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# Assessing contamination from maritime trade and transportation on Iberian waters: Impact on *Platichthys flesus*

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ABSTRACT

Maritime trade and transportation of hazardous and noxious substances (HNS) have been increasing in European waters, augmenting the risk of accidental spills from ships or in harbours. Despite their reported toxicity and hazardousness, information on HNS levels in the aquatic environment is still lacking. Therefore, an assessment combining a chemical and a multi-biomarker evaluation on HNS contamination was done in NW Iberian estuaries of Rivers *Minho*, *Lima* and *Douro* using *Platichthys flesus* (flounder). Of the twenty-five HNS measured, fifteen were found in flounder liver and muscle, and a few in sediments, though at generally low levels. Principal component analysis produced a clear distinction among sites, with *Douro* River estuary arising as the most impacted. Oxidised proteins and antioxidant enzymes (superoxide dismutase, catalase, glutathione peroxidase) were the biomarkers contributing to site discrimination. Correlations between biomarkers and HNS levels provided important baseline information for the study area and potential biological effects of HNS on this sentinel species.

## 1. Introduction

Over the past five decades maritime trade and transport of chemical compounds have suffered a remarkable growth, making up at present to more than 90 per cent of world trade (MKC 2012). Apart from oils, an assortment of other chemicals and goods ranging from vegetable oils to highly toxic compounds (Harold et al., 2014) are regularly transported by sea. Altogether, they are globally connoted as hazardous and noxious substances (HNS); in other words, "any substance other than oil, which, if introduced into the marine environment, has the potential to create hazards to human health, to harm living resources and marine life, to damage amenities or to interfere with other legitimate uses of the sea" (CEDRE 2012). Maritime transportation of HNS has also been increasing over the years (MKC 2012), comprising 11% of global chemical trade (around 165 million tonnes of bulk trade per year). In particular, in the European Union, around 50 000 HNS are carried by sea within which 2000 are transported on a regular basis (Purnell 2009; Harold et al., 2014). This increase on maritime trade augments the probability of spill occurrence due to accidents involving ships or transfer in harbours. Several events have been already recorded in European waters since the seventies (Neuparth et al., 2011).

Hazardous and Noxious Substances present different physico-chemical characteristics and behave differently once released into the aquatic environment (ITOPF 2011; Neuparth et al., 2012). This diversity makes it difficult to predict HNS behaviour and evaluate the consequences of a spill. Literature reporting the toxicological effects of polycyclic aromatic hydrocarbons (PAHs), which are present in oils, in marine and freshwater organisms is extensive (Almeida et al., 2012; Vieira and Guilhermino 2012; Abdel-Shafy and Mansour 2016). On the contrary, for HNS, their toxicity and modes of action are still little understood especially for marine organisms (Rocha et al., 2016). Recent evidence indicates, however, that HNS (in particular organic

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compounds) can induce biochemical, histopathological, reproductive and behavioural changes, as well as, carcinogenicity, and eventually mortality on estuarine and marine animals, depending on the concentration and time of exposure (Rocha et al., 2016; Abreu et al., 2018).

Located at the south-western end of Europe and owner of a considerable large Economical Exclusive Zone (EEZ), the Iberian Peninsula presents a very exposed geographical position, at the convergence of important worldwide maritime routes (Carvalho 2015). Over the last decade, more than 12 000 ships per year have moored in Portuguese harbours which involved, in 2013, the manoeuvre of 154 891 GT of cargo (Carvalho 2015). The north-western (NW) Portuguese coast has two harbour facilities in *Lima* and *Douro* River estuary: *Viana do Castelo* harbour, a secondary facility which handles around 500 000 tonnes per year of goods (AMT 2015); and *Leixões* harbour, which received in 2015 almost 3000 entries of cargo ships and handled more than 18 million tonnes of cargo, 43.3% of which regards liquid cargo (Carvalho 2015). Portugal has no oil or other chemical substances production, so it depends on the importation of large quantities of these goods (Jorge 2011). The imported crude oil and refined derivates are mainly received at *Leixões* and *Sines* (South coast of Portugal) harbour facilities (AMT 2015). Furthermore, important routes of transportation of oil and other chemicals cross the Portuguese EEZ. For these reasons, the Iberian coastal and estuarine ecosystems are susceptible to the deleterious consequences associated with accidental spills near coast or within harbour areas. Moreover, meteorological, hydrographic and oceanographic conditions of the Iberian coast can aggravate the deleterious effects of accidental spills, leading to the spread of spilled chemicals (Jorge 2011). The occurrence of several accidental spills of crude oil and other chemicals have been in fact registered in or near the Iberian coast, with more than 25 incidents since 1970 (Nunes 2003; Jorge 2011). With regard to the NW Portuguese coast, it is worth to mention the incidents involving *Jacob Maersk* in 1975, *Reijin* in 1980, *Cercal* in 1994, *Carol Bulker* in 2000 and *Prestige* in 2002 (Jorge 2011) which led to the spill of crude oil, fuel and lubricants into the sea.

Metals and PAHs contamination have been previously reported in the NW Iberian coast (Mucha et al., 2004; Guimarães et al., 2009; Gravato et al., 2010; Rocha et al., 2011; Mil-Homens et al., 2013; Reis et al., 2013). Nevertheless, there is still lack of information about the presence of HNS in Iberian coastal ecosystems (in several matrices, e.g. water, sediments, biological tissue), with only one very recent study (Gouveia et al., 2018). With that in mind and considering the economical and leisure activities occurring in the Iberian coast, the characterisation of the environmental state of the NW Iberian coast should be performed combining chemical characterisation of environmental matrices, namely the presence of HNS, and a multi-biomarker approach. So, monitoring campaigns were carried out, in the same season, in three River estuaries (*Minho*, *Lima* and *Douro*). A sentinel species was selected for this research, *Platichthys flesus* (flounder), since this eurihyaline fish species inhabits estuaries for most of the year, migrating to deeper water in the reproduction period or in winter (Hylland et al., 1996). Additionally, as a benthic species, it is especially vulnerable to pollution in sediments (Ferreira et al., 2004). The levels of a myriad of HNS were determined in sediments and tissues of flounder collected at the three estuaries. The sole measurement of pollutant levels in different environmental matrices is now recognised as insufficient for assessing the environmental risk of a contaminant (van der Oost et al., 2005). So, biomarkers of neurotoxicology, biotransformation, antioxidant defences and oxidative damage were also evaluated. Chemical and biochemical data were thereafter compared and integrated with multivariate analysis aiming to identify response profiles typical of each estuary. Biochemical responses are recognised as sensitive indicators of contamination and a rapid, easy to handling and cost effective approach to assess animal and environmental health. Changes on these parameters have therefore been commonly used in environmental risk assessment (ERA) procedures (van der Oost et al., 2003). This study increases knowledge about the presence of contaminants originated from maritime transportation in Iberian waters and their possible repercussions on the inhabiting organisms. To the best of our knowledge, this is the first study monitoring simultaneously the levels of twenty-five HNS in two different environmental matrices (biota and sediment).

## 2. Material and methods

### 2.1. Study area and sampling procedures

The study area was located on the north-western (NW) Atlantic coast of Portugal. Sampling campaigns were conducted in *Minho*, *Lima* and *Douro* River estuaries in April/May 2014 (Fig. 1). Along the NW Atlantic coast, different urban and industrial patterns of activity are observed. The most southern estuaries (*Lima* and *Douro*) and their adjoining areas are under a more intensive anthropic pressure owing to local oil refining industry and two maritime harbours (located at *Leixões* and *Viana do Castelo*), high population density and the development of several other industrial and urban activities. *Minho* River estuary, included in the Natura 2000 network, shows low susceptibility to human influence and low levels of environmental pollution (Guimarães et al., 2012; Rodrigues et al., 2014).

Adult flounders were captured by trawl fishing along the sampling area, summing up a total of 11, 13 and 8 flounders sampled in *Minho*, *Lima* and *Douro* River estuaries, respectively. Flounder specimens were transported to the laboratory in estuarine water collected at the site and with aeration. In the laboratory, the collected flounder specimens were sacrificed within 4 h after the capture and anaesthetization in a 10% solution of MS222. Each fish was measured and weighed. Liver, muscle and bile were sampled, weighed and frozen in liquid nitrogen. These samples were subsequently stored at – 80 °C until they were assayed. The condition factor (CF) and hepato-somatic index (HSI) were calculated based on total length/total fish weight and on liver weight/total fish weight, respectively.

Water and sediments were sampled within the sampling area of flounder, with four sampling sites in each estuary (Fig. 1). Sediments were collected by means of a Petite ponar dredge. Sediments were transported to the laboratory in portable coolers. In the laboratory, part of the sediments was frozen at – 20 °C for HNS and two other portions were dried, until constant weight, for PAHs and metal analysis. Part of sediment analysis (*Minho* and *Douro* River estuaries sediments) was done in the frame of Gouveia et al. (2018) study and which data are also presented in Table 3. Water samples were collected by means of a Niskin bottle at low and high tide (LT and HT, respectively) and, whenever there was enough water depth, surface, medium and bottom water samples were collected at each site. Water samples were transported to the laboratory in portable coolers and frozen at – 20 °C until analysis, for HNS and PAHs, and acidified with $HNO_3$ for metal analysis.

### 2.2. Chemical analysis

For HNS determination (2-propenenitrile, 1,1-dichloroethane, 1,2-dichloroethane, 1,1,2-trichloroethane, 1,1,1-trichloroethane, chloroform, benzene, carbon tetrachloride, trichloroethylene, tetrachloroethylene, 1,2-dichloropropane, cis-1,3-dichloropropene, trans-1,3-dichloropropene, toluene, chlorobenzene, ethylbenzene, m-xylene, p-xylene, styrene, o-xylene, butylacrylate, styrene, 1,2-dichlorobenzene, 1,3-dichlorobenzene, 1,4-dichlorobenzene and cyclohexylbenzene), 1 g of fresh tissue or fresh sediment was weighed into 20 mL amber glass vials with 10 mL of deionised water. Composite samples of tissue of flounder from several individuals were used to allow for five replicate measurements and homogenisation was carried out with an Ultra-turrax blender (Ika). A headspace solid phase microextraction (SPME) with a fiber of polydimethylsiloxane-divinylbenzene (PDMS-DVB, polar, Supelco) was carried out using a CombiPal model (CTC Analytics) autosampler. HNS quantification was done in a Varian Saturn 2000 mass spectrometer (Walnut Creek, CA) with a Varian 3900 gas chromatograph

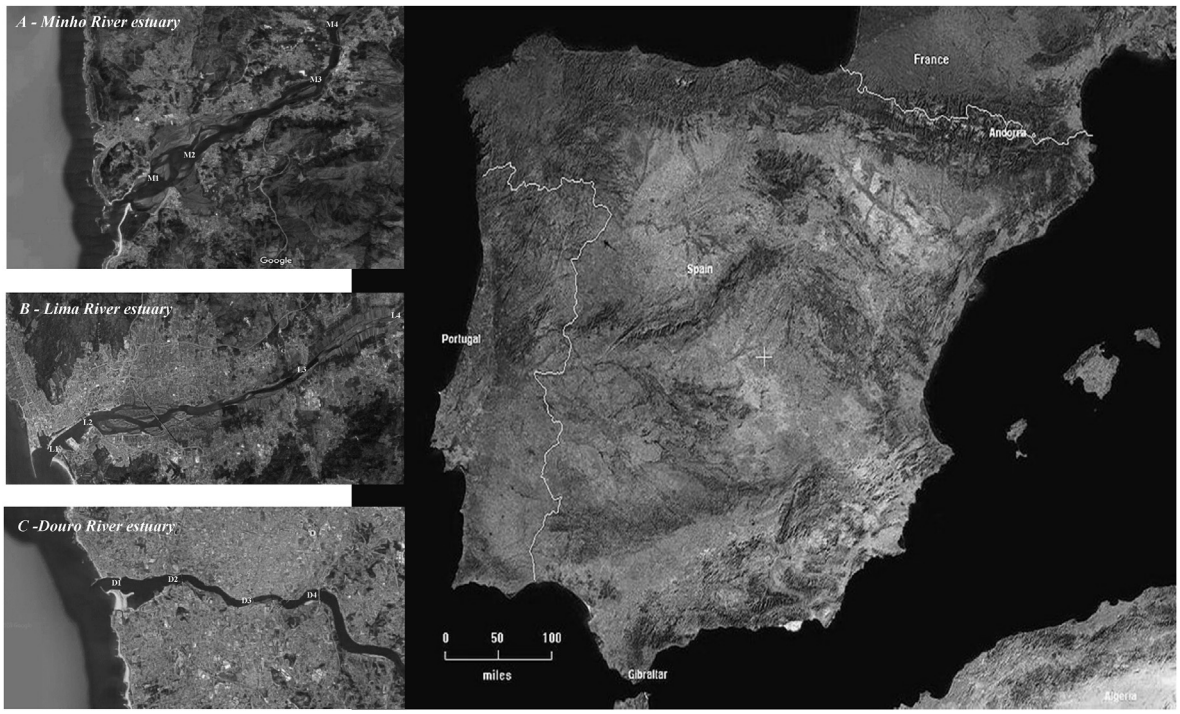


**Fig. 1.** Location of the sampling sites: specimens of flounder and estuarine sediments were collected in *Minho* (4 sampling points), *Lima* (2 sampling points) and *Douro* River (4 sampling points) estuaries (sampling campaigns carried out in the spring of 2014).

**Table 1**
Levels of hazardous and noxious substances (HNS) detected in estuarine sediments collected in *Minho*, *Lima* and *Douro* River estuaries. Values represent average concentrations of 3 replicates (standard deviation between brackets). Data from *Minho* and *Douro* River estuary adapted from Gouveia et al. (2018).

| | Sampling site | | | |
|---|---|---|---|---|
| **HNS (ng/g)** | | | | |
| ***Minho River estuary*** | **M1** | **M2** | **M3** | **M4** |
| *Toluene* | <0.1[a] | <0.1[a] | 7 (2) | <0.1[a] |
| ***Lima River estuary*** | **L1** | **L2** | **L3** | **L4** |
| *Chloroform* | 2.1 (0.2) | 3 (1) | n.c.[b] | n.c.[b] |
| ***Douro River estuary*** | **D1** | **D2** | **D3** | **D4** |
| *Toluene* | <0.1[a] | <0.1[a] | <0.1[a] | 0.83 (0.02) |

Other HNS measured in the sediments were below the limit of detection (values in brackets for each compound, in ng/g).
2-Propenenitrile (75), 1,1-Dichloroethane (2), Chloroform (1), 1,1,1-Trichloroethane(0.9), 1,2-Dichloroethane (2), Benzene (0.4), Carbon Tetrachloride (0.8), Trichloroethylene (22), 1,2-Dichloropropane (2), cis-1,3-Dichloropropene (0.9), trans-1,3-Dichloropropene (0.9), 1,1,2-Trichloroethane (1), Tetrachloroethylene (0.3), Chlorobenzene (0.7), Ethylbenzene (0.1), m-Xylene (0.1), p-Xylene (0.1), Butylacrylate (0.1), Styrene (0.1), o-Xylene (0.1), 1,3-Dichlorobenzene (0.8), 1,4-Dichlorobenzene (0.8), 1,2-Dichlorobenzene (0.3), Cyclohexylbenzene (0.1).

[a] Limit of detection.
[b] n.c. – not collected.

and a VF-5ms column. Limits of detection were in the range 0.01–6 ng/g wet weight for solid samples, depending on the individual compound. Detailed information can be found in (Rocha et al., 2019).

For PAHs determination (naphthalene, acenaphthylene, acenaphthene, fluorene, phenanthrene, anthracene, fluoranthene, pyrene, benz(a)anthracene, crysene, benz(b)fluoranthene, benz(k)fluoranthene, benz(a)pyrene, indene(1,2,3-cd)pyrene, dibenz(ah)anthracene and benz(ghi)perylene), a procedure adapted from Gonçalves et al. (2016) was used. For that, lyophilised sediment was extracted with methanol (5 mL, CHROMASOLV grade, Sigma-Aldrich) and afterwards cleaned up with Florisil (Fluka). An internal standard solution (containing deuterated PAHs) was added to all extract solutions. Sediment extracts were concentrated by SPME employing a 100 μm PDMS fibre and analysed in the chromatograph described above for HNS. Limits of detection below 6 ng/g dry weight were achieved for all the analysed PAHs. More details are provided in Rocha et al. (2019).

For metal determination (Zn, Cu, Pb, Ni, Cd and Hg), atomic absorption spectrophotometry with flame or electrothermal atomization (depending on metal levels) was used after an acidic (with HNO3) digestion of the dried sediments (three replicates per sample) in a high-pressure microwave with PARR vessels. Aqueous standard calibrations were used for metal quantification (Reis et al., 2013). Metals selected were those commonly found in sediments of the Northwest (NW) Portuguese coast (e.g. Reis et al., 2013).

### 2.3. Biochemical analysis

A portion (20–30 mg) of flounder liver was homogenised in ice-cold sodium phosphate buffer 100 mM $K_2HPO_4/KH_2PO_4$: 150 mM KCl: 1 mM dithiothreitol (DTT): 0.1 mM phenylmethanesulfonylfluoride (PMSF): 1 mM $Na_2EDTA$ (pH 7.4) solution, using a Precellys homogenizer. Mitochondrial fractions were obtained after centrifugation at 12 000 × g for 20 min. The supernatant was collected and divided in several aliquots for biochemical analysis.

Catalase (CAT) activity was evaluated through the measurement of $H_2O_2$ consumption at 240 nm ($\varepsilon = 40\ mM^{-1}\ cm^{-1}$) as described in (Aebi 1974). Superoxide dismutase (SOD) activity was measured using the indirect method based on the inhibition of cytochrome c reduction (Ferreira et al., 2005). In this, superoxide anion is generated by the reaction of hypoxanthine and xanthine oxidase; SOD competes with cytochrome c for the superoxide anion. The mitochondrial fraction was used to evaluate SOD activity, through the degree of inhibition of cytochrome c reduction measured at 550 nm (McCord and Fridovich 1969). The enzyme activity is expressed in SOD units such that 50% inhibition of the xanthine oxidase reaction is equivalent to one SOD unit.

Glutathione reductase (GR) activity was determined by measuring the decrease of nicotinamide adenine dinucleotide phosphate (NADPH)

**Table 2**
Levels of polycyclic aromatic hydrocarbons (PAHs) in estuarine sediments collected in *Minho*, *Lima* and *Douro* River estuaries. Values represent average concentrations of 3 replicates (standard deviation between brackets). Data from *Minho* and *Douro* River estuary adapted from Gouveia et al. (2018).

| PAHs (ng/g) | Sampling site | | | |
|---|---|---|---|---|
| ***Minho River estuary*** | **M1** | **M2** | **M3** | **M4** |
| *Naphthalene* | 2.3 (0.6) | 2.6 (0.2) | 4 (1) | <0.3[a] |
| *Phenanthrene* | 8 (2) | 11.9 (0.7) | 13 (2) | 3.2 (0.2) |
| *Anthracene* | 1.0 (0.3) | <0.5[a] | <0.5[a] | <0.5[a] |
| *Fluoranthene* | 4.7 (0.4) | 6 (3) | 11 (5) | <1[a] |
| *Pyrene* | 5 (1) | 6 (3) | 11 (4) | 2.2 (0.3) |
| *Benz(a)anthracene* | 7.46 (0.04) | 7.7 (0.2) | 9 (1) | 7.21 (0.08) |
| *Indene(1,2,3-cd)pyrene* | 4.9 (0.2) | 3.1 (0.4) | <1[a] | <1[a] |
| *Dibenz(ah)anthracene* | 4.5 (0.1) | 4.5 (0.2) | <0.8[a] | <0.8[a] |
| *Benz(ghi)perylene* | 4.6 (0.1) | 4.1 (0.3) | 4.7 (0.2) | <0.8[a] |
| ***Total PAHs*** | **40 (1)** | **45 (7)** | **52 (9)** | **13.6 (0.6)** |
| ***Lima River estuary*** | **L1** | **L2** | **L3** | **L4** |
| *Naphthalene* | 2.45 (0.03) | 2.5 (0.6) | n.c.[b] | n.c.[b] |
| *Phenanthrene* | 15.9 (0.7) | 10.1 (0.8) | | |
| *Anthracene* | 2.62 (0.01) | 1.6 (0.5) | | |
| *Fluoranthene* | 27 (4) | 8 (1) | | |
| *Pyrene* | 25 (4) | 9.1 (0.9) | | |
| *Benz(a)anthracene* | 14 (3) | 8.3 (0.2) | | |
| *Crysene* | 8 (2) | <1.0[a] | | |
| *Benz(b)fluoranthene* | 5 (2) | <1.4[a] | | |
| *Indene(1,2,3-cd)pyrene* | 4.9 (0.1) | 3.80 (0.03) | | |
| *Dibenz(ah)anthracene* | 4.2 (0.1) | 3.90 (0.02) | | |
| *Benz(ghi)perylene* | 4.8 (0.1) | 3.6 (0.1) | | |
| ***Total PAHs*** | **115 (12)** | **52 (4)** | | |
| ***Douro River estuary*** | **D1** | **D2** | **D3** | **D4** |
| *Naphthalene* | 1.2 (0.5) | 5.2 (0.4) | 6.2 (0.3) | 5.7 (0.6) |
| *Fluorene* | <0.6[a] | 2.9 (0.5) | 7 (3) | 2 (1) |
| *Phenanthrene* | 2.3 (0.2) | 31 (2) | 103 (49) | 25 (2) |
| *Anthracene* | <0.5[a] | 5.8 (0.5) | 28 (15) | 4.5 (0.6) |
| *Fluoranthene* | 1.9 (0.3) | 37 (7) | 200 (68) | 32 (2) |
| *Pyrene* | 2.6 (0.2) | 34 (4) | 188 (56) | 35 (3) |
| *Benz(a)anthracene* | 3.8 (0.2) | 16 (1) | 98 (17) | 15 (0.9) |
| *Crysene* | <1[a] | 8 (1) | 72 (8) | 7 (0.1) |
| *Benz(b)fluoranthene* | <1[a] | 4 (2) | 37 (7) | 4 (1) |
| *Benz(k)fluoranthene* | <0.6[a] | <0.6[a] | 18 (4) | <0.6[a] |
| *Benz(a)pyrene* | <1[a] | 1.9 (0.3) | 32 (9) | 2.5 (0.3) |
| *Indene(1,2,3-cd)pyrene* | 2.0 (0.6) | 4.7 (0.2) | 10 (1) | 5.9 (0.1) |
| *Dibenz(ah)anthracene* | 2.8 (0.1) | 4.61 (0.02) | 5.4 (0.1) | 4.8 (0.2) |
| *Benz(ghi)perylene* | 2.7(0.5) | 4.5 (0.3) | 8.8 (0.7) | 5.0 (0.3) |
| ***Total PAHs*** | **19 (2)** | **156 (18)** | **811 (233)** | **146 (8)** |

Other PAHs measured in the sediments were below the limit of detection (values in brackets for each compound, in ng/g).
Acenaphthylene (0.6), Acenaphthene (1), Fluorene (0.6), Crysene (1), Benz(b) fluoranthene (1), Benz(k)fluoranthene (0.6), Benz(a)pyrene (1).
[a] Limit of detection.
[b] n.c. – not collected.

levels due to the reduction of oxided glutathione (GSSG) to reduced glutathione (GSH), according to Cribb's method (Cribb et al., 1989), adapted to microplate. GR activity was expressed in nmol of oxidised NADP + per min per mg of protein.

Glutathione peroxidase (GPx) activity was determined by measuring the decrease in NADPH using hydrogen peroxide as the substrate, according to (Mohandas et al., 1984) method adapted to microplate. The activity of GPx is presented in nmol per minute per mg of protein.

Glutathione-s-transferase (GST) activity was evaluated according to the method of (Habig et al., 1974), adapted to microplate (Menezes et al., 2006). GST activity is presented in nmol of the substrate conjugated per min per mg of protein.

The total concentration of protein in the samples was measured in microplates through the Lowry method.

**Table 3**
Levels of metals in estuarine sediments collected in *Minho*, *Lima* and *Douro* River estuaries. Values represent average concentrations of 3 replicates (standard deviation between brackets). Data from *Minho* and *Douro* River estuary adapted from Gouveia et al. (2018). The effect-low range (ERL) guidelines established by (Long et al., 1995) for sediments are also presented.

| Metals (μg/g) | Sampling site | | | | ERL Long et al. (1995) |
|---|---|---|---|---|---|
| ***Minho River estuary*** | **M1** | **M2** | **M3** | **M4** | |
| *Zinc* | 44 (2) | 40.8 (0.7) | 6 | 20 (2) | 150 |
| *Copper* | 3.8 (0.1) | 4.3 (0.3) | 7 (1) | 1.3 (0.2) | 34 |
| *Lead* | 0.30 (0.03) | <0.2[a] | <0.2[a] | <0.2[a] | 46.7 |
| *Nickel* | 10 (2) | 12 (2) | 19 (2) | 6.1 (0.9) | 20.9 |
| *Cadmium* | <0.04[a] | <0.04[a] | 0.09 (0.01) | <0.04[a] | 1.2 |
| *Mercury* | 0.18 (0.03) | 0.193 (0.007) | 0.159 (0.006) | 0.074 (0.001) | 0.15 |
| ***Lima River estuary*** | **L1** | **L2** | **L3** | **L4** | |
| *Zinc* | 60.2 (0.5) | 52 (2) | n. c.[b] | n. c.[b] | |
| *Copper* | 7.1 (0.7) | 4.9 (0.2) | | | |
| *Lead* | 2.68 (0.05) | 2.0 (0.4) | | | |
| *Nickel* | 15 (2) | 10 (3) | | | |
| *Cadmium* | 0.05 (0.01) | 0.05 (0.02) | | | |
| *Mercury* | 0.34 (0.02) | 0.27 (0.02) | | | |
| ***Douro River estuary*** | **D1** | **D2** | **D3** | **D4** | |
| *Zinc* | 18 (1) | 87 (4) | 97 (5) | 108 (2) | |
| *Copper* | 1.7 (0.6) | 9.6 (0.2) | 16 (3) | 22 (1) | |
| *Lead* | 5 (1) | 3.6 (0.7) | 3 (2) | 6 (2) | |
| *Nickel* | 7 (2) | 15 (1) | 12 (1) | 14 (2) | |
| *Cadmium* | <0.04[a] | 0.076 (0.001) | 0.3 (0.2) | 0.22 (0.05) | |
| *Mercury* | 0.07 (0.01) | 0.30 (0.02) | 0.25 (0.02) | 0.27 (0.02) | |

[a] Limit of detection.
[b] n.c. – not collected.

### 2.4. Neurotransmission

A portion (10–15 mg) of flounder's muscle was homogenised in a Precellys homogenizer, in ice-cold phosphate buffer containing $K_2HPO_4$/$KH_2PO_4$ (100 mM, pH 7.4), KCl (150 mM) and $Na_2EDTA$ (1 mM). The supernantant was centrifuged at 6000 × g for 5min and divided in several aliquots. Total protein was measured in microplates using the method of Bradford.

The determination of cholinesterase (ChE) activity was done through the method of (Ellman et al., 1961). The activity was estimated by the formation of conjugates between thiocholine with 5,5′-dithio-bis-2-nitrobenzoate (DTNB), which result in an increase of absorbance measured at 412 nm. The measurements were done in a microplate reader, every 20 s, for a period of 5 min, after the addition of the reaction mixture containing acetylthiocholine (0.5 mM) and DTNB (0.3 mM) in the phosphate buffer. The activity of ChE/AChE is presented in nmol of substrate hydrolised per min per mg of protein.

### 2.5. Oxidative damage

Peroxidative damage to lipids (LPO) that occurs with free radical generation and results in the production of malondialdehyde (MDA) was assessed using the thiobarbituric acid method. For this, the liver homogenates were incubated with 100% trichloroacetic acid (TCA) and

then centrifuged. The supernatant was incubated with TBA (1%), NaOH (0.05 M) and BHT (0.025%) for 30 min at 100 °C (Niki and N Taniguchi, 2000). Absorbance was then measured at 532 nm. The levels of lipid peroxidation are presented as MDA equivalents per mg of protein.

Oxidative damage to protein (PCO) was determined spectrophotometrically as described in (Ferreira et al., 2008). The method is based on the reaction of dinitrophenylhydrazine (DNPH) with protein carbonyls to form protein hydrazones, which forms a Schiff base. In brief, the liver homogenate was divided in two aliquots to which 2 M HCl (control blank) or 10 mM DNPH prepared in 2 M HCl were added. After incubation (1 h, room temperature), protein was precipitated by adding 20% TCA and centrifuged at 10 000 g at 4 °C for 10 min. The pellet was washed three times with 1:1 ethanol-ethylacetate, being the suspension centrifuged after each washing procedure. The final pellet was dissolved in 6 M guanidine hydrochloride. The change in absorbance resulting from the formation of the carbonyl group was measured at 370 nm. The carbonyl content determined in the samples is presented in nmol/mg protein. The concentration of protein in the samples was measured in microplate using the Lowry method.

### 2.6. Statistical analysis

Data of each biomarker were first checked for normality with Shapiro-Wilk test and for homogeneity of variances with Levene's test. When normal distribution was not met, data were logged or square root-transformed and normality was again checked (ChE, CAT and LPO). Differences among estuaries for flounder specimens were investigated using a one-way analysis of variance (ANOVA) with a Tukey's multiple comparison test at a 5% significant level. Krustal-Wallis test with Dunn's multiple comparison test at a 5% significant level was carried out when normal distribution was not met (in the case of CAT and LPO). All statistical analyses were carried out in Graph Prism 5.

Patterns of biomarker responses measured and their relation to medium contamination were sought by doing a Principal Component Analysis (PCA) carried out in FactoMineR. Concentrations of contaminants above the limit of quantification and the selected biomarkers were entered as quantitative variables. Sampling sites were taken as a supplementary qualitative variable. The interpretation of PCA results was based on principal components (PC) showing Eigen values $> 1$. Correlations between the quantitative variables and their respective PC were used to interpret the solution obtained. Significant differences were accepted for $p < 0.05$.

## 3. Results

### 3.1. Levels of contaminants in sediments

Tables 1–3 exhibit the levels of HNS, PAHs and metals measured in sediment samples collected at several locations in *Minho*, *Lima* and *Douro* River estuaries (Fig. 1). Two out of 26 HNS were found at low levels (ng/g): toluene in *Minho* and *Douro* River estuaries and chloroform in *Lima* River estuary. PAHs were quantified (Table 2) in sediments collected at the three estuaries. Higher amounts of total PAHs, with major contribution of phenanthrene, fluoranthene, pyrene, benz(a)anthracene, were found in *Douro* River estuary (exception of D1 site). Low metal concentrations were generally found, the higher concentrations being measured in *Douro* River estuary sediments.

### 3.2. Levels of contaminants in tissues

Table 4 presents a list of the HNS measured in flounder tissues. Very low levels (below 30 ng/g) were found for all compounds but tetrachloroethylene. Generally, higher concentrations were found in liver when compared to flounder's muscle. 1,2-Dichloropropane and 1,4-dichlorobenzene were only found in flounder's liver from *Douro* River estuary whereas 1,1,2-trichloroethane was only detected on those collected at *Minho* River estuary. Flounder's muscle from *Lima* and *Douro* estuaries (those with higher anthropogenic pressure) contained tetrachloroethylene and significant higher amounts were found in specimens from *Lima* River estuary (near 1 μg/g). In addition, flounders from the same estuary showed higher amounts of toluene. On the other hand, the concentrations of m-, p- and o-xylene and ethylbenzene were generally higher in liver of flounders from *Minho* River estuary. Other HNS, such chloroform, benzene, trichloroethylene, trans-1,3-dichloropropene, chlorobenzene, styrene, were found in either muscle or liver.

### 3.3. Biomarker levels

Results concerning the condition factor (CF), hepato-somatic index (HSI), the measured levels of activity of antioxidant and neurotoxicity

**Table 4**
Levels of hazardous and noxious substances (HNS) measured in the liver and muscle of flounders (*Platichthys flesus*) captured in *Minho*, *Lima* and *Douro* River estuaries, Northwest Portugal. Values represent average concentrations of 3 replicates (standard deviation between brackets).

| | Minho River estuary | | Lima River estuary | | Douro River estuary | |
|---|---|---|---|---|---|---|
| | Muscle[a] | Liver[b] | Muscle[a] | Liver[b] | Muscle[a] | Liver[b] |
| HNS (ng/g) | | | | | | |
| Chloroform | 0.18 (0.09) | 2 (1) | <0.02* | <0.02* | 0.4 (0.2) | <0.02* |
| Benzene | 0.19 (0.01) | 2.3 (0.4) | <0.01* | 3 (1) | 1.44 (0.20) | 4.3 (0.7) |
| Trichloroethylene | <0.04* | 2.7 (0.4) | <0.04* | 2.2 (0.2) | 0.82 (0.05) | 1.8 (0.1) |
| 1,2-Dichloropropane | <0.1* | <0.1* | <0.1* | <0.1* | <0.1* | 5.7 (0.9) |
| trans-1,3-Dichloropropene | <0.02* | 3.6 (0.4) | <0.02* | 3.9 (0.9) | <0.02* | <0.02* |
| Toluene | 0.09 (0.01) | 5.1 (0.7) | <0.01* | 28 (24)[A] | 0.21 (0.02) | 1.9 (0.5)[A] |
| 1,1,2-Trichloroethane | <0.02* | <0.02* | <0.02* | 2.2 (0.2) | <0.02* | <0.02* |
| Tetrachloroethylene | <6* | <6* | 944 (207)[A] | <6* | 194 (75)[A] | <6* |
| Chlorobenzene | <0.01* | 0.4 (0.1) | <0.01* | <0.01* | <0.01* | 0.26 (0.03) |
| Ethylbenzene | 0.21 (0.02) | 5.0 (0.5) | <0.01* | 0.6 (0.4) | 0.06 (0.01) | 2 (1) |
| m-Xylene | 1.06 (0.08) | 21 (3) | <0.01* | 1.6 (0.6) | 1.07 (0.05) | 13 (10) |
| p-Xylene | 0.29 (0.08) | 8 (1) | <0.01* | <0.01* | 0.47 (0.03) | 4 (3) |
| Styrene | 0.26 (0.01) | 1.9 (0.2) | 0.18 (0.04) | 1.4 (1.2) | 0.37 (0.01) | 1.0 (0.4) |
| o-Xylene | 0.35 (0.04) | 9 (1) | <0.01* | 0.8 (0.4) | <0.01* | 4 (2) |
| 1,4-Dichlorobenzene | <0.01* | <0.01* | <0.01* | <0.01* | <0.01* | 0.39 (0.05) |

Other HNS measured were below the limit of detection (values in brackets for each compound).
2-Propenenitrile (149 ng/g), 1,1-Dichloroethane (0.1 ng/g), 1,1,1-Trichloroethane (0.03 ng/g), 1,2-Dichloroethane (0.04 ng/g), CarbonTetrachloride (0.03 ng/g), cis-1,3-Dichloropropene (0.02 ng/g), Butylacrylate (0.01 ng/g), 1,3-Dichlorobenzene (0.01 ng/g), 1,2-Dichlorobenzene (0.01 ng/g), Cyclohexylbenzene (0.01 ng/g).
*Limit of detection.
[a] Muscle: values represent average concentrations of 5 replicates of a composite sample.
[b] Liver: values represent average concentrations of 5 sets of individual livers.

**Table 5**
Condition factor (CF) and hepato-somatic index (HSI) of *Platichthys flesus* collected in spring in *Minho*, *Lima* and *Douro* River estuaries (n = 11, 12 and 8, respectively). Levels of lipid peroxidation (LPO) and proteins carbonyls (PCO), and activities of biotransformation (glutathione-s-transferase (GST)) and antioxidant enzymes (catalase (CAT), glutathione reductase (GR), glutathione peroxidise (GPx) and superoxide dismutase (SOD)) were determined in the liver of *Platichthys flesus*, as well as, the activity of a neurologic enzyme (cholinesterase (ChE)) determined in muscle of the same organism (Values represent average concentrations (standard error between brackets); different letters indicate statistical significance among data ($p < 0.05$)).

| | Minho River Estuary | Lima River Estuary | Douro River Estuary |
|---|---|---|---|
| **CF** | 1.06 (0.03)$^{a,b}$ | 0.998 (0.017)$^{a}$ | 1.15 (0.02)$^{b}$ |
| **HSI** | 1.6 (0.1)$^{a}$ | 0.71 (0.04)$^{b}$ | 1.4 (0.1)$^{a}$ |
| **GST** (nmol/min/mg protein) | 46 (4) | 48 (2) | 46 (2) |
| **CAT** (μmol/min/mg protein) | 16.6 (0.7)$^{a}$ | 34.1 (5.3)$^{b}$ | 14.6 (0.7)$^{a,b}$ |
| **GR** (nmol/min/mg protein) | 5.2 (0.4) | 6.5 (0.5) | 5.5 (0.3) |
| **GPx** (nmol/min/mg protein) | 3.4 (0.1)$^{a}$ | 4.5 (0.4)$^{a}$ | 6.6 (0.5)$^{b}$ |
| **SOD** (U/mg protein) | 4.2 (0.6)$^{a}$ | 13.1 (1.3)$^{b}$ | 2.6 (0.3)$^{a}$ |
| **LPO** (pmol MDA/mg protein) | 56 (18) | 38 (4) | 100 (41) |
| **PCO** (nmol C-Carbonil/mg protein) | 1.7 (0.3)$^{a}$ | 5.7 (0.9)$^{b}$ | 1.5 (0.4)$^{a}$ |
| **ChE** (nmol/min/mg protein) | 129 (16)$^{a}$ | 98 (14)$^{a,b}$ | 69 (9)$^{b}$ |

enzymes, and oxidative damage to cell macromolecules for flounder specimens are displayed in Table 5. Significantly lower values of CF and HSI were generally found for flounders from *Lima* River estuary in comparison to the other two River estuaries.

Analysing and comparing the data obtained for flounders from *Minho*, *Lima* and *Douro* River estuaries, considerable differences in several biochemical parameters were observed. Glutathione *S*-transferases activity was similar among the specimens collected at all three River estuaries. With regard to antioxidant defences CAT and SOD activity were significantly higher in flounders from *Lima* River estuary (34.1 ± 5.3 μmol/min/mg protein and 13.1 ± 1.3 U/mg protein, respectively) comparing to *Minho* and *Douro* River estuary. The activity of GPx augmented according to the following order: *Minho* River estuary < *Lima* River estuary < *Douro* River estuary (*Douro River estuary* show significantly higher levels than *Minho* and *Lima* estuaries). Conversely, no significant differences among estuaries were found for GR activity. In contrast to antioxidant enzymes, flounder specimens from *Minho* River estuary presented the highest activity level of ChE, which was significantly different from that measured in flounder from *Douro* River estuary. No significant differences were found for LPO levels, although the peroxidation of cellular membrane appeared to be lower in specimens from *Lima* River estuary and more pronounced in those from *Douro* River estuary. On the other hand, the oxidation of proteins was significantly higher in tissue of flounders collected at *Lima* River estuary, comparing to the other two River estuaries.

### 3.4. Principal component analysis

Two PCAs were performed for flounder combining biochemical parameters with either contamination levels in sediments or in fish tissue.

The analysis combining biomarker with sediment data showed that two components summarised 82% of total variability observed in the data. PCA interpretation was therefore based on these principal components (PC) (Fig. 2 A). PC1 (horizontal axis) was mainly related to PAHs, Cd and Pb contamination: correlations with the component ranged from 0.858 to 0.998. Zn and Cu were also highly associated to this component (0.964 and 0.977, respectively). Biomarkers associated to the component were related to neurotoxicity, antioxidant defences (GR and GPx) and oxidative stress. The axis established a gradient opposing *Minho* and *Lima* to *Douro* River estuary ($p < 0.001$). Sediments from *Douro* River estuary tended to present higher accumulation of PAHs on average and flounder specimens from this sampling site tended to show activation of GPx antioxidant activity. Hg ($r = 0.989$), Ni ($r = 0.974$) and chloroform ($r = 0.891$) were positively correlated with the PC2 (vertical axis) while toluene and dibenz(ah)anthracene was negatively correlated ($r = -0.915$ and $r = -0.955$, respectively). In addition, the activity of CAT ($r = 0.673$) and SOD ($r = 0.735$) and levels of PCO ($r = 0.728$) were correlated with the axis. PC2 further discriminated the upper northern estuaries, opposing *Lima* to *Minho* ($p < 0.001$). Flounders from *Minho* River estuary tended to exhibit higher ChE activity while those from *Lima* River estuary presented higher antioxidant activity (CAT and SOD) and PCO levels, as well as contamination by Hg, Ni and chloroform.

With regard to bioaccumulation of contaminants, in the PCA performed, two components expressed 84% of the total variability observed in the data, thus both were selected to interpret the PCA results (Fig. 2 B). Several HNS measured either in muscle or liver (e.g. m-xylene, p-xylene, o-xylene in both tissues, toluene and styrene in muscle and chlorobenzene in liver) showed significant high correlations with the axis (*r* values ranging from 0.733 to 0.987). The activity of CAT ($r = -0.689$) and SOD ($r = -0.833$), and PCO ($r = -0.789$) levels were negatively correlated with this axis, as well as, the levels of toluene ($r = -0.969$) and trichloroethane ($r = -0.991$) measured in liver, and tetrachloroethylene ($r = -0.996$) measured in muscle. Data indicates association among these contaminants and activity of CAT and SOD, and PCO levels. PC2 was significantly and positively correlated with styrene ($r = 0.953$) and trichloroethylene levels ($r = 0.953$) in liver, and to a lesser extent to ChE ($r = 0.510$). Negative correlations were also found between benzene (liver, $r = -0.996$, and muscle, $r = -0.879$), trichloroethylene (muscle, $r = -0.934$), 1,2 – dichloropropane (liver, $r = -0.934$) and GPx activity ($r = -0.724$) with PC2. The PCs established a clear gradient of sampling locations as in the PCA combining biomarker data and contaminants levels (Fig. 2 A and B).

## 4. Discussion

In this study, the multi-biomarker approach in conjunction to chemical characterisation of environmental compartments indicates that, in general, *Minho*, *Lima* and *Douro* River estuaries are not severely contaminated, although the occurrence of HNS in these ecosystems was confirmed.

A variety of HNS were found in flounder's tissue, liver and muscle. These organic chemicals can be lipophilic and thus concentrate mainly in lipid-containing organs (Sullivan and Krieger 2001; ATSDR 2007, 2010, 2014), such as the liver. Nevertheless, many of these organic HNS have low to moderate potential to bioconcentrate or bioaccumulate in aquatic organisms (ATSDR 2007; 2010; 2014; Rocha et al., 2016; Rocha et al., 2018). HNS were found in flounder's tissue at generally rather low levels, < 5 ng HNS/g $_{\text{wet tissue}}$, with exception of toluene in flounders from *Lima* River estuary and tetrachloroethylene in flounders from *Lima* and *Douro* River estuaries. Both *Lima* and *Douro* River estuaries are considerably impacted due not only to the harbour facilities but also to other anthropogenic activities which results in important inputs of pollutants. Among these are industrial and urban effluents, agriculture runoff and discharge of domestic sewage (Ferreira et al., 2004; Mucha et al., 2004; Ferreira et al., 2005; Gravato et al., 2008; PGBHa 2012; PGBHb 2012). *Minho* River estuary has, on the other hand, been regularly found as an area less impacted by human activities (Gravato et al., 2010; Guimarães et al., 2012; Rodrigues et al., 2014).

Toluene was found in tendentiously higher amounts in flounder's tissue from *Lima* River estuary. The quantification of this HNS was probably linked to the proximity of the estuary to a harbour facility. Toluene is a natural compound of crude oil. It also originates from the process of production of gasoline and other fuels from crude oil, as well

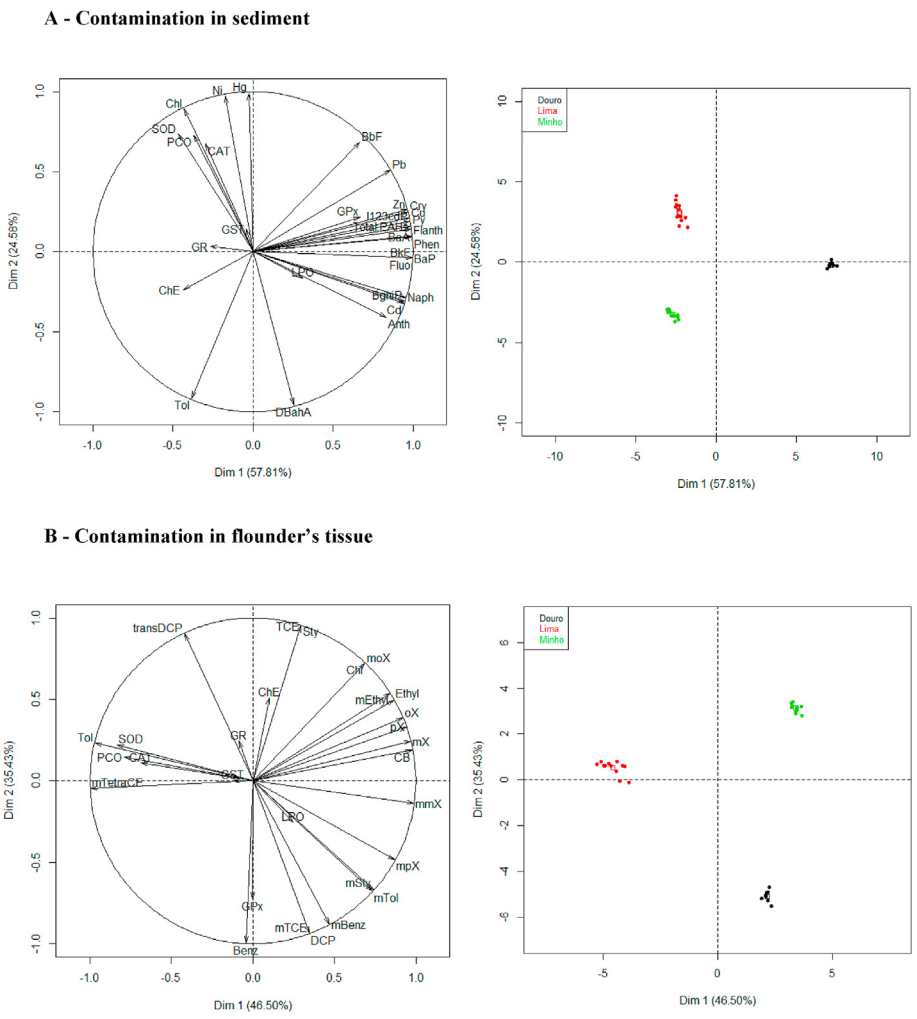


**Fig. 2.** Results of the Principal component analysis (PCA) combining biochemical parameters measured with contamination levels in estuarine sediments (A) and in flounder' tissue (B). Variable factor map (left) and Individuals factor map (right). Lipid peroxidation (LPO), proteins carbonyls (PCO), glutathione-s-transferase (GST), catalase (CAT), glutathione reductase (GR), glutathione peroxidise (GPx), superoxide dismutase (SOD) and acetylcholinesterase (AChE). Toluene (Tol), chloroform (Chl), Benzene (Benz), trichloroethylene (TCE), 1,2 – dichloropropane (DCP), trans-1,3-dichloropropene (transDCP), tetrachloroethylene (tetraCE), chlorobenzene (CE), ethylbenzene (Ethyl), m-xylene (mX), p-xylene (pX), styrene (Sty), o-xylene (oX), naphthalene (Naph), fluorene (Fluo), phenanthrene (Phen), anthracene (Anth), fluoranthene (Flanth), pyrene (Py), benz(a)anthracene (BaA), crysene (Cry), benz(b)fluoranthene (BbF), benz(k)fluoranthene (BkF), benz(a)pyrene (BaP), indene(1,2,3-cd)pyrene (I123cdP), dibenz(ah)anthracene (DBahA), benz(ghi)perylene (BghiP), polycyclic aromatic hydrocarbons (PAHs), zinc (Zn), copper (Cu), lead (Pb), nickel (Ni), cadmium (Cd) and mercury (Hg). For B (variable factor map), contaminant abbreviations initiated by the letter m refer to the level of contaminant found in flounder's muscle. The remaining contaminant abbreviations refer to the level of contaminant found in flounder's liver.

as, from the making of coke from coal. To improve octane ratings toluene, along with benzene and xylene, is also added to gasoline (ATSDR 2017). Apart from toluene, tetrachloroethylene accumulated in significantly higher amounts in flounder's muscle from *Douro* (194 ± 75 ng/g $_{\text{wet tissue}}$) and *Lima* (944 ± 277 ng/g $_{\text{wet tissue}}$) River estuaries than in those from *Minho*. With a density of 1.62 g/cm$^3$, when released in aquatic medium this HNS can sink, despite its high volatilisation (ATSDR 2014). It is therefore likely to be in contact with benthic organisms. Tetrachloroethylene is a commercially important chlorinated hydrocarbon commonly used as a dry cleaning agent, degreasing, as a heat-transfer medium and as a solvent for fats, greases, waxes, rubber, etc (ATSDR 2014). Bioaccumulation of tetrachloroethylene has been reported before in bluegill sunfish (*Lepomis macrochirus*) (Barrows et al., 1980), in rainbow trout (*Oncorhynchus mykiss*) (Neely et al., 1974), fathead minnows (*Pimephales promelas*) (Ahmad et al., 1984) and common dab (*Limanda limanda*), a marine fish (Pearson and McConnell 1975), in laboratory bioassays. Particularly, accumulation of tetrachloroethylene in fish muscle was verified for rainbow trout and common dab. Nevertheless, Pearson and McConnell (1975) reported that the bioconcentration of the HNS in the liver of *Limanda limanda* was two orders of magnitude greater than in muscle, which is in accordance with their lipophilic character. In addition, a more recent study (Wittlingerová et al., 2016) reported the accumulation of tetrachloroethylene in tissue of several fish species in a contaminated part of a Czech Republic river relative to fish from an unaffected area. The content of tetrachloroethylene found was considerably below that registered herein in flounder's muscle. The authors inferred that, although no linear dependence had been found, accumulation of tetrachloroethylene in fish tissue is indicative of contamination of the habitat, because after remedial action, there was a progressive decrease in the average concentration of the HNS in the fish muscle (Wittlingerová et al., 2016). The concentrations found herein in flounder's muscle are below the human-equivalent low observable adverse effect level (LOAEL) dose calculated from an 8-week study using rats (1800 ng/kg/day) (ATSDR 2014).

Flounder is a pleuronectiform species which lives generally covered by sand or mud and feeds from the bottom of coastal and estuarine areas. It is therefore particularly vulnerable to chemical pollution in the sea bottom. A few HNS were detected in the sediments collected from each estuary although at low levels. These chemicals have different physico-chemical characteristics, many of them being readily degradable, chemically and biologically (ITOPF 2011; Rocha et al., 2016). Moreover, HNS have low soil organic carbon – water partition coefficients (Poulson et al., 1997; WHO 2004; ATSDR 2014) and most of the studied HNS are floaters or dissolvers (ITOPF 2011; Rocha et al., 2016). Low levels of HNS were also registered for surface, medium and bottom waters from each estuary, with a higher variety and levels of HNS (<1000 ng/L) in bottom waters, probably due to resuspension of sediments (unpublished data). Other contaminants, as metals and PAHs, more frequently found in the aquatic environment were also determined in sediments. Levels found were considerably lower than the established effect-low range (ERL) estimated by (Long et al., 1995), except for Hg, in all three River estuaries. Previously reported metal concentrations found in these estuaries

were within the same order of magnitude as those registered in this study, except for Hg. For all River estuaries, Hg levels in sediments were higher than those published previously, e.g. [Hg] < 0.036 μg/g for *Minho*, *Lima* and *Douro* River estuaries (Guimarães et al., 2012) and [Hg] < 0.06 μg/g for *Minho* River estuary (Mil-Homens et al., 2013). In *Douro* River estuary, low levels of Hg were reported in sediments ranging from 0.06 to 0.18 μg/g, although suspended particulate Hg concentration could reach 6.5 μg/g (Ramalhosa et al., 2005). Mercury is a ubiquitous component of industrial and agricultural effluents, but can naturally occur in different regions (Lacerda and Malm 2008). Mercury is recognised as the most toxic metal causing harmful effects to microorganisms, plants and animals, including aquatic organisms (Boening 2000), and is capable of biomagnification in almost all food chains (Lacerda and Malm 2008). The Hg concentrations found are probably associated to human activities. Despite the relatively low Hg concentrations found in sediments from *Minho* River estuary, Mil-Homens et al. (2013) verified that Hg presented the highest enrichment factor relatively to Al, with higher values found in areas affected by diverse anthropogenic activities or with favourable environmental conditions for Hg accumulation. As expected, PAHs levels were higher in sediments from *Lima* and *Douro* River estuary than from *Minho* River estuary. Levels and variety of PAHs previously reported by other authors for all three River estuaries (Guimarães et al., 2012; Capela et al., 2016; Gonçalves et al., 2016) were generally lower than those measured in this study. However, sampling locations varied among studies so a direct and accurate comparison of results is not possible, except for sampling point D4, in *Douro* River estuary. This sampling site and S3 of Rocha et al. (2011) are located within the same river section. Comparing both data, sediments analysed in this study showed higher concentrations of phenantrene, anthracene, fluoranthene, pyrene and benz(a)anthracene than those sampled in 2009 (Rocha et al., 2011). Nevertheless, the levels of higher molecular weight PAHs (chrysene, benz(b)fluoranthene, benz(k)fluoranthene, benz(a)pyrene, indene(1,2,3-cd)pyrene, dibenz(ah)anthracene and benz(ghi)perylene) diminished significantly along these last years, as well as, naphthalene and fluorene levels (Rocha et al., 2011). In estuarine areas, anthropogenic activities are the main sources of PAHs: in fact, this sampling point (D4) is located near a recreational marina and the decline of high molecular weight PAHs is in line with the petrogenic nature of the PAHs found (Souza et al., 2015). The degree of sediment contamination can be evaluated based on the 16 priority PAHs. According to the classification currently in use (Souza et al., 2015), which is supported on more limitative parameters than those set in Long et al. (1995), the sediments of all three River estuaries are identified as weakly contaminated (Σ16 PAHs concentration below 250 ng/g). The only exception is D3 in *Douro* River estuary, with PAHs concentration above 500 ng/g, and therefore considered as highly contaminated. This site is located within the old town centre, a very touristic area which concentrates a variety of commercial and catering establishments and is subjected to intense traffic of both cars and tourist tour boats, which might explain the higher accumulation of pollutants in this area.

Evidence that flounder can accumulate contaminants (e.g. (Vinagre et al., 2004; Kopecka et al., 2006)) in its tissue can be found in the literature. Nevertheless, for HNS, knowledge is still scarce and possible and relevant bioindicators of HNS presence in aquatic ecosystems have not yet been identified. The ability to bioaccumulate contaminants is an important criterion for selecting aquatic bioindicators, nevertheless, fish also can efficiently metabolise and excrete contaminants (Reynolds and Feist, 2003; Dupuy and Galland, 2014). Thus, integrative approaches are imperative for an accurate classification. Herein, the combination of chemical and biochemical analyses revealed significant differences among River estuaries and the health status of flounder clearly indicated the anthropogenic pressure exerted on its habitat. Multi-biomarker approaches combining parameters related to the mode of action of toxicants and involved in vital physiological functions have in fact produced valuable information and effective evaluations (Guimarães et al., 2012; Rodrigues et al., 2014), and are recommended for use within the scope of the Marine Strategy Framework Directive in integrated chemical and biological effects monitoring programmes aiming at assessing the good environmental status (GES). Biomarkers of neurotransmission, antioxidant responses and indicative of oxidative stress were chosen in resemblance to previous studies (Ferreira et al., 2004; Guimarães et al., 2009; Capela et al., 2016) for comparative temporal analysis. PCA analysis highlighted such differences so that when combining data from chemical monitoring and the biomarker approach a clear gradient was established either when considering sediment or tissue contamination together with the biomarkers. In fact, both analyses discriminated *Minho*, *Lima* and *Douro* River estuaries. Data herein presented indicate flounder as a suitable bioindicator of HNS contamination.

GPx is an antioxidant enzyme responsible for the conversion of hydrogen peroxide ($H_2O_2$), formed during cellular metabolism, into water and oxygen (Mesquita et al., 2011). LPO is an oxidative damage biomarker related to the oxidation of polyunsaturated fatty acids caused by reactive oxygen species (ROS) generated during the detoxification processes and which leads to the destruction of the lipid structure of cellular membranes and later to cell death (Lima and Abdalla 2001; Mesquita et al., 2011). Both GPx and LPO augmented from the least to the most impacted River estuaries (*Minho* < *Lima* < *Douro* River estuary) as indicated by the PCA analysis. The increase of anthropogenic pressure inhibited, on the other hand, the activity of ChE in flounders, an enzyme involved in the cholinergic neurotransmission which catalyses the hydrolysis of choline esters (Mesquita et al., 2011). A clear opposition of ChE regarding *Lima* and *Douro* River estuaries is evinced in PCA analysis. The inhibition of ChE has been frequently used as a biomarker of exposure to anticholinesterasic agents, such as pesticides, some PAHs and metals (Napierska et al., 2009; Kirby and Law 2010), and can affect locomotion and ventilation, influencing nourishing ability and escape to predators.

Moreover, the PCA seems to indicate that tetrachloroethylene and toluene levels in fish tissue could be one factor affecting CAT and SOD activities, and PCO levels. A significant SOD and CAT induction has in fact been observed in previous field surveys (van der Oost et al., 2003). Catalase and SOD are antioxidant enzymes: CAT acts on the conversion of $H_2O_2$ into water and oxygen and SOD alternately catalyses the dismutation (or partitioning) of the superoxide radical ($O_2^{\cdot-}$) into either molecular oxygen or $H_2O_2$. If not regulated through the enzymatic antioxidant defences both $O_2^{\cdot-}$ and $H_2O_2$ can cause many types of cell damage (Abele et al., 2011). The increased levels of CAT and SOD activities probably contributed to minimise oxidative damage to cell membranes. Despite the more active enzymatic activity, higher levels of PCO, a measure of protein carbonylation, were observed. Toxicity of HNS towards aquatic organisms is still little understood, especially at biochemical level. Generally, most works found relied on organisms' mortality as tested endpoint as reviewed in (Rocha et al., 2016). Sublethal effects, particularly on enzymatic activity, of acrylonitrile and xylene towards European seabrass and amphipod *Gammarus locusta* have been studied (Neuparth et al. 2013, 2014). No more works have been found on this subject despite increased HNS transportation and probability to occur in nature.

Taking into consideration data from previous years, an important amelioration of the environmental state of River estuaries was denoted. Flounders from *Minho* River estuary used herein presented significantly higher activity of phase I and phase II biotransformation enzymes, GST and 7-ethoxy-resorufin-*O*-deethylase (EROD), and significantly lower activity of SOD and LPO levels in comparison to a monitoring program performed in the river in 2012 (Capela et al., 2016). In *Douro* River estuary, the differences were remarkable for CAT and SOD activity, as well as, LPO levels, when comparing to those reported in 2001 and 2002 (Ferreira et al., 2005), with clearly lower values registered nowadays. In fact, there was a severe decrease of several orders of magnitude in CAT activity (2001: CAT activity – 11 900 ± 1800 μmol/min/mg protein

(Ferreira et al., 2005); 2014: CAT activity 14.6 ± 0.7 μmol/min/mg protein). Reduced activity of antioxidant enzymes and low contents of lipid peroxidation are associated to low or non-stressful conditions (van der Oost et al., 2003).

Over the last decades, there has been an increasing concern about water quality and an effort from European countries to apply the Water Framework Directive and Marine Strategy Framework Directive to achieve good environmental status. Thus, stricter laws concerning water quality for human consumption and agricultural and industrial use, and the release of effluents from industrial, rural and urban wastewater treatment facilities have been therefore elaborated. Furthermore, the construction of urban wastewater treatment plants, especially in the case of *Douro* River, and implementation of several national, regional and local actions may have also contributed to reduce the inputs of nutrients and contaminants from inland and therefore explain the reduction of stress in flounders. Our results indeed evince this improvement in water quality.

## 5. Conclusion

A chemical assessment in conjunction with a biomarker approach showed to be a suitable tool for monitoring contamination by HNS. A clear distinction among sampling sites in terms of impact and degree of anthropogenic pressure was found. The dissimilarity among sampling areas was clearly evinced, with *Douro* River estuary arising as the most impacted area, in line with historical data available for these NW Iberian areas. Flounder was shown to be a suitable bioindicator of HNS contamination. Chemical assessment of contamination is no longer sufficient to environmentally characterise the ecological status of aquatic ecosystems. Data herein highlight therefore the urge to lean on integrative approaches for a more accurate assessment of environmental status.

Both chemical monitoring and biomarker analysis showed that there has been a considerable amelioration of the environmental state of each River estuary, with a decrease on the levels of contaminants in environmental matrices comparing to previous years. Coastal and river management activities implemented over the last decade may explain this amelioration. Nevertheless, contamination by toluene, Hg and PAHs in *Douro* River estuary, and tetrachloroethylene in *Lima* River estuary, deserves further attention and future monitoring for assessing the evolution of sediment contamination and identifying the origin of these enrichments, information that is important for supporting the elaboration of further mitigation measures. Overall, despite the location near crucial maritime routes for transportation of HNS, the diversity and levels of these contaminants detected in sediments and fish tissues from the study estuaries were moderate to low.

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.indic.2020.100098.

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