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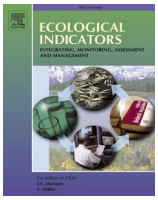

Assessing contamination from maritime trade and transportation on Iberian waters: Impact on *Mytilus* sp

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ABSTRACT

Contamination derived from maritime transportation is not only linked to petroleum-based substances but also to transportation of other substances such as hazardous and noxious nature (HNS). Over the last decade, the maritime trade of HNS has been augmenting. However, levels of HNS in aquatic environment are still quite unknown. So, an integrative study, combining a chemical and a multi-biomarker evaluation, was done in the NW Atlantic Iberian coast where specimens of *Mytilus sp*. were collected. Ten HNS were found in the digestive gland of mussels from *Vila Chã* and *Foz*, though at generally low levels. Only measurable levels of benzene, trichloroethylene and 1,1,2-trichlorethane were found in other locations. Principal component analysis produced a clear distinction between *Vila Chã* and *Foz* and the remaining sampling sites. Biomarkers associated to contamination were the levels of lipid peroxidation (LPO) and activity of glutathione reductase (GR), superoxide dismutase (SOD) and glutathione peroxidase (GPx) enzymes. Correlations between biomarkers and HNS levels highlight the importance of performing biomonitoring campaigns integrating chemical analysis and a biomarker approach to accurately assess the environmental state of aquatic environments.

1. Introduction

Integrated risk assessment is a valuable approach for understanding the ecotoxicological effects of contaminants, offering a balanced view and more realistic results. Used as early warning signals, biomarkers provide a diagnostic assessment of an organism exposure to pollutants before visible adverse effects are observed (Abrahamson, 2007). Studies assessing biomarkers have been widely used both in vertebrates and invertebrates for environmental biomonitoring, with different biomarkers having been used to survey the toxicity of contaminants towards aquatic organisms (Garrigues et al., 2001; Bainy and Marques, 2003). The use of comprehensive approaches, comprising biomarkers, is recommended in the frame of the Marine Strategy Framework Directive in monitoring programmes with the purpose of evaluating the *Good Environmental Status* (GES) of aquatic ecosystems (Davies and Vethaak, 2012). Such integrative procedures will improve the quality of environmental measurements and risk assessment studies, which data will support the management and formulation of protection policies.

Marine contamination and toxicity of Hazardous and Noxious Substances (HNS) towards aquatic organisms is still little understood (Rocha et al., 2016) and far behind from the current knowledge about polycyclic aromatic hydrocarbons (PAHs) (Almeida et al., 2012; Vieira and Guilhermino, 2012; Rodrigues et al., 2013; Abdel-Shafy and Mansour, 2016). Nevertheless, HNS (in particular organic compounds) can induce several changes on aquatic organisms, such as biochemical, histopathological, reproductive, behavioural, depending on the concentration and period of exposure (Rocha et al., 2016). These substances have different physico-chemical characteristics and, once released into the

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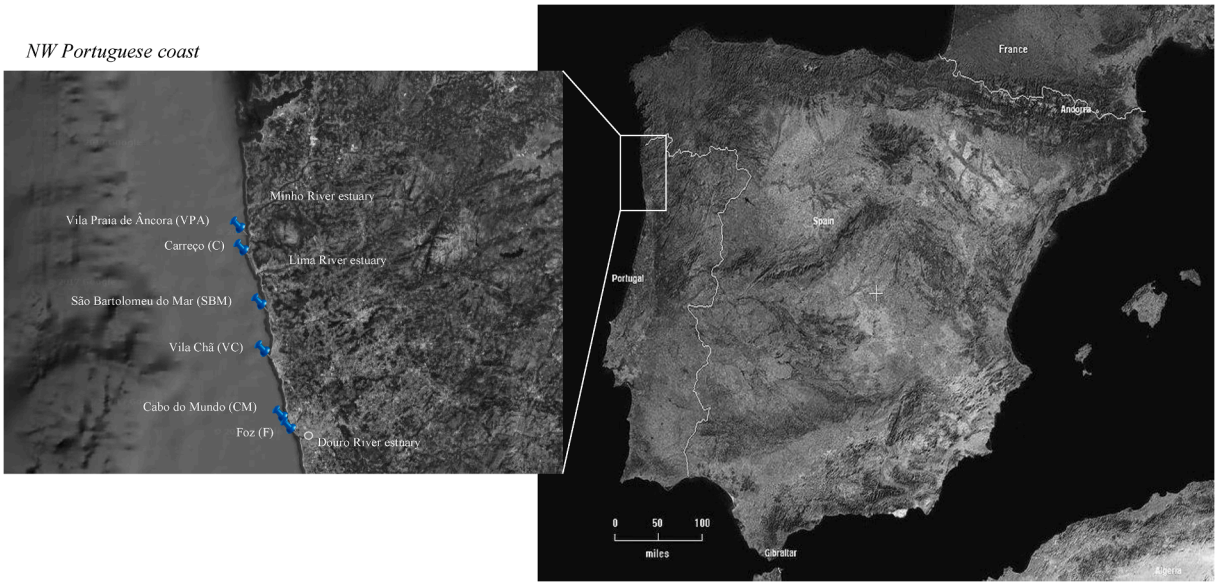

Fig. 1. Location of the sampling sites: specimens of mussels, water samples were collected in six beaches along the Northwest Iberian coast (sampling campaigns carried out in the spring of 2014).

aquatic environment, can behave differently (ITOPF, 2011; Neuparth et al., 2012). The prediction of HNS behaviour and consequences of a spill are therefore difficult, as well as, the preparation of appropriate emergency and contingency plans. Despite the increasing transportation and handling of these substances, little is known about the presence of HNS in different environmental matrices worldwide and their impact on ecosystems.

With that in mind, this work aimed at assessing the occurrence of HNS over the north-western (NW) Atlantic Iberian coast and evaluating the potential consequences on aquatic organisms, complementing the study performed in NW Iberian River estuaries, where flounder *Platichthys flesus* was used. The studied area comprises two important harbour facilities: *Viana do Castelo* harbour (trade of around 500 000 tonnes per year of goods (AMT, 2015)); and *Leixões* harbour (trade of more than 18 million tonnes of cargo, 43.3% of which regards liquid cargo (Carvalho, 2015)), located within the convergence of important worldwide maritime routes (Carvalho, 2015). Due to its exposed geographical location, this area is susceptible to accidental spills of toxic chemical substances transported by cargo ships. Actually, since 1970, more than 25 incidents (Nunes, 2003; Jorge, 2011) have been reported in or near the Iberian coast, such as *Jacob Maersk* (1975), *Reijin* (1980), *Cercal* (1994), *Carol Bulker* (2000) and *Prestige* (2002) (Jorge, 2011), which led to the spill of crude oil, fuel and lubricants into the sea. Maritime transportation of HNS has been increasing over the years (around 165 million tonnes of bulk global trade per year) (MKC, 2012). In the European Union, 2 000 HNS are transported by sea regularly (Purnell, 2009; Harold et al., 2014). One should be aware that HNS spills can affect several areas, as rivers and estuarine areas and coastal zones, including coastal beaches. In areas of the NW Iberian coast, located in the maritime route of a lot of ships transporting different kinds of chemical compounds, the risk of negative effects of HNS is considerable. In fact, measurable levels of chloroform and tetrachloroethylene were detected in sediments (Gouveia et al., 2018), and tetrachloroethylene in water (Rocha et al., 2019), sampled along this coastal area. Nevertheless, no information about the accumulation of such contaminants in aquatic organisms was found, as well as, regarding their possible repercussions on the organisms' health. In this ambit, an integrative approach, combining a chemical and a multi-biomarker characterisation, was carried out in six different locations along the coast between *Minho* and *Douro* Rivers. For this work, a sentinel species was chosen as biological matrix, namely, *Mytilus* sp. This bivalve mollusc, used as an indicator organism in a myriad of monitoring surveys, is widely distributed and present a sedentary lifestyle and tolerance to a large range of environmental conditions. In addition, it is a filter-feeder with very low metabolism, so it can bioaccumulate contaminants in its tissues (Lima et al., 2007). *Mytilus* sp. is widely used in marine contamination and ecotoxicology studies and is the bioindicator of choice in environmental monitoring programmes, with many studies assessing effects at molecular and cellular level (Moreira and Guilhermino, 2005; Kopecka et al., 2006; Lima et al., 2007; Giarratano et al., 2011). Nevertheless, to our knowledge, no studies with HNS are available. Multivariate analysis comparing chemical and biochemical data was performed to identify response profiles potentially triggered by contamination.

Table 1
GPS coordinates of the sampling sites in which specimens of mussels (along the Northwest Portuguese coast: *Vila Praia de Âncora, Carreço, São Bartolomeu do mar, Vila Chã, Cabo do Mundo and Foz*) were collected in spring of 2014.

Sampling Location	Tide*	GPS coordinates
Vila Praia de Âncora (VPN)	LT	41° 49.375′ N, 8° 52.466′ W
Carreço (C)	LT	41° 44.537′ N, 8° 52.651′ W
São Bartolomeu do Mar (SBM)	LT	41° 34.431′ N, 8° 47.9296′ W
Vila Chã (VC)	LT	41° 17.902′ N, 8° 44.257′ W
Cabo do Mundo (CM)	LT	41° 13.221′ N, 8° 42.898′ W
Foz (F)	LT	41° 9.117′ N, 8° 40.706′ W

* Low tide (LT) and high tide (HT).

2. Material and methods

2.1. Study area and sampling procedures

A sampling campaign was carried out in June 2014, at low tide, in six beaches along the NW Atlantic Iberian coast: *Vila Praia de Âncora*, *Carreço*, *São Bartolomeu do Mar*, *Vila Chã*, *Cabo do Mundo* and *Foz* (Fig. 1 and Table 1). The study area is located between *Minho* River and *Douro* River, presenting different patterns of urban and industrial activity.

This littoral area presents high population density and oil refining industry and two maritime harbours (located at *Leixões* and *Viana do Castelo*). Metals and PAHs have been previously reported, at moderate concentrations, in sediments (Mucha et al., 2004; Rocha et al., 2011; Guimarães et al., 2012; Mil-Homens et al., 2013; Reis et al., 2013).

Table 2
Levels of hazard and noxious substances (HNS), polycyclic aromatic pollutants (PAHs) and metals measured in *Mytilus* sp.'s tissue collected from marine beaches along the northern Iberian coast between *Minho* and *Douro* River estuaries (six sampling beaches: *Vila Praia de Âncora*, *Carreço*, *São Bartolomeu*, *Vila Chã*, *Cabo do Mundo* and *Foz*). Values represent average concentrations of 3 replicates (standard deviation between brackets).

	Sampling Site					
	Vila Praia de Âncora	Carreço	São Bartolomeu do Mar	Vila Chã	Cabo do Mundo	Foz
HNS (ng/g)						
Benzene	24 (2)	27 (7)	30 (4)	40 (5)	15.1 (0.8)	20 (3)
Trichloroethylene	1.8 (0.6)	<0.03*	<0.03*	<0.03*	<0.03*	<0.03*
Toluene	<0.01*	<0.01*	<0.01*	0.5 (0.1)	<0.01*	0.7 (0.1)
1,1,2-Trichloroethane	0.20 (0.03)	0.22 (0.01)	0.26 (0.02)	0.14 (0.01)	0.24 (0.01)	0.10 (0.01)
Ethylbenzene	<0.01*	<0.01*	<0.01*	0.56 (0.08)	<0.01*	0.57 (0.02)
m-Xylene	<0.01*	<0.01*	<0.01*	2.4 (0.3)	<0.01*	2.1 (0.1)
p-Xylene	<0.01*	<0.01*	<0.01*	0.42 (0.08)	<0.01*	0.365 (0.006)
Styrene	<0.01*	<0.01*	<0.01*	2.1 (0.3)	<0.01*	3.3 (0.3)
o-Xylene	<0.01*	<0.01*	<0.01*	1.4 (0.2)	<0.01*	1.2 (0.1)
1,4-Dichlorobenzene	<0.01*	<0.01*	<0.01*	0.023 (0.002)	<0.01*	0.032 (0.001)
PAHs (ng/g)						
Naphthalene	8 (1)	8.0 (0.4)	9 (1)	8.2 (0.7)	9 (1)	11.0 (0.9)
Acenaphthylene	12 (1)	12 (4)	15 (5)	11 (1)	46 (26)	<1.1*
Fluorene	1.8 (0.6)	<0.7*	<0.7*	5.4 (0.3)	2.2 (0.4)	3.7 (0.2)
Phenanthrene	5.7 (0.9)	5.8 (0.9)	6.0 (0.7)	11.1 (0.7)	7 (1)	12 (1)
Anthracene	<0.3*	<0.3*	<0.3*	1.3 (0.5)	<0.3*	0.9 (0.1)
Fluoranthene	4.8 (0.6)	2.2 (0.6)	2.0 (0.2)	2.4 (0.8)	3.0 (0.3)	3.2 (0.3)
Pyrene	2.6 (0.3)	1.4 (0.3)	1.7 (0.2)	1.6 (0.3)	1.9 (0.2)	3.6 (0.4)
Total PAHs	34 (3)	30 (4)	34 (5)	41 (3)	69 (25)	35 (1)
Metals (μg/g)						
Zinc	261 (88)	287 (29)	439 (65)	360 (16)	307 (24)	342 (17)
Copper	7 (2)	6 (3)	8 (1)	5.4 (0.4)	4 (2)	6 (1)
Lead	4 (1)	4 (1)	2.3 (0.8)	4.4 (0.2)	4.8 (0.5)	5.2 (0.2)
Nickel	1.7 (0.4)	1.2 (0.2)	1.9 (0.3)	1.3 (0.3)	0.7 (0.2)	1.2 (0.2)
Cadmium	0.38 (0.08)	0.43 (0.07)	0.53 (0.05)	0.28 (0.09)	0.16 (0.04)	0.15 (0.03)
Mercury	<0.16*	0.4 (0.1)	0.28 (0.02)	<0.16*	<0.16*	<0.16*

HNS as 2-Propenenitrile (149 ng/g), 1,1-Dichloroethane (0.1 ng/g), Chloroform (0.02 ng/g), 1,1,1-Trichloroethane (0.03 ng/g), 1,2-Dichloroethane (0.09 ng/g), Carbon Tetrachloride (0.02 ng/g), 1,2-Dichloropropane (0.09 ng/g), *cis*-1,3-Dichloropropene (0.01 ng/g), *trans*-1,3-Dichloropropene (0.01 ng/g), Tetrachloroethylene (6 ng/g), Chlorobenzene (0.01 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) were below the limit of detection (indicated in brackets).
PAHs as Acenaphthene (1.4 μg/g), Benz(a)anthracene (0.2 μg/g), Crysene (0.2 μg/g), Benz(b)fluoranthene (1.5 μg/g), Benz(k)fluoranthene (1.0 μg/g), Benz(a)pyrene (1.0 μg/g), Indene(1,2,3-cd)pyrene (6.0 μg/g), Dibenz(ah)anthracene (1.9 μg/g), Benz(ghi)perylene (0.4 μg/g) were below the limit of detection (indicated in brackets).
* Limit of detection.

Nevertheless, rocky shore beaches, such as *Carreço* and *Vila Chã*, have been used as reference sites since they are less susceptible to human influence and present low levels of contamination (Cairrão et al., 2004; Rodrigues et al., 2013). This part of the NW Atlantic coast is located near important maritime traffic routes (CNADS, 2001), so accidents and spills of chemical substances can also occur as, for example, the *Prestige* oil spill in 2002 (CNADS, 2001, Cairrão et al., 2004).

Water was collected in every sampling point, at low tide, to glass containers and transported to the laboratory in cooled portable freezers. In the laboratory, water was filtered either through 0.45 μm porosity acetate cellulose filters (Millipore) for metal analysis or 1.2 μm porosity glass fibre filters for PAHs. All were afterwards frozen at – 20 °C until analysis.

Twenty specimens of mussel were collected from each beach and transported to the laboratory in cooled portable coolers. At the laboratory, mussels' tissues were removed after a 24-hour depuration period. Digestive gland and adductor muscle were removed and frozen in liquid nitrogen and kept at – 80 °C until they were assayed.

2.2. Chemical analysis

For HNS determination, five replicate measurements were performed from composite samples of tissue of mussel. The tissue was homogenised with an Ultra-turrax blender (Ika), then 1 g was added to 10 mL of deionised water. Headspace solid phase microextraction (SPME) was performed using an autosampler CombiPal model (CTC Analytics) fitted with a 65 μm thickness of polydimethylsiloxane-divinylbenzene (PDMS-DVB, polar) fibre from Supelco. HNS, such as 1,1-dichloroethane, 2-Propenenitrile, chloroform, 1,2-dichloroethane, 1,1,1-trichloroethane, carbon tetrachloride, benzene, 1,2-dichloropropane, trichloroethylene, toluene, *cis*-1,3-dichloropropene, *trans*-1,3-dichloropropene, 1,1,2-trichloroethane, chlorobenzene,tetrachloroethylene, m-xylene, p-xylene, ethylbenzene, styrene, styrene, o-xylene, butylacrylate, 1,4-dichlorobenzene, 1,2-dichlorobenzene, 1,3-dichlorobenzene, and cyclohexylbenzene, were quantified using a Varian Saturn 2000 mass spectrometer (Walnut Creek, CA) coupled to a Varian 3900 gas chromatograph. Analytical details are described in Rocha et al. (2019).

For PAHs determination, freeze-dried tissue (0.25 g) was extracted with 1 mL of ethyl acetate and 250 μL of deionised water and 50 μL of PAHs deuterated internal standard solution (20 mg/L) was added. After extraction and clean-up, 100 μL of isooctane was added and the extract was evaporated with N_2 until dryness and re-suspended in 500 μL acetonitrile. The analysis of PAHs, such as acenaphthene, naphthalene, fluorene, acenaphthylene, anthracene, pyrene, phenanthrene, fluoranthene, benz(a)anthracene, benz(ghi)perylene, benz(b)fluoranthene, crysene, dibenz(ah)anthracene, benz(a)pyrene, benz(k)fluoranthene and indene(1,2,3-cd)pyrene, was performed using a Varian Saturn 2000 mass spectrometer (Walnut Creek, CA) coupled to a Varian 3900 gas chromatograph (Rocha et al., 2019). Further details regarding the extraction method and analytical GC parameters are also described in (Rocha et al., 2019).

For metal determination (Zn, Cu, Pb, Ni, Cd and Hg), water samples were preconcentrated in a metal chelating resin (Chelex-100). Metals were then eluted with nitric acid and measured through atomic

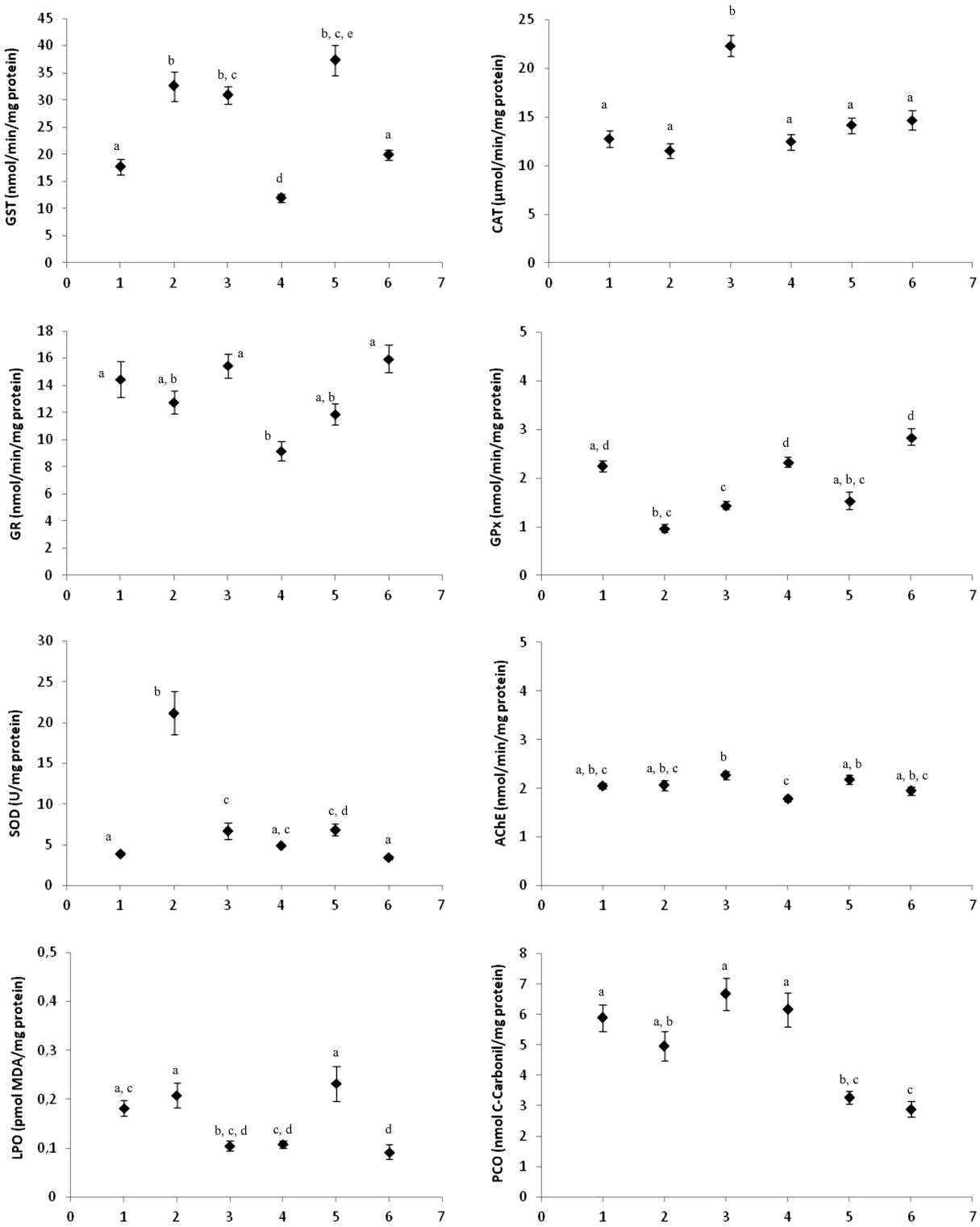

Fig. 2. Levels of lipid peroxidation (LPO) and proteins carbonyls (PCO), activities of biotransformation (glutathione-s-transferase (GST)) and antioxidant enzymes (catalase (CAT), glutathione reductase (GR), glutathione peroxidise (GPx) and superoxide dismutase (SOD)) determined in digestive gland of *Mytilus sp*. collected in spring from six beaches of NW Iberian coast (*Vila Praia de Âncora* (1), *Carreço* (2), *São Bartolomeu do Mar* (3), *Vila Chã* (4), *Cabo do Mundo* (5) and *Foz* (6), n = 20), as well as, the activity of a neurologic enzyme (acetylcholinesterase (AChE)) in adductor muscle of the same organism (error bars represented standard error (SE); different letter indicates statistical significance among data ($p < 0.05$)).

absorption spectrophotometry with flame or electrothermal atomization (depending on the levels of the metal). More details are described in (Reis et al., 2013).

2.3. Biochemical analysis

Biomarkers were determined using established methods as described previously in Semedo et al. (2014) and Abreu et al. (2018): i) glutathione-s-transferase (GST), superoxide dismutase (SOD), catalase

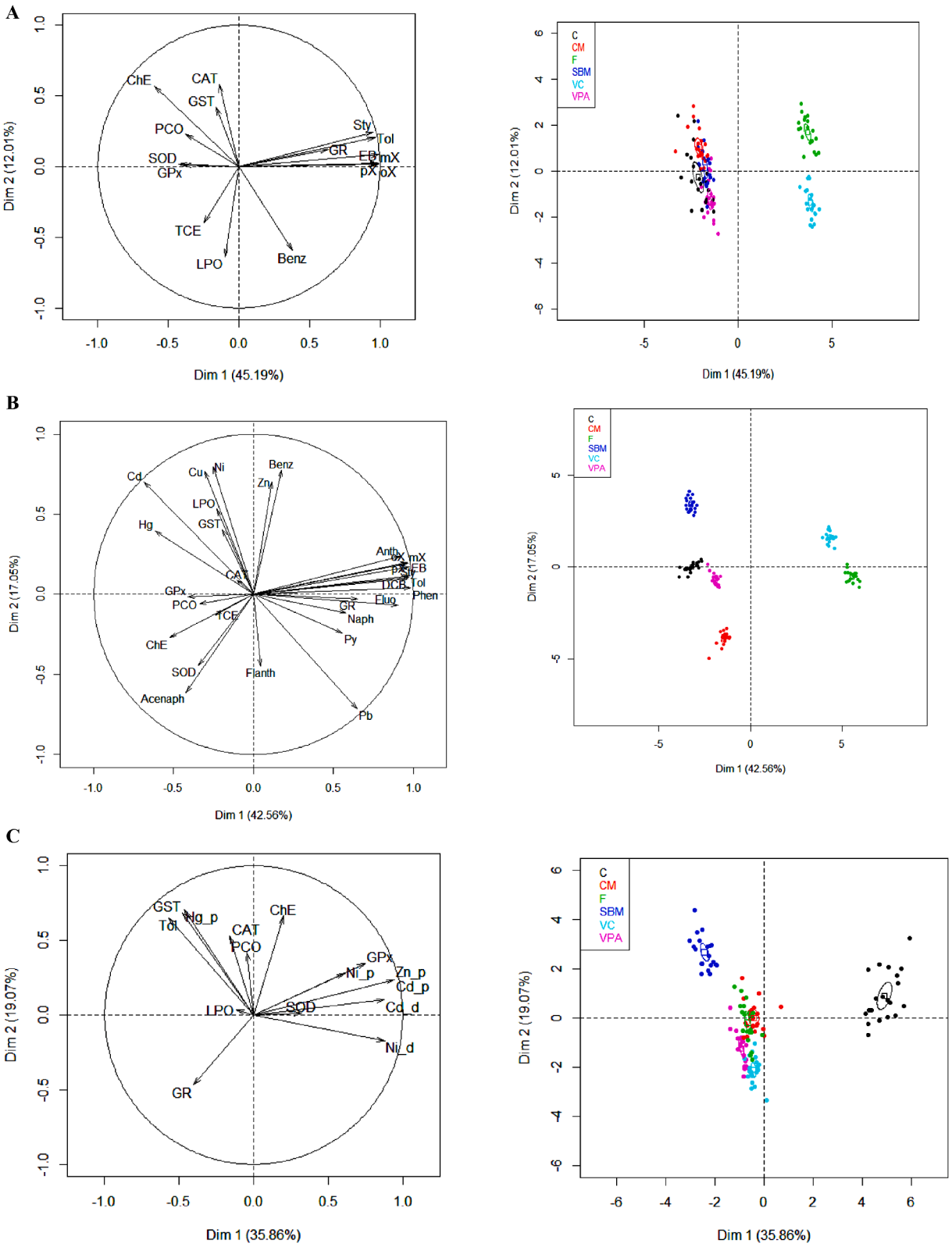

Fig. 3. Principal component analysis (PCA) combining (A) biochemical parameters measured with hazardous and noxious substances (HNS) levels in mussels' tissue, (B) biochemical parameters measured with HNS, PAHs and metals levels in mussel's tissue, and (C) biochemical parameters measured with contaminants levels in water (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 peroxidase (GPx), superoxide dismutase (SOD) and acetylcholinesterase (AChE) Benzene (Benz), trichloroethylene (TCE), toluene (Tol), ethylbenzene (EB), m-xylene (mX), p-xylene (pX), styrene (Sty), o-xylene (oX), 1,4-Dichlorobenzene (1,4 – DCB), Naphthalene (Naph), acenaphthylene (Acenaph), fluorene (Fluo), phenanthrene (Phen), anthracene (Anth), fluoranthene (Flanth), pyrene (Py), zinc (Zn), copper (Cu), lead (Pb), nickel (Ni), cadmium (Cd) and mercury (Hg) *Vila Praia de Âncora* (VPA), *Carreço* (C), *São Bartolomeu do Mar* (SBM), *Vila Chã* (VC), *Cabo do Mundo* (CM) and *Foz* (F).

(CAT), glutathione peroxidase (GPx) and glutathione reductase (GR) activities as antioxidant defences; ii) acetylcholinesterase (AChE) activity for neurotoxicity; and iii) peroxidative damage to lipids (LPO) and oxidative damage to proteins (PCO) as measures of oxidative stress.

2.4. Statistical analysis

Data normality was first checked for each biomarker with Shapiro-Wilk test. Since normal distribution was not met, data were log or square root-transformed and normality was rechecked. Differences among sampling locations 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, GR, GPx and AChE). All statistical analyses were carried out in Graph Prism 5.

Principal Component Analysis (PCA), carried out in FactoMineR, was performed to assess the relationship between biomarker patterns of response and contamination. Contaminants concentrations and the studied biomarkers were used as quantitative variables. Sampling sites were considered as a qualitative variable and included as supplementary factor. Only Principal Components (PC) with eigen values greater than 1 were accepted. Correlations between the variables and the PCs obtained were analysed and used to interpret PCA results. Significant differences were accepted for $p < 0.05$.

3. Results

3.1. Levels of contaminants in water

Table S1 exhibits the levels of HNS, PAHs and metals measured in water samples collected at six beaches along the NW Iberian coast (Fig. 1). The samples generally showed low levels of HNS, only toluene being determined in *São Bartolomeu do Mar*, *Cabo do Mundo* and *Foz*. None of the 16 EPA's priority PAHs were detected (Table S1) in these samples. With regard to metals, particulate and dissolved metal levels were considerable low in all coastal samples.

3.2. Levels of contaminants in tissues

Low levels of benzene and 1,1,2-trichloroethane were detected in the tissues of mussels collected at all selected beaches. Furthermore, mussels' tissues from *Vila Chã* and *Foz* also presented measurable concentrations of several other HNS, namely, toluene, ethylbenzene, m-xylene, p-xylene, styrene, o-xylene and 1,4-dichlorobenzene. Trichloroethylene was only detected in mussels from *Vila Praia de Âncora* at considerable low levels (Table 2). Regarding PAHs, measurable levels of naphthalene, acenaphthylene, fluorene, phenanthrene, anthracene, fluoranthene and pyrene were generally determined in mussels from all beaches. Total PAHs were higher in samples from *Cabo do Mundo*, probably due to the proximity to an oil refinery plant. Zinc, Cu, Cd, Pb and Ni were found in mussels' tissues from all sites whereas Hg was only detected in mussels from *Carreço* and *São Bartolomeu do Mar*.

3.3. Enzymatic activity and parameters related to oxidative stress

Fig. 2 exhibits data regarding the biomarkers quantified in mussels' tissues from six beaches located along the NW Iberian coast. Statistical differences among mussel specimens collected at the selected beaches were found in most cases. In particular, the activity of GST was significantly more pronounced in mussels from *Carreço*, *São Bartolomeu do Mar* and *Cabo do Mundo* in comparison to those from the remaining beaches, with mussels from *Vila Chã* registering the significantly lowest level of activity. In resemblance to GST, GR activity was, in most cases, significantly lower in mussels from *Vila Chã* (Fig. 2). Higher GPx activity was registered in mussels from *Vila Praia de Âncora*, *Vila Chã* and *Foz*, the lowest and highest levels being usually registered for *Carreço* and *Foz*. The activity of enzymes CAT and SOD were generally statistically similar among beaches apart from *São Bartolomeu do Mar* and *Carreço*, respectively, which presented significantly higher activities in comparison ($p < 0.001$, Fig. 2). The activity of the neurotoxicity enzyme, AChE, did not differ significantly among mussels from most of the selected sampling sites; statistical differences were found between *São Bartolomeu do Mar* and *Vila Chã*, as well as between *Cabo do Mundo* and *Vila Chã*. With regard to oxidative stress biomarkers, LPO levels showed significant differences between *Foz* (the lowest level) and *Vila Praia de Âncora*, *Carreço* and *Cabo do Mundo*. For PCO levels, mussels from *Cabo do Mundo* and *Foz* presented significantly lower rates of protein oxidation ($p < 0.01$) (Fig. 2).

3.4. Principal component analysis

Principal component analysis was performed for mussels combining biomarker responses with tissue accumulation of either HNS (PCA1, Fig. 3 A) or all contaminants (PCA2, Fig. 3 B), as well as biomarker responses with water contamination (PCA3, Fig. 3 C).

In PCA1, four components expressing 77% of the total variability observed in the data were retained. The first two principal components (PC) summarised 57% of the total inertia so these were used for PCA interpretation (Fig. 3 A). The principal component in the horizontal axis (PC1) was mainly linked to HNS contamination; r values ranging between 0.965 and 0.989. Biomarkers associated to this component were mainly related to antioxidant defences (GR ($r = 0.6296$) and neurotoxicity (ChE, $r = -0.6015$). The axis established a gradient opposing *Vila Chã* and *Foz* to the remaining sampling sites ($p < 0.001$). Mussels' tissues collected at these sampling sites tended to accumulate toluene, m-xylene, p-xylene, o-xylene and ethylbenzene in higher amounts and showed activation of GR activity. In PC2 (vertical axis), ChE, CAT and GST enzymes were positively associated with this axis (r values of 0.5789, 0.5678, 0.4192, respectively) whereas LPO presented negative correlations (r value of −0.6367).

In PCA2 (Fig. 3 B), four components expressed 81% of the total variability. PCA interpretation was focused on the first two components, which summarised 60% of the total inertia (Fig. 3 B). Significant very strong correlations with PC1 axis were found for HNS (r values above 0.963) as shown in PCA1. Furthermore, some PAHs, namely, phenanthrene ($r = 0.986$), anthracene ($r = 0.921$), fluorene ($r = 0.904$) and pyrene ($r = 0.557$), as well as Pb ($r = 0.649$), showed significantly high correlations with the axis. Hg and Cd showed, however, negative correlations with the axis (−0.616 and −0.683, respectively). Antioxidant biomarkers (mainly GR, $r = 0.652$, GPx, $r = -0.410$) and neurotoxicity (ChE, $r = -0.523$) parameters were associated with this axis. PC2 was positively associated with Zn ($r = 0.701$), Ni ($r = 0.797$), Cu ($r = 0.767$), Cd ($r = 0.701$) and benzene ($r = 0.776$), whereas fluoranthene ($r = -0.446$), acenaphthylene ($r = -0.614$) and Pb ($r = -0.713$) presented negative correlations with this component. In addition, biomarkers GST and SOD showed significant positive and negative correlations with the axis of 0.405 and −0.442, respectively. Both axes contributed significantly to discriminate sites within the supplementary variable (sampling site, $r^2 = 0.995$ for PC1 and $r^2 = 0.981$ for PC2). PC1 established a gradient opposing *Carreço* and *São Bartolomeu do Mar* to *Vila Chã* and *Foz*, which tended to present higher levels of HNS in mussel tissues. PC2 established a gradient opposing *Cabo do Mundo* to *São Bartolomeu do Mar*, which mussels tended to show higher levels of metals (Fig. 3 B).

In PCA3 (Fig. 3 C), the first two components (expressing 54% of the total variability) were chosen for PCA interpretation. Particularly, Zn ($r = -0.9449$) and Cd ($r = 0.9449$), dissolved Ni ($r = 0.8782$) and Cd ($r = 0.8763$) and GPx ($r = 0.7463$) showed significant strong correlations with PC1. The axis established a gradient opposing *Carreço* and *São Bartolomeu do Mar* ($p < 0.001$). Contaminants associated to PC2 were particulate Hg ($r = 0.7051$) and toluene ($r = 0.6520$). GST ($r = 0.6843$) and AChE were also associated with this axis ($r = 0.6647$). Furthermore,

this axis produced a clear distinction among *Vila Praia de Âncora*, *Carreço*, *São Bartolomeu do Mar* and *Vila Chã*.

4. Discussion

Chemical monitoring of HNS, PAHs and metals in different environmental matrices revealed that, despite the maritime traffic and anthropogenic activities settled on the area, the NW Atlantic Iberian coast can be considered a low contaminated area. Low contents of HNS and metals were generally found in water collected along the coast, although Hg found at *São Bartolomeu do Mar* presented levels slightly above the limit value set on Decree of Law n° 103 of 2010 (DL, 2010). In sediments, few HNS (chloroform and tetrachloroethylene) and PAHs (naphthalene and phenanthrene) were found as reported in a complementary work within this monitoring campaign (Gouveia et al., 2018). Considering the guidelines established by Long et al. (1995), the content of PAHs and metals found was considerably lower than their estimated Effect-low Range (ERL) values (Long et al., 1995).

Likewise, low levels of contaminants were generally found in tissues of *Mytilus* specimens. A higher variability of HNS was however registered in mussels from *Vila Chã* and *Foz*, indicating a previous presence of these chemicals in the area, which was not reflected in the collected water samples. As a filter feeder and sedentary organism, *Mytilus sp*. has been recognised as a good bioindicator of water-column contamination (Moreira and Guilhermino, 2005, Lima et al., 2007). Moreover, mussels are present throughout the year, so they compose relevant indicators of previous or immediate contamination.

Mussels' tissues also presented measurable levels of PAHs and metals. Total PAHs were in general higher in tissue samples of *Mytilus* from *Cabo do Mundo*. The pattern of accumulation of PAHs in an organism can be affected by the exposure period, the concentration of PAHs, the salinity level, the nature of the hydrocarbon, the type of body tissue and the presence of algal food cells (Widdows et al., 1982). *Cabo do Mundo* and *Foz* sampling sites are located within areas of intense anthropogenic pressure close to an important petrol refinery plant. *Mytilus* specimens from those sampling sites are more likely exposed to contamination. Northern rocky shore beaches, on the other hand, are not expected to present major problems of contamination, except in the event of a spill, as these areas are generally less impacted by human activities. In fact, some of the sampling sites (e.g. *Carreço, Vila Chã*) have been considered a reference (Cairrão et al., 2004, Lima et al., 2007). Therefore, the levels registered in mussels from *Vila Chã*, an open beach inserted in an urban parish with low susceptibility to human influence (PELN, 2007), were not expected. The main human activities found in this area are small-scale traditional agriculture and fishery. Severe anthropogenic contamination is unlikely to occur. Nevertheless, this sampling site, as well as *São Bartolomeu do Mar*, is inserted in the hydrographic network of *Cávado*, *Ave* and *Leça* rivers, which are high industrialised areas (PGRH, 2015). Cadmium, nickel, lead, mercury, benzene, dichloromethane and trichloroethylene can be discharged into the hydrographical network (PGBHb, 2012), fact that might explain the data found in water and mussel samples from *Vila Chã* and *São Bartolomeu do Mar*.

Chemical monitoring of petrochemical products or other seaborne products, such as HNS, have not been regularly performed in Iberian waters, so that temporal trends are difficult to establish. To our best knowledge, this is the first assessment study of HNS, frequently transported by sea, in mussels. Metal content found in mussels were similar in relation to previous data (Lima et al., 2008). On the other hand, with regard to PAHs, the accumulated amounts have diminished significantly over the years in comparison to surveys performed in 1998 (Salgado and Serra, 2001) and 2005 (Lima et al., 2007), denoting a decreasing presence of such contaminants in coastal waters. In comparison with a monitoring campaign carried out by the *Agència Portuguesa do Ambiente*, the ΣPAHs found herein in *Cabo do Mundo* were slightly higher than those registered in 2008 (APA, 2010). No more reports of PAHs contamination in mussels were found after 2008. European legislation regarding contaminants in foodstuff provides legal limits for some metals (Pb, Cd) and benzo(a)pyrene and the sum of benzo(a)pyrene, benzo(a)anthracene, benzo(b)fluoranthene and chrysene in molluscs (EC, 2006), but does not stipulate a maximum limit for the remaining PAHs and HNS. Benzo(a)anthracene, benzo(a)pyrene, chrysene and benzo(b)fluoranthene were not detected on the collected mussels and the levels of metals were significantly lower than the maximum limits (EC, 2006), reinforcing the low contaminated state of these coastal organisms.

Biomarker responses measured in mussels' tissues showed some differences among sampling sites. *Mytilus sp*. is known as tolerant, but not insensitive organism, to several contaminants (Moreira and Guilhermino, 2005; Lima et al., 2007): therefore, it is suitable for ecotoxicological assays and monitoring surveys. Altered values of antioxidant enzymatic activity and oxidative stress biomarkers have been registered before in aquatic organisms in response to organic contaminants (van der Oost et al., 2003; Lima et al., 2007; Cravo et al., 2009; Sureda et al., 2011). Herein, despite the generally low levels of contaminants found in mussels' tissues, the integration of biochemical data and those from chemical analysis showed a clear distinction among sampling sites. A plain opposition is set between *Vila Chã* and *Foz* and the other sampling sites, evincing the presence of higher levels of HNS in those areas. A clear distinction among the remaining four sampling sites was also registered as function of PAHs and metals contamination accumulated in tissues.

Vila Chã and *Foz*, which presented a tendency for accumulation of higher HNS amounts in mussels, showed important alterations on GR and GPx activities. In fact, PCA analysis indicated a negative correlation between the activity of GR and GPx. Cell membrane damage can be related to the depletion of GSH (Ault and Lawrence, 2003), an important ROS scavenger and intermediate in detoxification of contaminants (Abele et al., 2011). Glutathione reductase and GPx are two antioxidant enzymes involved in the GSH cycle within cells. Gluthathione peroxidase leads to the oxidation of GSH into GSSG to eliminate organic and inorganic peroxides from the organism. To maintain cellular redox balance, GR reduces GSSG to GSH at the expense of NADPH (Ault and Lawrence, 2003; Abele et al., 2011). Lima et al. (2007) reported higher activity of GPx in mussels from *Carreço*, *Vila Chã* and *Cabo do Mundo*, collected in winter than in other sites of the NW Iberian coast. The activity of GR was however within the same order of magnitude. PAHs levels in mussels collected in 2005 were more elevated, as mentioned before, probably due to contamination from spillage originating from *Prestige* in 2002. The authors found significant correlations between PAHs levels and the activities of GPx and GR (Lima et al., 2007). Results suggest therefore the involvement of GSH as a possible detoxification mechanism. Given their low content in contaminants, mussels might have privileged other pathways of detoxification of PAHs and HNS, resulting in concomitant reduction of GSH. Detoxification of xenobiotics, such as PAHs, HNS and metals, with the involvement of GSH is in fact a common route of detoxification and is well underpinned in published literature (ATSDR, 1999; Kirman et al., 2005; Faria et al., 2009). Furthermore, mussels present a remarkable ability to depurate and eliminate contaminants from their tissues. For instance, Sureda et al. (2011) verified that, after the *Don Pedro* oil spill, in Eivissa Island, Spain, the levels of PAHs augmented significantly in soft tissues of mussels, returning to normal values after six months. Furthermore, the authors found that oxidative damage and biochemical biomarkers also returned to basal values (Sureda et al., 2011). Herein, LPO levels were notably lower than those published by Lima et al. (2007), which carried out a study within the same sampling area, during winter, and registered a significantly higher contamination by PAHs. In addition, PCO and LPO levels tended to be lower in mussels from more impacted areas than in the remaining areas. In Lima et al. (2007), higher LPO levels in digestive gland were found in areas within urban centres in comparison to those from low-impacted beaches. These parameters are connected to

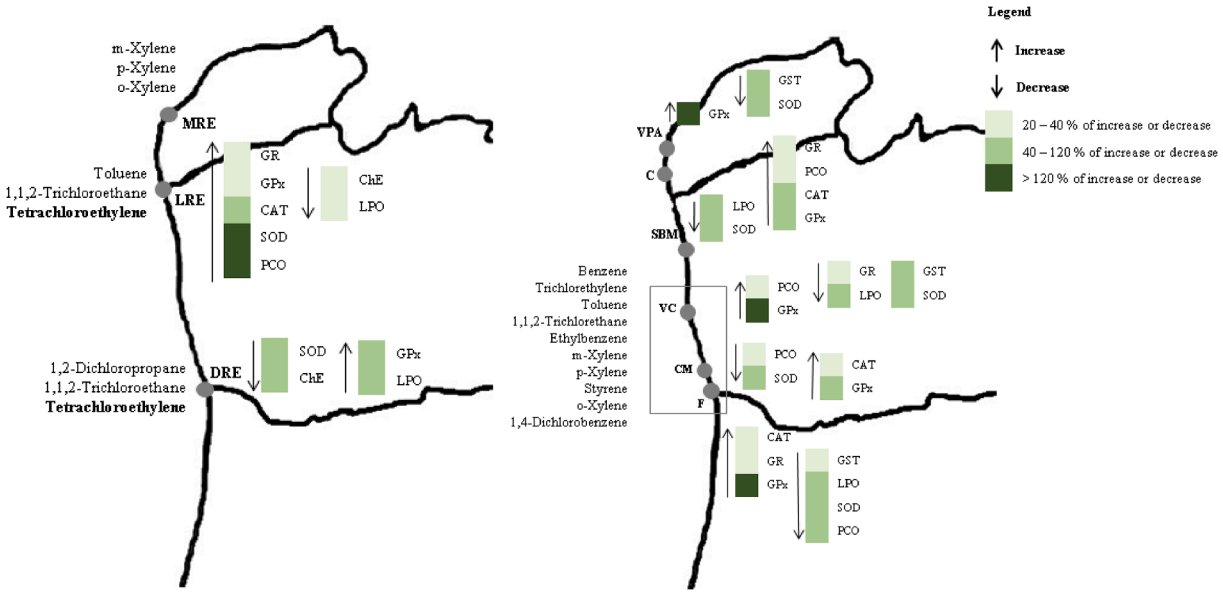

Fig. 4. Differences found in biomarkers response in flounders (left) (data from Rocha et al. submitted) collected in NW Iberian *Minho, Lima* and *Douro* estuaries and mussels (right) collected at each sampling site with regard to the response measured in each organism collected in the least impacted sampling site. For estuary monitoring campaign (left), *Minho* river estuary was taken as reference while for coast monitoring campaign, *Carreço* was taken into consideration. The increase or decrease of enzymatic activity and levels of oxidative damage is presented in percentage (values below 20% difference were not considered in this representation). The most relevant hazardous and noxious substances found in each sampling campaign are indicated along the NW Iberian coast in each map. Lipid peroxidation (LPO), proteins carbonyls (PCO), glutathione-s-transferase (GST), catalase (CAT), glutathione reductase (GR), glutathione peroxidase (GPx), superoxide dismutase (SOD) and acetylcholinesterase (AChE). *Minho* river estuary (MRE), *Lima* river estuary (LRE), *Douro* river estuary (DRE), *Vila Praia de Âncora* (VPA), *Carreço* (C), *São Bartolomeu do Mar* (SBM), *Vila Chã* (VC), *Cabo do Mundo* (CM) and *Foz* (F).

oxidative damage associated with injuries at cellular and organ level (Livingstone, 2001), and have been used as biomarkers of the chemical stress triggered by exposure to contamination (Lima et al., 2008, Guimarães et al., 2012). The low organic contamination registered herein can be a possible explanation for the results achieved: the organisms seem to be coping with the accumulation of organic contaminants preventing therefore oxidative damage of cellular membrane and proteins.

Apart from PAHs, which presence has been reported before (Rocha et al., 2011), this monitoring campaign confirms the occurrence of HNS in the NW Atlantic Iberian coast. Fewer of these chemicals were registered in water than in mussels' tissues. Despite the low levels, the fact that a variety of HNS were found in mussels' tissues suggests a continuous exposure of the animals to HNS. The NW Iberian coast is considerably hydrodynamic which, along with the volatile or sinking chemical nature of the HNS under study, hampers the quantification of HNS in water in which these chemicals are prompt to quickly disappear.

The integration of chemical analysis and a biomarker approach proved to be valid and crucial for the assessment of the good environmental state of coastal areas with elevated hydrodynamics. This approach was also suitable for the monitoring campaign of river estuaries (Rocha et al., submitted). Fig. 4 clearly highlights, for both monitoring campaigns, the changes in biomarkers response of flounders and mussels in relation to HNS detection. For river estuaries and coastal monitoring campaigns (Fig. 4), *Minho* estuary and *Carreço*, respectively, were taken as reference since they were the least impacted sites. In flounder, antioxidant (SOD, CAT and GPx), neurotoxicity (ChE) and oxidative damage to proteins (PCO) seem to be the biomarkers indicative of contamination by HNS (Fig. 4, left). In contrast, in mussels (Fig. 4, right), the most relevant biomarkers were oxidative damage to lipids (LPO) and antioxidant enzymes (GR, SOD and GPx). Bioaccumulation of contaminants and biomarker responses are dependent upon the species, the anthropogenic activities in the area, transportation of pollutants from urban centres, marine streams and the nature of seabed among other factors (Rocha et al., 2018). Moreover, contamination of HNS in the coastal area and the river estuaries seems to be associated to different sources and/or anthropogenic activities, while propagation of estuarine contamination to coastal area appears to be limited. Data obtained in these studies denote therefore the importance of performing biomonitoring campaigns at a local scale, selecting sentinel species relevant for each particular area.

5. Conclusion

An integrative evaluation of the NW Iberian coastal contamination by maritime trade substances was performed combining chemical monitoring and a biomarker approach. Such evaluation showed a clear distinction between *Vila Chã* and *Foz* and the remaining sampling sites associated to HNS contamination. Furthermore, PAHs and metals contamination seem to contribute for differences found among the remaining sampling sites. Important alterations were registered in GR and GPx activity, as well as, SOD activity. These results suggest the involvement of GSH as a possible detoxification mechanism to cope with contamination.

Overall, contamination level in the NW Iberian coast was moderate to low. Despite the low levels, the accumulation of HNS in organisms' tissues validates the presence of these contaminants. Mussels and flounder were also found suitable to investigate exposure to and effects of HNS contamination. To the best of our knowledge, this is the first monitoring campaign focused on HNS and involving two sentinel species. Furthermore, the evaluation of biomarker responses proved to be essential for data interpretation and for a more accurate assessment of the environmental status of aquatic ecosystems and highlights the advantages of combining chemical contamination with biological responses.

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

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