Cátia Vanessa Caetano Gonçalves

Licenciada em Ciências de Engenharia do Ambiente

Can the complex mixture of sediment contaminants cause endocrine disruption on a benthic fish? A case study with *Solea senegalensis*

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

Pollution in coastal ecosystems is a serious threat to the biota and human populations there residing. Anthropogenic activities in these ecosystems are the main cause of contamination by endocrine disruption compounds (EDCs), which can interfere with hormonal regulation and cause adverse effects to growth, stress response and reproduction. Although the chemical nature of many EDCs is unknown, it is believed that most are organic contaminants. Under an environmental risk assessment for a contaminated estuary (the Sado, SW Portugal), the present work intended to detect endocrine disruption in a flatsfish, *Solea senegalensis* Kaup, 1858, and its potential relationship to organic toxicants. Animals were collected from two areas in the estuary with distinct influences (industrial and rural) and from an external reference area. To evaluate endocrine disruption, hepatic vitellogenin (VTG) concentrations in males and gonad histology were analysed. As biomarkers of exposure to organic contaminants, cytochrome P450 (CYP1A) induction and the ethoxyresorufin-*O*-deethylase (EROD) activity were determined. The results were contrasted to sediment contamination levels, which are overall considered low, although the area presents a complex mixture of toxicants. Either males or females were found sexually immature and showed no significant evidence of degenerative pathologies. However, hepatic VTG concentrations in males from the industrial area in estuary were superior than those from the Reference, even reaching levels comparable to those in females, which may indicate an oestrogenic effect resulting from the complex contaminant mixture. These individuals also presented higher levels of CYP1A induction and EROD activity, which is consistent with contamination by organic substances. The combination of the results suggest that the exposure of flatfish to an environment contaminated by mixed toxicants, even at low levels, may cause endocrine disruption, therefore affecting populations, which implies the need for further research in identification of potential EDCs, their sources and risks at ecosystem scale.

Keywords: flatfish, EDCs, organic contaminants, hepatic vitellogenin, cytochrome P450, ethoxyresorufin-*O*-deethylase.

Resumo

A poluição nos sistemas aquáticos é uma séria ameaça para o biota e as populações humanas aí residentes. As atividades antropogénicas nestes ecossistemas são a principal causa de contaminação por compostos desreguladores endócrinos, os quais podem interferir com a regulação hormonal e causar efeitos adversos ao nível do crescimento, resposta ao *stress* e reprodução. Embora a natureza química dos desreguladores endócrinos permaneça desconhecida, crê-se que a maior parte pertença aos contaminantes orgânicos. No âmbito da avaliação de risco ambiental de um estuário contaminado (o Sado), o presente trabalho pretendeu detetar desregulação endócrina numa espécie de linguado, *Solea senegalensis* Kaup, 1858, e a sua potencial relação com contaminantes orgânicos. Os animais foram recolhidos de duas zonas no estuário com distintas influências (industrial e rural) e de uma zona de referência. Como avaliação de desregulação endócrina foram determinados os valores hepáticos de vitelogenina (VTG) nos machos e realizou-se a histologia às gónadas. Como biomarcadores de exposição foram avaliadas a indução do citocromo P450 (CYP1A) e a atividade da enzima etoxiresorufina-*O*-deetilase (EROD). Os resultados foram contrastados com os níveis de contaminação dos sedimentos, os quais são considerados globalmente baixos, apesar da área apresentar uma complexa mistura de contaminantes. Machos e fêmeas revelaram-se sexualmente imaturos e não apresentaram evidência significativa de patologias degenerativas. No entanto, as concentrações de VTG nos machos da zona do estuário com influência industrial, são superiores às dos machos da referência, atingindo níveis equiparáveis aos das fêmeas, o que poderá indicar um efeito estrogénico resultante da complexa mistura de contaminantes. Estes indivíduos revelaram, também, valores mais elevados de CYP1A e da atividade da EROD, o que é consistente com os níveis de contaminantes orgânicos nos sedimentos. A conjugação dos resultados obtidos sugere que a exposição de linguados a ambientes contaminados com poluentes orgânicos, mesmo com níveis baixos, pode causar desregulação endócrina e afetar a população, o que indica a necessidade de realizar estudos futuros para identificação de potenciais substâncias desreguladoras endócrinas, as suas fontes e riscos à escala do ecossistema.

Palavras-Chave: peixes chatos, compostos desreguladores endócrinos, contaminantes orgânicos, vitelogenina hepática, citocromo P450, etoxiresorufina-*O*-deetilase.

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Abbreviation List

AhR − Aryl hydrocarbon Receptor

As − Arsenic

BSA − Bovine Serum Albumin

Cd − Cadmium

Cr − Chromium

Cu − Copper

CYP − Cytochrome

CYP1A − Cytochrome P450

DDT − Dichloro-Diphenyl-Trichloroethane

DTT − Dithiothreitol

EC − European Commission

EDCs − Endocrine Disruption Compounds

EDTA − Ethylenediamine tetra-acetic acid

Eh − Redox Potential

ELISA − Enzyme-Linked Immunosorbent Assay

EQSs − Environmental Quality Standards

ER − Oestrogen receptor

ERA − Environmental Risk Assessment

EROD − Ethoxyresorufin-*O*-deethylase

EU − European Union

H&E − Haematoxylin and Eosin

HCB − Hexachlorobenzene

IPCS − International Programme on Chemical Safety

L_s − Standard length

MFOs − Mixed function oxidases

MSFD − Marine Strategy Framework Directive

Ni − Nickel

Pb − Lead

PCBs − Polychlorinated Biphenyls

PEL − Probable Effects Level

PMS − Postmitochondrial supernatant

PS − Priority Substances

Se − Selenium

SW − South-west

TC − Tetrachrome

TEL − Threshold Effects Level

VTG − Vitellogenin

WFD − Water Framework Directive

WHO − World Health Organization

ww_t − Total wet weight

Zn − Zinc

1. Introduction

Coastal transition ecosystems (e.g. estuaries and coastal lagoons) are very dynamic and complex ecosystems that serve as nurseries, spawning and feeding grounds for a wide range of organisms. The settlement of human populations near estuaries and, consequently, their anthropogenic pressures set onto them, has become a critical problem in these environments. One of the greatest concerns is the release of xenobiotics to the water bodies, either through diffuse sources (e.g. atmosphere, urban and rural runoffs) or through the effluents of industries and wastewater treatment plants (see for instance Sumpter and Jobling, 1995). Altogether, monitoring the environmental quality in these areas is long deemed priority. Nevertheless, the complexity and variability of these ecosystems, combined with the multiple sources and types of polluting agents hamper risk assessment, thus mandating caution in the interpretation of findings and the development of specific approaches to address this issue, as well as the choice of adequate indicators.

The real impact of contaminants on the biota of estuaries and the human populations settled upon these areas is still being investigated, however several studies reported already that some contaminants (in its individual form or in a complex mixture) can interfere with hormonal regulation and cause adverse effects at the development level, responses to stress and, moreover, reproduction, which implies a direct impact to populations. These contaminants are denominated as endocrine disruptor compounds (EDCs) and they are considered an emerging environmental threat.

Through the International Programme on Chemical Safety (IPCS) the World Health Organization (WHO) establishes the scientific basis for assessing the risk to human health and the environment from exposure to chemicals, EDCs included. In order to summarize the current state of the scientific knowledge regarding the potential effects of exposure to EDCs in humans and wildlife, the IPCS developed the Global Assessment of the State-of-the-Science of Endocrine Disruptors, in 2002. In this assessment is identified the lack of knowledge about the real effects of wildlife EDCs exposures and issued specific research recommendations.

In 1999, the European Commission (EC) issued the Community Strategy for Endocrine Disruptors, where the key requirements of further research were proposed, including, international co-operation and recommendations for short-, medium- and long-term actions. In 2000, the European Parliament released a Resolution on endocrine disruptors, emphasising the application of the precautionary principle and calling to the identification of substances for immediate action. The scientific concern and public debate about EDCs has been growing in the last two decades, which stimulated many national governments, international organizations, scientific societies, the chemical industry and public interest groups to establish research programs. European Union (EU)

legislation emerged in order to stipulate guidelines to manage and protect the aquatic environment. The Water Framework Directive (WFD, Directive 2000/60/EC) establishes all information about how to address water contamination problems, by introducing a list of Priority Substances (PS), such as hazardous hydrocarbons and organophosphorous compounds and metals. In the WFD, the EDCs are not considered as a whole group of contaminants, however, in 2012 emerged a proposal to an amendment of the WFD that included "emerging" pollutants, as EDCs, that should be implemented by 2014. Although the proposal revised the list of PS and Environmental Quality Standards (EQSs), at least regarding EDCs, the proposed guidelines are based on the average range of concentrations of a few compounds in the environment and not on their real effects to the biota, meaning that, further investigation is still needed to identify and quantify the potentially wide array of chemicals with endocrine-active properties, as well as their mechanism of action.

Numerous chemical substances belong to the EDC category, and most are organic substances, although some metals and metallic compounds may interfere with the endocrine system. In this class are included industrial compounds such as dioxins, bisphenol-A, and some polychlorinated biphenyls (PCBs); chemical substances that are widely used in cosmetics (such as phthalates, ultraviolet filter constituents and parabens); pesticides such as chlorinated insecticides, imidazoles, and triazoles; estradiol as an ecological contaminant from urban effluents; natural chemicals such phytoestrogens and fungal estrogens (Damstra et al., 2002; Iavicoli et al., 2009).

In contact with EDCs, the functioning of the endocrine system may be compromised by alterations in biosynthesis, transport, availability and metabolism of hormones (Lister and Van Der Kraak, 2001). These compounds may also interact with hormone receptors and modify the natural response patterns of this critical system. The impact of oestrogenic and androgenic contaminants may trigger feminization or masculinization, respectively, which may have repercussions at population development level by interfering with the animals' reproductive system (Jobling et al., 1998). Some studies demonstrated the existence of effects related to EDCs exposure and the disruption of the reproductive system in many fish species, including flatfish (see, for instance, Lye et al., 1997; Hashimoto et al., 2000; Vethaak et al., 2002; Kirby et al., 2004).

Many developed countries established long–term biomonitoring programmes for the environmental risk assessment (ERA) of pollutants in coastal and transitional ecosystems. Many of these programmes employ fish, especially flatfish, as sentinels (due to their ecological and economical importance, sensitivity and close contact to sediment floors), which are analysed through a set of biomarker responses (Kirby et al., 2007; Costa et al., 2012). Altogether, it is aimed at establishing the potential relationship between environmental toxicants and the effects caused by exposure, in order to retrieve some measure of risk. Several biomarkers, measured in fish tissues, are used to assess the effects of exposure to a given toxicant or class of toxicants. For example, the cytochrome

P450 (CYP1A) induction and ethoxyresorufin-*O*-deethylase (EROD) activity are acknowledged biomarkers of exposure to some organic compounds (e.g. polycyclic aromatic hydrocarbons and dioxins). The biomarkers are based on the principle that, following exposure to this specific class of contaminants, the CYP1A gene is overexpressed, resulting in higher synthesis of CYP1A enzymes (including EROD) increasing global CYP mixed function oxidase activity, responsible for the biotransformation of many organic toxicants. The determination of vitellogenin (VTG), which is a protein precursor of egg yolk in oviparous vertebrates, in male fish is considered one of the most sensitive biomarker of exposure to EDCs, more specifically the oestrogenic compounds (van der Oost et al., 2003). It must also be noted that, like many organic and inorganic toxicants EDCs may be dissolved in water, bound to suspended matter or trapped in aquatic sediments. The latter thus acts as a storage compartment of xenobiotics, with emphasis on sediments of transition ecosystems, which are usually complex in respect to their geochemical composition and combination of toxicants, whose interactions may yield in vivo additive, synergistic or antagonist effects, further compromising the interpretation of results (see, e.g., Waring and Harris, 2005). Thus, reading ecotoxicological data from impacted transitional ecosystems is biased by many confounding factors, from the presence of mixed toxicants to the many noise variables that affect these ever–changing systems. As a consequence, studies attempting to link a specific effect, such as endocrine disruption, to a given set of stressors under such challenging scenarios are scarce.

In Portugal, full biomonitoring programs are yet relatively incipient, in spite of the recommendations of the WFD and, more recently, the Marine Strategy Framework Directive (MSFD, Directive 2008/56/EC). In order to develop the first biomonitoring EDCs in the Sado estuary (SW Portugal, Fig. 2.1), the flatfish *Solea senegalensis* was chosen as sentinel species. This species is a common estuarine fish in South-West (SW) of Europe, holding high commercial value, for both fisheries and aquaculture. This species typically occupies sandy-muddy bottoms of coastal waters (estuaries included) and it feeds on small invertebrates (Cabral and Costa, 1999). Its benthic behaviour makes *Solea senegalensis* vulnerable to sediment contamination. This species has already been employed in risk assessment studies of coastal ecosystems in Portugal and other SW European countries, especially in the Sado estuary (Costa et al., 2009, 2010; Gonçalves et al., 2013), where, however, EDCs have yet to be surveyed and their effects validated as potential indicators of exposure.

1.1. Objectives

In spite of the recent efforts to determine and characterize the contamination pattern of the study area and its effects towards the biota, the Sado estuary (SW Portugal) has not been surveyed for potential endocrine disruption caused by aquatic pollutants. This particular ecosystem holds many constraints towards risk assessment strategies, in most part owing to its size, biogeographical

variation and heterogeneity regarding sources and nature of environmental toxicants. As such the main objectives of the present work can be summarized as follows:

- To detect the possible effects of endocrine disruption in juvenile male soles, *Solea senegalensis* Kaup, 1858, collected from a contaminated estuary, the Sado;
- To evaluate if endocrine disruption may be associated with gonadal alterations in both male and female soles;
- To investigate which contaminant classes are potentially related to endocrine disruption in an estuarine environment characterized by complex mixtures of contaminants, using the profiles of sediment contamination;
- To correlate the biomarkers of organic contamination (e.g. CYP1A induction and EROD activity) to a potential biomarker of exposure to chemicals that are direct oestrogen agonists (i.e. able to induce VTG production in males), in order to establish a connection between endocrine disruption and organic compounds;
- To compare two distinct areas of the estuary impacted by different sets of stressors, namely urban/industrial and rural/riverine, in order to contribute to risk assessment strategies in an area already classified as ecotoxicologically heterogeneous.

2. Material and Methods

2.1. Study area and sampling

The Sado estuary is the second largest estuary in Portugal and is located in South-West of Portugal. This coastal ecosystem has approximately 240 km^2 and is characterized by its high ecological importance, mainly due to the existence of spawning and nursery areas for many fish and others species, and its high biogeographical diversity (Fig. 2.1). The estuary comprises the city of Setúbal and several industrial units which form a large heavy-industry belt. The estuary is also important for tourism, fisheries, aquaculture and part is classified as a national reserve (see for instance Costa et al., 2012 and Carreira et al., 2013). Moreover, the fields along the banks of the Sado River may constitute an additional input of contaminants for the estuary, such as pesticides and fertilizers, since they are used to agricultural activity. To these contamination hazards are added the metallic contamination conducted by river transportation, once the Sado River crosses an important pyrite mining region (Cortesão and Vale, 1995, 1996). Altogether, the various human activities constitute an important potential source of contamination, either point or diffuse. Previous studies revealed the existence of contamination levels capable of inducing adverse effects to the biota, albeit the estuary being globally judged as moderately contaminated (e.g. Caeiro et al., 2009; Costa et al., 2012).

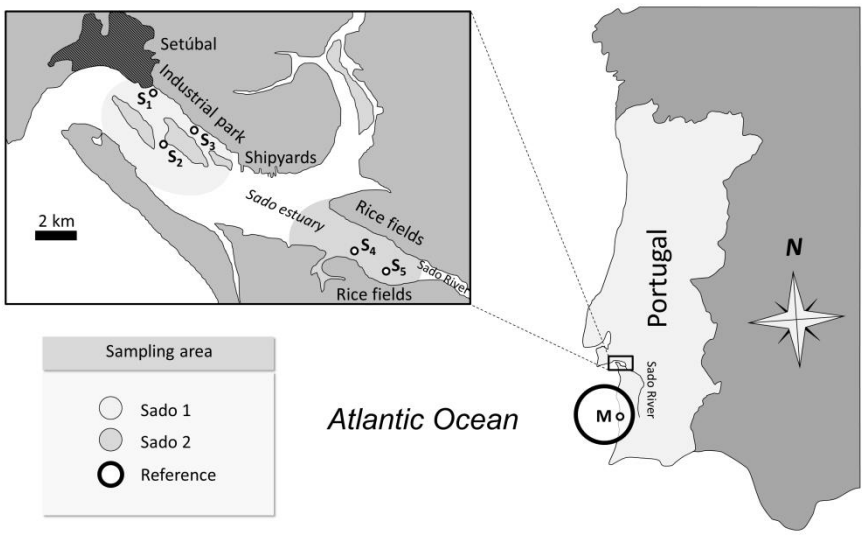

Fig. 2.1. Location of the three fish collection sites (Sado 1, Sado 2 and Reference). Sediment collection sites of the Sado and Mira (Reference) estuaries, identified by S1−S5 and M, respectively.

Recent research on contamination profiles of estuarine sediments revealed the distinction between the northern (urban and industrial) and southern (rural and riverine) areas of the Sado estuary (Carreira et al., 2013). The overall contamination status of the Sado estuary was inferred from

available sediment contamination data (gathered from 2007 to 2011), consisting of sediment samples collected from five locations distributed within the northern and southern areas (acknowledged fishing grounds for flatfish in the estuary) plus a reference site (M), located at an oceanic beach off the Mira estuary (Fig. 2.1), one of the least impacted coastal areas in Portugal (e.g. Vasconcelos et al., 2007) and one of the most important coastal fishing areas in the SW Portuguese coast. Sediment contamination data were gathered from Costa et al., 2011 (sediment sample S_1) and Carreira et al., 2013, op. cit. (S_2 to S_5 and M). Sediment contamination levels were contrasted to the Threshold Effects Level (TEL) and Probable Effects Level (PEL) sediment quality guidelines (Macdonald et al., 1996) to determine the potential to cause adverse effects to organisms. Sediment contamination was determined for metalloids (arsenic − As and selenium − Se); metals (chromium − Cr, nickel − Ni, copper − Cu, zinc − Zn, cadmium − Cd and lead − Pb); polycyclic aromatic hydrocarbons (PAHs) and organochlorines, namely polychlorinated biphenyls (PCBs) plus the pesticides dichloro-diphenyl-trichloroethane (*pp'*DDT) and its metabolites (*pp'*DDE and *pp'*DDD), and hexachlorobenzene (HCB). Metals and metalloids were analysed by inductively coupled plasma mass spectrometry after acid (HNO_3) and peroxide (H_2O_2) digestion of samples in closed TFE vials and organic contaminants by gas-chromatography techniques following extraction with mixed organic solvents. Standard sediment parameters (organic matter, fine fraction and sediment redox potential) were also determined. Procedural specifications and validation procedures are given in detail by Costa et al. (2011) and Carreira et al. (2013). Table 2.1 summarizes the main parameters and contamination profiles of the surveyed sediments. Further details about sediment contamination are found in Annex 1.

Table 2.1. Physico-chemical characterization and main contaminants of the surveyed sediments from Sado estuary (S_1, S_2, S_3, S_4 and S_5) and Reference (M) that exceed the TEL or PEL values. Data from sediment sample S_1 were gathered from Costa et al. (2011) and samples S_2 to S_5 and M were adapted from Carreira et al. (2013).

	Site							
	S_1	S_2	S_3	S_4	S_5	M		
TOM (%)	10.2	0.9 ± 0.1	10.4 ± 0.0	6.9 ± 0.1	8.8 ± 0.0	0.7 ± 0.01		
FF (%)	95.6	3.5	52.9	63.7	74.3	0.78		
Eh (mV)	-300	–	-359 ± 2	-260 ± 28	-315 ± 7	184 ± 13		
Contaminant class							**TEL**	**PEL**
Inorganic (μg.g^{-1})								
Nonmetal								
As	23.98 ± 0.48*	0.34 ± 0.26	19.7 ± 5.21*	26.44 ± 2.68*	25.02 ± 8.84*	0.88 ± 0.43	7.24	41.6
Metal								
Cr	80.73 ± 1.61*	2.36 ± 0.36	77.67 ± 4.57*	62.22 ± 4.45*	87.61 ± 2.97*	1.81 ± 0.12	52.3	160
Ni	33.30 ± 0.67*	4.10 ± 1.66	16.67 ± 1.1*	17.15 ± 1.21*	22.79 ± 9.47*	3.04 ± 0.65	15.9	42.8
Cu	172.72 ± 3.45**	4.51 ± 1.05	178.64 ± 7.01**	74.15 ± 13.16*	92.3 ± 5.63*	2.31 ± 0.36	18.7	108
Zn	364.83 ± 7.30**	13.10 ± 1.51	327.51 ± 1.16**	269.79 ± 7.81*	385.11 ± 35.69**	1.04 ± 0.51	124	271
Pb	55.19 ± 1.10*	3.50 ± 0.48	56.45 ± 3.1*	25.3 ± 0.91	32.7 ± 1.21*	1.48 ± 1.64	30.2	112
Organic (ng.g^{-1})								
PAH								
3-ring								
Acenaphthylene	2.38 ± 0.40	<DL	9.77 ± 1.66*	1.98 ± 0.34	0.90 ± 0.15	<DL	5.87	128
Acenaphthene	12.25 ± 2.08*	0.40 ± 0.07	9.00 ± 1.53*	1.78 ± 0.30	1.16 ± 0.20	<DL	6.71	88.9
4-ring								
Fluoranthene	315.71 ± 53.67*	2.07 ± 0.35	207.29 ± 35.24*	38.99 ± 6.63	14.38 ± 2.45	<DL	113	1 494
Pyrene	263.18 ± 44.74*	1.93 ± 0.33	175.12 ± 29.77*	36.23 ± 6.16	14.89 ± 2.53	<DL	153	1 398
Benz[a]anthracene	81.25 ± 13.81*	0.86 ± 0.15	72.16 ± 12.27	13.01 ± 2.21	4.44 ± 0.76	<DL	74.8	693
5-ring								
Benzo[a]pyrene	101.86 ± 17.32*	0.62 ± 0.10	86.29 ± 14.67	12.83 ± 2.18	4.05 ± 0.69	<DL	88.8	793
Dibenzo[a,h]anthracene	13.32 ± 2.26*	<DL	15.09 ± 2.57*	0.70 ± 0.12	0.04 ± 0.01	<DL	6.22	135

[<DL] – Below detection limit; TOM, total organic matter; FF, sediment fine fraction (particle size < 0.063 mm); Eh, sediment redox potential; TEL, threshold effects level guideline; PEL, probable effects level guideline; *, value above TEL; **, value above PEL. Contaminant concentration ranges indicate the standard quantification error.

Fish were collected during the fall/winter 2010/2011 at two sites with distinct characteristics: an impacted site in the northern area of Sado estuary (Sado 1) and an important commercial area for fisheries in the SW coast of Portugal, located off the Mira estuary, which is the reference area for this present study (Fig. 2.1). From Sado 1 and the reference sites were obtained twenty-one and nineteen animals respectively. In order to provide additional ecotoxicological information it was included another site located in the southern part of the Sado estuary (Sado 2). The sampling at this third site was done in the spring 2011, with ten animals being collected. Animals were measured and dissected following arrival at the laboratory. Liver portions were frozen (-80 ºC) for subsequent biochemical analyses. Gonad samples were processed immediately for histological procedures.

2.2. Histological analysis

Freshly dissected gonads samples from each fish (at least two random samples per individual) were fixed in Bouin's solution for c.a. 36 h at room temperature. Details on standard histological sample preparations and staining are described, for instance, in Martoja and Martoja-Pierson (1967). In brief: samples were dehydrated in a progressive series of ethanol, intermediately infiltrated in xylene and embedded in paraffin. Samples were then sectioned at 5 μm thickness using a Jung RM 2035 BioCut Microtome (Leica Microsystems), stained with Haematoxylin and Eosin (H&E) and with Tetrachrome staining (TC), dehydrated, cleared with xylene and mounted with DPX resin (see for TC stainig procedure, Costa and Costa, 2012). Two slides with at least eight serial sections from each gonad sample were examined with a DMLB model microscope (Leica Microsystems) to characterize the maturation stage and to search for gonad abnormalities such as signs of inflammation, oocyte atresia, intersex or necrosis. The stages of spermatogenesis were determined according to García-López et al. (2006a) and the oocyte developmental stages were identified in accordance with the description in García-López et al. (2007), both specific to *Solea senegalensis*. Female maturity stages were classified into six different stages (Shinkafi et al., 2011): immature (I), maturing (II), mature (III), ripe and running (IV), spent (V) and resting (VI).

2.3. Biomarker analysis

The present study analysed two biomarkers of exposure (cytochrome P450 induction, ethoxyresorufin-*O*-deethylase activity) and one potential biomarker of exposure (hepatic vitellogenin concentration). All biomarkers were determined from approximately 100 mg of liver samples homogenized with 400 μL of homogenization buffer for retrieval of the postmitochondrial supernatant (PMS), defined in Nilsen et al. (1998), containing 0.1 M sodium phosphate, pH 7.4,

0.15 M KCl, 1mM ethylenediamine tetra-acetic acid (EDTA), 1 mM dithiothreitol (DTT), 10% (v/v) glycerol. Fractionated samples were then prepared according the protocol of each biomarker, as described below.

Cytochrome P450 induction was measured by enzyme-linked immunosorbent assay (ELISA) in the microsomal fraction of liver homogenates, according to the protocol of Nilsen et al. (1998). In brief: after the post-microsomal fraction ultracentrifugation, the microsomal pellet was resuspended and diluted to 1:10 (v/v). The blocking phase was mediated by addition of 2% (w/v) Bovine Serum Albumin and followed by an incubation period of 60 minutes at 37°C. It was used a rabbit anti-fish CYP1A peptide polyclonal antibody (Biosense Laboratories) for primary antibody and the anti-rabbit alkaline phosphatase antibody (Sigma-Aldrich) for the secondary, both diluted to 1:1000 (v/v). The procedure was followed by addition of the development solution in each well. The color reaction developed for 90 minutes and was stopped by adding 3N sodium hydroxide. Due to the absence of a commercial CYP (cytochrome) protein for absolute quantifications, CYP1A induction was determined semi-quantitatively and was performed in duplicate per sample. The plate was read at 405 nm with a Benchmark model microplate reader from Bio–Rad and the results are given in $Abs \cdot ng^{-1}$ total microsomal protein.

The activity of mixed function oxygenases was inferred from ethoxyresorufin-*O*-deethylase activity, measured in the hepatic microsomal fraction according to (Gagné and Blaise, 1993). Briefly: approximately 10 µL of each sample were placed in fluorescence-compliant microplate wells, followed by 150 µL of EROD reaction mix [67.5% Tris (50mM, pH 7.4), 20.0% Bovine Serum Albumin [BSA (5.32 mg/mL in 50mM Tris)], 5.0% Ethoxyresorufin (100 µM in MeOH/50mM Tris 15%/85%) and 7.5% NADPH (6.7mM in 50mM Tris)]. After 15 minutes of incubation at 37°C, the reaction was stopped by adding 100 µL of 2M glycine, pH 10.3−10.4. The plate was read at 560 nm excitation and 610 nm emission wavelength with a Tecan Infinite 200 microplate reader. The amount of ethoxyresorufin converted to resorufin was determined from a standard curve for resorufin (Sigma). The assay was performed in triplicate for samples, standards and blanks. The results are given in ng $resorufin \cdot mg^{-1}$ total microsomal protein.

Vitellogenin (VTG) was determined by direct ELISA in the post-microsomal fraction of liver homogenates according to Denslow et al. (1999). To the ELISA microplate plate wells were added approximately 50 µL of samples previously diluted to 1:50 (v/v) in phosphate-buffered saline (PBS). Rabbit anti-fish Vitellogenin Polyclonal was used as the primary antibody (Biosense Laboratories) and the anti-rabbit alkaline phosphatase antibody (Sigma-Aldrich) as the secondary (polyclonal) antibody. Determination followed a VTG standard curve, using carp VTG as standard (Biosense Laboratories). The plate was read at 405 nm with a Benchmark model microplate reader from Bio–Rad and the results are given in ng $VTG \cdot ng^{-1}$ total post-microsomal protein.

The Bradford (1976) method was used for the measurement of microsomal and post-microsomal total protein, taking BSA as standard and using a Benchmark microplate reader (Bio-Rad).

2.4. Statistical analysis

All data were examined for their fit to a normal distribution and homogeneity of variances using the Kolmogoroff-Smirnoff's and Levene's tests, respectively. After the invalidation of at least one of these assumptions for parametric tests, non-parametric statistics were employed, namely the Mann-Whitney U test to search for inter-site differences and the Spearman rank-order correlation ρ to search for individual links between biomarkers. The three biomarkers of exposure and the maturation stage were modeled through multivariate statistics, namely discriminant analysis, to determine the significance of each variable in site differentiation. A significance level $\alpha = 0.05$ was set for all analyses. All statistics were performed using Statistica (StatSoft).

3. Results

3.1. Animal size

Animals collected from Sado sites exhibited significantly lower total wet weight (ww_t) and standard length (L_s) than those from the reference location. Fish from Sado 2 were larger in weight but not length than those from Sado 1 (Mann-Whitney U, $p < 0.05$). There were no significant differences between male and female weight and length of any fish from all sites under consideration.

3.2. Gonadal status

The histological analysis on gonads revealed that most of the female individuals were immature (stage I), with exception of a single stage IV female collected from the reference site that was considered as positive control for VTG analyses. The males collected from the Sado estuary (sites Sado 1 and Sado 2) were classified as immature (stage I); while those from the reference site were in maturing/mature stages (stage II and stage III). The male:female ratios differed between sites: at Sado estuary the number of female individuals was higher than males (2:1 at Sado 1 and 1:4 at Sado 2) and at reference site all individuals were males, except the single mature female (18:1).

Histological observations revealed the existence of melanomacrophage aggregates in the male gonads of fish collected from reference site and the indication of low-moderate gonadal tissue inflammation (as small hyperaemic foci) in eight males of the nineteen individuals from the same location (Fig.3.1A). Four females collected from Sado 1 also revealed low-moderate evidence for hyperaemia (Fig. 3.1B). No other significant pathologies were found in the gonads of males and females collected for the present study.

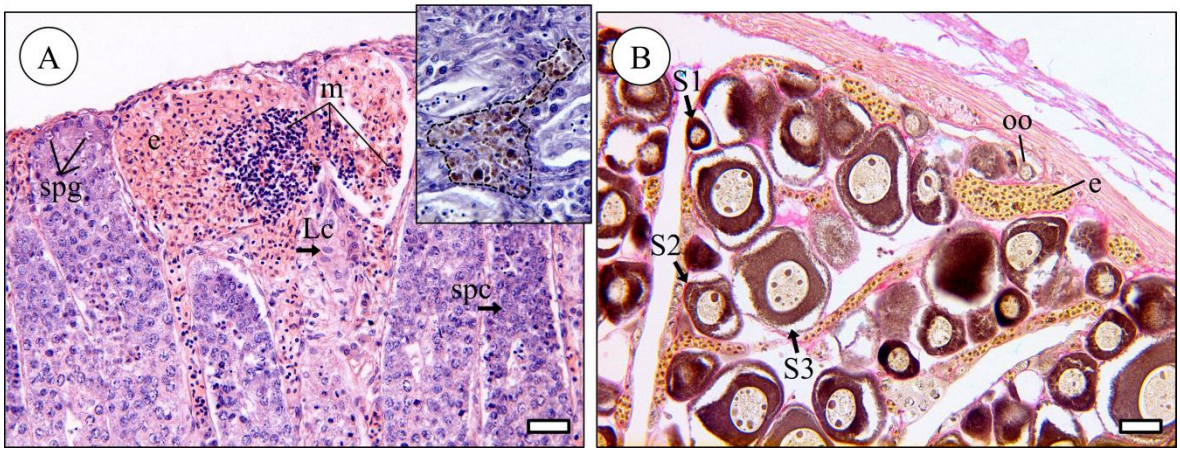

Fig. 3.1. Representative photomicrographs of gonads of sampled Senegalese sole. Scale bars: 25µm. (A) Small hyperaemic focus located in a gonad from male fish collected from Reference (H&E). Note the influx

of defense cells mainly macrophages (m) in the blood vessel, surrounded by erythrocytes (e). Spermatogonia (spg), spermatocyte (spc) and Leydig cells (Lc) are also present. Inset: detail of a melanomacrophage centre (broken line). (B) Low-moderate evidence for hyperaemia in a gonad from a female sole collected in Sado 1 (TC). Presence of oogonia (oo) and oocytes at different developmental stages: chromatin nucleolar stage (S1), early perinucleolus stage (S2) and late perinucleolus stage (S3).

3.3. Biomarker analysis

Cytochrome P450 (CYP1A) induction measured in soles from Sado 1 was about 2-fold higher than that of animals from the reference location, while in the animals from Sado 2 the proportion was approximately 1.5-fold higher than in Reference animals (Fig.3.2.A). A similar pattern was found for EROD activity (Fig.3.2.B): the highest concentration was in animals from Sado 1 followed by fish from Sado 2 and those from reference location. Both CYP1A induction and the EROD activity did not show significant differences between soles from Sado 2 and the other sites. On the other hand, Sado 1 animals always yielded significantly higher values than those from the reference site (Mann-Whitney U, $p < 0.05$).

Hepatic vitellogenin (VTG) levels in males revealed significant differences between sites (Mann-Whitney U, $p < 0.05$). The highest VTG levels in males were found in fish from Sado 1, whereas males from Sado 2 had VTG values 2-fold lower than Reference males (Fig.3.2C). It was also observed that the VTG levels between females and males from Sado 1 were similar, with no significant differences being found (Mann-Whitney U, $p > 0.05$). The same statistical result was registered for both male and female individuals from Sado 2. The highest female VTG value was observed in the single mature female from the reference site, taken as positive reference for comparison (0.090 ng VTG·ng^{-1} total post-microsomal protein), while the male with the highest VTG value was collected from Sado 1 (0.083 ng VTG·ng^{-1} total post-microsomal protein).

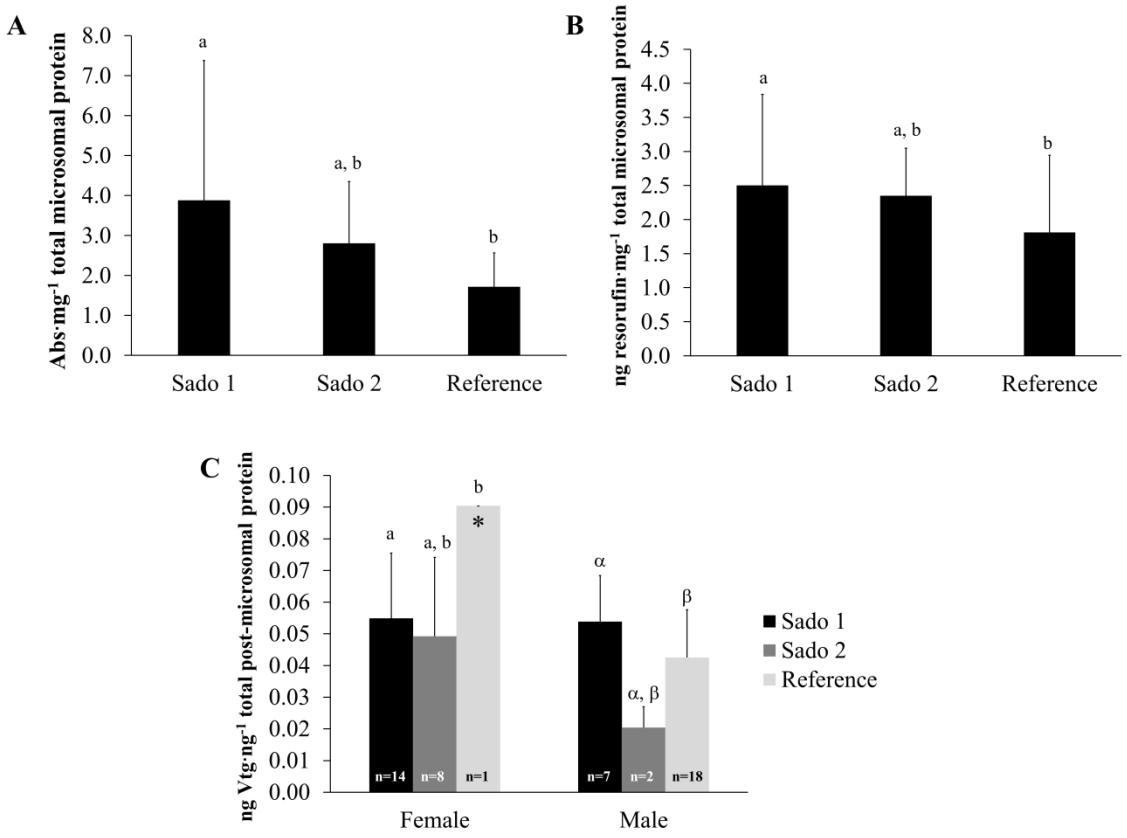

Fig. 3.2. Mean results of hepatic biomarkers of exposure per site (Sado 1, Sado 2 and Reference). Error bars indicate 95% confidence intervals. Different letters indicate significant differences (Mann-Whitney U, $p < 0.05$). A) Cytochrome P450 induction (males and females combined); B) Ethoxyresorufin-*O*-deethylase activity (males and females combined); C) Hepatic vitellogenin concentration. The single maturation stage IV female collected from the reference station is shown as positive reference (*). Latin letters indicate significant differences between females and greek letters indicate significant differences between males. n indicates the number of animals sampled.

3.4. Statistical integration of data

The two models resulting from discriminant analysis are presented in Table 3.1 In model A (including both females and males), the variables that significantly contributed to site differentiation were the maturation stage and CYP1A. In model B, the best variables to distinguish sites are the maturation stage, VTG and CYP1A.

Table 3.1. Results from discriminant analysis. Model A) Both sexes: females and males (total Wilk's λ = 0.3429, p<0.0000); Model B) Males only (total Wilk's λ = 0.2296, p<0.0001).

Variables	Model A		Model B	
	Wilk's λ	p value	Wilk's λ	p value
Maturation stage	0.7808	0.0000*	0.4901	0.0003*
VTG	0.3658	0.2399	0.3436	0.0145*
CYP1A	0.3964	0.0409*	0.3397	0.0163*
EROD	0.3446	0.8962	0.2443	0.5192

VTG, hepatic vitellogenin concentration; CYP1A, Cytochrome P450 induction; EROD, Ethoxyresorufin-*O*-deethylase activity; ∗ Significant variables ($p < 0.05$).

Correlation between all biological data (biomarkers and size) is presented in (Table 3.2). It was verified that, in male soles from Sado 1, the highest correlation occurred between EROD and CYP1A, followed by correlations between weight and length, EROD and VTG (both concerning female fish from the same site). Significant correlations were also found between female weight and length, plus between female weight and CYP1A, in fish collected from Sado 2. When considering correlations at reference location, the higher significant correlations ($\rho > 0.8$ and $\rho > 0.7$, respectively) were obtained between male's weight and length, CYP1A and VTG.

Table 3.2. Results from Spearman rank-order correlation ρ statistic, between all biological responses for Senegalese sole collected from all prospected sites (Sado 1, Sado 2 and Reference), females (F) and males (M) segregated. All correlations listed were positive and statistically significant ($\rho > 0.6$ and $p < 0.05$).

			L_s	ww_t	VTG	CYP1A	EROD
Sado 1	L_s	F					
		M					
	ww_t	F	0.7776				
		M					
	VTG	F					
		M					
	CYP1A	F					
		M					
	EROD	F			0.6659		
		M				0.8571	
Sado 2	L_s	F					
		M					
	ww_t	F	0.8743				
		M					
	VTG	F					
		M					
	CYP1A	F		0.8810			
		M					
	EROD	F					
		M					
Reference	L_s	F					
		M					
	ww_t	F					
		M	0.8223				

Table 3.2. (Continued)

			L_s	ww_t	VTG	CYP1A	EROD
Reference	**VTG**	F					
		M					
	CYP1A	F					
		M			0.7647		
	EROD	F					
		M					

L_s, standard length; ww_t, total wet weight; VTG, Vitellogenin concentration in the liver; CYP1A, Cytochrome P450 induction; EROD, Ethoxyresorufin-*O*-deethylase activity.

4. Discussion

The present results revealed the existence of distinct patterns of biomarker responses in fish collected from each sampled location. Overall, the findings are in agreement with the sediment contamination levels of Sado 1 and Sado 2 (see Annex 1), since animals from Sado 1 (the globally most contaminated), yielded greater biomarker responses, namely CYP1A induction and EROD activity, comparatively to soles from Sado 2 and reference sites, which is consistent with contamination by organic substances. In addition, the results also suggest moderate endocrine disruption effects occurring in males from Sado 1, who exhibited higher hepatic VTG contents than those from Sado 2 and, moreover, the Reference fish, an observation that should also be consistent with higher impact from pollutants, especially organic, as indicated by CYP1A responses. It must be noticed that, although the fish sampling from Sado 1 and Sado 2 occurred in distinct seasons, the seasonality should not be a critical factor impairing the comparison between sites, since the intermediate biomarker response levels (i.e. CYP1A induction and EROD activity) observed in Sado 2 animals are in accordance with the intermediate levels of sediment contamination registered in this area. In fact, previous studies already reported negligible seasonal fluctuations of biomarker responses for this species (Fonseca et al., 2011b). Furthermore, considering that the individuals from both Sado sites were classified as immature (stage I) and the *S. senegalensis* has been described to exhibit high nursery fidelity in this maturation stage (Vinagre et al., 2008, 2011), thus meaning limited interchange of individuals between Sado 1 and Sado 2 areas.

The concern about novel contaminants with potential to disrupt hormonal function in wildlife has been increasing since the 1980s and consequently several techniques were developed to search the effects of these compounds mainly in aquatic environments. Vitellogenin, a phospholipoglycoprotein, is synthesised in all oviparous female vertebrates, in response to normal cycles of estradiol during oogenesis (Denslow et al., 1999; Jones et al., 2000). In ecotoxicology, the analysis of VTG in males can be used as a biomarker of exposure to chemicals that are direct oestrogen agonists (Jones et al., 2000). With regard to estuarine environments, several studies indicate industrial effluents and treated domestic sewage discharges as the main causes of oestrogenic effects on fish. For instance, Allen et al. (1999) already reported higher plasma VTG in male flounder in several industrialised estuaries. Roy et al. (2003) also observed an increase in plasma and hepatic VTG levels in males from three flatfish species collected near a wastewater outfall. Other studies described analogous results with flatfish sampled from coastal environments proving evidence of oestrogenic activity in waters with strong urban influence (Lye et al., 1997; Hashimoto et al., 2000; Vethaak et al., 2002; Kirby et al., 2004). In the present work, male soles sampled from Sado 1, i.e. the area most contaminated by organics, accused higher hepatic VTG concentrations than those obtained from the Reference fish, which is in agreement with previous

authors performing research on contaminated estuaries and other confined coastal waters. Nevertheless, it is important to notice that VTG production is affected by a variety of other environmental variables that can act as confounding factors. The synthesis of VTG can be modulated by water temperature and salinity, the age of the fish, exposure time, type of oestrogenic contaminants and their concentrations, and, above all, if an exposure to single or combined toxicants is involved. To understand the effects of mixtures of oestrogenic chemicals, Sumpter and Jobling (1995) conducted some preliminary experiments with non-flatfish species that revealed higher VTG synthesis in hepatocytes treated with a mixture of five oestrogenic chemicals, compared with the response of hepatocytes exposed to a single oestrogenic chemical at the same concentration. Matthiessen et al. (2002) also reported elevated VTG levels in flounder males (*Platichthys flesus*) from contaminated/industrialized estuaries, which are characterized by the presence of mixed contaminants with and without oestrogenic properties, as likely occurring in the Sado estuary.

Water temperature can play an important role in the reproductive process of Senegalese sole. During the spawning period, water temperature below 16ºC can stop egg emission (Dinis et al., 1999), and the exposure to constant temperature can disrupt gonadal development, as reported by García-López et al. (2006b). Although there is no clear agreement about the optimum temperature for the VTG synthesis, Guzmán et al. (2008) suggested an impairment of the VTG synthesis at colder temperatures. Therefore, from the current findings it may be inferred that VTG production in males may have not been upregulated by water temperature, once soles collected in winter season (Sado 1) obtained greater hepatic VTG levels than soles from Sado 2 (collected in Spring), as seen in Fig.3.2C. This fact leads to admit that VTG levels on soles from Sado 1 were upregulated by other environmental factors. Although there are metal contaminants that can act like endocrine disruptors (Iavicoli et al., 2009; Tan et al., 2009), their levels in sediment samples S1 and S2 (Annex 1) are relatively similar, which may suggest that the VTG levels from Sado 1 soles is due to the presence of organic contamination caused by the urban and industrial influence.

Vitellogenesis, one of the main processes of oogenesis in oviparous animals, comprises the synthesis of vitellogenin (a yolk precursor protein) in the liver and its uptake by maturing oocytes (Tyler and Sumpter, 1996). Under specific environmental conditions like water temperature, food availability and/or photoperiod, the brain stimulates the pituitary gland to produce gonadotropins, which are released into the bloodstream (Nicolas, 1999; Jones et al., 2000). After, these hormones are carried to the ovaries where they promote the synthesis of oestrogens (primarily estradiol), which enter the liver cells (hepatocytes) by diffusion, bind to oestrogen receptor (ER) and activates the transcription of the vitellogenin gene (see Nicolas, 1999, for a review). In males, this gene is normally silent due to low oestrogen concentrations but under exposure to pro–oestrogens, VTG

transcription can be induced and vitellogin be synthetised (Sumpter and Jobling, 1995; Denslow et al., 1999).

The EDCs can act through different and complex mechanisms that are not completely understood (Lister and Van Der Kraak, 2001). However, these mechanisms modify the natural response patterns of the endocrine system and can trigger either agonistic or antagonistic effects. In the first case, the EDC binds to the receptor and activates a response, thus acting as a hormone mimic. On the other hand, if the chemical (then called a hormone blocker) binds to a receptor, preventing natural hormone from interacting, the response is inhibited and an antagonistic effect occurs (Lister and Van Der Kraak, 2001; Baker, 2001). Several compounds have been highlighted as oestrogenic mimic agents, most of which are organic substances, as some natural chemicals such as phytoestrogens and mycoestrogens, to which are added man-made substances, such as some organochlorine pesticides and their derivatives (like *o,p*-DDT), PCBs, alkylphenols, ethoxylates, bisphenol-A, some phthalates and even pharmaceuticals such as ethinylestradiol (Sumpter and Jobling, 1995; Jones et al., 2000). Polycyclic aromatic hydrocarbons (PAHs) were the most significant organic toxicants in Sado 1, however, it must be highlighted there are likely many other substances that could not be determined in the present study, among which may lie known or novel endocrine disruptors. Still, as far as PAHs are concerned, there is no consensus about their real effect on VTG synthesis. As Nicolas (1999) has reviewed, elevated levels of PAHs have been found to have a deleterious effect on the plasma vitellogenin of fish and can even have a significant antioestrogenic effect. Nevertheless, relative induction of oestrogenic effects of some priority PAHs in human cells has been reported by Villenueve et al. (2002). Even assuming the antioestrogenic effect by PAHs, the present results indicate that the oestrogenic effect is more evident in soles from Sado 1, which is the area with the most significant contamination by organic toxicants.

The histological analysis revealed a low-moderate degree of inflammation in the gonads of female soles from Sado 1 and male soles from the reference location, otherwise, no noticeable lesions and alterations were found (Fig. 3.1). Still, Jones et al. (2000) indicated that environmental agents can cause injuries or damage the structure of the tissues responsible for hormone production. Allen et al. (1999) adds that excessive production of VTG in males will damage their general fitness to compete for resources and fight disease. The histology results also revealed the absence of gonadal abnormalities, such as ovotestis, however all fish (with exception of one female) were in the early stages of maturation, which is in agreement with the observations from Kirby et al. (2004), who concluded that juvenile males from areas contaminated by EDCs may not develop ovotestis even when VTG synthesis is abnormally upregulated.

The soles from the Sado estuary were exposed to a complex mixture of contaminants, comprising organic toxicants, metals and metalloids. Although little research has been able to relate adverse effects and responses to mixed sediment contaminants, some studies have already been performed in non-flatfish species (Monteverdi et al. 1999; Aerni et al., 2004; Silva et al., 2012). In this study, the male soles from the sampling area with strong anthropogenic influence and consequently higher concentrations of contaminants (Sado 1) yielded a more pronounced induction of hepatic VTG levels compared with those of soles from a rural area (Sado 2). This difference in hepatic VTG levels in juvenile male soles may be due to the different profiles of sediment contamination in these sediments. However, it should be noted that the males from Sado 2 may not be representative of that contamination, since this site presents a low number of males, which may explain the fact of hepatic VTG levels from this site are below the levels of the Reference animals. Moreover, Sado 2 may be influenced by VTG production inhibiting compounds and/or the soles from the Reference may be influenced by its advanced maturation stage relatively to soles from Sado estuary. Nevertheless, the integrations of VTG results with CYP1A induction and EROD activity (both related with biotransformation of organic compounds) support the hypothesis that the exposure to organic contamination (or to a mixture of different classes of organic and inorganic contaminants) was the main cause of increased hepatic VTG levels in fish from the Sado estuary, indicating that endocrine disruption is indeed occurring.

The induction of CYP1A in fish has been evaluated as a sensitive biomarker for organic contaminants such PAHs, PCBs, dioxin-like compounds and even pesticides (Nilsen et al., 1998; Nebert and Dalton, 2007). Exposure to these compounds may increase CYP1A gene expression via the aryl hydrocarbon receptor (AhR) pathway. On the other hand, increased production of active CYP enzymes and availability of substrate (i.e. CYP–metabolizable compounds), increase the catalytic activity of CYP mixed function oxidases (MFOs), which may be inferred from EROD activity. Thus, both CYP1A induction and EROD activity have long been regarded as consistent indicators of contamination by many organic compounds and have been shown to be a useful tool for monitoring marine pollution involving complex mixtures of organic contamination (Förlin and Celander, 1993; Haasch et al., 1993; Peters et al., 1997; Collier et al., 1998; Costa et al., 2009; Fonseca et al., 2011a). Globally, the present results indicate that the organic contamination response (CYP1A induction and the EROD activity) attained higher levels in the soles from Sado 1, where PAHs were the most significant organic sediment contaminants (see Annex 1). Fish from Sado 2, on the other hand, show an intermediate level of CYP1A and EROD between Sado 1 and Reference, which is also in accordance with the surveyed levels of contamination. The lowest levels of CYP1A and EROD were found in the animals from Reference, which is in accordance with the contamination levels in this area and the statements of previous authors, which revealed that these biomarkers are significantly related to the contaminant levels in the environment (Haasch

et al., 1993; Bucheli and Fent, 1995). Although there are studies that revealed EROD inhibition by metals in non-flatfish species (Roméo et al., 1994; Guilherme et al., 2008; Vieira et al., 2009), elevated EROD activity was reported by Fonseca et al. (2011a) in *Solea senegalensis* from estuaries contaminated by both metals and PAHs. Also, Trisciani et al. (2011) confirmed that EROD activity is an effective biomarker of exposure for petroleum products in common sole (*Solea solea*).

As for most biomarker responses, in natural fish populations the influence of abiotic (e.g. temperature, photoperiod, salinity and season) factors may influence the levels and the activity of the P-450 system (Goksøyr and Förlin, 1992; Bucheli and Fent, 1995). Although the abiotic parameters were not analysed in the present study, Fonseca et al. (2011b) concluded that, in *S. senegalensis*, EROD activity is more related to PAH exposure than to environmental variables. Plus, it is important to notice that, in a previous work, the same fish were surveyed for oxidative stress through several biochemical biomarkers (Gonçalves et al., 2013). In this study, Gonçalves et al. (2013) found that animals from Sado 1 revealed higher oxidative stress, inferred from increased hepatic lipid peroxidation, than animals from Sado 2 and the Reference, which was in accordance with the organic contamination in the area and the response pattern from the biomarkers of exposure analysed in the current study.

Altogether, the present findings sustain that endocrine disruption may be occurring in fish from Sado 1, which has a strong urban and industrial influence and has the highest levels of CYP1A induction and EROD activity, which suggests stronger impact by organic, CYP-activatable compounds, like PAHs. The comparison between fish from Sado 1 and Sado 2 may also indicate that the organic contamination caused endocrine disruption in juvenile males. Still, the effects of contaminant mixtures remain yet weakly investigated and may not be neglected. It should still be considered that confounding factors may arise once the fish from the Reference were not collected in inshore waters and further research on the endocrine disruption developing, in this and other contaminated estuaries, is required.

5. Conclusion

The present study revealed that endocrine disruption may occur in environments contaminated by low levels of background contamination of mixed toxicants. It has also been shown that aquatic animals in a confined environment can present different degrees of endocrine disruption, in accordance with the levels of contaminants and their potential sources. As such, estuarine biogeographical heterogeneity may result in significant differences between the effects sustained by organisms residing in adjacent areas, depending on the specific type of anthropogenic stressors, which has been demonstrated for industrial and rural sites in the present study. The current findings also indicate that the degree of endocrine disruption may not be reflected at histological level, at least in juvenile fish, since gonadal abnormalities were not observed in any male or female soles from the Sado estuary. The positive link found between oestrogenic effects, CYP1A induction and EROD activity highlight that a multi-biomarker approach is an effective strategy in endocrine disruption assessment even when the full range of background contaminants is unknown, permitting establishing a link between effects and a more specific class of pollutants.

Although at this stage it is not possible to pinpoint the specific substance or substances responsible for endocrine disruption in the study area, the results indicate that complex mixtures of sediment contaminants may cause endocrine disruption effects to the biota. It must, at this point, be stressed that the disruption of the endocrine system may adversely affect resident populations of the impacted estuarine ecosystem by compromising reproduction. Moreover, the endocrine disruptor compounds (EDCs) may have a dual function (inducers and/or inhibitors of the normal hormonal responses), meaning that further research in the area should be conducted to 1) identify the potential EDCs; 2) check for androgenic EDCs and characterize their effects on females and 3) to sample fully adult animals to survey for pathological effects in the reproductive system, since these should be more prone to develop and accumulate in mature animals or in animals at later maturation stages. Finally, the current study highlights the need to broaden the scope of effects elicited by background contamination of intricate systems like estuaries, since the identification of specific substances is often unfeasible and biomarker responses allow the identification of physiological alterations that can aid steering risk assessment strategies for these areas.

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7. Annexes

Annex 1. Physico-chemical characterization and contamination profiles of the sediments collected in Sado estuary (S1, S2, S3, S4 and S5) and Reference (M). Data from sediment sample S1 were gathered from Costa et al. (2011) and samples S2 to S5 and M were retrieved from Carreira et al. (2013). The TEL and PEL sediment quality guidelines were obtained from MacDonald et al. (1996). Refer also to Fig. 2.1.

	Site							
	S_1	S_2	S_3	S_4	S_5	M		
TOM (%)	10.2	0.9 ± 0.1	10.4 ± 0.0	6.9 ± 0.1	8.8 ± 0.0	0.7 ± 0.01		
FF (%)	95.6	3.5	52.9	63.7	74.3	0.78		
Eh (mV)	-300	-	-359 ± 2	-260 ± 28	-315 ± 7	184 ± 13		

Contaminant class								TEL	PEL
Inorganic ($\mu g.g^{-1}$)	Nonmetal								
	As	23.98 ± 0.48*	0.34 ± 0.26	19.7 ± 5.21*	26.44 ± 2.68*	25.02 ± 8.84*	0.88 ± 0.43	7.24	41.6
	Se	1.21 ± 0.02	1.84 ± 0.84	1.92 ± 1.45	0.59 ± 0.21	0.72 ± 0.08	0.43 ± 0.39	N/A	N/A
	Metal								
	Cr	80.73 ± 1.61*	2.36 ± 0.36	77.67 ± 4.57*	62.22 ± 4.45*	87.61 ± 2.97*	1.81 ± 0.12	52.3	160
	Ni	33.30 ± 0.67*	4.10 ± 1.66	16.67 ± 1.1*	17.15 ± 1.21*	22.79 ± 9.47*	3.04 ± 0.65	15.9	42.8
	Cu	172.72 ± 3.45**	4.51 ± 1.05	178.64 ± 7.01**	74.15 ± 13.16*	92.3 ± 5.63*	2.31 ± 0.36	18.7	108
	Zn	364.83 ± 7.30**	13.10 ± 1.51	327.51 ± 1.16**	269.79 ± 7.81*	385.11 ± 35.69**	1.04 ± 0.51	124	271
	Cd	0.26 ± 0.01	0.03 ± 0.02	0.27 ± 0.03	0.33 ± 0.13	0.43 ± 0.19	0.10 ± 0.05	0.68	4.21
	Pb	55.19 ± 1.10*	3.50 ± 0.48	56.45 ± 3.1*	25.3 ± 0.91	32.7 ± 1.21*	1.48 ± 1.64	30.2	112
Organic ($ng.g^{-1}$)	PAH								
	3-ring								
	Acenaphthylene	2.38 ± 0.40	<DL	9.77 ± 1.66*	1.98 ± 0.34	0.90 ± 0.15	<DL	5.87	128
	Acenaphthene	12.25 ± 2.08*	0.40 ± 0.07	9.00 ± 1.53*	1.78 ± 0.30	1.16 ± 0.20	<DL	6.71	88.9
	Fluorene	15.33 ± 2.61	0.30 ± 0.05	8.41 ± 1.43	2.80 ± 0.48	1.18 ± 0.20	0.11 ± 0.02	21.2	144
	Phenanthrene	63.87 ± 10.86	11.44 ± 1.94	66.02 ± 11.22	35.03 ± 5.96	15.86 ± 2.70	6.62 ± 1.12	86.7	544
	Anthracene	21.00 ± 3.57	<DL	9.00 ± 1.53	1.54 ± 0.26	1.15 ± 0.20	<DL	46.9	245
	4-ring								
	Fluoranthene	315.71 ± 53.67*	2.07 ± 0.35	207.29 ± 35.24*	38.99 ± 6.63	14.38 ± 2.45	<DL	113	1 494
	Pyrene	263.18 ± 44.74*	1.93 ± 0.33	175.12 ± 29.77*	36.23 ± 6.16	14.89 ± 2.53	<DL	153	1 398
	Benz[a]anthracene	81.25 ± 13.81*	0.86 ± 0.15	72.16 ± 12.27	13.01 ± 2.21	4.44 ± 0.76	<DL	74.8	693
	Chrysene	41.06 ± 6.98	<DL	40.88 ± 6.95	7.87 ± 1.34	3.44 ± 0.59	<DL	108	846
	5-ring								
	Benzo[b]fluoranthene	98.00 ± 16.66	1.02 ± 0.17	78.17 ± 13.29	14.24 ± 2.42	4.94 ± 0.84	<DL	N/A	N/A
	Benzo[k]fluoranthene	30.76 ± 5.23	<DL	49.74 ± 8.46	7.02 ± 1.19	3.15 ± 0.54	<DL	N/A	N/A
	Benzo[e]pyrene	74.95 ± 12.74	0.96 ± 0.16	70.04 ± 11.91	13.54 ± 2.30	4.50 ± 0.77	<DL	N/A	N/A
	Benzo[a]pyrene	101.86 ± 17.32*	0.62 ± 0.10	86.29 ± 14.67	12.83 ± 2.18	4.05 ± 0.69	<DL	88.8	793
	Dibenzo[a,h]anthracene	13.32 ± 2.26*	<DL	15.09 ± 2.57*	0.70 ± 0.12	0.04 ± 0.01	<DL	6.22	135

Annex 1. (Continued)

Contaminant class								TEL	PEL
Organic ($ng.g^{-1}$)	PAH								
	6-ring								
	Indeno[1,2,3–cd]pyrene	82.06 ± 13.95	<DL	101.85 ± 17.31	14.43 ± 2.45	3.67 ± 0.62	<DL	N/A	N/A
	Benzo[ghi]perylene	51.93 ± 8.83	<DL	78.14 ± 13.28	13.03 ± 2.22	4.72 ± 0.80	<DL	N/A	N/A
	tPAH	**1 365.20 ± 232.08**	**19.60 ± 3.33**	**1 076.98 ± 183.09**	**215.03 ± 36.55**	**82.47 ± 14.02**	**6.72 ± 1.14**	1 684	16 770
	Pesticides								
	pp 'DDE	<DL	0.02 ± 0.00	0.19 ± 0.03	0.12 ± 0.02	0.11 ± 0.02	<DL	2.07	374
	pp 'DDD	0.37 ± 0.06	<DL	0.08 ± 0.01	0.06 ± 0.01	0.01 ± 0.00	<DL	1.22	7.81
	pp 'DDT	<DL	<DL	0.95 ± 0.16	0.04 ± 0.01	<DL	<DL	1.19	4.77
	tDDT	**0.37 ± 0.06**	**0.02 ± 0.00**	**1.22 ± 0.21**	**0.21 ± 0.04**	**0.13 ± 0.02**	**0.00 ± 0.00**	3.89	51.7
	HCB	–	**0.04 ± 0.01**	**0.04 ± 0.01**	**0.05 ± 0.01**	**0.06 ± 0.01**	**0.02 ± 0.00**	20	480
	PCBs								
	Trichlorinated								
	18	0.27 ± 0.05	<DL	0.01 ± 0.00	<DL	0.02 ± 0.00	<DL	N/A	N/A
	26	1.80 ± 0.31	<DL	<DL	<DL	<DL	<DL	N/A	N/A
	31	0.26 ± 0.04	0.02 ± 0.00	0.05 ± 0.01	0.01 ± 0.00	0.07 ± 0.01	0.02 ± 0.00	N/A	N/A
	Tetrachlorinated								
	44	0.17 ± 0.03	<DL	<DL	<DL	<DL	<DL	N/A	N/A
	49	0.13 ± 0.02	<DL	0.08 ± 0.01	<DL	0.02 ± 0.00	<DL	N/A	N/A
	52	0.10 ± 0.02	<DL	0.19 ± 0.03	<DL	0.03 ± 0.00	<DL	N/A	N/A
	Pentachlorinated								
	101	0.25 ± 0.04	0.01 ± 0.00	0.52 ± 0.09	<DL	<DL	<DL	N/A	N/A
	105	0.26 ± 0.04	<DL	<DL	<DL	<DL	<DL	N/A	N/A
	118	0.55 ± 0.09	<DL	0.42 ± 0.07	0.01 ± 0.00	0.01 ± 0.00	<DL	N/A	N/A
	Hexachlorinated								
	128	0.26 ± 0.04	<DL	0.11 ± 0.02	<DL	<DL	<DL	N/A	N/A
	138	0.71 ± 0.12	<DL	1.04 ± 0.18	<DL	<DL	<DL	N/A	N/A
	149	0.05 ± 0.01	0.02 ± 0.00	0.85 ± 0.14	0.05 ± 0.01	0.05 ± 0.01	<DL	N/A	N/A
	151	0.77 ± 0.13	<DL	<DL	<DL	<DL	<DL	N/A	N/A
	153	0.98 ± 0.17	<DL	1.08 ± 0.18	0.12 ± 0.02	0.01 ± 0.00	<DL	N/A	N/A
	Heptachlorinated								
	170	0.20 ± 0.03	<DL	0.05 ± 0.01	<DL	<DL	<DL	N/A	N/A
	180	0.73 ± 0.12	<DL	0.86 ± 0.15	0.08 ± 0.01	0.06 ± 0.01	<DL	N/A	N/A
	187	0.29 ± 0.05	<DL	<DL	<DL	<DL	<DL	N/A	N/A
	194	0.12 ± 0.02	<DL	0.11 ± 0.02	<DL	<DL	<DL	N/A	N/A
	tPCB	**7.91 ± 1.34**	**0.05 ± 0.01**	**5.37 ± 0.91**	**0.26 ± 0.04**	**0.27 ± 0.05**	**0.02 ± 0.00**	34.1	277

[<DL] – Below detection limit; TOM, total organic matter; FF, sediment fine fraction (particle size < 0.063 mm); Eh, sediment redox potential; PEL, probable effects level guideline; TEL, threshold effects level guideline; N/A, no guideline available; *, value above TEL; **, value above PEL. Contaminant concentration ranges indicate the standard quantification error.