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Licenciada em Ciências de Engenharia do Ambiente

Development of an integrative approach for the characterization of ecological risk on marine transition ecosystems: the Sado Estuary as a case study

Dissertação para obtenção do Grau de Mestre em Engenharia do Ambiente

Orientador: Prof. Doutora Maria Helena Ferrão Ribeiro da Costa, Professora Associada com Agregação, Faculdade de Ciências e Tecnologia da Universidade Nova de Lisboa

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Abstract

Estuaries and other transitional waters are complex ecosystems critically important as nursery and shelter areas for organisms. Also, humans depend on estuaries for multiple socio-economical activities such as urbanism, tourism, heavy industry, (taking advantage of shipping), fisheries and aquaculture, the development of which led to strong historical pressures, with emphasis on pollution. The degradation of estuarine environmental quality implies ecologic, economic and social prejudice, hence the importance of evaluating environmental quality through the identification of stressors and impacts. The Sado Estuary (SW Portugal) holds the characteristics of industrialized estuaries, which results in multiple adverse impacts. Still, it has recently been considered moderately contaminated. In fact, many studies were conducted in the past few years, albeit scattered due to the absence of true biomonitoring programmes. As such, there is a need to integrate the information, in order to obtain a holistic perspective of the area able to assist management and decision-making. As such, a geographical information system (GIS) was created based on sediment contamination and biomarker data collected from a decade-long time-series of publications. Four impacted and a reference areas were identified, characterized by distinct sediment contamination patterns related to different hot spots and diffuse sources of toxicants. The potential risk of sediment-bound toxicants was determined by contrasting the levels of pollutants with available sediment quality guidelines, followed by their integration through the Sediment Quality guideline Quotient (SQG-Q). The SQG-Q estimates per toxicant or class was then subjected to georreferencing and statistical analyses between the five distinct areas and seasons. Biomarker responses were integrated through the Biomarkers Consistency Indice and georreferenced as well through GIS. Overall, in spite of the multiple biological traits surveyed, the biomarker data (from several organisms) are accordant with sediment contamination. The most impacted areas were the shipyard area and adjacent industrial belt, followed by urban and agricultural grounds. It is evident that the estuary, although globally moderately impacted, is very heterogeneous and affected by a cocktail of contaminants, especially metals and polycyclic aromatic hydrocarbon. Although elements (like copper, zinc and even arsenic) may originate from the geology of the hydrographic basin of the Sado River, the majority of the remaining contaminants results from human activities. The present work revealed that the estuary should be divided into distinct biogeographic units, in order to implement effective measures to safeguard environmental quality.

Keywords:

Risk Assessment, Transitional Ecosystems, Estuaries, Sediment contamination, Biomarkers, Environmental toxicology.

Resumo

Os estuários assim como outras águas de transição são ecossistemas complexos, vitalmente importantes como viveiros e áreas de abrigo para os organismos. O Homem também depende dos mesmos devido a múltiplas atividades sócio-económicas, tal como o desenvolvimento urbano, o turismo, a indústria pesada (tirando partido da navegação), as pescas e a aquacultura, o desenvolvimento dos quais conduziu a fortes pressões ao longo do tempo, com enfase na poluição. A degradação da qualidade do ambiente estuarino implica prejuízo ou dano ecológico, económico e social, como tal é importante avaliar a qualidade ambiental, identificando os agentes stressores e seus impactes. O estuário do Sado (SO Portugal) possui as características de um estuário industrializado, o qual resulta em múltiplos impactes adversos. Contudo, foi recentemente considerado como moderadamente contaminado. De facto, ao longo dos anos muitos estudos foram conduzidos, embora dispersos devido à ausência de um verdadeiro programa de biomonitorização. Como tal, existe uma necessidade de integrar informação, por forma a obter uma perspetiva holística da área possibilitando a gestão e a tomada de decisão. Desta maneira, foi criado um sistema de informação geográfica (SIG) baseado na contaminação sedimentar e nos dados dos biomarcadores recolhidos em publicações da última década. Foram identificadas e caracterizadas por distintos padrões de contaminação sedimentar, relativos a diferentes "hot spots" e a fontes difusas de tóxicos, quatro áreas impactadas e uma área de referência. O risco potencial da ligação dos tóxicos ao sedimento foi determinado pelo contraste entre os níveis de poluentes com os valores guia disponíveis de qualidade sedimentar, seguidos pela sua integração através do "Sediment Quality guideline Quotient" (SQG-Q). O SQG-Q estima por toxico ou classe de tóxicos, foi então sujeito a georreferenciação e a análises estatísticas entre as distintas cinco áreas e entre estações do ano. As respostas dos Biomarcadores foram integradas através do "Biomarlers Consistency Indice" e foi igualmente georreferenciado através dos SIG. No geral, apesar das múltiplas características biológicas pesquisadas, os dados dos biomarcadores (a partir de vários organismos) estão em concordância com a contaminação sedimentar. As áreas mais impactadas foram, a área dos estaleiros e a cintura industrial adjacente, seguida das áreas urbanas e agrícola. É evidente que o estuário, apesar de na sua globalidade estar moderadamente impactado, é muito heterogéneo, sendo perturbado por um "cocktail" de contaminantes, em especial por metais e hidrocarbonetos aromáticos. Apesar de elementos (como o cobre, o zinco e até o arsénio) poderem vir da geologia da bacia hidrográfica do rio Sado, a maioria dos restantes contaminantes resulta das atividades humanas. O presente trabalho revelou que o estuário deve ser dividido em unidades biogeográficas distintas, por forma a implementar medidas efetivas que salvaguardem a qualidade ambiental.

Palavras-Chave:

Avaliação de Risco, Ecossistemas de Transição, Estuários, Contaminação Sedimentar, Biomarcadores, Toxicologia Ambiental.

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Abbreviation List

AChE - Acetylcholinesterase
As − Arsenic
Bi – Biomarker Consistency Indice
CAT – Catalase
CASP3 – Caspase 3
Cd − Cadmium
Cr − Chromium
Co – Cobalt
Cu − Copper
CYP − Cytochrome
CYP1A − Cytochrome P450
DDE - Dichlorodiphenyldichloroethylene
DDT − Dichloro-Diphenyl-Trichloroethane
DNA - SB – Deoxyribonucleic acid strand breakage
DTT − Dithiothreitol
EC − European Commission
EcoQ - Ecological quality status
EDCs − Endocrine Disruption Compounds
EDTA − Ethylenediamine tetra-acetic acid
ELISA − Enzyme-Linked Immunosorbent Assay
ENA - Erythrocyte Nuclear Abnormalities
ERA − Environmental Risk Assessment
EU − European Union
FF – Fine fraction
GPx - Glutathione peroxidase isoform 1
GR – Glutathione reductase
GSH – Reduced glutathione
GSH/GSSG – Reduced/oxidised glutathione ratio
GST – Glutathione *S*-transferase
GSSG - Oxidized glutathione
HCB − Hexachlorobenzene
Hg – Mercury
HSP90AA - Heat-shock protein 90 kDa alpha
IDW – Inverse Distance Weight Algorithm
Ih - Histopathological condition indice

LDH – Lactate dehydrogenase

LOE – Line of evidence

LPO - Lipid peroxidation

Mn – Manganese

MSFD − Marine Strategy Framework Directive

MT – Metallothionein induction

Ni − Nickel

PAHs – Polycyclic aromatic hydrocarbons

Pb − Lead

PCBs − Polychlorinated Biphenyls

PCR – Polymerase chain reaction

PEF - Postexposure feeding

PEL − Probable Effects Level

RT-PCR – Reverse transcription polymerase chain reaction

Rf - Reference

SCGE – Single cell gel electrophoresis

Se − Selenium

SOD - Superoxide dismutase

SQG-Q – Sediment Quality Guideline Quotient index

SW − South-west

TBARS - Thiobarbituric acid reactive substances

TEL − Threshold Effects Level

TOM – Total organic matter

tGSH – Total glutathione

V - Vanadium

WFD − Water Framework Directive

WHO − World Health Organization

Zn − Zinc

1. Introduction

Transitional aquatic ecosystems, such as estuaries are important areas from which organisms and humans are highly dependent on, being one of the most threatened coastal environments on the account of multiple anthropogenic pressures (see for instance Cunha et al., 2007; Vasconcelos et al., 2007). They are especially important as nursery areas, providing shelter, reproductive and feeding grounds for many aquatic organisms, from fish to a variety of migratory bird life (Ridgway and Shimmield, 2002). On the other hand, Humans long depend on estuaries for settlement, tourism, heavy industry, aquaculture, shipping and agricultural activities, which inevitably dictates a variaty of impacts, among which pollution is one of the most critical. Overall, the degradation of estuarine environmental quality implies ecologic, economic and social prejudice, so it is important to evaluate the state of its environment to assist management, mandating the need to build solid knowledge upon which legislation and guidelines can be draw to ensure sustainability (Freitas et al., 2008).

The European Water Framework Directive (WFD; Directive 2000/60/EC) and the Marine Strategy Framework Directive's (MSFD; Directive 2008/56/EC) have been set as a series of norms and guidelines designed for the protection of groundwater plus inland (surface), estuarine (transitional) and coastal waters (Ducrotoy, 2010). These frameworks require that Member States assess the Ecological Quality Status (EcoQ) of transitional and coastal waters by 2006 (in the case of WFD) and achieve at least Good Environmental Status (in the case of the MSFD) in marine systems by 2020. However, the directives do not provide direct information about the matrices to be sampled or which specific pollutants should be monitored among the environmental stressors potential impacting aquatic ecosystems. Nonetheless many authors acknowledge that, in order to monitor the ecological status of aquatic ecosystems, both the contaminant matrices (such as sediments) and the toxicants per se should be take in to account (Borja et al., 2004).

Aquatic sediments are considered to be paramount in the assessment of anthropogenic impacts in coastal and estuarine environments (see for instance Chapman and Wang, 2001; Ridgway and Shimmield, 2002). Contaminants tend to be trapped in sediments, which may thus act as a reservoir of hazardous xenobiotics while the toxicants may accumulate in invertebrates, thus entering in the food chain (Lee et al., 2000; Garcês and Costa, 2009). Still, it must be known that, some contaminants are naturally present in aquatic ecosystems, posing a threat when their levels are raised above the background, up to the threshold of eliciting adverse effects, therefore becoming agentes of pollution (see Rainbow et al., 1990; Garcês and Costa, 2009).

The bioavailability of sediment-bound contaminants to the biota is ultimately determined by physical, chemical and biological factors, from the type of substance to sediment pH, anoxia and, most

importantly, particle characteristics and organic load. As such, it is very important to evaluate the proportions of total organic matter (TOM) and fine fraction particle (FF) because, besides being strongly correlated, high levels of both characteristics operate as traps for contaminants (Counihan et al., 2014), especially organic toxicants, such as PAHs, which are markedly hydrophobic and tend to be trapped in sediments for large periods (see Eggleton and Thomas, 2004 for a review). Therefore, the sediments of transitional waters, whose particular hydrodynamics and balance between ocean and riverine inputs typically dictate high TOM and FF, tend to act as deposits of contaminants may be released to the water column or entre the food chains, making them available to the biota (see Klosterhaus et al., 2011 and Chapman et al., 2013 for a review).

Marine environments and, moreover, estuaries and other confined waters, are particularly difficult to monitor because of their natural variability trough seasons, hydrology, geology and biogeography. They are not steady systems, meaning that natural variation is the main driving force of these ecosystems (which is term the "Estuarine Quality Paradox"), so it is difficult to evaluate the actual levels of contamination from point and diffuse sources and if whether these are natural or anthropogenic (Elliot and Quintino, 2007; Dauvin and Rullet, 2009; Chapman et al., 2013). As such it is necessary to implement integrative approaches that combine contamination, toxicity and ecology in order to gather a consistent, holistic approach, as has been suggested for instance, through the Sediment Quality Triad approach (Long and Chapman, 1985).

Biomarkers are long acknowledge tools to evaluate the effects/or exposure of the biota to contaminants, being one of the potential lines-of-evidence (LOE) that should be integrated within a weight-of-evidence (WOE) holistic approaches for Environmental Risk Assessment (ERA). In theory, biomarkers can be specific to a contaminant ore to a mixture of contaminates, providing information early warning, sub-individual clues on the impacts and, potentially, the causes of contamination to the biota (see Galloway et al., 2004 and Chapman et al., 2013 for a review). Among the vast set of biomarkers surveyed in ecologically-relevant organisms of models, oxidative stress-related biomarkers, such as the activity or relevant enzymes (like catalase) or biomarkers related to exposure to organic toxicants such as PAHs and dioxins (e. g. CYP1A induction and EROD activity), or metallothionein induction for metal toxicants, are some of the most common. These examples are typically considered biomarkers of exposure. Conversely biomarkers of effect tend to reflect lesions or metabolic impairment. Within this group are allocated for instance, DNA strand breakage and nuclear anomalies for the evaluation of genotoxicity and histopathological alterations and lipid peroxidations (see, e.g., Carreira et al., 2013; Costa et al., 2011a, 2011b and 2013; Gonçalves et al., 2013 and Rodrigo et al., 2013).

The case study of the present work, the Sado Estuary, SW Portugal (Fig. 1.1.), is characterized for its large area, high biogeography heterogeneity, and multiple sources of point and diffuse contamination resulting from its multiple anthropogenic usages and alterations, from urbanism to heavy-industry. All these characteristics pose significant constraints for the determination of environmental risk, as addressed below.

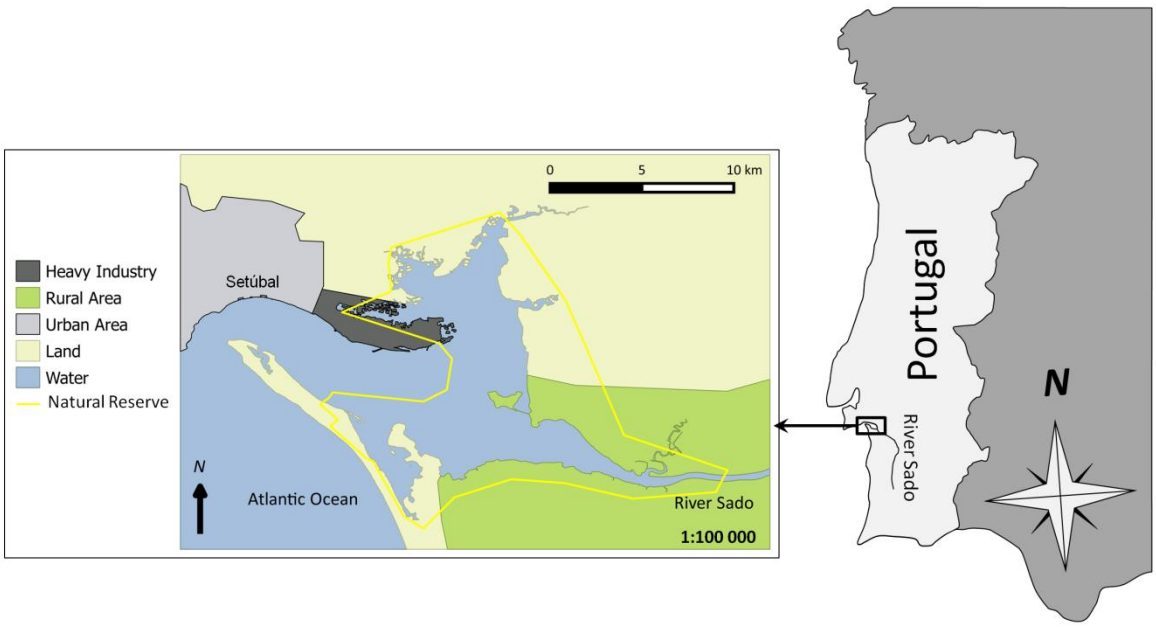

Fig. 1.1. Map of the case study (Sado Estuary, SW Portugal), including an overview of human settlement and land use (Hydrographic Institute of the Portuguese Navy).

The Sado Estuary is a warm-temperate mesotidal system located on the European Atlantic coast at the transitional zone between temperate and tropical climates (Quevauviller et al., 1989). Tides are semidiurnal, with a tidal range of about 1.6 m in spring tides and 0.6 m in neap tides (Martins et al., 2000). The mean river flow is 40 $m^3.s^{-1}$ (Vasconcelos et al., 2007). However riverine input displays strong seasonal variability, with freshwater discharges occurring mostly during the winter (Rocha, 2000). Thus, the system has also been referred to as a lagoon-type estuary (Cabeçadas et al., 1999), with a water volume of 500×106 m^3 and mean water residence time of 30 days (Vasconcelos et al., 2007).

It is one of the largest estuarine areas in Europe, with an approximate area of 240 km^2, that consists of a large confined coastal area located in the west coast of Portugal, that holds high socioeconomical and ecological value, however it is subjected to various sources of anthropogenic contamination, both point and diffuse. To preserve environmental quality and sustain human development, has dictated

several attempts to monitor environmental contamination and its effects on organisms (see for instance Caeiro et al., 2009; Costa et al., 2012 and Carreira et al., 2013 and references therein).

The estuary comprises the city of Setúbal (≈100 000 inhabitants) at the north and adjacent urban areas, a large heavy-industry belt characterised by many potential polluting activities, including a large paper mill, shipyards, chemical plant, mineral ore deployment activities and a thermoelectrical power plant, Eurominas, yeast factory, outfall of the City of Setúbal, fishing and urban ports, being one of Portugal's highest concentration of heavy industries (Caeiro et al., 2005b; Costa et al., 2008a, 2008b, 2012a; Carreira et al., 2013). In the southern areas, near the Sado's river mouth, the runoffs from agriculture grounds, mostly rice fields, nearby, potentially contribute to the transport of a particular set of xenobiotics such as pesticides and fertilizers, to which is added the river itself as a source of these substances from the extensive agriculture ground upstream (Costa et al., 2009a). Also, the river is an important source of metals since it crosses an important pyrite-mining region (Cortesão and Vale, 1995; Caeiro et al., 2005b; Carreira et al., 2013). At the entrance of Águas de Moura, aquaculture and rice field are the relevant activities (Caeiro et al., 2005b). There are other important activities such as maritime transport and tourism. These anthropogenic usages often collide with the preservation of the ecosystems that supports the life of many species like a resident population of bottlenose dolphins (*Tursiops truncatus*) (Van Bressem et al., 2003). Altogether the abovementioned human activities represent a critically large portion of the local society's income and economy.

Part of the estuary is classified as natural reserve (Costa et al., 2009b) and is the only Portuguese underwater reserve on the mainland territory, which is located just off the estuary. This reflects the importance of ecological conservation and biomonitoring and contributes for the conflict between natural environment preservation and anthropogenic usage and alterations (see Costa et al., 2009; Costa et al., 2012a and Carreira et al., 2013).

Recent findings (Caeiro et al., 2005b, 2009; Costa et al. 2012a) concluded that the Sado estuary is very heterogeneous with respect to sediment contamination; however, the area has generally been considered moderately impacted. Still, Neuparth et al. (2005), Caeiro et al. (2005) and Costa et al. (2012), for instance, disclosed that sections located near the industrial areas and the lower estuary hold levels of concern for many contaminants (both organic and inorganic), with adverse toxicological consequences to organisms.

2. Objectives

The main objective of this work is to develop and apply an integrative methodology for the characterization of the potential ecological risk of a contaminated estuary, through the combination of historical data collection, Sediment Quality Guidelines (SQG), biomarker index, statistics and Geographical Information Systems (GIS) tools.

Specifically, it is aimed at (see also Fig. 2.1.):

i) Determining the spatial distribution of sediment-bound toxicants (organic and inorganic) and link these to potential sources of toxicants.

ii) Contrasting the spatial distribution of contaminants to biological effects and responses inferred from an integrative biomarker approach.

iii) Using geospatial referring systems to aid identifying particular abiotic factors modulating contamination and risk, namely ocean/river influence, proximity to pollution hotspots and seasonality.

iv) Identifying the main areas of risk within the estuary and its potential causes as a preliminary tool to define several functional areas for future biomonitoring and management endeavours.

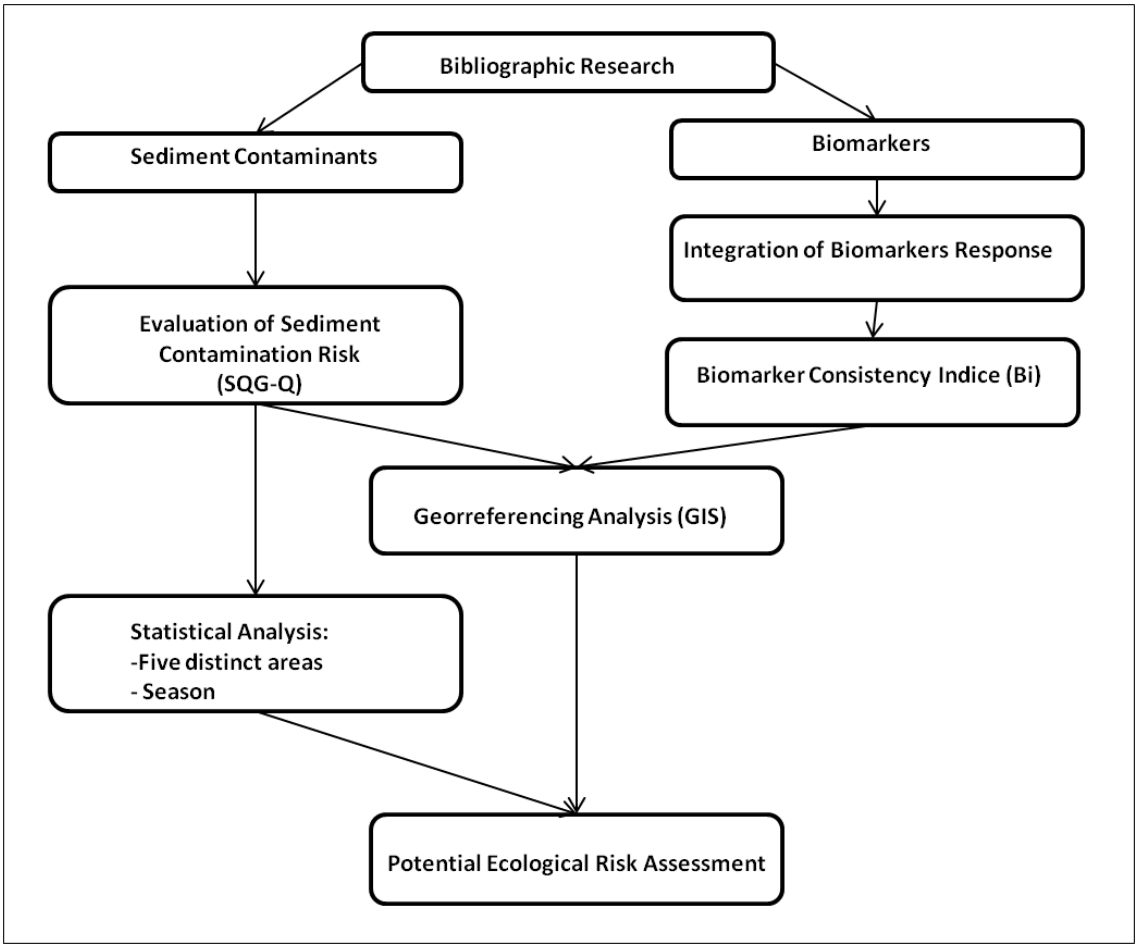

Fig. 2.1. Research and thesis layout

3. Material and methods

3.1. Bibliographic research and data collection

The collection of historical data was based on a bibliographic research (on Pub-Med, Science Direct and Springer) from which twenty-one published peer-reviewed articles related to risk assessment analysis in the Sado Estuary were selected. The publications, mostly, from the last decade, reported sediment contamination data and biological/and/or biomarker responses from wild and bioassayed organisms (fish and invertebrates). Table 3.1 summarizes the origin of the data included in the present research, see also Annexes 1 and 2 for further details.

Table 3.1. Sources of the data included and analysed in the present work, sorted by date.

Reference	Year	Season	Analysed sediment contaminants	Biomarkers	Organism and organ
Gil and Vale (2001)	1993	Spring/ Summer	PCBs		
Moreira, et al. (2006)	2003	Summer	As, Se, Cr, Ni, Cu, Zn, Cd, Pb, Hg	CAT, GST, tGSH, GSSG, GSH/GSSG, GPx, GR, AChE, LDH, SOD, LPO and PEF.	*Hediste diversicolor* (Whole body and head)
Neuparth, et al. (2005)	2004	Spring	Cu, Zn, Cd, PAHs, PCBs		
Costa, et al. (2008a)	2004	Spring	As, Cu, Cd, Pb	MT	*Sparus aurata* (Liver)
Lobo et al. (2010)	2006	Fall	As, Ni, Cu, Zn, Cd, Pb, PAHs, DDTs, PCBs		
Lillebø et al. (2011)	2006	Spring	Hg		

Table 3.1. (Continued)

Reference	Year	Season	Analysed sediment contaminants	Biomarkers	Organism and organ
Costa, et al. (2008b)				ENA and DNA-SB	*Solea senegalensis*
Costa, et al. (2009a)	2006	Fall	As, Cr, Ni, Cu, Zn, Cd, Pb, PAHs, DDTs, PCBs	Ih	(Peripheral blood, liver and gills)
Costa, et al. (2009b)				Bioaccumulation, MT and CYP1A.	
Costa et al. (2010)				Ih	
Costa et al. (2011a)				LPO, ENA, DNA-SB	*Solea senegalensis*
Costa et al. (2011b)	2007	Spring	As, Se, Cr, Ni, Cu, Zn, Cd, Pb, Co, Hg, Mn, PAHs, DDTs, PCBs	CYP1A, MT, GPx, CAT, GST and HSP90AA	(Peripheral blood and liver)
Costa et al. (2011c)				Ih	
Costa et al. (2012a)	2006/ 2007	Fall/ Spring	As, Se, Cr, Ni, Cu, Zn, Cd, Pb, Co, Hg, Mn, PAHs, DDTs, PCBs		
Costa et al. (2012c)	2007	Spring	As, Se, Cr, Ni, Cu, Zn, Cd, Pb, Co, Hg, Mn, PAHs, DDTs, PCBs		
Carvalho P. N. et al. (2009b)	2007/ 2008	Spring	DDTs		
Serafim et al. (2013)	2009	Fall	Cr, Ni, Cu, Zn, Cd, Pb, Hg, PAHs		

Table 3.1. (Continued)

Reference	Year	Season	Analysed sediment contaminants	Biomarkers	Organism and organ
Carreira et al. (2013)				LPO, CAT and MT	*Ruditapes decussates*
Costa et al. (2013)				Ih	(Gills and digestive glands)
Gonçalves et al. (2013)	2010/ 2011	Fall/ Winter	As, Se, Cr, Ni, Cu, Zn, Cd, Pb, PAHs, DDTs, PCBs	Ih, MT, CAT, GST, LPO	*Solea senegalensis* (Liver)
Rodrigo et al. (2013)				MT, LPO, GST, tGSH and GSH/GSSG	*Sepia officinalis* (Gills and digestive glands)

AChE, Acetylcholinesterase; As, Arsenic; Cd, Cadmiunm; CAT, Catalase; Cr, Chromium; Co, Cobalt, Cu, Copper; CYP1A, Cytochrome P450; DNA-SB, DNA strand breakage; ENA, Erythrocyte nuclear abnormalities; GPx, Glutathione peroxidise isoform 1; GR, Glutathione reductase; GSH/GSSG, Reduced/oxidised glutathione ratio; GSSG, Oxidized glutathione; GST, Glutathione *S*-transferase; HSP90AA, Heat-shock protein 90 kDa alpha; Hg, Mercury; Ih, Histopathological indice; LDH, Lactate dehydrogenase; LPO, Lipid peroxidation; Mn, Manganese; MT, Metallothionein induction; Ni, Nickel; Pb, Lead; PEF, Post-exposure feeding; Se, Selenium; SOD, Superoxide dismutase; tGSH, Total glutathione; Zn, Zinc.

3.2. Evaluation of sediment contamination risk

The potential risk to cause adverse effects to the biota of sediment-bound contamination was inferred by contrasting the Sediment Quality Guidelines proposed by MacDonald et al. (1996), namely the Threshold Effects Level (TEL) and the Probable Effects Level (PEL). The Sediment Quality Guideline Quotient (SQG-Q) was then estimated for total contaminants or class of contaminants according to the formula proposed by Long and MacDonald (1998):

$$SQG - Q = \frac{\sum_{i=1}^{n} \frac{Ci}{PELi}}{n} \quad (1)$$

Where *Ci* is the measured concentration of the *i* contaminant, *PELi* is the *PEL* guideline value for contaminant *i* and *n* is the number of contaminants under analysis.

To all sediments analysed a score was then attributed, according to the SQG-Q for total or grouped toxicants (per class) as proposed by MacDonald et al. (2004): SQG-Q<0.1—unimpacted; 0.1≤SQG-Q<1— moderately impacted; SQG-Q≥1—highly impacted. Although the guidelines and the SQG-Q analyses were designed for coastal waters and not transitional ecosystems, previous research been reported that the SQG-Q approach has provided the most consistent risk assessment classifications for sediment contamination in estuaries, including the Sado estuary, probably accounting, in most part, to its low range of salinity (from 29 to 37%) (see Rodrigues and Quintino, 1993 and Caeiro et al., 2005b).

3.3. Integration of biomarkers responses

Due to the high diversity of organisms and biomarker analyses reported in the different studies, these were integrated into a combined indice, namely the Biomarker Consistency Indice (*Bi*) proposed by Costa et al. (2012a). This indice measures the relative ability of a given set of biomarker response to achieve a statistically significant difference between an impacted and a reference site. The indice ranges between -1 (maximum consistency of a set of biomarkers yielding values below the reference situation), 1 (for values above the reference) and 0 (for values equals the reference), and is estimated as:

$$Bi = \frac{\sum_{j=1}^{n} sj}{n} \quad (2)$$

Where *n* is the number of surveyed biomarker responses and *sj* is the evaluation for the *j* case.

Unlike other approaches, such as the Integrated Biomarker Response (Beliaeff and Burgeot, 2002) the *Bi* ranges between a limited interval [-1,1], regardless of the number of organisms and biomarkers surveyed, therefore being more apt for comparisons between different assessment scenarios.

The present work is based on multiple animals organisms (namely fish, crustaceans and molluscs), types of assessment (wild organisms versus bioassays) and biomarker responses, hence the needs for an integrative approach.

The biomarkers surveyed in this work were of exposure and of effect. The biomarkers of exposure were acetylcholinesterase (Ache), catalase (CAT), cytochrome P450 (CYP1A), glutathione peroxidise isoform 1 (GPx), glutathione reductase (GR), reduced/oxidised glutathione ratio (GSH/GSSG), oxidized glutathione (GSSG), glutathione S-transferase (GST), hear-shock protein 90 kDa alpha (HSP90AA), lactate dehydrogenase (LDH), metallothionein induction (MT), superoxide dismutase (SOD) and total glutathione (tGSH). The biomarkers of effect were DNA strand breakage (DNA-SB) and erythrocyte nuclear abnormalities (ENA), histopathological indice (Ih) and Lipid peroxidation (LPO). It was also taken into account post-exposure feeding (PEF) in *Hediste diversicolor* and a bioaccumulation in the liver of *Solea senegalensis*. See table 3.1. for further details.

3.4. Georeferencing Analysis

A Geographic Information System (GIS) analysis of the Sado Estuary was undertaken using QGis 2.0. Digital maps were obtained from the Hydrographic Institute of the Portuguese Navy (http://www.hidrografico.pt), for mainland coastal waters (EPSG: 4326 – WGS 84 coordinate system) with base scale 1:100 000. The analysis aimed at surveying the spatial distribution of SQG-Q for total sediments toxicants and for major classes of toxicants; sediment physico-chemical properties (namely organic matter and fine fraction contents); the raw data of main toxicants of concern and the *Bi* scores as an estimate for biological effects. Interpolation was achieved by using the Inverse Distance Weight Algorithm (IDW), normalized for maximum/minimum scores for each plotted parameter.

The division of the Sado Estuary into distinct biogeographical/ecotoxicological subareas was achieved by analysing historical data (see Table 3.1) and empirical information on the distribution of anthropogenic activities and potential sources of point and diffuse contamination. The division into functional subareas was integrated within GIS and subsequent analyses. Whenever achievable, according to available data, data were georreferenced by season. For the purpose, seasonal patterns were surveying by dividing available data by “cold” (autumn and winter) and “hot” (spring and summer) seasons.

3.5. Statistical Analysis

Following invalidation of assumptions to perform parametric statistics (normality and homocedasticity) through the Levene’s and Komogoroff-Smirnoff’s test, respectively, the non-parametric Kruskall-Wallis ANOVA by ranks *H* statistic was computed to verify overall differences between the different areas and seasons for all studies parameters. A significance level of $\alpha = 0.05$ was considered for all analyses. All statistics were performed with the software Statistica (Statsoft). Statistics follow Zar (1998).

4. Results

4.1. Division of the estuary into functional subareas

The estuary was divided into five distinct areas (Fig. 4.1), based the abovementioned preliminary survey based. Each area was observed to allocate distinct socio-economical activities, such as industry, urbanism, agriculture and mariculture. As such, the five areas consisted of a reference location (Rf) and other four probably impacted areas termed S1, S2, S3 and S4.

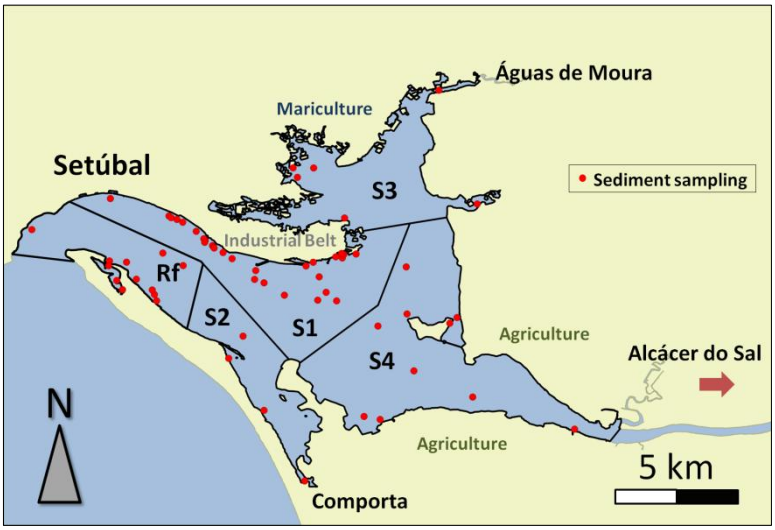

Fig. 4.1. The five distinct areas of Sado Estuary, the reference area (Rf) and impacted areas (S1, S2, S3 and S4). The Rf area is clean with very strong ocean influence. The S1 area is very influenced by the urban area of the city of Setúbal and the Industrial Belt of the Mitrena peninsula which has low hydrodynamics. The S2 area is at the Comporta channel and it is characterise by agriculture influence. The S3 area is at the Águas de Moura channel has low hydrodynamics and has some mariculture. The S4 area is at the mouth of the river and has strong influence of agriculture runoffs.

4.2. Description of sediment contamination

From the Fig. 4.2. it could be inferred that the Sado Estuary is very heterogeneous regarding the relative contents of fine fraction (FF, particle size < 63 µm) and total organic matter (TOM) in sediments. Higher percentages of FF were registered in area S4 (near the river mouth). Conversely, the sediments from the reference area (Rf) are almost devoid of FF, with a difference of two orders of magnitude from highest values from the other areas. On its turn, TOM it is evident some points of deposition in S1 (near the city and the harbour of Setúbal) and S3 (Águas de Moura channel), however the S2 (Comporta channel) and S4 are mostly clean of organic matter differing from Rf that is totally clean, with no TOM.

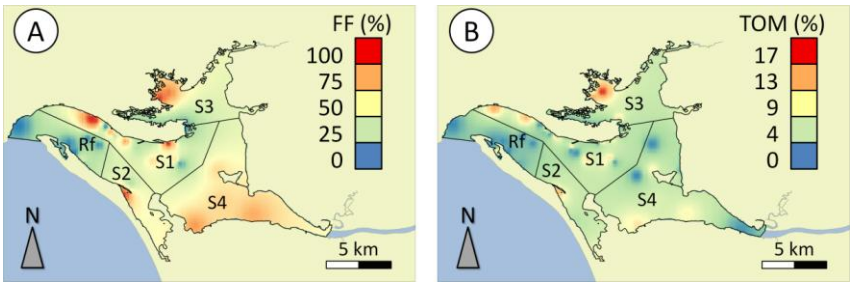

Fig. 4.2. Spatial distribution of the percentage of A) fine fraction (FF) and B) total organic matter (TOM) in the sediments of the Sado Estuary (in % relatively to dry sediment mass). Interpolation by IDW

The different areas of the Sado Estuary revealed also distinct patterns of sediment contamination. Regardless of season, significant differences were found between SQG-Q total contaminants between all areas (Kruskall-Wallis H, $p<0.05$), as shown on Fig. 4.3.A. According to this, the Rf area could be considered unimpacted (average SQG-Q = 0.06±0.08) while the S1, S2, S3 and S4 areas were found to be moderately impacted. The average SQG-Q for total contamination can be ranked as S1>S2>S3>S4>Rf. The high SQG-Q was retrieved for area S1 (0.38±0.22). In general no significant differences were found between the hot and cold season for SQG-Q total (Kruskall-Wallis H, $p>0.05$), as shown on Fig. 4.3.B. and 4.3.C. There was no data available for S2 area in the cold season (Autum/Winter).

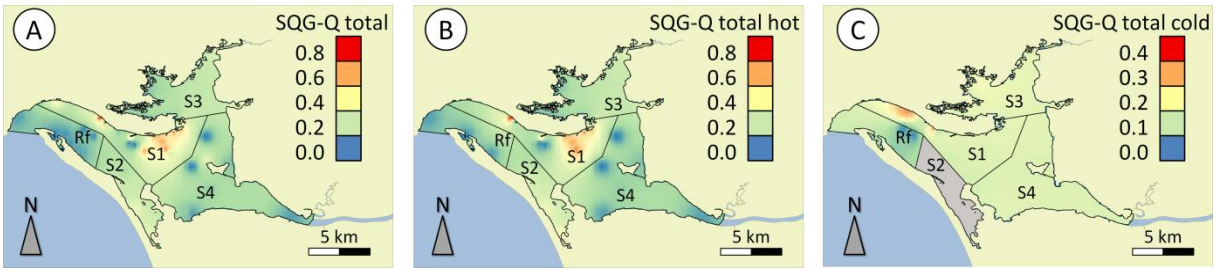

Fig. 4.3. Spatial distribution of Sediment Quality Guideline Quotients (SQG-Q) for total contaminants (metallic and organic). A) All data combined. B) Hot season. C) Cold season. Grey-shaded areas indicate unavailable data. Interpolation by IDW.

As previous SQG-Q for inorganic toxicants yielded significantly different patterns between different areas (Kruskall-Wallis H, $p<0.05$), as shown on Fig. 4.4.A. The overall potential of observing adverse biological effects was again lowest for Rf (0.10±0.10) leading to the classification as unimpacted. The sediments from areas S1, S2, S3 and S4 were mostly moderately impacted with one point in S1 (0.47±0.33) attaining the rank of highly impacted (1.43). The average SQG-Q for inorganic contamination can be ranked as previous. Again no significant differences were found between hot and cold season (Kruskall-Wallis H, $p>0.05$), as shown on Fig. 4.4.B and 4.4.C.

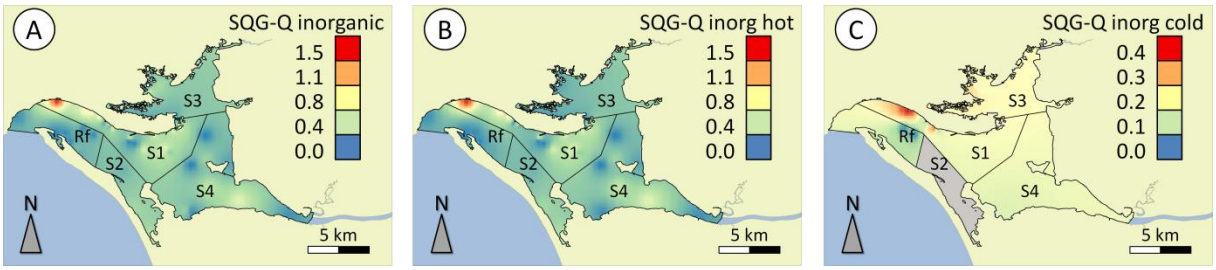

Fig. 4.4. Spatial distribution of Sediment Quality Guideline Quotient (SQG-Q) for inorganic contaminants. A) All metallic/metalloid toxicants combined. B) Hot season. C) Cold season. Grey-shaded areas indicate unavailable data. Interpolation by IDW.

There were significant differences between the SQG-Q organic among the different areas (Kruskall-Wallis H, $p<0.05$), as shown on Fig. 4.5.A. For the overall potential of observing adverse biological effects the Rf, S3 and S4 areas were considered unimpacted and the S1 and S2 considered moderately impacted. The average SQG-Q for organic contaminants can be ranked as S2>S1>S3>Rf>S4. The highest SQG-Q was retrieved for area S1 (0.90). There were significant differences between the SQG-Q organic in the different seasons (Kruskall-Wallis H, $p<0.05$), as shown on Fig. 4.5.B and 4.5.C.

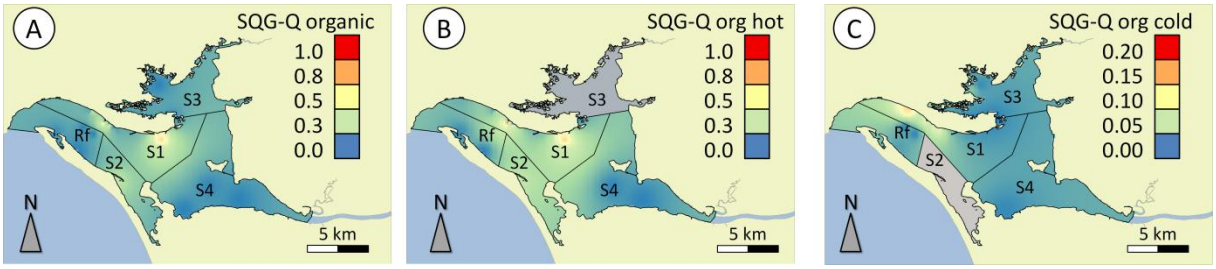

Fig. 4.5. Spatial distribution of Sediment Quality Guideline Quotient (SQG-Q) for organic contaminants. A) All organic combined. B) Hot season. C) Cold season. Grey-shaded areas indicate unavailable data. Interpolation by IDW

4.3. Sediment contaminant of particular interest

From the recollected data it was observed that the metals that exceeded the Probable Effect Level (PEL) (see MacDonald et al., 1996) were copper (Cu) (PEL> 108 µg/g), zinc (Zn) (PEL>271 µg/g) and the metalloid arsenic (As) (PEL>41.6 µg/g). The high significance of these elements as contaminants in the estuary's sediments was also referenced in Costa et al. (2010 and 2012a) that they were consistently the elements of most concern.

From the Fig. 4.6.A it may be inferred that the distribution of As in sediments was uniform between all areas, with the exception of the reference area (Rf) in which the levels of this metalloid were, on average, lower than in other areas by a order of magnitude. The high level was found to be of 44 µg As/g of dry sediment in the industrial belt (S1 area) from the hot season and the low level was of 0.34 µg/g at the reference area (Rf) at the cold season.

Copper (Fig. 4.6.B) has his hot spot (361 µg/g in the hot season) from the runoff of the city of Setúbal (S1 area) that differs from the reference area in tree orders of magnitude (0.21 µg/g in the hot season). For the rest of the areas it seems to be clean with levels below PEL.

The Zn (Fig. 4.6.C) it is most representative on the runoff of the Sado river (S4 area) and also from the industrial belt (S1area). The high level was found to be of 385.11 µg/g in the mouth of river (S4 area) from the hot season and the low level was of 8.2 µg/g at the reference area (Rf) on the hot season.

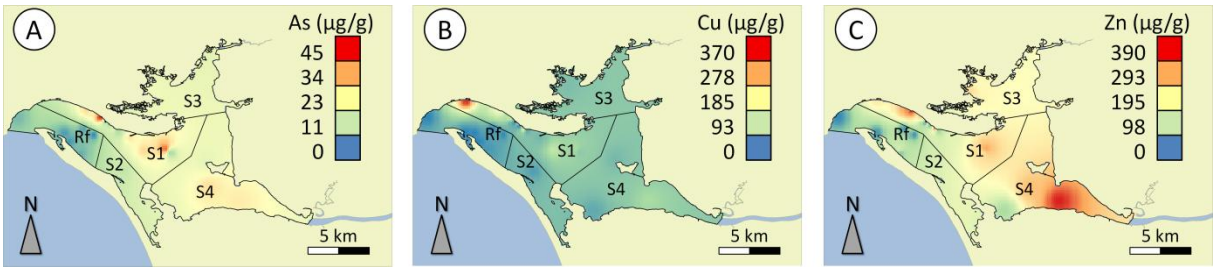

Fig. 4.6. Spatial distribution of the most representative elements in sediments from the Sado Estuary. A) arsenic, PEL>41.6 µg/g; B) copper, PEL>108 µg/g; C) zinc, PEL>271 µg/g. Interpolation by IDW.

There are no data available for PAHs in sediments from S2 area (Fig. 4.7.). Still it was observed that there were differences between the hot season and the cold season for SQG-Q PAH total, a difference from one order of magnitude, however all this levels are below the PEL. The high level was found to be of 0.90 in the industrial belt (S1 area) from the hot season and the low level was of 0.003 at the reference area (Rf) on the cold season. For the overall potential of observing adverse biological effects in total PAH only S1 has points that can be considered to be moderate impacted, all the other areas are unimpacted. There was also no data available for S3 area for Fig. 4.7.B.

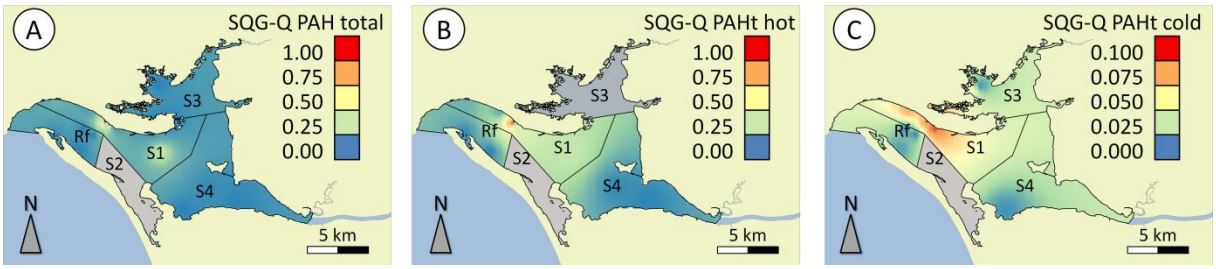

Fig. 4.7. Georreferencing of Sediment Quality Guideline Quotient (SQG-Q) for total PAHs. A) All PAH total combined. B) Hot season. C) Cold season. Grey-shaded areas indicate unavailable data. Interpolation by IDW.

4.4. Global biomarker response

In general the Biomarker Consistency Indice (*Bi*) (Fig. 4.8.) was in accordance with the sediment contamination, with the most impacted area being the S1 and the clean area being the reference area (Rf). There was some response from the biomarkers at the mouth of the river (S4), the S2 area had no biomarker response and there was unavailable data for S3 area.

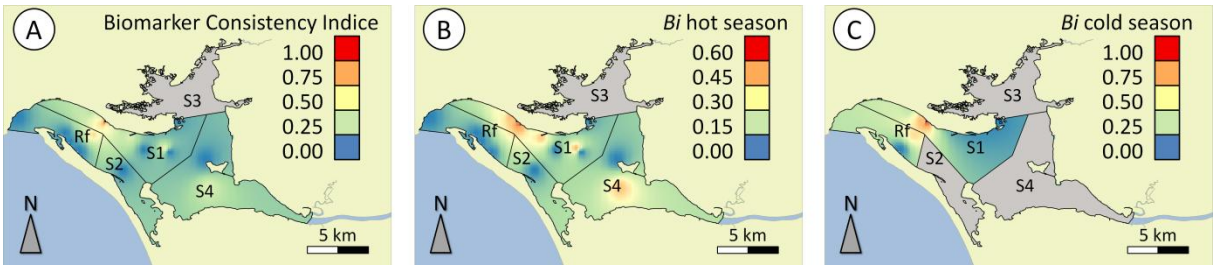

Fig. 4.8. Spatial distribution of the Biomarker Consistency Indice from the Sado Estuary. Grey-shaded areas indicate unavailable data. Interpolation by IDW.

The Biomarker Consistency Indice revealed differences between sites with respect to yielded significant biological responses in comparison to any given reference scenario. Through the Table 4.1 it was observed that in the hot season for the reference area has no biomarkers responses in contrast for the cold season has responses. For S1 area there was effects in both seasons with more significance in cold season. For S2 there was no overall significant biological response in the hot season and in the cold season there was not available data. For S3 there was no data available in both seasons. In S4 for the hot season there were biological responses and in cold season there was no data available.

Table 4.1. The Biomarker Consistency Indice (Bi) ± standard deviation per season (hot and cold), for each area of the Sado Estuary: reference (Rf) and contaminated (S1-S4).

Areas	**Season**			
	Hot		**Cold**	
	Bi X±Dv	**n**	**Bi X±Dv**	**n**
Rf	0	4	0.30±0.42	2
S1	0.34±0.20	10	0.64±0.34	3
S2	0	1	N/A	0
S3	N/A	0	N/A	0
S4	0.20±0.28	2	N/A	0

(N/A – Not available); n, number for surveyed sites per area; Bi, Biomarker Consistency Indice.

5. Discussion

5.1. Main observations

Marine environments are diversified yet singular ecosystems due to their complex biogeographical, geochemical and geophysical characteristics, with particular respect to transitional ecosystems such as estuaries. To these factors are added various anthropogenic stressors, including multiple point and diffuse sources of pollutants. Due to their complex characteristics, every marine ecosystem may be regarded as unique which, therefore, dictates the need to develop specific monitoring programs as basis for specific environmental management plans. Also, even within each such ecosystem, natural biogeographical heterogeneity may imply specific management plans for the different spatial subunits within.

The present study showed that the Sado Estuary is indeed very heterogeneous, not only in respect to its ecological and geographical characteristics but also to the sources, distribution and biological effects of toxicants. In fact, concerning the spatial distribution of pollutants in sediments alone it was possible to confirm that there is a range between clean and very significantly contaminated sediments. The SQG-Q approach confirmed, at least theoretically, the heterogeneity of the estuarine basin, in terms of risk to the biota, meaning that it ranged between 0 (unimpacted) for the reference area and 0.79 (impacted) for area S1 (Fig. 4.2A). Previous studies (namely Caeiro et al., 2005b) had already stated that for metals and metalloids the reference area is unimpacted, although contrary to the present findings, it was also concluded that the S3 area and the sandbanks (S1) were unimpacted. The differences between areas are also likely caused by distinct hydrodynamics along the estuary, causing higher deposition of fine sediments from the river and near the city of Setúbal. Also the contamination gradient of estuarine sediments is a consequence of the different sources of pollutants from the multiple human activities (industrial, agricultural and urban), settled along the banks of the estuary, although preferentially localized per specific areas. It is also important to notice that natural contamination of estuarine sediments may also occur, in particular by some metals (such as Cu and As), due to the geological characteristics of Sado river basin, which crosses an important iron ore mining region (Quevauviller et al., 1989). Altogether, the complexity of the estuary confirms the importance of integrative approaches on complex ecosystems for Environmental Risk Assessment. In fact, previous research has already stressed the high ecotoxicological heterogeneity of the Sado Estuary, and thus the need to implement specific monitoring and management actions for each biogeographical unit within (Caeiro et al., 2005b and 2009; Costa et al., 2012a).

The main impacted areas revealed in this work were S1 (urban and industrial area) and S4 (mouth of river). The S1 area is the most impacted area, most likely due to Setúbal's heavy industry area, which

includes shipyard, chemical plants, thermoelectrical power plant and other potentially polluting facilities. On its turn the S4 area has a strong influence of the river runoff, which may imply contamination by metals from natural erosion processed of the watershed of the Sado River plus fertilizers and pesticides from the agriculture grounds upstream, especially from extensive river cultivation. As confirmed in the previous work, Caeiro et al. (2005b) and Costa et al. (2012a) already concluded that the sediments most contaminated by metals and metalloids were located on the North Channel and near the City of Setúbal.

The main sediments contaminants in the Sado Estuary were found to be Cu, Zn, As and PAHs. Other surveyed toxicants, namely PCBs, DDTs and the other elements (Cd, Cr, Pb Hg, Ni, Ag) were consistently below the PEL threshold, implying reduced environmental risk. Nonetheless, older works on the Sado Estuary (see Caeiro et al., 2005b, 2009) found that the metal and metalloids of concern were Cd, Cu, Zn and As, since these exceeded the PEL guidelines, together with organochlorine pesticides (DDTs) which is not accordant with the present work. It is possible that the Sado Estuary is undergoing a process of recovery following, for instance, changes in wastewater treatment implemented in the past years (see Caeiro et al., 2009), leading to come changes in sediment contamination profiles. The overall level of impact was estimated through SQG-Qs for total sediments contaminants (organic and inorganic combined), revealing that the most impacted areas were those close to the shipyard facilities and also those closer to the city of Setúbal.

The percentages of FF and TOM in the sediments were found to be in general accordance with the levels of contaminants in sediments. The highest levels of FF in sediments were recorded in area S1 and TOM were in area S3 (Fig. 4.1.) which were precisely the areas were the highest levels of toxicants were found, especially, Cu and As (mostly in S1). The relationship between sediment FF, TOM and the levels of sediment-bound contaminants is long known (see for reviews, Sigleo and Means, 1990; Chapman et al., 2013), which, together with the current results, stresses the importance of understanding the geodynamics of estuarine sediments and the sediments' critical role in the trapping and storing of hazardous substances and the difficulties in accurately assessing the true environmental risk of sediment-bound xenobiotics.

Nonetheless, the current findings showed a closer link between sediment FF and SQG-Q for total contamination than TOM (Figs. 4.1A and 4.2A). If fact, the relation between FF and SQG-Q (for total contaminants) was more obvious in sediments from area S3, probably due to its higher time of residence of the sediments particles (as a function of hydrodynamics), relatively to other areas.

Overall, the major sources of inorganic contaminants in the estuary should be located in the urban/industrial zone (area S1) with some contamination hotspot located close to the porto f the city of

Setúbal (Fig. 4.3A). Along the remaining estuary the presence of these contaminants in the sediments is more uniformly distributed (in low-moderate levels). Among these toxicants As is seemingly the most ubiquitous, likely resulting from natural erosion processes along the Sado River basin. In fact, natural erosion of river basin bedrocks has been found to be one, if not the most, important sources of metals in aquatic ecosystems (e.g. Preda & Cox, 2002).

Sediment organic contaminants seemingly have their major sources located of the industrial belt (S1), which implies anthropogenic origins. Most of these substances should hold pyrolitic (combustion) and petrolytic origins (see Cardellicchio et al., 2007 and Neser et al., 2012). Oppositely, contamination by PAHs in sediments from areas with greater agricultural/riverine influence (and the reference area) is low or null (Fig.4.4.). No significant dissimilarities were found between the distributions of high and low molecular weight PAHs, which sustains the notions that these compounds contaminate ecosystems as mixtures. This fact, on its turn, stressed the importance of evaluating the inputs of the PAHs in to the environment because of their potential carcinogenic and mutagenic capabilities (Pashin & Bakhitova, 1979; Larcher et al., 2014). It must be noted, though, that the SQG-Qs for total PAH indicate that in the hot season the concentrations of PAH in the sediments increase in comparison to the winter/fall months, likely due to reduced ocean hydrodynamics and freshwater inputs, favouring the deposition of FF and TOM to which these hydrophobic contaminants are preferentially bound to (Fig. 4.6.). Conversely, during the winter, the increased freshwater inputs and higher ocean driven tidal hydrodynamics tend to wash-off the FF and TOM from the superficial layers of sediments, thus reducing toxicant concentrations. In fact, seasonality in transitional waters has long been acknowledged as paramount, inclusively regarding its effects on sediment dynamics (Li et al., 2014; Chapman et al., 2013). Nonetheless, the present findings contradict previous studies in estuarine and riverine basins that state that stormwater and heavy runoffs are responsible for increased contamination (and related toxicity) of sediment-bound organic toxicants, including PAHs (e.g. Anderson et al., 2007; Nemirovskaya, 2011). Still, it is highly likely that, in the Sado Estuary, a typical Mediterranean climate basin, thus presenting low hydrodynamics during summer in combination with reduced freshwater input, the deposition of particulate suspended matter during summer is increased, leading to a concomitant increase of global PAH in sediments. Thus, the present findings indicate the importance of considering the specific climate and hydrodynamics for each particular case study.

No significant differences between seasons concerning the distribution of metals and metalloids in sediments, which may be due at least in part, to the different chemicals proprieties between these (more hydrophilic) and organic contaminants like PAHs and other highly hydrophobic compounds. As such, it is likely that the trapping of metals in sediments is less dependent of FF and TOM, unlike organic pollutants. On the other hand, the origin of metals, specifically those from natural origins, may be more diffuse in this estuary. Still, Vale and Cortesão (1989) concluded that the effect of season is

important for the distribution of metals in the sediments of the Sado Estuary since the river drains an active pyrite mining area, which is not entirely accordant with the present findings, showing that the distribution and concentration of inorganic elements does not vary significantly with season. Nonetheless Quevauviller et al. (1989) noted seasonal variation, especially for Zn and Cu, in sediments from the estuary. It is plausible, though, that the decrease of mining activities in the area have been contributing to a reduction in metals inputs in the estuary. Still, there are other reports of the reduced effects of season the distribution of these substances in impacted coastal areas. For instance, DelValls et al. (2002) affirmed that in the Gulf of Cadiz (Spain), an area historically affected by metals, seasonality didn't affect sediment quality for metals and metalloids. Still, further research is needed on the subject for the Sado Estuary, since the lacked of focused monitoring programmes clearly led to many gaps in the information on sediment contamination along the year-course.

Through the Biomarker Consistency Indice (Bi) it was observed that the biological responses were generally in accordance with sediment contamination, with the most consistent responses being retrieved from area S1 (Fig. 4.7.A). Moreover, the current findings indicate that during the cold season organisms are more responsive to stressors, likely toxicants, even in the reference area which is not in accordance with the expected increase of metabolic rates during the hot season. Nonetheless, because of the little data available for the biological responses the conclusions regarding this subject must be limited. Still, for instance, Bucher et al. (1996) found out that SOD activity increased during the summer on the polychaete *Arenicola marina*. Shaw et al. (2004) also found that the CYP1A – like immunopositive protein levels had its maximum in May and the DNA strand breaks had its lowest in December on the mussel *Mytilus edulis*. Geffard et al. (2005) also found that the MT in mussels, *Mytilus edulis* increased during the summer season. Bocchetti & Regoli (2006) noticed season-related effects in wild *Mytilus galloprovincialis* for several biological responses with major variation in spring and summer months due to probably to temperature, reproductive cycle and food availability. Altogether, it is clear that seasonality of biological responses to environmental stressors is key issue in environmental risk assessment that should be targeted for further studies in the Sado Estuary.

5.2. Description of the different areas

The present work showed that it is relevant to know the individual history and present problems of each ecosystem, especially those of transitional waters. For instance in Lake Macquire, Australia, the presence of coal-fired power stations also with other heavy industry input elevated concentrations of Cu, Se and, especially, Cd in the sediments (Schneider et al., 2014). In Er-Ren estuary, Taiwan, Chen et al. (2014) described that the sediments were mainly contaminated by the metals Cu and Zn, originated from industrial waste. In Wabamun Lake, Canada, sediments were contaminated, presumably by the activities from the coal industry, with metals and PAHs (Donahue et al., 2006). In

another example, the Todos-os-Santos Bay (Brazil) suffered from several anthropogenic activities such as industrial and untreated domestic effluents, solid wastes, ports and agriculture, resulting in mixed contamination (Krull et al., 2014). Also in Sydney Estuary (Australia) rapid urbanization and industrialization contaminated the sediments with metals, especially Cu, Pb and Zn (Nath et al., 2014). As such, each area needs to be evaluated individually in face of its specific sets of stressors and biogeographical characteristics, which should also apply for each individual subarea in highly heterogeneous ecosystems.

The reference area of the present study (Rf) is located at the entrance of the estuary, has a strong ocean influence and is considered in this work to be unimpacted. In accordance with the reduced levels of sediment contaminants, the levels of FF and TOM were low. The absence of direct pollutant sources and relatively high oceanic influence should be the major factors involved in the reduced impacts observed in this area, accountable through reduced biological effects to stressors. As such, this area chiefly important for tourism and leisure activities in the estuary (part being classified as natural reserve) shown no immediate concerns regarding environmental contaminants and appears to be an adequate internal reference for comparative purposes.

The S1 area (Northern channel) is the most impacted due the urban influence (city of Setúbal) and the industrial belt. The FF and TOM percentages and the SQG-Q total were consistently high in this area, confirming its relatively high impact in comparison to the remaining areas of the estuary. In a sediment collection point near the city of Setúbal, the SQG-Q for inorganic pollutants attained the classification of highly impacted (1.4), the highest score in the study. Moreover, the highest levels of As in sediments were also recorded in this area (highest value 44 µg/g), of the industrial belt. In the past, even the sediments from nearby sandbanks (and acknowledged fishing grounds in the estuary), high levels of As were found, hitherto related to the use of pesticides and herbicides (Caeiro et al., 2005b). For the SQG-Q organic the contamination comes mostly from the industrial belt, mostly concerning PAHs Although the levels of PAHs were higher in this area, albeit without attaining significant risk, according to SQG-Qs, these substances have been linked to very significant adverse effects to organisms in past studies (see Costa et al., 2012), which mandates caution when interpreting risk from PAH levels in the environment alone and indicates that these substances should be target for monitoring as well, together with As and other metals of concern.

The S2 area (Comporta channel) has a stronger agricultural influence, in particular from the adjacent rice fields. The information on sediment contamination for this area is incomplete, nevertheless, with only data for metals and metalloids in the sediments being available. Also, there is no information available for colder months, critically compromising the interpretations of season-related effects. Overall, the S2 area may be considered to be moderately impacted in face of the existing data. Further

research is clearly needed for this area, because paddy fields and other adjacent agricultural areas are acknowledged sources of toxicants to rivers and estuaries (see for a review Ueji and Inao, 2001).

The S3 area (Águas de Moura channel) has low hydrodynamics, therefore favouring particle deposition and high water residence time. The main anthropogenic stressors are related to mariculture, although most facilities are currently deactivated. In accordance with the area's reduced hydrodynamics, the percentages of FF and TOM were high while the area may be deemed low-moderately impacted. However the scarce ecotoxicological information available for the area grossly limits the evaluation. From the SQG-Q for organic contaminants, the area may be judged to be unimpacted (no data is available for the hot season). The levels of As, Cu and Zn (the main toxicants in the estuary) the levels were below PEL which suggest low risk. This area needs further research as well. In recent studies it was verified that the presence of mariculture increases the inputs of organic matter in to the sediments of transitional waters, therefore increasing the trapping of contaminants, especially organic (more hydrophobic) like PAHs (Yu et al., 2012), PCBs (Wang et al., 2011) and even toxic metals like Hg (Liang et al., 2012).

The S4 area (Alcácer do Sal channel) holds the strongest influence from river runoffs according to the SQG-Q approach, this area may be considered moderately impacted by metals and unimpacted by organic toxicants and there were significant differences between seasons. Still, for As and Cu there were no significant contamination, which may indicate that the sediments from this area do not tend to concentrate metals and As resulting from the river drainage basin (refer to Quevauviller et al., 1989 and Cortesão and Vale, 1995), unlike the high FF and TOM sediments further downstream, especially those from area S1. Still, there is a lack of information on important organic toxicants for this area, especially pesticides and fertilizers. As such, the findings should be interpreted with caution.

6. Conclusions

The present work confirmed that the Sado Estuary is very heterogeneous in terms of sediment contamination, both from inorganic and organic toxicants. It has been shown through spatial analyses, that the distribution of toxicants in estuarine sediments is caused by point and diffuse sources of anthropogenic, to which are added natural sources of contamination. Nonetheless, human activities constitute the major sources of toxicants. Very importantly, it has been shown that the distribution of sediment contaminants is modulated by the complex biogeography of the area, with particular respect to hydrodynamics and the balance between ocean and riverine inputs, rendering patterns of contamination that are not solely explained by proximity to pollution hotspots. In addition to the aforementioned constraints, management policies for the Sado Estuary have to deal with many conflicting situations between the socio-economical activities and the need to preserve environmental quality. In fact, whereas the estuary comprises a dense heavy-industry belt, a very significant part of it is classified as a natural reserve. Also the levels of contamination in the sediments were found to range between low-medium and revealed complex mixtures of toxicants, which altogether poses yet another constraint when attempting to identify sources of pollution. These circumstances imply that the decision-makers and other stakeholders need to consider a differentiated management between the different areas of the estuary.

In general the most problematic area is the one closer to the shipyard facilities (S1 area). Within this area the sediments collected of the urban region were the most impacted by inorganic toxicants (i.e., metals and metalloids) whereas the sediments closer to the shipyards were the most contaminated by organic toxicants. Regarding specific substances of concern, it was noted that As attained higher levels and high risk in S1 area, while the metallic was found to be diffusely distribute along the sediments of the entire estuary, reaching concentrations of moderate concern, which indicates the natural origins of this element from river inputs. Copper is another element of concern especially in sediments adjacent to the urban area (although its distribution is diffuse as well), possibly resulting from wastewaters, urban runoffs, maritime transport but also from river, from the natural erosion processes along the river bed (which crosses important pyrite mining grounds. Similar findings were obtained for zinc, whose main source is likely riverine input. The PAHs were found to be the most important organic toxicants, with some increase in sediment contamination by these substances being observed during the hot season, most likely due to a shift in hydrodynamic process that increase sediment fine particle and organic matter deposition. Still, although PAH levels did not attain high risk levels, the higher levels were found of the industrial belt.

The use of Geographic Information Systems (GIS) was proven to be particular very useful as an integrative tool, since it assisted a holistic approach to the estuary, specifically through the geographic

distribution of contaminants in the sediments and biomarkers responses on the Sado Estuary. The approach was clearly advantageous to identify differences between areas and probable sources of contamination to assist future management by the competent entities. However, computational limitations resulting from the IDW algorithm itself (that may hinder contrasts) and much missing data indicate that further research with this tool should be endeavoured in the future.

The present work demonstrated the importance of performing integrative approaches in environmental risk assessment (ERA), since they permit circumventing information gaps inconclusive responses and findings of difficult interpretation in complex ecosystems, thus permitting a more solid groundwork to draw conclusions about the true environmental status of transitional waters. In fact, many other authors stressed the importance of surveying several endpoints in ERA. As an example, it has been mentioned the importance of surveying at least three lines of evidence (LOE), for adequate risk assessment for sediment contamination, namely such sediment contamination profiling toxicity assessment and infaunal community determination (Long and Chapman, 1985), although the last LOE (the ecological component) may be more difficult to achieve due to the natural complexity of ecological networks. As such multiple LOEs permit a weight of evidence (WOE) approach on complex scenarios.

The current work also endure some constraints, especially those related to in the lack of historical information for some of the studied areas and moreover between seasons. These limitations hindered the analyses of a few areas namely S2 and S3, which clear need further research. Overall, these constraints indicate the need to perform an integrated biomonitoring programme for the estuary.

Finally, it must be stressed that this work confirmed the uniqueness of a marine ecosystem and the importance of designing specific, permanent biomonitoring programmes upon which effects management can be set. It is clear that, in the near future permanent monitoring in the Sado Estuary needs to be designed put in motion, which is actually one of the demands of the MSFD for European marine waters as an instrument meet the ambitious goals of attained Good Environmental Status for European coasts by 2020 (see Borja et al., 2010; Lyons et al., 2010).

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8. Annexes

Annex 1. Physico-chemical characterization and inorganic contamination profiles of the sediments collected in Sado Estuary.

										Inorganic (µg/g)										
										Non metals				Metal						
Longitude	**Latitude**	**Station**	**Area**	**Reference**	**Year**	**Season**	**TOM (%)**	**FF (%)**	**Eh (mV)**	**As**	**Se**	**Cr**	**Ni**	**Cu**	**Zn**	**Cd**	**Pb**	**Co**	**Hg**	**Mn**
-8.862338	38.492232	S1	Rf	Carreira et al. 2013, Costa et al. 2013, Gonçalves et al. 2013, Rodrigo et al. 2013.	2010/2011	Fall/Winter	0.80	2.50	-	3.5	0.63	2.3	1.71	4.04	14.51	0.13	5.73	-	-	-
-8.852725	38.48632	S2	Rf				0.90	3.50	-	0.34	1.84	2.36	4.1	4.51	13.1	0.03	3.50	-	-	-
-8.833843	38.4925	S3	S1				10.40	52.90	-	19.7	1.92	77.67	16.67	178.64	327.51	0.27	56.45	-	-	-
-8.743549	38.436586	S4	S4		2011	Spring	6.90	63.70	-	26.44	0.59	62.22	17.15	74.15	269.79	0.33	25.30	-	-	-
-8.715739	38.424214	S5	S4				8.80	74.30	-	25.02	0.72	87.61	22.79	92.30	385.11	0.43	32.70	-	-	-
-8.879504	38.487932	S6	Rf	Costa et al. 2008a	2004	Spring	0.38	0.64	-	3.34	-	-	-	1.75	-	0.08	2.25	-	-	-
-8.87487	38.47987	S7	Rf				0.46	0.35	-	2.39	-	-	-	1.97	-	0.08	2.47	-	-	-
-8.780113	38.469656	S8	S1				1.44	9.21	-	10.94	-	-	-	72.04	-	0.09	32.19	-	-	-
-8.804832	38.472344	S9	S1				10.38	58.79	-	27.44	-	-	-	120.69	-	0.08	57.86	-	-	-
-8.74681	38.463473	S10	S4				8.63	60.30	-	19.37	-	-	-	56.05	-	0.08	41.71	-	-	-
-8.824401	38.452988	S11	S2				6.07	28.83	-	14.7	-	-	-	27.50	-	0.08	22.41	-	-	-
-8.86631	38.472658	S12	Rf	Costa et al 2008b, 2009a, 2009b, 2010, 2012a	2006	Fall	3.00	37.00	-233	7.25	-	24.2	12.97	22.57	147.48	0.04	23.70	-	-	-
-8.859444	38.509742	S13	S1				12.00	98.00	-290	27.43	-	76.33	33.67	167.32	312.23	0.22	66.49	-	-	-
-8.842278	38.498189	S14	S1				8.00	76.80	-316	12.38	-	21.85	9.03	41.18	87.75	0.15	45.17	-	-	-
-8.86528	38.469702	S15	Rf	Costa et al 2011a, 2011b, 2011c, 2012a, 2012c	2007	Spring	2.00	23.00	-140	5.2	0.27	18.14	7.31	28.20	72.29	0.06	18.57	3.37	0.11	100.75
-8.855667	38.50813	S16	S1				10.00	96.00	-300	23.98	1.21	80.73	33.3	172.72	364.83	0.26	55.19	13.94	0.69	464.34
-8.838844	38.495771	S17	S1				7.00	76.00	-312	20.69	0.8	51.7	20.49	95.31	269.31	0.29	43.76	9.43	0.71	362.47
-8.798676	38.528008	S21	S3	Lobo et al. 2010	2006	Fall	12.40	94.00	-187	21	-	-	26	64.00	233	0.2	31.00	-	-	-
-8.86734	38.474809	S22	Rf				3.20	37.00	-233	7.3	-	-	13	23.00	147	0.04	24.00	-	-	-
-8.858414	38.508936	S23	S1				11.80	98.00	-290	27	-	-	34	167.00	312	0.2	66.00	-	-	-
-8.829575	38.48959	S24	S1				7.70	77.00	-316	12	-	-	9	41.00	88	0.2	45.00	-	-	-

Annex 1. (Continued)

										Inorganic (µg/g)										
										Non metals				Metal						
Longitude	**Latitude**	**Station**	**Area**	**Reference**	**Year**	**Season**	**TOM (%)**	**FF (%)**	**Eh (mV)**	**As**	**Se**	**Cr**	**Ni**	**Cu**	**Zn**	**Cd**	**Pb**	**Co**	**Hg**	**Mn**
-8.924308	38.503315	S25	Rf				0.50	0.00	-	7.00	-	2.00	-	3.50	8.20	0.60	3.20	-	0.07	-
-8.846545	38.502509	S26	S1				1.00	0.70	-	44.00	-	51.00	-	124.00	243.00	7.00	32.00	-	0.58	-
-8.838306	38.494986	S27	S1	Moreira et al. 2006	2003	Summer	1.80	7.30	-	13.00	-	14.00	-	24.00	67.00	2.00	9.40	-	0.22	-
-8.818393	38.483968	S28	S1				9.50	29.90	-	26.00	-	32.00	-	54.00	165.00	4.20	23.00	-	0.43	-
-8.785092	38.473688	S29	S1				10.00	57.50	-	39.00	-	48.00	-	94.00	295.00	6.40	48.00	-	0.35	-
-8.887596	38.488515	S30	Rf				0.70	-	-	-	-	-	-	0.21	20	0	-	-	-	-
-8.789063	38.469971	S31	S1				8.70	-	-	-	-	-	-	85.00	221	0.32	-	-	-	-
-8.842535	38.497297	S32	S1	Neuparth et al. 2005	2004	Spring	0.40	-	-	-	-	-	-	14.00	27	0.13	-	-	-	-
-8.838072	38.49461	S33	S1				0.40	-	-	-	-	-	-	11.00	62	0.12	-	-	-	-
-8.887167	38.517983	S34	S1				12.70	-	-	-	-	-	-	361.00	217	0.6	-	-	-	-
-8.794594	38.486149	S35	S1				2	-	-	-	-	-	-	-	-	-	-	-	0.4	-
-8.780518	38.490449	S36	S1				10.5	-	-	-	-	-	-	-	-	-	-	-	0.35	-
-8.777428	38.489912	S37	S1				6.50	-	-	-	-	-	-	-	-	-	-	-	0.55	-
-8.776741	38.491524	S38	S1				4.50	-	-	-	-	-	-	-	-	-	-	-	0.2	-
-8.777771	38.492061	S39	S1				4.50	-	-	-	-	-	-	-	-	-	-	-	0.1	-
-8.770905	38.491793	S40	S1				5.00	-	-	-	-	-	-	-	-	-	-	-	0.4	-
-8.731766	38.569142	S41	S3				6.00	-	-	-	-	-	-	-	-	-	-	-	0.25	-
-8.71357	38.515436	S42	S3	Lillebø et al. 2011	2006	Spring	8.00	-	-	-	-	-	-	-	-	-	-	-	0.25	-
-8.800735	38.532448	S43	S3				8.50	-	-	-	-	-	-	-	-	-	-	-	0.1	-
-8.77636	38.50881	S44	S3				10.00	-	-	-	-	-	-	-	-	-	-	-	0.15	-
-8.814468	38.478177	S45	S1				0.50	-	-	-	-	-	-	-	-	-	-	-	0	-
-8.818932	38.479789	S46	S1				1.00	-	-	-	-	-	-	-	-	-	-	-	0	-
-8.760567	38.457748	S47	S4				0.50	-	-	-	-	-	-	-	-	-	-	-	0	-
-8.723145	38.46178	S48	S4				3.00	-	-	-	-	-	-	-	-	-	-	-	0.1	-
-8.726578	38.459092	S49	S4				7	-	-	-	-	-	-	-	-	-	-	-	0.1	-

Annex 1. (Continued)

										Inorganic (µg/g)										
										Non metals		Metal								
Longitude	**Latitude**	**Station**	**Area**	**Reference**	**Year**	**Season**	**TOM (%)**	**FF (%)**	**Eh (mV)**	**As**	**Se**	**Cr**	**Ni**	**Cu**	**Zn**	**Cd**	**Pb**	**Co**	**Hg**	**Mn**
-8.818932	38.479789	S46	S1				1.00	-	-	-	-	-	-	-	-	-	-	-	0	-
-8.760567	38.457748	S47	S4				0.50	-	-	-	-	-	-	-	-	-	-	-	0	-
-8.723145	38.46178	S48	S4				3.00	-	-	-	-	-	-	-	-	-	-	-	0.1	-
-8.726578	38.459092	S49	S4				7	-	-	-	-	-	-	-	-	-	-	-	0.1	-
-8.747177	38.485702	S50	S4				0.50	-	-	-	-	-	-	-	-	-	-	-	0	-
-8.887939	38.486239	S51	Rf				1.00	-	-	-	-	-	-	-	-	-	-	-	0	-
-8.881416	38.474952	S52	Rf	Lillebø et al. 2011	2006	Spring	1.00	-	-	-	-	-	-	-	-	-	-	-	0.05	-
-8.884163	38.479252	S53	Rf				8	-	-	-	-	-	-	-	-	-	-	-	0.2	-
-8.795242	38.384563	S54	S2				9	-	-	-	-	-	-	-	-	-	-	-	0.35	-
-8.759537	38.413622	S55	S4				10	-	-	-	-	-	-	-	-	-	-	-	0.05	-
-8.667526	38.409048	S56	S4				1	-	-	-	-	-	-	-	-	-	-	-	0.05	-
-8.527451	38.368145	S57	S4				7.5	-	-	-	-	-	-	-	-	-	-	-	0.4	-
-8.814468	38.417926	S58	S2				5	-	-	-	-	-	-	-	-	-	-	-	0.25	-
-8.7912	38.4879	S59	S1	Carvalho P. N. et al. 2009b	2007/2008	Spring	9.2	99	-	-	-	-	-	-	-	-	-	-	-	-
-8.8312	38.4425	S60	S2				12.1	97	-	-	-	-	-	-	-	-	-	-	-	-
-8.790989	38.532437	S61	S3	Serafim et al. 2013	2009	Fall	16.4	83.01	-	-	-	40	20	60	200	0	60	-	0.05	-
-8.767128	38.415041	S62	S4				8.51	77.87	-	-	-	50	30	40	100	0	50	-	0.05	-

As, Arsenic; Cd, Cadmium; Co, Cobalt; Cr, Chromium; Cu, Copper; Eh, sediment redox potential; FF, sediment fine fraction (particle size <0.063 mm); Hg, Mercury; Mn, Manganese; Ni, Nickel; Pb, Lead; Se, Selenium; TOM, total organic matter; Zn, Zinc.

Annex 2. Organic contamination profiles of the sediments collected in Sado Estuary.

							Organic (ng/g)							
							PAH							
Longitude	**Latitude**	**Station**	**Area**	**Reference**	**Year**	**Season**	**PAH2R**	**PAH3R**	**PAHlow**	**PAH4R**	**PAH5R**	**PAH6R**	**PAHhigh**	**PAHt**
-8.862338	38.492232	S1	Rf	Carreira et al. 2013, Costa et al. 2013, Gonçalves et al. 2013, Rodrigo et al. 2013.	2010/2011	Fall/Winter	-	11.4	11.4	7.49	4.99	0	12.48	23.88
-8.852725	38.48632	S2	Rf				-	12.14	12.14	4.86	2.6	0	7.46	19.6
-8.833843	38.4925	S3	S1				-	102.2	102.2	495.45	299.33	179.99	974.77	1076.97
-8.743549	38.436586	S4	S4		2011	Spring	-	43.13	43.13	96.1	48.33	27.46	171.89	215.02
-8.715739	38.424214	S5	S4				-	20.25	20.25	37.15	16.68	8.39	62.22	82.47
-8.86631	38.472658	S12	Rf	Costa et al 2008b, 2009a, 2009b, 2010, 2012a	2006	Fall	-	11.96	11.96	39.44	29.04	5.99	74.47	86.43
-8.859444	38.509742	S13	S1				-	81.32	81.32	395.45	314.03	91.56	801.04	882.36
-8.842278	38.498189	S14	S1				-	83.6	83.6	479.4	475.22	62.26	1016.88	1100.48
-8.86528	38.469702	S15	Rf	Costa et al 2011a, 2011b, 2011c, 2012a, 2012c	2007	Spring	-	15.29	15.29	50.9	26.64	8.97	86.51	101.8
-8.855667	38.50813	S16	S1				-	114.83	114.83	701.2	415.18	133.99	1250.37	1365.2
-8.838844	38.495771	S17	S1				-	100.71	100.71	772.24	423.91	150.85	1347	1447.71
-8.798676	38.528008	S21	S3	Lobo et al. 2010	2006	Fall	-	35	35	362.5	251.1	89	702.6	737.6
-8.86734	38.474809	S22	Rf				-	11.9	11.9	39.7	29.1	6	74.8	86.7
-8.858414	38.508936	S23	S1				-	81.9	81.9	396	314.5	91	801.5	883.4
-8.829575	38.48959	S24	S1				-	83.2	83.2	479	475	62	1016	1099.2
-8.887596	38.488515	S30	Rf	Neuparth et al. 2005	2004	Spring	17	77	94	259	81	0	340	434
-8.789063	38.469971	S31	S1				41	371	412	2362	1521	1017	4900	5312
-8.842535	38.497297	S32	S1				4	1762	1766	6406	2184	644	9234	11000
-8.838072	38.49461	S33	S1				65	385	450	3721	2501	657	6879	7329
-8.887167	38.517983	S34	S1				5	163	168	1056	518	0	1574	1742
-8.790989	38.532437	S61	S3	Serafim et al. 2013	2009	Fall	6.34	8.42	14.76	10.8	2.94	1.28	15.02	29.78
-8.767128	38.415041	S62	S4				6.59	6.66	13.25	6.03	4.07	1.22	11.32	24.57

Annex 2. (Continued)

							Organic (ng/g)							
							Organochlorines		PCB					
Longitude	**Latitude**	**Station**	**Area**	**Reference**	**Year**	**Season**	**DDTt**	**HCB**	**PCB3C**	**PCB4C**	**PCB5C**	**PCB6C**	**PCB7C**	**PCBt**
-8.862338	38.492232	S1	Rf	Carreira et al. 2013, Costa et al. 2013, Gonçalves et al. 2013, Rodrigo et al. 2013.	2010/2011	Fall/Winter	0.02	0.02	0.05	0.03	0.02	0.04	0.04	0.18
-8.852725	38.48632	S2	Rf				0.02	0.04	0.02	0	0.01	0.02	0	0.05
-8.833843	38.4925	S3	S1				1.22	0.04	0.06	0.27	0.94	3.08	1.02	5.37
-8.743549	38.436586	S4	S4		2011	Spring	0.22	0.05	0.01	0	0.01	0.17	0.08	0.27
-8.715739	38.424214	S5	S4				0.12	0.06	0.09	0.05	0.01	0.06	0.06	0.27
-8.86631	38.472658	S12	Rf	Costa et al 2008b, 2009a, 2009b, 2010, 2012a	2006	Fall	0.85	-	0.73	0.14	0.07	0.43	0.51	1.88
-8.859444	38.509742	S13	S1				4.94	-	0.33	0.58	1.49	1.57	1.67	5.64
-8.842278	38.498189	S14	S1				2.43	-	0.18	0.81	6.76	7.22	0.38	15.35
-8.86528	38.469702	S15	Rf	Costa et al 2011a, 2011b, 2011c, 2012a, 2012c	2007	Spring	0	-	0.05	0.03	0.03	0.41	0.27	0.79
-8.855667	38.50813	S16	S1				0.37	-	2.33	0.4	1.06	2.77	1.34	7.9
-8.838844	38.495771	S17	S1				2.52	-	2.75	1.08	1.62	4.28	2.23	11.96
-8.788376	38.48099	S18	S1	Gil and Vale 2001	1993	Spring/Summer	-	-	-	-	-	-	-	114
-8.842964	38.498995	S19	S1				-	-	-	-	-	-	-	32
-8.852921	38.506787	S20	S1				-	-	-	-	-	-	-	58
-8.798676	38.528008	S21	S3	Lobo et al. 2010	2006	Fall	0.09	-	2.1	0.18	0.14	0.64	0.26	3.32
-8.86734	38.474809	S22	Rf				0.85	-	0.6	0	0	0.35	0.47	1.42
-8.858414	38.508936	S23	S1				5	-	0.34	0.58	1.4	1.58	1.57	5.47
-8.829575	38.48959	S24	S1				2.5	-	0.18	0.9	6.8	7.3	0.4	15.58
-8.7912	38.4879	S59	S1	Carvalho P. N. et al. 2009b	2007/2008	Spring	14.3	-	-	-	-	-	-	-
-8.8312	38.4425	S60	S2				6.5	-	-	-	-	-	-	-

DDT, Dichloro-Diphenyl-Trichloroethane; HCB, Hexachlorobenzene; PAHs, Polycyclic aromatic hydrocarbons; PCBs, Polychlorinated Biphenyls.