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The use of biomarkers as integrative tools for transitional water bodies monitoring in the Water Framework Directive context — A holistic approach in Minho river transitional waters

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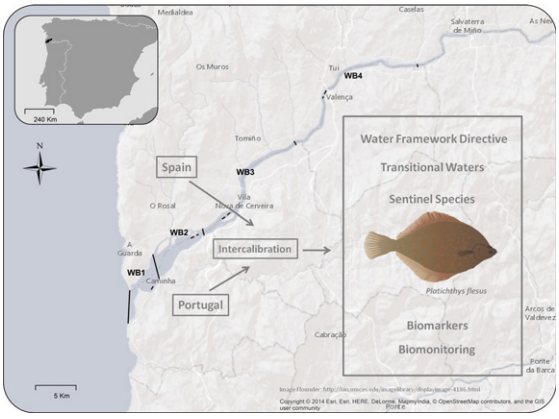
HIGHLIGHTS

- We need to establish biological monitoring in transitional waters.
- Biomarkers can be used as whole tools to monitor ecological status in estuaries.
- We studied patterns of biomarker response in juvenile flounder in the Minho estuary.
- Juvenile flounder showed a robust biomarker response over physical trait gradients.
- The use of juvenile flounder instead of adults eliminated confounding factors.

GRAPHICAL ABSTRACT



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ABSTRACT

The Water Framework Directive (WFD) provides an important legislative opportunity to promote and implement an integrated approach for the protection of inland surface waters, transitional waters, coastal waters and groundwaters. The transitional waters constitute a central piece as they are usually under high environmental pressure and by their inherent characteristics present monitoring challenges. Integrating water quality monitoring with biological monitoring can increase the cost-effectiveness of monitoring efforts. One way of doing this is with biomarkers, which effectively integrate physical–chemical status and biological quality elements, dealing holistically with adverse consequences on the health of water bodies. The new Marine Strategy Framework Directive (MSFD) already incorporates the biomarker approach. Given the recent activities of OSPAR and HELCOM to harmonize existing monitoring guidelines between MSFD and WFD the use of similar methodologies should be fostered. To illustrate the potential of the biomarker approach, juveniles of flounder (*Platichthys flesus*) were used to evaluate the quality of the

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Minho river-estuary water bodies. The use of juveniles instead of adults eliminates several confounding factors such changes on the biological responses associated with reproduction. Here, a panel of well-established biomarkers, EROD, AChE, SOD, CAT, GST, LPO, ENA and FACs (1-Hydroxyrene) were selected and measured along with a gradient of different physical conditions, and integrated with trace elements characterization on both biota and sediments. In general, a clear profile along the water bodies was found, with low seasonal and spatial variation, consistent with a low impacted area. Overall, the results support the use of both the battery of biomarkers and the use of juvenile flounders in the monitoring of the water quality status within the WFD.

1. Introduction

The Water Framework Directive 2000/60/EC (WFD) was approved in 2000, by the European Union (EU) members, with the purpose of establishing a framework for the protection of inland surface waters, groundwater, transitional waters and coastal waters. It constitutes a new view of the water resources management in Europe, that for the first time is mainly based on biological elements (ecosystems at the center of management decisions), (Borja, 2005).

For the directive implementation, new specific measures for the control of the ecological status and the development of an integrated EU water policy are required. Implicitly, the EU acknowledges the problems of judging the system's chemical and ecological quality and has delegated this to the member states although there are Common Implementation Strategies to ensure a comparable approach (de Jonge et al., 2006). It requires the monitoring and classification of all European surface and ground waters on several biological and physical–chemical criteria in order to ensure a 'Good Ecological Quality' of all water bodies (WFD 2000/60/CE). Therefore, the criteria harmonization becomes a crucial factor in its implementation and constitutes a complex call for the member states, as it also requires a close cooperation for a homogeneous, harmonic and coordinated effort, in order to interpret and apply the directive basis in the same way (Borja, 2005; Borja and Dauer, 2008; Van Hoey et al., 2010).

This directive provides an important legislative opportunity to promote and implement an integrated approach to risk assessment of chemicals. In this context, the transitional waters constitute a central piece; they are usually under high environmental pressure and by their inherent characteristics present several monitoring difficulties. Those areas have a relatively lower amount of studies performed within this context compared to the other water resources (Martinez-Haro et al., 2015). Another central question is whether the present monitoring can only detect large statistically significant changes and unusual occurrences or whether it has the ability, in the dynamic estuarine and coastal systems, to measure and detect subtle changes.

Given the budget limitations, a cost-effective monitoring approach will have to be set in place, so in general we need to integrate water quality monitoring and biological monitoring with applied system research (de Jonge et al., 2006). The integrated risk assessment for environmental and ecological issues may be the answer to that. It can provide early signs of previously unidentified risks, with both human and wildlife species in the role of sentinel species (Valavanidis and Vlachogianni, 2010). Biomarkers can offer a good connection between both classical (physical–chemical) and biological approaches, dealing holistically with the adverse consequences on health status caused by the possible exposures. In recent years OSPAR and ICES have increased their focus on the integration of biological and chemical data (OSPAR Commission, 2015), and the Marine Strategy Framework Directive 2008/56/EC (MSFD) already incorporates biomarker responses as early warning signals of pollution in order to anticipate potential impacts at higher levels of biological organization (Allan et al., 2006; Sanchez and Porcher, 2009). Furthermore, the estuarine areas due to their inherent characteristics present several additional challenges if we aim to perform a correct impact evaluation even by traditional approaches. The acquired information can be relevant to the future application of the proposed tools within the WFD context, as suggested in the few available studies (Serafim et al., 2012; Basset et al., 2013; Chapman et al., 2013). The integration of such data forms an important methodology, linking contaminants and ecological responses aiming at assessing the overall quality of the marine environment. Given the connectivity between both directives (WFD and MSFD) the use of similar monitoring methodology should be fostered. Over the past decades, a wide range of biomarkers have been employed in monitoring as early-warning indicators of contamination and ecosystem health (Giltrap et al., 2013). Combining the use of biomarkers with chemical determinations and ecological evaluation offers a huge scope to extend their use to reduce uncertainty in the risk assessment of hazardous substances (Hagger et al., 2008).

Biomarkers can be considered 'functional measures of exposure to stressors expressed at the sub-organismal, physiological or behavioural level' (Galloway, 2006). However, their use needs to be accompanied by an understanding of the significance of these measurements to ensure the adequate and reliable interpretation of results by water quality managers (Allan et al., 2006).

The current work aims at establishing and applying a methodology that is able to provide information that may allow the classification of the international transitional water bodies of the Minho river-estuary within the WFD context. The magnitude of river discharges as well as tidal and seasonal fluctuations pose several difficulties on settling proper monitoring programs and are particularly challenging on the managing perspective (Ferreira et al., 2006a, 2007).

The main objective of the presented work was to validate biomarkers, which are sensitive, easy to apply and cost-effective to use in the ecological state evaluation of transitional water bodies in a practical monitoring situation. Biomarkers can provide valuable information both for establishment of reference conditions and the environmental quality assessment of the Minho river transitional waters.

A set of tools based on ecotoxicological biomarkers was validated with the purpose of fostering its integration in the WFD. The WFD is a 'dynamic Directive' which allows further incorporation of new methodologies, changes in the previous definitions and classification of sites, etc., because any increase in knowledge should feedback into any further assessments (Borja, 2005). As it will be shown ahead, the practical application of the described methodology could be pertinent in order to provide new insights of the biomarkers use within this context. For the present study, flounder (*Platichthys flesus*) juveniles were selected as model organism because of their presence throughout the whole study area. The flatfishes of the Pleuronectidae family are a worldwide spread group of fishes, occurring in fresh, brackish and marine waters. The population in the Minho estuary is largely dominated by juveniles being the upstream zones (freshwater) of the estuary preferred by the species (Souza et al., 2013) allowing the results validation on all the sampling points of a wide range of hydrographical and geomorphologic characteristics. Several studies propose this species as a primary species for monitoring programs (Davies and Vethaak, 2012; Giltrap et al., 2013; Nunes et al., 2014). However, all have used adult animals. Here, we propose the juveniles as study model, in order to eliminate several confounding factors (maturation and physiological status and spawning

periods) validating the biomarker responses on a monitoring context. Given this, a set of biomarkers was chosen based on the their mechanistic understanding and the applicability in this specific context: Cytochrome P-450 (Ethoxyresorufin *O*-Deethylase — EROD; indicator of exposure to organic contaminants such as polycyclic aromatic hydrocarbons — PAHs, polychlorinated biphenyls — PCBs, etc.); Acetilcholinesterase activity — AChE (organo-phosphorous, carbamates, metals, etc.); Antioxidant/Biotransformation enzymes (Superoxide Dismutase — SOD, Catalase — CAT, Glutathione S-Transferase — GST; exposure to ROS, free radicals, pollutants causing oxidative stress); Lipid peroxidation — LPO (oxidants, metals, etc.); Erythrocytic nuclear abnormalities — ENA (exposure to genotoxic agents); Bile metabolites (Fluorescent aromatic compounds — FACs, 1-Hydroxyrene; indicator of PAHs exposure). Major and trace elements (aluminum — Al, vanadium — V, chromium — Cr, manganese — Mn, cobalt — Co, nickel — Ni, copper — Cu, zinc — Zn, selenium — Se, cadmium — Cd and lead — Pb) were determined in liver paralleled with biomarker characterization. PCB congeners (18, 26, 31, 44, 49, 52, 101, 105, 118, 138, 149, 151, 153, 180 and 187) and PAH (Fluorene — F, Phenanthrene — P, Anthracene — A, Fluoranthene — FL, Pyrene — PY, Benzo[a]anthracene — BAA, Chrysene — C, Benzo[b]fluoranthene — BBF, Benzo[k]fluoranthene — BKF, Benzo[e]pyrene — BEP, Benzo[a]pyrene — BAP, Indeno[1,2,3-cd] pyrene — IN, Dibenzo[a,h]anthracene — DBA, Benzo[g,h,i]perylene — BPE) were determined in fish tissues (PCBs in muscle) and in sediments (Metals, PCBs and PAHs), characterizing the contaminant content of the main substratum for *P. flesus* as well as the tissue accumulation.

2. Material and methods

2.1. Samples and handling

The WB division criteria were based on hydrographical and geomorphologic criteria, predicted in the WFD. The Minho estuarine basin is characterized by a narrow channel with high flow river discharge, presenting high hydrodynamic energy and lower water residence time (Saraiva et al., 2007; Brito et al., 2012). It is 300 km long and has 17,100 km^2 watersheds that extend mostly over Spain. The river has a freshwater discharge of 300 $m^3\ s^{-1}$ and the estuary is classified as a meso-tidal stratified with an average residence time of 1.5 days (Ferreira et al., 2005a). Seawater and freshwater mixed generally in the lower estuary, although the physical tidal influence extends to 40 km upstream (Sousa et al., 2005). The morphological and salinity conditions were combined to identify the water bodies (WB1, WB2, WB3 and WB4), common to Spain and Portugal under the Water Framework Directive (Fig. 1).

Juvenile flounders (*P. flesus*) were captured in the four water bodies (WB) that compose the Minho river-estuary system by beam trawl during 10 min (in triplicate) at night in spring tides of May and November 2012. The fishing effort was similar in all the sampling areas.

After the collection, the animals were transported alive to the laboratory in a refrigerated and aerated recipient. In the laboratory, the fish were anaesthetized in ice cold water and their body total length and weight recorded. The Fulton's condition index [CF = (Weight/Length^3) × 100], indicative of general fish condition was also calculated. Blood from the caudal vein was collected and the animals euthanized by decapitation to avoid further suffering. Blood was smeared on clean slides and allowed to air-dry. The slides were then processed for the determination of ENA. After dissection, the liver was weighted for calculation of the hepatossomatic index [HSI = (Liver weight/Weight) × 100], gonads for calculation of the gonadossomatic index [GSI = (Gonad weight/Weight) × 100] and the gender of the fish determined. The liver and a portion of muscle tissue (without skin) were removed for biochemical and chemical analysis and immediately frozen in liquid nitrogen and subsequently stored at −80 °C and −20 °C, respectively. The brain and bile were also removed, frozen in liquid nitrogen and stored at −80 °C until analysis.

Eighteen grab sediments were collected in the same fishing areas in May 2012, using a Van-Veen grab. The surface layer (approximately

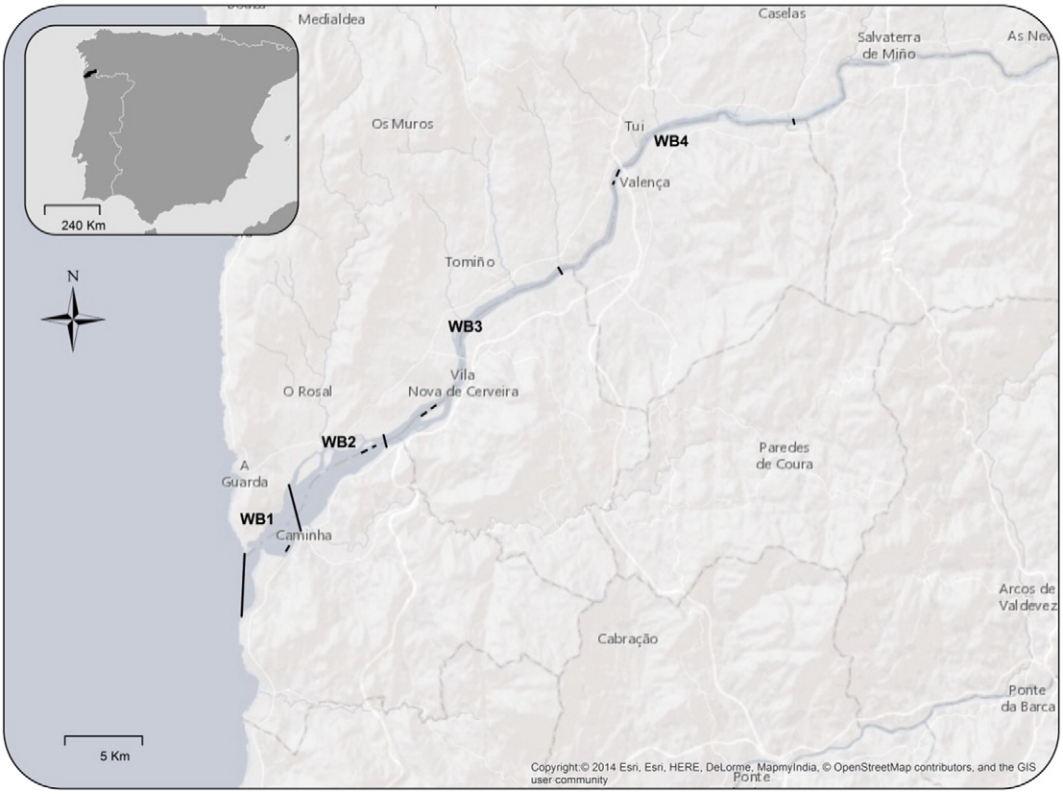

Fig. 1. Study area with the water bodies (WB) and sampling places (dashed).

5-cm thickness) was sampled and kept in individual plastic bags for trace-element, PCB and PAH analysis.

2.2. Biochemical determinations

Livers were homogenized and mitochondrial fractions extracted. Microssomes were obtained at high speed according to Fent and Bucheli (1994) and stored at −80 °C until use. Hepatic EROD activity was measured according to Lima et al. (2008). GST was determined by the method of Habig (Habig et al., 1974) as described in Ferreira et al. (2010). SOD activity was determined by an indirect method involving the inhibition of Cytochrome C reduction in the mitochondrial fraction, according to Ferreira et al. (2010). CAT activity was determined by measuring the consumption of H_2O_2 as indicated in Ferreira et al. (2010). AChE activity was determined by the Ellman's method (Ellman et al., 1961) adapted to microplate (Guimarães et al., 2012). The LPO was determined by the thiobarbituric acid method according to Ferreira et al. (2010). Protein content in the fractions was assayed by the Lowry method using bovine serum albumin (BSA) as a standard.

2.3. Erythrocytic nuclear abnormalities (ENA)

Genotoxic damage was evaluated using the ENA assay, performed according to Micael et al. (2007) and Santos et al. (2010). Four thousand erythrocytes per fish were scored for the presence of ENA under a 1000× magnification lens. Slides were coded and scored blindly by the same observer, with two replicate slides per fish. The considered abnormalities were: micronuclei, small (<1/3 of the main nucleus) non-refractive, circular or ovoid chromatin bodies, showing the same pattern as the main nucleus (Al-Sabti and Metcalfe, 1995); cells presenting two nuclei were considered as binucleated; nuclei divided in two lobes were classified as segmented nuclei; nuclei with a central and unilateral constriction were classified as kidney shaped nuclei.

2.4. Fluorescent aromatic compounds (FACs)

FACs in the bile were determined by Fixed Wavelength Fluorescence (FF). The supernatant was used for FF determination at the excitation/emission wavelength 341/383 nm, denoted FF341/383, when Pyrene type metabolites are detected (Santos et al., 2010). A calibration curve for this metabolite, using 1-hidroxypyrene, was made according to Lima et al. (2008). Biliverdin content was estimated spectrophotometrically at 380 nm in the same samples to normalize FAC concentrations.

2.5. Polycyclic aromatic biphenyls (PAHs)

Polycyclic aromatic hydrocarbons (PAHs) were determined in muscle and sediment samples spiked with surrogate standards (from Supelco), after extraction by ASE 200. To determine PAHs in biological samples, the dried material were mixed with diatomaceous earth and extracted using a mixture of acetone/hexane (v/v) with an ASE 200 accelerated solvent extraction system and concentrated with a N_2 stream (Martins et al., 2008). To quantify PAHs in sediments about 5 g of dried sediment were used following the same methodology. The determination of sixteen individual PAHs (3 to 6-ring) was performed on a Thermo DSQ gas chromatography–mass spectrometry (GC–MS) system in selected ion monitoring (SIM) mode. Before analysis, relevant standards were run to check column performance, peak height and resolution. With each set of samples to be analyzed, a solvent blank, a standard mixture and a procedural blank were run in sequence to check for contamination, peak identification and quantification. None of the target compounds were detected in the procedural blanks. Identification of acenaphthylene, acenaphtene, fluorene, phenantrene, anthracene, fluoranthene, pyrene, benzo(a)anthracene, chrysene, benzo(*b*)fluoranthene, benzo(*k*)fluoranthene, benzo(*a*)pyrene, benzo(*e*)pyrene, dibenzo(*ah*)anthracene, indeno(1,2,3-cd)pyrene and benzo(*g,h,i*)perylene was based on the comparison of their GC-retention times and mass spectrum with appropriate individual standards. Concentrations of individual PAHs were measured by the internal standard peaks area method and a 9-point calibration curve for each compound used. The detection limit was calculated with a signal-to-noise ratio of 3:1 in a blank sample (n = 5) and varied within a narrow interval of 0.001 μg g^{-1}. Quality control was obtained analyzing the certified material SRM 1941b (NIST, USA) Recoveries of analyzed PAHs in the certified material ranged from 81% to 123%. Results of PAH compounds are expressed in nanogram per gram of sample.

2.6. Polychlorinated biphenyls (PCBs)

Polychlorinated biphenyls (PCBs) were Soxhlet-extracted from wet muscle and sediment samples with n-hexane, for 6 and 16 h, respectively (Ferreira et al., 1990, 2003). The extracts were fractionated with a Florisil chromatographic column and purified with sulphuric acid. Seventeen PCB congeners (tri- to hepta-CB) were quantified by gas chromatography with an electron-capture detector and a capillary column. Quantification was performed by gas chromatography (Hewlett-Packard 6890) with an electron-capture detector and a capillary column (DB5, J&W, 60 m). Quantification was obtained by the external standard method, using a seven-point calibration curve for each compound. Procedural blanks were analyzed each batch of 10 samples to monitor possible laboratory contamination. Certified reference mussel tissue SRM 2977 and SRM 1941b (NIST, USA) was analyzed to validate the procedure. The obtained PCB values were found within certified range, with the recoveries being 73–112% and 80–110%, respectively. Detection limits calculated from three times the peak height in blank samples, ranged from 0.01 to 0.04 ng.g^{-1}. Results are expressed in nanogram of PCB congener per gram of sample (dry weight for fish tissues).

2.7. Trace elements

Trace elements (V, Cr, Mn, Co, Ni, Cu, Zn, As, Se, Cd and Pb) in muscle and liver were analyzed in lyophilized, grinded and homogenized samples after digestion with a mixture of HNO_3 (sp, 65% v/v) and H_2O_2 (sp, 30% v/v) at different temperatures according to the method described by Ferreira et al. (1990). Sediment samples were dried, grounded with an agate mortar and completely digested according to the method described by Caetano et al. (2009). Aluminum (Al) was analyzed by flame atomic absorption spectrometry with a nitrous oxide-acetylene flame. Concentrations of V, Cr, Mn, Co, Ni, Cu, Zn, As, Se, Cd and Pb were determined by a quadrupole ICP-MS, equipped with a Peltier Impact bead spray chamber and a concentric Meinhard nebulizer. Procedural blanks and Quality Control solutions were run every 10 samples. The precision and accuracy of the analytical procedures was controlled through repeated analysis of the elements studied in international (National Research Council of Canada) certified reference materials (DORM-3, DOLT-3 and DOLT-4 for organisms and MESS-2 and PACS-2 for sediments). The results obtained were in good agreement with the certified values ($p > 0.05$). Procedural blanks always accounted for less than 1% of the total element in the samples. Detection limits were 0.0030 μg g^{-1} for V, 0.0019 μg g^{-1} for Cr, 0.0016 μg g^{-1} for Mn, 0.0020 μg g^{-1} for Co, 0.0010 μg g^{-1} for Ni, 0.0015 μg g^{-1} for Cu, 0.0032 μg g^{-1} for Zn, 0.74 μg g^{-1} for As, 0.0030 μg g^{-1} for Se, 0.0009 μg g^{-1} for Cd and 0.0088 μg g^{-1} for Pb. All concentrations in fish samples are given in microgram of trace element per gram of tissue dry weight (μg g^{-1}; dw).

2.8. Statistical analysis

Differences between groups were tested using a One-Way ANOVA followed by the Fisher's Least Significant Difference (LSD) multiple comparisons test, at a 5% significance level. Some data had to be log-

transformed in order to fit ANOVA assumptions. All the statistical tests were performed using the software Statistica7.0 (Statsoft, Inc., 2004). Principal component analysis (PCA) was used to investigate patterns of response of the biomarkers assessed in the liver in relation to the studied water bodies and seasons. Chemical, morphometric and biomarker data entered in the PCA as quantitative variables to provide a profile of each water body. The water body and season were entered as supplementary qualitative variables. Only principal components (PCs) with Eigen values >1 were extracted. PCA interpretation was based on the examination of the correlations between the variables and the PCs obtained. Confidence ellipses were drawn around the qualitative supplementary units (i.e., around the barycentre of the samples assessed on each unit) to enable visualizing whether or not the groups differed significantly. PCA was carried out in FactoMineR.

3. Results

3.1. Biometrics

Flounder weight, length, and values of the morphometric condition factor (CF), hepato-somatic index (HSI) and gonadossomatic index (GSI) are presented in Table 1. In both sampling campaigns heavier and longer specimens were found near the estuary mouth (WB1) while smaller ones on the upstream areas (WB4). Although larger differences of those parameters were recorded in November, the CF and HSI were not statistically different among the WB's in May and November. The GSI in females was always low, only significant differences ($p < 0.05$) were found in fish from November in WB1. In males GSI was also low on all stations, although in WB1 and WB2 from November presented higher values, yet without statistically significant differences.

3.2. Trace elements, PAHs and PCBs in sediment samples

Table 2 gives the Al content and the ratios to Al of V, Cr, Mn, Co, Ni, Cu, Zn, As, Se, Cd and Pb in sediment samples from the four WBs. No significantly differences were observed for Al and all the element/Al ratios among WB's, although enhanced values were observed in WB3 and WB4 for Mn, Co, Ni, Zn and Cd ratios, and in WB1 for Pb ratio. Ratios to Al of Mn, V and Cr were relatively constant in the four WBs. Trace element concentrations in all sites are far below the values considered to have a low effect on biota (ERL) proposed in Long et al. (1995).

Among the PAH compounds, only fluoranthene (0.5–2.0 ng g^{-1}) and pyrene (<0.8–1.9 ng g^{-1}) were quantified in sediments from WB1, WB2 and WB3, the other PAH being below the detection limits. No significant differences were found among water bodies. The median concentrations (and range) of total PCB were: 0.12 ng g^{-1} (0.09–0.90) in WB1, 0.49 ng g^{-1} (<0.07–1.4) in WB2, 0.37 ng g^{-1} (0.20–1.6) in WB4, and 1.30 ng g^{-1} (<0.07–2.5) in WB3. All the samples showed concentrations far below 23 ng g^{-1}, the Effect Range Limit proposed by Long et al. (1999).

Table 2
Aluminum (%) concentrations and the ratios to Al (Aluminum) of V (Vanadium), Cr (Chromium), Mn (Manganese), Co (Cobalt), Ni (Nickel), Cu (Copper), Zn (Zinc), As (Arsenic), Cd (Cadmium) and Pb (Lead) in sediment samples from the four WBs.

Elements	WB1	WB2	WB3	WB4
Al (%)	6.6 ± 2.8	7.4 ± 1.4	9.4 ± 2.3	8.0 ± 2.2
V/Al ($\times 10^{-4}$)	4.6 ± 2.1	4.3 ± 0.90	4.7 ± 0.44	4.5 ± 1.0
Cr/Al ($\times 10^{-4}$)	4.0 ± 1.2	3.5 ± 0.61	3.8 ± 0.77	3.9 ± 0.49
Mn/Al ($\times 10^{-4}$)	24 ± 10	20 ± 4.0	28 ± 3.6	27 ± 5.8
Co/Al ($\times 10^{-4}$)	0.52 ± 0.18	0.65 ± 0.21	0.98 ± 0.31	1.1 ± 0.49
Ni/Al ($\times 10^{-4}$)	1.2 ± 0.49	1.6 ± 0.52	2.1 ± 0.34	2.4 ± 0.76
Cu/Al ($\times 10^{-4}$)	1.0 ± 0.11	1.0 ± 0.18	1.3 ± 0.35	1.4 ± 0.49
Zn/Al ($\times 10^{-4}$)	6.4 ± 0.62	6.1 ± 1.5	7.9 ± 1.8	8.6 ± 2.6
As/Al ($\times 10^{-4}$)	1.1 ± 0.2	1.8 ± 0.51	1.7 ± 0.5	1.5 ± 0.53
Cd/Al ($\times 10^{-4}$)	0.011 ± 0.005	0.010 ± 0.005	0.016 ± 0.006	0.018 ± 0.007
Pb/Al ($\times 10^{-4}$)	2.7 ± 0.41	2.8 ± 0.48	2.4 ± 0.22	2.3 ± 0.21

3.3. Trace elements in flounder tissues

3.3.1. Liver

Mean and standard deviation of V, Cr, Mn, Co, Ni, Cu, Zn, As, Se, Cd and Pb concentrations in liver of the flounders captured in the four water bodies are presented in Table 3. Values ranged from 0.040 μg g^{-1} of Pb to 304 μg g^{-1} of Zn in WB2 during the November survey. Noteworthy were the significant differences of each element concentration between water bodies and season. In May three different patterns were observed: (i) WB1 and WB2 showed higher concentrations of V, Cr, Co, Cu, As and Se; (ii) WB2 presented enhanced values of Cd; (iii) WB4 displayed higher values of Mn. In November, enhanced concentrations were registered in: WB1 for As, WB2 for Cd, Zn and Cu, WB3 and WB4 for Cr and Ni, and WB4 for Mn, Co and Pb. In short, results obtained in the two seasons indicate higher As concentrations in liver of specimens captured in WB1, while enhanced Cd was found in flounders from WB2. In general, flounders captured in May in the four water bodies presented higher concentrations of Mn, Co, Ni and Pb in comparison to organisms from November. Conversely, As showed enhanced values in organisms captured in November. Vanadium presented higher levels in May only in WB1 and WB2, but the opposite was observed for WB3 and WB4 with enhanced values in November. Concentrations of Cr were also higher in WB1 and WB2 in May, but similar levels were registered in the two surveys in WB3 and WB4. All differences were statistically valid ($p < 0.05$).

3.3.2. Muscle

Mean and standard deviation of V, Cr, Mn, Co, Ni, Cu, Zn, As, Se, Cd and Pb concentrations observed in the muscle of the flounder captured in the four water bodies are presented in Table 4. Mean concentrations varied up to four orders of magnitude from 0.002 μg g^{-1} of Ni in WB1 to 68 μg g^{-1} of Zn in WB4. In May, the comparison between water bodies showed four patterns with elevated values of: (i) V and Se in WB1; (ii) Cr, Ni and As in WB1 and WB2; (iii) Mn, Co and Zn in WB3 and WB4; and (iv) Cu, Cd and Pb in flounders from WB3. In November variations between water bodies were as follows: (i) high concentrations of As

Table 1
Biometrics from captured animals (*P. flesus*) by water bodies (WBs) in May and November. Data are presented as Mean ± SE. CF — Foulton Condition Factor; HSI — Hepatosomatic Index; GSI — Gonadossomatic Index.

	May				November			
Biometrics	WB1	WB2	WB3	WB4	WB1	WB2	WB3	WB4
N	13	13	9	14	6	13	10	14
Weight (g)	31.28 ± 5.50	28.62 ± 3.43	27.21 ± 7.23	12.89 ± 0.79	158.95 ± 57.16	50.88 ± 12.40	35.54 ± 7.35	26.31 ± 6.93
Length (cm)	14.36 ± 0.82	14.32 ± 0.52	13.67 ± 1.25	11.00 ± 0.23	22.62 ± 3.33	16.48 ± 1.21	15.07 ± 0.96	13.66 ± 0.76
CF	0.97 ± 0.02	0.92 ± 0.02	0.89 ± 0.02	0.96 ± 0.03	1.01 ± 0.03	0.93 ± 0.02	0.93 ± 0.02	0.89 ± 0.03
HIS	0.96 ± 0.05	0.72 ± 0.04	0.77 ± 0.05	0.91 ± 0.06	0.96 ± 0.08	0.78 ± 0.03	0.74 ± 0.04	0.83 ± 0.02
GSI (Males)	0.065 ± 0.008	0.061 ± 0.008	0.075 ± 0.012	0.089 ± 0.017	0.833 ± 0.460	0.285 ± 0.160	0.079 ± 0.007	0.088 ± 0.005
GSI (Females)	1.01 ± 0.07	1.00 ± 0.06	0.98 ± 0.25	0.67 ± 0.05	1.13 ± 0.11	0.89 ± 0.07	0.81 ± 0.05	1.12 ± 0.06

Table 3
Metals in Liver of animals (*P. flesus*) captured in each water body both in May and November. Data are presented as Mean ± SE. WB2 data from November are lacking SE due to sampling constrains, the quantification was performed in pools of captured animals, not on individual samples correspondent to each animal. V — Vanadium; Cr — Chromium; Mn — Manganese; Co — Cobalt Ni — Nickel; Cu — Copper; Zn — Zinc; As — Arsenic; Se — Selenium; Cd — Cadmium; Pb — Lead.

Liver	May				November			
Metals (μg/g)	WB1	WB2	WB3	WB4	WB1	WB2	WB3	WB4
V	0.52 ± 0.060	0.43 ± 0.044	0.13 ± 0.050	0.19 ± 0.14	0.37 ± 0.015	0.36	0.34 ± 0.081	0.39 ± 0.058
Cr	1.5 ± 0.18	1.6 ± 0.17	1.2 ± 0.11	1.1 ± 0.17	0.37 ± 0.029	0.38	1.2 ± 0.20	1.328 ± 0.21
Mn	8.64 ± 1.4	4.4 ± 0.27	8.0 ± 0.72	13 ± 2.5	2.6 ± 0.13	3.46	1.3 ± 0.29	24.580 ± 4.377
Co	1.85 ± 0.32	2.6 ± 0.28	0.78 ± 0.14	1.1 ± 0.20	0.92 ± 0.32	1.42	1.4 ± 0.15	2.3 ± 0.18
Ni	1.5 ± 0.11	2.1 ± 0.35	1.8 ± 0.32	1.6 ± 0.35	0.17 ± 0.021	0.30	0.62 ± 0.11	0.861 ± 0.145
Cu	49 ± 8.4	54 ± 5.6	18 ± 3.6	19 ± 5.6	50 ± 22	172	92 ± 11	41 ± 8.4
Zn	117 ± 9.2	111 ± 7.4	114 ± 10	112 ± 1.9	161 ± 11	303	216 ± 12	172 ± 7.2
As	4.5 ± 0.88	3.1 ± 0.32	1.8 ± 0.60	1.2 ± 0.48	11 ± 1.1	7.3	9.6 ± 0.35	3.83 ± 0.44
Se	7.5 ± 1.4	8.6 ± 1.0	6.2 ± 1.0	2.3 ± 0.73	5.7 ± 0.89	4.52	4.7 ± 0.72	3.893 ± 0.29
Cd	0.31 ± 0.036	0.89 ± 0.14	0.29 ± 0.086	0.15 ± 0.082	0.39 ± 0.034	0.57	0.37 ± 0.040	0.31 ± 0.086
Pb	0.65 ± 0.11	1.2 ± 0.13	1.4 ± 0.40	1.2 ± 0.32	0.10 ± 0.030	0.040	0.062 ± 0.012	0.419 ± 0.062

in WB1 and WB2; (ii) elevated levels of V, Co and Zn in WB3 and WB4; (iii) lower values of Cr and Cd in WB1. Differences between seasons in the muscle were comparable to the ones observed for the liver. Flounders captured in May presented enhanced concentrations of Mn, Co, Ni and Pb in comparison to November, but the opposite was observed for As with higher levels in November. Vanadium presented higher levels in May only in WB1 and WB2, and enhanced values in November in WB3 and WB4. Concentrations of Cr were also higher in WB1 and WB2 in May, but similar levels were registered in the two surveys in WB3 and WB4. Copper showed higher concentrations in November for WB2, WB3 and WB4. All differences were statistically significant ($p < 0.05$).

3.4. PAHs and PCBs in muscle samples

All concentrations of the PAH compounds in muscle tissue of flounders captured in the WBs were below the detection limit (0.01 ng g^{-1}). The mean and standard deviation of total PCBs in muscle of flounders captured on the water bodies were: WB1 (0.20 ± 0.10 ng g^{-1}), WB2 (0.76 ± 0.14 ng g^{-1}) and WB3 (1.03 ± 0.56 ng g^{-1}). The congeners CB18, CB153 and CB187 were the major contributors to the total PCBs. Statistical differences were observed between values of the three water bodies.

3.5. Biotransformation enzymes (EROD and GST)

Results for phase I and II biotransformation enzymes (EROD and GST) are displayed in Fig. 2 and Supplementary Table 5. EROD activity ranged from 1.60 ± 0.32 pmol/min/mg protein in WB4 in November to 5.82 ± 1.61 pmol/min/mg protein in WB2 in May (WB2 values were significantly higher than the other water bodies, $p < 0.05$). In November the values were similar in WB1, WB2 and WB3 and significantly lower in WB4, compared to WB3 (1.60 ± 0.32 and 4.64 ± 1.01 pmol/min/mg protein, respectively, $p < 0.05$). No seasonal significant differences were found for this biomarker. GST ranged from 38.44 ± 2.01 nmol/min/mg protein in WB1 to 51.20 ± 1.33 nmol/min/mg protein in WB3 in November. Activities in May were significantly higher in WB2 and WB3 (46.98 ± 1.79 and 47.17 ± 2.56 nmol/min/mg protein, respectively) compared to WB1 (38.45 ± 1.59 nmol/min/mg protein, $p < 0.05$). November GST activities were similar on all water bodies except for WB3, being significantly higher ($p < 0.05$). Seasonal differences were found in WB2 (46.98 ± 1.79 and 39.29 ± 1.23 nmol/min/mg protein, respectively), being significantly lower in November ($p < 0.05$).

3.6. Antioxidant enzymes (SOD and CAT)

The antioxidant defenses (SOD and CAT) results are displayed in Fig. 2 and Supplementary Table 5. SOD activity ranged from 6.65 ± 1.81 sU/min/mg protein in WB4 in November to 16.81 ± 1.68 U/min/mg protein in WB1 in May. The activities in May were higher in the river mouth (WB1) compared to upstream areas. Statistical significant differences were observed in fish from WB1, activities were higher than WB3 and WB4 (10.06 ± 1.39 and 8.27 ± 1.82 U/min/mg protein, respectively, $p < 0.05$) and values in WB2 (12.61 ± 1.33 U/min/mg protein) were higher than in WB4 ($p < 0.05$). In November WB1 activity values (11.51 ± 1.09 U/min/mg protein) were significantly higher than in WB4 ($p < 0.05$). Also seasonal differences were found in WB1. CAT activities ranged from 11.23 ± 3.88 μmol/min/mg protein in WB3 in May to 22.91 ± 2.10 μmol/min/mg protein in WB1 in November. The activities in May were higher in WB1 and WB4 (19.97 ± 2.67 and 21.80 ± 3.87 μmol/min/mg protein, respectively) with significant differences to WB3 (11.23 ± 3.88 μmol/min/mg protein, $p < 0.05$). No significant seasonal differences were found for CAT. November WB3

Table 4
Metals in muscle of captured animals (*P. flesus*) by water bodies both in May and November. Data are presented as Mean ± SE. V — Vanadium; Cr — Chromium; Mn — Manganese; Co — Cobalt Ni — Nickel; Cu — Copper; Zn — Zinc; As — Arsenic; Se — Selenium; Cd — Cadmium; Pb — Lead.

Muscle	May				November			
Metals (μg/g)	WB1	WB2	WB3	WB4	WB1	WB2	WB3	WB4
V	0.18 ± 0.034	0.12 ± 0.015	0.11 ± 0.011	0.12 ± 0.020	0.051 ± 0.010	0.045 ± 0.010	0.19 ± 0.021	0.16 ± 0.014
Cr	0.91 ± 0.14	1.0 ± 0.16	0.40 ± 0.026	0.28 ± 0.023	0.12 ± 0.015	0.31 ± 0.12	0.34 ± 0.031	0.37 ± 0.020
Mn	3.7 ± 1.14	4.7 ± 0.82	6.5 ± 1.1	9.4 ± 1.7	0.72 ± 0.12	2.0 ± 0.56	3.0 ± 0.60	7.4 ± 1.5
Co	0.070 ± 0.020	0.075 ± 0.011	0.18 ± 0.019	0.28 ± 0.033	0.017 ± 0.006	0.041 ± 0.007	0.073 ± 0.013	0.169 ± 0.022
Ni	0.81 ± 0.22	0.87 ± 0.14	0.52 ± 0.15	0.47 ± 0.051	0.002 ± 0.001	0.32 ± 0.15	0.077 ± 0.019	0.076 ± 0.012
Cu	0.98 ± 0.11	1.2 ± 0.096	1.6 ± 0.076	1.3 ± 0.12	0.99 ± 0.11	1.2 ± 0.11	1.2 ± 0.078	1.1 ± 0.067
Zn	31 ± 2.6	42 ± 3.0	67 ± 3.6	55 ± 5.5	32 ± 8.1	44 ± 3.0	65 ± 4.7	67 ± 7.8
As	7.4 ± 1.8	3.8 ± 0.44	2.4 ± 0.34	0.89 ± 0.11	13 ± 3.2	8.4 ± 1.5	6.3 ± 1.1	4.004 ± 0.721
Se	1.5 ± 0.20	0.81 ± 0.066	0.98 ± 0.097	1.0 ± 0.095	1.3 ± 0.16	1.1 ± 0.099	1.0 ± 0.067	1.428 ± 0.095
Cd	0.002 ± 0.001	0.004 ± 0.001	0.010 ± 0.002	0.004 ± 0.001	0.001 ± 0.0001	0.003 ± 0.002	0.003 ± 0.001	0.004 ± 0.001
Pb	0.037 ± 0.008	0.026 ± 0.003	0.055 ± 0.004	0.034 ± 0.005	0.014 ± 0.004	0.011 ± 0.001	0.018 ± 0.004	0.016 ± 0.002

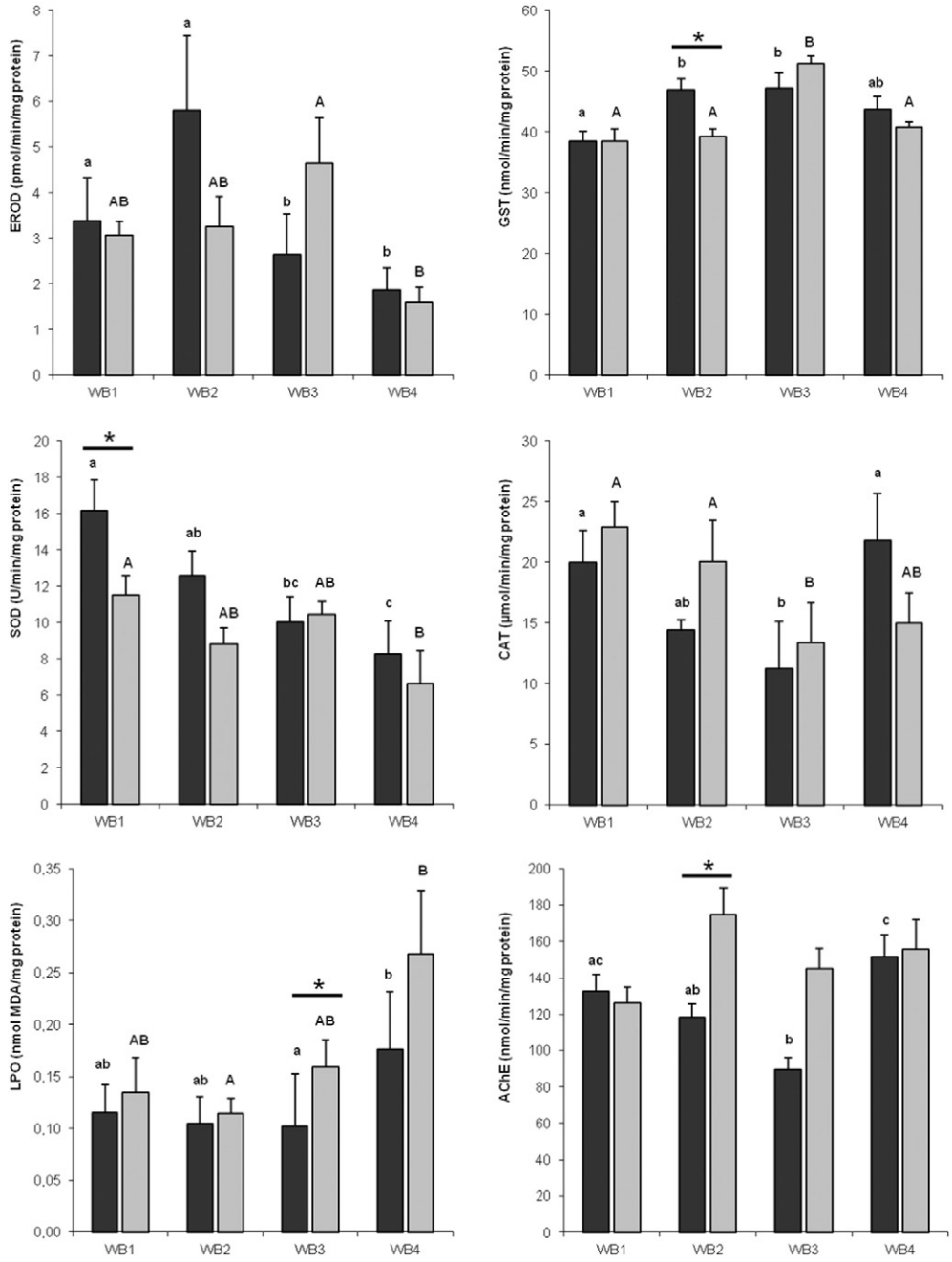

Fig. 2. Graphs represent the phases I and II biotransformation enzymes (EROD and GST), antioxidant defenses (CAT and SOD), lipid peroxidation (LPO) and cholinergic neurotransmission in brain (AChE). Bars represent mean values by station (■ — May; □ — November). Error bars represent the Standard Error of the mean. Different letters represent significant differences between stations (lowercase — May; uppercase — November), $p < 0.05$; Asterisk/bar means significant differences between campaigns ($p < 0.05$).

activities (13.37 ± 3.30 µmol/min/mg protein) also had the lower values, significantly different than WB1 and WB2 (22.91 ± 2.10 and 20.04 ± 3.42 µmol/min/mg protein, respectively, $p < 0.05$).

3.7. Neurotransmission (AChE)

Fig. 2 displays the results obtained for AChE activity; values ranged from 89.57 ± 6.50 nmol/min/mg protein in WB3 in May to 174.90 ± 14.69 nmol/min/mg protein in WB2 in November. Brain AChE activity in May was lower in WB3, with significant differences to WB1 and WB4 (132.52 ± 9.43 and 151.78 ± 11.80 nmol/min/mg protein, respectively, $p < 0.05$). Significant differences were also recorded between WB2 (118.09 ± 7.57 nmol/min/mg protein) and WB4 (Fig. 3). In November no significant geographical differences were found. Seasonal differences were found in WB2 ($p < 0.05$), the higher values were in November.

3.8. Lipid peroxidation (LPO)

Lipid peroxidation was generally low; the results are represented in Fig. 2 and Supplementary Table 5. LPO values ranged from 0.103 ± 0.050 nmol MDA/mg protein in WB3 in May to 0.268 ± 0.062 nmol MDA/mg protein in WB4 in November. In May peroxidation levels were similar in the first three water bodies (WB1 to WB3) and higher in WB4. Significant differences were observed between WB3 and WB4 (0.103 ± 0.050 and 0.176 ± 0.056 nmol/MDA/mg protein, respectively, $p < 0.05$). In November we could notice that the LPO levels were higher, with significant seasonal variation in WB3. On this campaign the LPO

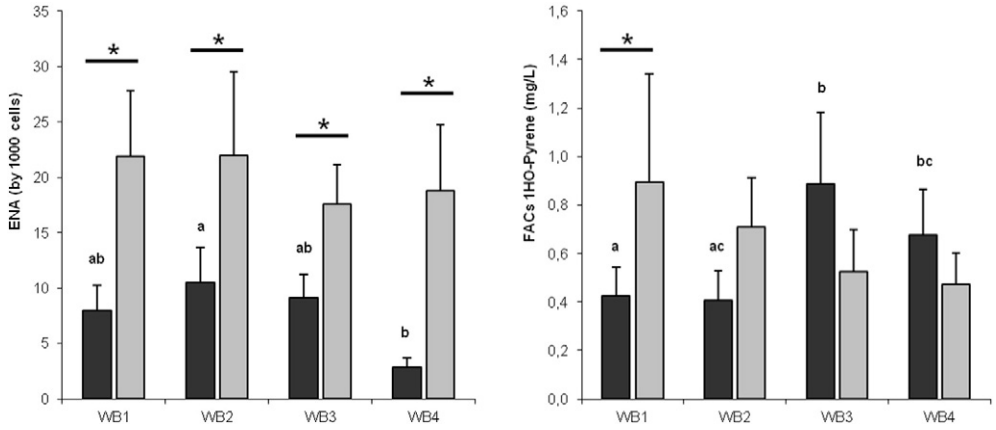

Fig. 3. Results from fluorescent aromatic compounds, 1-hidroxypirene (FAC) and genotoxic effects, evaluated as erythrocytes nuclear abnormalities (ENA). Bars represent mean values by station (■ — May; □ — November). Error bars represent the Standard Error of the mean. Different letters represent significant differences between stations (lowercase — May; uppercase — November), $p < 0.05$; Asterisk/bar means significant differences between campaigns ($p < 0.05$).

levels in WB4 were significantly higher than in WB2 (0.268 ± 0.062 and 0.115 ± 0.015 nmol/MDA mg protein, respectively, $p < 0.05$).

3.9. Genotoxicity (ENA)

Genotoxic effects, evaluated as ENA frequency in mature erythrocytes are displayed in Fig. 3 and Supplementary Table 5. ENA values ranged from 2.83 ± 0.79 abnormalities by 1000 cells in WB4 in May to 21.96 ± 7.59 abnormalities by 1000 cells in WB2 in November. In May the ENA values were higher in the first three water bodies (WB1 to WB3), with WB2 showing significant differences to WB4 (10.50 ± 3.21 and 2.83 ± 0.79 abnormalities by 1000 cells, respectively, $p < 0.05$). In November the ENA levels showed a significant increment in all water bodies; WB1 and WB4 values were significantly higher than in May ($p < 0.05$). However, on this campaign no significant geographical differences were reported.

3.10. Bile metabolites (FAC)

The FAC concentrations in bile (1-Hidroxipyrene) were very low along the study area, ranging from 0.41 ± 0.12 mg/L in WB2 in May to 0.90 ± 0.45 mg/L in WB1 in November. In May the river mouth areas showed lower values than the upriver areas. The WB3 values (0.89 ± 0.30 mg/L) were significantly higher than in WB1 and WB2 (0.43 ± 0.12 and 0.41 ± 0.12 mg/L, $p < 0.05$), as it is depicted in Fig. 3 and Supplementary Table 5. In November the highest values were reported in the river mouth, however, no significant differences were found along the estuary. Seasonal differences were found in WB1, with higher levels in November ($p < 0.05$).

3.11. PCA analyses

In the PCA carried out on liver measurements the components extracted expressed 79% of the total variability observed in the data. The first two summarized a representative amount (42%) of the total inertia. Hence, PCA interpretation was based on these two PCs (Fig. 4 and Supplementary Tables 7 and 8). PC1 established a gradient of seasonality, opposing mainly the May to the November campaign. It is mainly linked to several metals, neuromotor transmission and DNA damage. Accumulation of Ni, Pb and Cr ($r > 0.8$, $p < 0.001$), as well as Temperature ($r > 0.7$, $p < 0.001$), show high positive correlation to the axis. Cu, As and Zn ($r > 0.7$; $p < 0.001$), and to a slightly less extent the levels of AChE and ENA ($r > 0.5$; $p < 0.001$) are negatively correlated with this axis. In particular, fish from WB1, WB2 and WB3 collected during November tend to exhibit higher than average levels of these biomarkers. PC2 established a geographical gradient opposing downstream stations (WB1 and WB2; $p < 0.001$) to upstream stations (WB3 and WB4; $p < 0.001$). The component is highly associated to biotransformation and antioxidant defenses as well as to the accumulation of some metals. The elements Se and V were positively correlated to PC2 ($r > 0.8$;

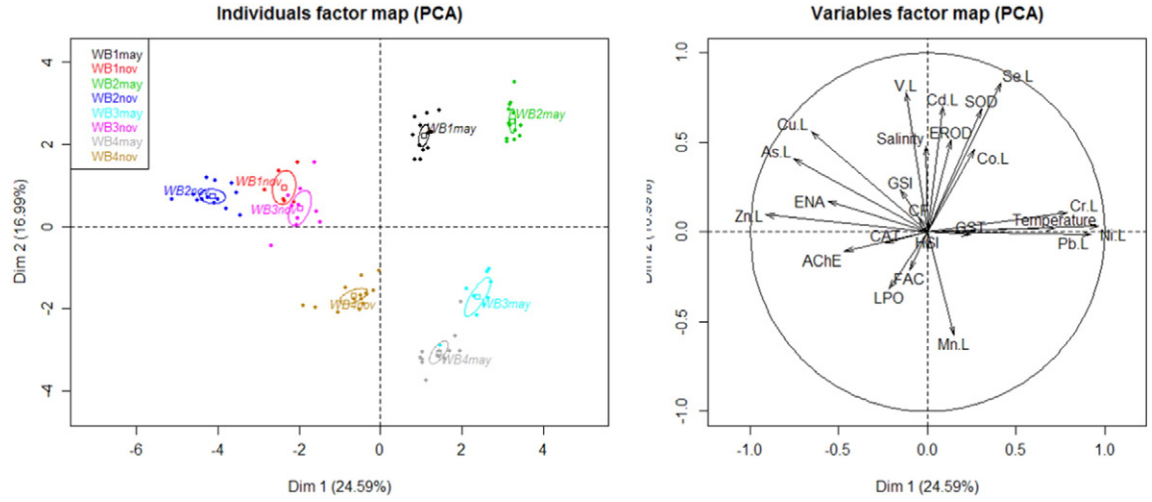

Fig. 4. Results of the PCA carried out with the liver measurements of accumulated metals and biochemical determinations, biometrics and abiotic factors.

$p < 0.001$), SOD activity and Cd ($r > 0.7$; $p < 0.001$), EROD activity and Cu ($r > 0.5$; $p < 0.001$). As, Co and Salinity also showed relevant significant positive correlations with this axis ($r > 0.40$; $p < 0.001$). Fish from WB1 and WB2 tend to have higher concentrations of Se, Cd, V, Co and Cu in the liver and higher activities of the biotransformation and antioxidant enzymes. Those from WB3 and WB4 tend to exhibit lower levels of these parameters and higher accumulation of Mn.

4. Discussion

The European Commission aims at reaching good ecological and chemical quality status for all European inland, transitional and coastal water bodies by 2015. In order to achieve this, the WFD is a key legal instrument that has been adopted by all member states. It sets the need to consider both chemical and ecological quality criteria in the water management context. Here we aimed at applying biomarkers as a set of tools to bridge the chemical and ecological approach and provide a holistic view of the monitoring concept in transitional waters. The present results in Minho transitional waters, points both to a low level of contaminants in sediments and biota and a low level of induction of biomarkers.

The presence of contaminants in sediments and flounders from Minho provides a weak signal of anthropogenic impact. Retention of trace elements in the bottom is low partially because sediments are composed by coarser particles to which trace elements have low affinity (Eggleton and Thomas, 2004). Accumulation of trace elements in fish is organ-specific. Essential elements such as Fe, Zn, Cu, Mn, Co and Se show distinct affinities for the tissues where they play their main metabolic roles (Monteiro et al., 2009). The low concentrations observed in muscle are of less relevance. Even muscle of fish from metal-contaminated sites shows generally low values relatively to other organs such as the liver (Jezierska and Witeska, 2006; Vinodhini and Narayanan, 2008). In the present study, both muscle and liver of flounders were analyzed and the results were in accordance with the previous claims, showing higher metal concentration in the liver. Trace elements registered both in sediments and tissues of flounders captured in water bodies of Minho were low with respect to the reported concentrations in other organisms from estuaries in the region (Gravato et al., 2010; Rodrigues et al., 2014). Also, organic pollutants presented low concentrations compared to other estuaries in southern Europe, such as Douro and Lima (Ferreira et al., 2004, 2005b; Gravato et al., 2010). The low values of 1-Hydroxypyrene, considered as a biomarker for total PAH metabolism (van der Oost et al., 2003), in *P. flesus* bile were in line with other considered low impacted reference sites for this biomarker (Santos et al., 2010), and are consistent with the residual PAH values present in sediments. Hence, these results indicate that the Minho river estuary is a low impacted area in terms of priority organic and inorganic contaminants.

Biomarkers reflecting the impact of all the stressors to which organisms may be exposed, provide direct evidence of alterations occurred in the ecosystem due to environmental pollution. They integrate variation on water quality, chemical pollution and natural stressors, allowing for a better understanding of the potential effects and actual toxicities (Zhou et al., 2008). As previously referred, the ability for distinguishing the biomarkers natural variability from pollution events is crucial on the application of these tools, so the sampling strategy is of main importance for the success of this approach (Kopecka and Pempkowiak, 2008; Chapman et al., 2013). Given this, the sampling strategy tried to narrow the size and age range of analyzed individuals; only juvenile animals were considered (mainly 0-, 1-year groups) in order to establish a uniform criteria throughout the study area and to avoid size effects on biochemical responses. Even though seasonal and spatial variability were found within the sample, both the CF and the GSI showed no significant variation among sites, which indicates similar physiological states. The Minho river estuary is considered a nursery area for the European flounder and the species distribution along all the study area allows the use of this species as a bioindicator/sentinel species. Also, younger animals tend to be more frequent in upriver areas, increasing in age/size on the estuary mouth (Freitas et al., 2009; Morais et al., 2011; Souza et al., 2013). In November animals were generally larger than in May on all sampling sites, reflecting the reproductive and estuary colonization periods of the species, the 0-juveniles preferentially settle in the upstream areas migrating downwards as they grow, tending to leave the estuary in the adulthood (Souza et al., 2013). Those biological factors changed seasonally across the study area, thus reinforcing the need to account for this influence in monitoring programs. However, juvenile animals were present in all the study area and in both seasons, showing comparable biomarker responses, allowing a basal characterization for the different areas and seasons.

On a monitoring program aiming to establish the status of water bodies, biomarkers are considered integrative tools between chemical monitoring and their impacts on aquatic organisms. In the present study a set of biomarkers were used to assess the quality of the four water bodies.

CYP1A induction, measured by hepatic EROD activity levels in flounder sampled in the four water bodies, showed in general upriver areas with lower activity values and intermediate areas with higher variability. However, no significant induction able to cause biological effects was observed. Several studies reported EROD activity to be well correlated with contaminant gradients in areas that are influenced by river outflows (Stegeman et al., 1988; Eggens et al., 1995a; Eggens et al., 1995b) or between river downstream stations and upstream stations (Flammarion et al., 2002). Usually CYP1A plays a particular role in the metabolism of a large number of compounds, such as PCB and PAH that are relevant aquatic contaminants. In Douro estuary the increased EROD activities found in eel, mullet and flounder are in good agreement with the presence of these pollutants (Ferreira et al., 2004, 2006b). In the present study, although differences were observed in levels of this enzyme between water bodies and seasons, they are always low as compared with contaminated rivers or estuaries (Napierska and Podolska, 2005; Ferreira et al., 2010; Maceda-Veiga et al., 2012) and very close to the levels found in the sentinel species *Lipophrys pholis* (10 pmol/min/mg protein) sampled in the south of the Portuguese coast with low anthropogenic contamination (Lima et al., 2008; Santos et al., 2010). The variation on the levels of this enzymatic activity attributed to the reproductive cycle (Kopecka and Pempkowiak, 2008), is not considered as only juvenile flounders were used in this study. Furthermore, liver GST, an enzyme also involved in the detoxification of contaminants, including PCB and PAH compounds (Van Der Oost et al., 1996; Ferreira et al., 2010) showed the same pattern of that observed for EROD activity. Although some seasonal variability was found in the different water bodies, this may be attributed to seasonal fluctuations of environmental factors rather than to a relevant concentration of contaminants, due to the low magnitude of the responses obtained for the biomarkers tested.

The presence of oxidative stress in flounders was monitored by the determination of the levels of the antioxidant enzymes SOD and CAT as well as LPO. The antioxidant mechanisms related with oxidative stress have gained considerable interest in the field of ecotoxicology. Antioxidant enzymes are considered as sensitive biomarkers for environmental stress monitoring before hazardous effects occur in fish and are important parameters for testing the effects of toxicants in the water (Atli and Canli, 2010). Animals can adapt to low pollution conditions and, under these circumstances, seasonal factors might affect biomarker responses to a greater extent than pollution variations (Stoliar and Lushchak, 2012). However, in our study, despite some seasonality observed, it should be noted that the parameters assessed provided a clear structuring of the water bodies, thus allowing their baseline characterization. Antioxidant enzymes had seasonal and spatial differences, SOD and CAT activities were in general higher in the river mouth areas. LPO showed the inverse profile of antioxidant enzymes;

this could reflect a relative compensation by the higher enzymatic activities found for SOD and CAT. Nevertheless, the LPO values were much lower compared than those of previous studies in contaminated areas. Results presented in this work could reflect the organisms' normal physiological activity, providing basal values for this biomarker. It is known that among other pollutants, metals are oxidative stress inducers (Ferreira et al., 2005b). Several elements (i.e. Cr, Zn, Ni, Cd, Mn, V and Se) were correlated with antioxidant enzymes (Supplementary Table 6), most of them with important biological functions for the organisms, yet all could be oxidative stress inducers if the correct balance is altered (Atli and Canli, 2010). Selenium, an element known by its antioxidant properties, was highly correlated with SOD and presented negative correlation with LPO (Supplementary Table 6). This element influences the response pattern of the SOD–CAT system in animals (Monteiro et al., 2009). In the presence of low H_2O_2 levels, organic peroxides are the preferred substrate for GPx and at high H_2O_2 concentrations they are metabolized by CAT (Yu, 1994). In the present study no significant activation of antioxidant enzymes was reported, so presumably low values of H_2O_2 were present, which is not enough to cause significant CAT activation. Evidence thus support the good physiological condition of the organisms. Furthermore, PCA associated SOD activity with several essential metals (V, Co and Se), Cd and EROD. The phase I detoxification can produce reactive metabolites that could be influencing SOD activity. However, in the considered levels significant biological effects are not expectable, providing a basal characterization of these biomarkers for this species in a juvenile state.

For the neurotransmission evaluation, AChE activity in the brain was measured. This biomarker is usually associated with organophosphate and carbamates exposure, though various biotic and abiotic factors are known to affect the responses of this biomarker (Nunes, 2011). Here we observed significant correlations with the weight and length, but those were not the only preponderant factors explaining the seasonal or spatial differences found. Statistically significant differences among sites and studied seasons were found after inclusion of weight/length as covariate in an analysis of covariance. This suggests that other spatial and seasonal factors had also relevant influence. Those factors could be anticholinesterase agents as organophosphate and carbamate insecticides used in crop fields in the vicinity of the estuary. Also, metals and PAH may inhibit AChE in feral fish (Guimarães et al., 2009, 2012). Here, the lowest values were found in WB3 station in May. This water body is more affected by runoff than other downstream areas of the estuary, though levels of contaminants found in the present study were generally very low. Other studies found high seasonal variations for this enzymatic activity without fitness reduction for the species (Solé et al., 2006), or relating those to environmental condition variations; bottom water temperatures or physiological activity of fish (Kopecka et al., 2006). In this case, no inhibition of AChE is evident when compared to impacted water bodies (Quintaneiro et al., 2006; Solé et al., 2008; Guimarães et al., 2009; Rodrigues et al., 2014), which usually present higher inhibition profiles. The results, thus, support the good environmental condition of Minho estuarine waters in terms of the presence of anticholinergic agents.

Finally, exposure to genotoxic agents was evaluated using the presence of ENA. Cytogenetic assays, like ENA, are sensitive nonspecific indicators for mutagenic damage, which have shown promising results in laboratory experiments (Pacheco and Santos, 1998; van der Oost et al., 2003; Micael et al., 2007; Oliveira et al., 2007) and are also currently used in monitoring situations (Ergene et al., 2007; Santos et al., 2010). Available data from other studies indicate that the increase of ENA frequency may be associated with a set of sub-lethal effects and this technique is one of the most well accepted approaches to evaluate exposure to genotoxic agents (Ergene et al., 2007; Micael et al., 2007). Species from relatively clean areas show background ENA values within the range of 3–20‰ (Micael et al., 2007; Oliveira et al., 2007; Santos et al., 2010), which is similar to the levels observed in the study area. Although we could notice an increase of ENA on all the sampling stations from May to November, the observed values stand within the normal range. Galindo and Moreira (2009) also found a clear seasonal variation, namely an increase in ENA in the rainy months; the precipitation increases water runoff that promote chemical drainage into streams or rivers. As such, this may also explain the observed increase in ENA frequency in Minho.

In order to integrate the different variables, a PCA analysis was carried out including abiotic factors, trace elements in liver and biomarker responses. Overall, metal accumulation and biomarkers reflect the main structuring of the data, with clear seasonal structure resulting in the separation of the May and November campaigns. Besides, an evident geographical variation along the water bodies was found. In May higher levels of Cr, Ni and Pb were found in all water bodies. Oxidative stress and biotransformation enzymes tended to show higher values (SOD and EROD), contrasting with the lower values of FACs and LPO; higher levels of V, Cd, Se and Co in WB1 and WB2 were present. In November, the levels of Cu, As and Zn were higher contrasting with the lower levels of Cr, Ni and Pb; ENA and AChE activities were higher on WB1 to WB3. The geographical structure verified along the study area distinguished mainly the downstream stations WB1 and WB2 from the upriver stations WB3 and WB4.

The current evaluation indicates that contamination of Minho estuary by trace elements, PAH and PCB is low, which is in line with previous studies in this area (Guimarães et al., 2009; Gravato et al., 2010; Rodrigues et al., 2014). The magnitude of the biomarkers response was low in the surveyed sites and seasons, which is in accordance with a low contamination profile. Despite this, differences in biomarkers found in different sites seem to indicate their sensitivity to distinguish water bodies under low contamination. These results are in agreement with spatial and seasonal variations previously found for several biomarkers and environmental contaminants in the Minho estuary (Gravato et al., 2008; Guimarães et al., 2009, 2012).

5. Conclusions

In conclusion, the data supports the use of juvenile flounder as sentinel species for monitoring purposes. The species sensitivity on this development stage, the distribution along the study estuary and the coherent results support this claim. Given the broad geographical distribution of this species, the present study favors its use in monitoring programs in other geographical areas.

Chemical determinations were within the range for low impacted areas. The magnitude of the biomarkers response was similar between areas and seasons, and also sensitive enough to characterize the different water bodies, supporting both the use of the applied set of tools and the selected key species in a juvenile developmental stage on monitoring programs within the WFD context.

Also, this study sets the foundations for future monitoring campaigns, establishing basal values for the considered biomarkers in juvenile flounders. Historical records provide valuable information to understand the ecosystem evolution. Though, coordinated campaigns for this area are lacking and the available data is dispersed and poorly accessible for these purposes.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.scitotenv.2015.08.113.

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