an "Integrated Biomarker Response" (IBR) index was applied to data on biochemical responses of *Ulva* sp., *C. maenas* and *L. aurata* (Chapters IV, VI, VII and VIII). In general, IBR represented a potential measure of the health of those populations at the Óbidos lagoon, providing a better evidence of the existing impact. Hence, current IBR results substantiated the evidence that Barrosa branch is the most impacted site in the lagoon. Moreover, IBR varied temporally for the three target species. Gills, liver and kidney of *L. aurata* presented higher values in summer than winter, while the opposite pattern was found for crabs' hepatopancreas. The lack of values in summer for *Ulva* sp. made unviable the comparison of those surveys in terms of IBR, but the hierarchy found within other periods for Barrosa branch was the follow: autumn > spring > winter.

A central aim of the current work was to associate biological effects with metals accumulation in target species. Within this context, GPx activities in *Ulva* sp., as well as in gills and kidney of *L. aurata* were correlated with metal levels (manganese, zinc and copper, respectively). Moreover, significant Pearson correlations were obtained between GST measured in *Ulva* sp. and accumulated cadmium. Similarly, GST in gills, liver and kidney of *L. aurata* were correlated respectively with accumulated copper, lead and copper. At Barrosa branch oxidative stress parameters in the three target species were associated occasionally with accumulation of metals. In general, those associations were mainly provided by concomitant enhancements of metals accumulation and biochemical effects in other seasons rather than summer. A sole exception was found for *Ulva* sp. reinforcing the hypothesis of effects driven by sediment-related processes. Despite the obtained correlations for crabs and fish organs, other parameters related with eutrophication process were hypothesised to be on the basis of biological effects. For instance, the broad fluctuation of dissolved oxygen recorded in summer (Chapter III) could lead to the activation of antioxidant defences in mullet's gills (Chapter VII). Moreover, in winter the higher levels of ammonium and nitrite recorded at Barrosa may be associated with biochemical responses in *Ulva* sp., crabs and fish.

The comparison of biological effects measured in terms of oxidative stress points to a higher sensitivity of *Ulva* sp and gills of *L. aurata*. In fact, both organisms/organs have a wide surface area that is continuously in contact with the external medium. Thus, metals are mainly absorbed from the aqueous phase in both *Ulva* sp. and fish gills. Eutrophication related variables such as ammonium, nitrite and dissolved oxygen were also associated with biochemical responses. Because other factors rather than contaminants could influence responses, prudence should be taken in analysing the results. This relevant aspect was particularly considered in the works with *C. maenas* for which the responses showed to be conditioned by biological factors namely those related with gender.

9.4 References

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CHAPTER X

Final Remarks

10 Final remarks

The Óbidos lagoon presented a contamination scenario characterized mainly by moderate metal contamination that is inducing biological effects in inhabitant species.

The work brought new perspectives to biomonitoring by the identification of sensitive species and organs. In fact, *Ulva* sp., that had been underemployed as sentinel, displayed the ability to closely reflect the metal levels in water of the Óbidos lagoon on its body burden. In addition, the responsiveness of its oxidative stress parameters was comparable to aquatic animals (namely crabs and fish). Moreover, data of this thesis recommend genders separation in biomonitoring programs using crabs. Furthermore, metal levels and biochemical effects in fish gills reflected the environmental status of the Óbidos lagoon, showing this interface organ as an important route of entry for metals. Based on both evidences, *L. aurata* gills were considered as a "mirror door" in the context of water contamination assessment.

In field studies, the multitude of chemicals present in aquatic environments make difficult to correlate biomarker responses with specific compounds in a dose-response manner. Despite this complexity had occurred at the Óbidos lagoon, the adopted strategy had demonstrated its suitability for environmental health assessment. Through a combined analysis of external levels of exposure, accumulation levels and biochemical responses it was possible to confirm the existence of hazardous conditions at Barrosa branch. This was observed both in winter a summer and thus driven by distinct sources of metals - freshwater inputs or release from sediments. Summer data of *Ulva* sp. suggested that organisms can accumulate metal levels that were provided from the sediment-water exchanges. However, additional studies (e.g. field transplantation) will be required to better clarify the effect of pulse release of metals (recorded in summer) in target species both in terms of accumulation and biochemical responses. Intermittent exposures are generally overlooked in the literature, particularly those associated with sediment-related processes under eutrophic conditions. Nonetheless, a long series of peak events is probably what happens at Barrosa branch in summer nights. Whether or not the organisms react as it was a continuous exposure will be a function of the clearance time of the metals relative to the duration and intensity of the exposure. Until now, eutrophic environments had been mainly studied for nutrients dynamics and ecological appraisals.

However, current results suggest that under those disturbed conditions, metals are additional stressors for organisms, particular in summer months.

The adopted approach provided a wide range of data both on metals accumulation and on biological effects. Thus, common patterns (spatial and temporal) to the different target species were occasionally difficult to discern. Moreover, metals accumulation and antioxidant modulation showed to be organ-specific (among gills, liver and kidney) in *L. aurata*. This diversity and specificity of response profiles make more complex the data interpretation and represents a difficulty when, unrealistically, it is intended to define patterns extrapolable for different species or even for different organs of the same species. However, ecotoxicologists can take advantage from this complexity that, in turn, offers an array of biological sensors thereby increasing the biomonitoring efficacy. Additionally, under moderate contamination conditions subtle biological responses could occur making hard a cause-effect association which is a main goal of ecotoxicologists. This could be particularly relevant under moderate contamination where confounding factors (biotic and abiotic) could have higher expression.

The presence of contaminants in the Óbidos lagoon, even at sublethal concentrations, induced changes at biochemical level in several target species. These changes can have long-term effects in the wildlife namely populations of fish and epibenthic crustacea. Our goal is ultimately to improve the quality status of the Óbidos lagoon which in turn would get better organism health. In view of that, this study is a contribution for Óbidos lagoon health assessment and offered baseline information for local and regional management entities towards mitigation programs, in order to achieve a "good ecological" status. Conclusions of the thesis may be useful for designing future investigative monitoring programs in coastal lagoons suffering from eutrophication and moderate contamination by metals, particularly within the scope of the European Water Framework Directive.