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RESEARCH ARTICLE

Health status of *Pomatoschistus microps* populations in relation to pollution and natural stressors: implications for ecological risk assessment

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Abstract

Effects induced on wild populations by recurrent environmental contamination may difficult the ecological risk assessment of punctual pollution events such as oil spills. Here, the issue was addressed by comparing the health status of *Pomatoschistus microps* populations from four NW Iberian estuaries, using an integrated chemical–biological monitoring. Despite high seasonal variability, the parameters measured discriminated estuaries with different contamination levels and associated biological effects with chemical and abiotic stress. The decreased health status of fish from polluted sites strengthens the need of considering pollution-induced background effects and seasonal variability when assessing impacts and risks of oil and other chemical spills.

Keywords: Common goby, biomarkers, polycyclic aromatic hydrocarbons, metals, integrated chemical–biological monitoring, redundancy analysis

Introduction

When assessing the ecological risks of punctual pollution events, such as chemical spills in estuaries and coasts under anthropogenic stress, the presence of background contamination may act as a chief confounding factor. Separating negative effects induced by these events from those induced by a recurrently contaminated setting is a difficult task. The task becomes even more complex when the levels of background contamination are relatively low. In such cases, their effects on native populations may not be obvious yet at the time of the spill, though the populations might already have reduced health status. Another relevant confounding factor is natural variability (e.g. seasonal) caused by both abiotic and biotic variables, which also needs to be separated from the effects induced by chemical spills. These are major challenges requiring more research to improve the reliability and predictability of our models for ecological risk assessment. A way of addressing these issues is to compare contamination levels and effects induced by the chemical spill with data from previous long-term monitoring studies of the impacted area. Indeed, comparative approaches integrating the determination of chemical and biological parameters in populations of keystone species are particularly relevant for ecological risk assessment. Besides contributing to discriminate before- and after-impact effects, and natural variability, from changes induced by exposure to the spilled chemicals, they provide information at the population, community and/or ecosystem levels.

Among chemical spillages, oil spills in the marine environment are of major concern as they usually have great ecological and economic negative impacts.

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Immediate effects of oil spills and associated black tides, such as the incorporation of oil carbon into the planktonic food web (Graham et al. 2010), were evidenced once again by the recent accident in the Gulf of Mexico (April 2010). Long-term effects in exposed populations also occur as shown by recent studies on spills that happened several years ago (Esler et al. 2010).

Oils are complex mixtures of chemicals with variable composition, but including always several metals and polycyclic aromatic hydrocarbons (PAHs) as important components. Metals are persistent contaminants that can interact with several molecular targets, inducing a variety of toxic effects. Increased mortality (Giardina et al. 2009), growth delay (Hansen et al. 2004) and decreased reproduction (Dietrich et al. 2010), among other deleterious effects, have been reported in marine populations exposed to metals. PAHs are volatile compounds and part of them is rapidly lost after an oil spill. However, water dissolution occurs simultaneously (Díez et al. 2007), as well as processes such as the incorporation of oil and/or oil components in sediments when the spilled oil is a heavy one. Marine organisms may therefore be exposed to high PAH concentrations, at least for short periods of time. In fish, PAHs are rapidly metabolized by the cytochrome P450 system and subsequent pathways including, hydrolysis by epoxide hydrolase. However, some of the metabolites generated in this process are resistant to epoxide hydrolase action and able to cause DNA damage and carcinogenic effects (Parkinson & Ogilvie 2008). Reported effects in marine populations exposed to PAHs include increased mortality (Billiard et al. 2008), hepatic and carcinogenic lesions (Collier & Varanasi 1991; Hawkins et al. 1991), developmental abnormalities, decreased swimming ability (Ostrander et al. 1990), and reproductive alterations (White et al. 1999; Monteverdi & Di Giulio 2000). The NW Iberian coast is at high risk of impact by oil spills due to its proximity to main maritime traffic routes, adverse sea conditions especially in the autumn and winter, and particular hydrodynamic characteristics (Lima et al. 2007). The existence of important harbors and oil-related activities (e.g. oil transformation and storage) further makes this a hotspot area for oil-spills occurrence. This coast has several estuaries of high conservational, economical and social value (e.g. the estuary of Minho River that is included in NATURA 2000) which serve as vital nursery grounds for estuarine and marine species, including the common goby, and provide important ecosystem services and goods.

Gathering chemical- and biological-monitoring data on keystone species of such ecosystems is therefore essential to maintain or improve water quality in compliance with the objectives of the Water Framework Directive and the Marine Strategy Framework Directive. Likewise, these data are vital as before-impact information for the ecological risk assessment of oil and other chemical spills.

The main aims of this study were to investigate the health status of wild populations of the common goby (*Pomatoschistus microps*) from NW Iberian estuaries in relation to long-term environmental contamination and variation of natural stressors. We further discuss the implications of these potential confounding factors for the ecological risk assessment of oil spills. *P. microps* was chosen because it is a relevant intermediate predator and prey of European estuaries. It is a convenient sentinel species (Monteiro et al. 2007) for which a wealth of ecological and toxicological data is available in the literature (Salgado et al. 2004; Berrebi et al. 2005; Monteiro et al. 2005; Monteiro et al. 2006; Dolbeth et al. 2007; Vieira et al. 2008, 2009; Fonseca et al. 2011).

Methods

General

The study was conducted in 2006, in five sampling sites located in the estuaries of rivers Minho, Lima, Cávado and Douro (NW Portugal; Figure 1). Fifteen water abiotic variables were measured monthly; eight metals and 16 PAHs in sediments and fish samples, and 10 fish biomarkers, were measured seasonally. In total, 6,860 determinations were made: 2,700 for water variables, 2,880 for chemical analysis in sediments and fish, and 1,280 for biomarkers.

Estuaries and sampling sites

Minho and Lima are international rivers draining hydrological basins of 10,080 km^2 and 2,250 km^2, respectively (Bettencourt et al. 2004). The Minho estuary is considered as low impacted (Ferreira et al. 2003) and was used as reference in previous ecotoxicological studies (Monteiro et al. 2007; Guimarães et al. 2009), despite having a few localized sources of pollution. Two sampling sites were selected in this estuary: R1 (41° 52′ 26.33″ N; 8° 51′ 37.62″ W) located upstream, and R2 at the mouth of the estuary (41° 53′ 26.33″ N; 8° 49′ 30.98″ W; Figure 1). The Lima estuary receives contamination from untreated effluents of urban and industrial origin. It also has a harbor, and a shipyard at its mouth, and a paper mill upstream. Although low concentrations of organochlorine pesticides were found in this estuary (Carvalho et al., 2009), the main source of contamination is related to oil transport, storage and local use. The sampling site, S1 (41° 40′ 59.40″ N; 8° 49′ 43.32″ W), was near the harbor. The Cávado River drains a basin of approximately 1,600 km^2 (Bettencourt et al. 2004) and receives inputs from industrial and urban sources. The sampling site, S2 (41° 30′ 54.07″ N; 8° 46′ 25.58″ W), was downstream, close to the mouth of the estuary, near a shipyard and a nautical recreation area. The Douro river drains the largest watershed of the Iberian Peninsula (about 98,000 km^2; Bettencourt et al. 2004). Urban and industrial effluents, and residuals from agriculture activities, are discharged into its tributaries and the estuary, some of them still untreated. It is polluted by metals, pesticides (including dichlorodiphenyltrichloroethane and related compounds), polychlorinated

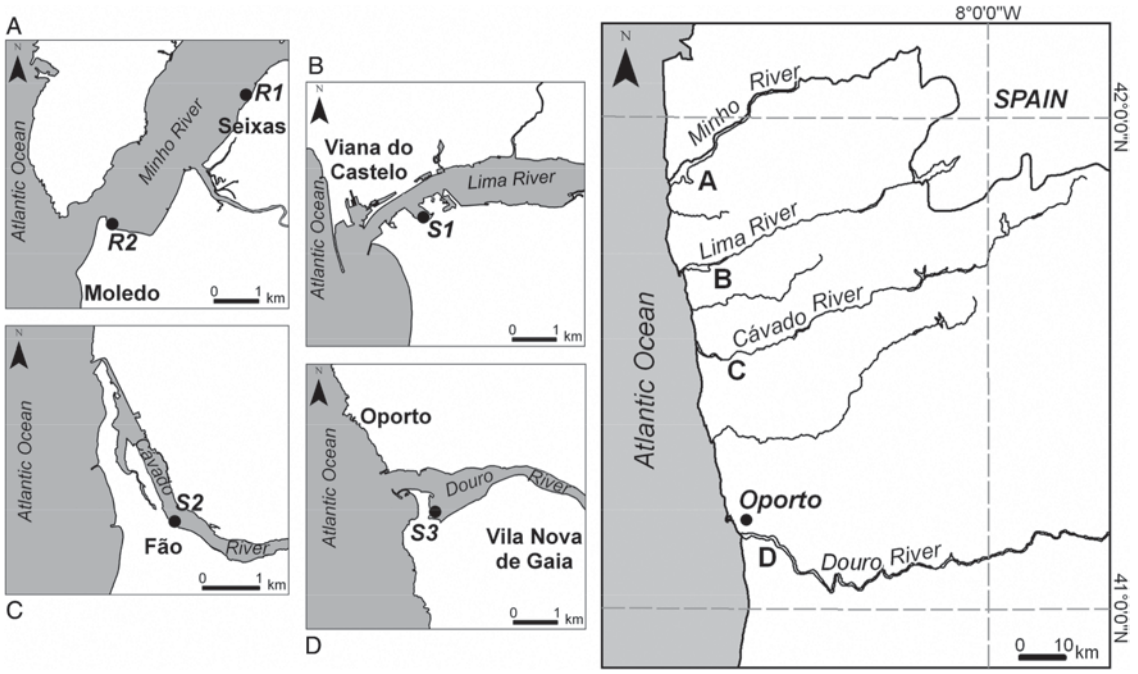

Figure 1. Location of the five sampling sites, including two reference stations (R1 and R2) at the estuary of (A) Minho River, and three impacted sites at the estuaries of rivers (B) Lima (S1), (C) Cávado (S2), and (D) Douro (S3).

biphenyls (PCBs), organotins and other endocrine disrupting compounds (Ferreira et al. 2004, 2006; Almeida et al. 2007; Ribeiro et al. 2009). The sampling site, S3 (41° 08′ 16.64″ N; 8° 39′44.16″ W) was located at the mouth of the estuary.

Sediments and fish sampling

Sediments for chemical analyses of metals and PAHs were collected (in triplicate) at each sampling site, from the top layer (1-cm depth) as indicated previously (Rubal et al. 2009). All samples were kept at –20°C until analysis.

P. microps juveniles 2–4 cm long were captured at low tide using hand operated nets. Specimens were immediately transported to the laboratory where they were ice-anaesthetized, measured (body length, cm) and weighed (body weight, g). Animals were then sacrificed by decapitation. For the quantification of metals and PAHs, three independent pools of fish whole body (10 g) were prepared per sampling site and season. Samples were kept at –20°C until analysis. For biomarkers, seven replicate samples (pools of selected organs/tissues from different fish) were prepared per sampling site and season, except for Douro estuary. Here, despite the higher capture effort, only four replicates could be obtained. These samples were stored at –80°C until analysis.

Sampling and sample treatment were conducted in compliance with the ethical guidelines of the European Union Council (86/609/EEC) and the Portuguese Ministry of Agriculture, Rural Development and Fisheries (General Directorate of Veterinary Medicine, Portaria n° 1005/92) for the protection of animals used for experimental and other scientific purposes.

Water variables

Temperature, salinity, conductivity, dissolved oxygen and pH were measured monthly at low tide with a multiparameter probe (Hydrolab DS5X). At the same time, subsurface water samples were collected into polyethylene-terephthalate bottles and stored at –20°C for later determination of water hardness, turbidity, and concentrations of ammonium, nitrates, nitrites, phosphates, iron, phenol and silicate using colorimetric methods in a Palintest 7000 interface photometer. Chlorophyll *a* was determined seasonally according to Strickland and Parsons (1972). The water-quality status was evaluated according to the criteria for assessment of nutrient levels in transitional, coastal and marine waters developed by Crouzet et al. (1999). These were based on the concentrations of nitrogen and phosphorus as key plant nutrients that can cause eutrophication. Based on the annual means of nitrates + nitrites, quality status is considered good for concentrations <6.5 μM, fair for values ranging between 6.5 and 9.0 μM, poor for values ranging between 9.0 and 16.0 μM, and bad for values >16 μM. Based on the annual means of phosphorus, quality status is considered good for concentrations < 0.5 μM, fair for values ranging between 0.5 and 0.7 μM, poor for values ranging between 0.7 and 1.1 μM, and bad for values >1.1 μM.

Chemical analyses

Total-recoverable metal contents were determined by atomic absorption spectrophotometry (SpectrAA 220FS, Varian), either with flame or electrothermal atomization (GTA 110 unit), depending on the concentration of metals in solution. Aqueous matched standards were used for external calibrations. Eight metals were analyzed

in each compartment: cadmium, chromium, copper, mercury, nickel, lead, zinc, and vanadium. Control quality of sediment samples was checked using reference material SRM 2702 (National Institute of Standards and Technology, USA). Control quality of fish samples was checked using reference materials Dorm-2 and Tort-2 (National Research Council of Canada), and SRM 2976 (National Institute of Standards and Technology, USA).

PAHs were determined by gas chromatography-mass spectrometry (GC-MS) using an autosampler and a DB-5 column. Identification of PAH compounds was based on the comparison of their GC-retention times and mass spectrum with appropriate individual standards. Sixteen PAHs were analyzed: acenaphethylene, fluorene, phenanthrene, anthracene, fluoranthene, pyrene, benzo[*a*]anthracene, chrysene, benzo[*b*]fluoranthene, benzo[*k*]fluoranthene, benzo[*e*]pyrene, benzo[*a*]pyrene, perylene, indeno[*1,2,3-cd*]pyrene, dibenzo[*a,h*]anthracene, and benzo[*g,h,i*]perylene. Reference materials used for quality control were: SRM 1941b (National Institute of Standards and Technology, USA) for sediments; NIST and SRM 2977 for fish samples.

Biomarkers

The 10 biomarkers assessed were as follows: Fulton's condition index (K) and the liver somatic index (LSI; Lloret et al. 2002) indicative of general fish condition; acetylcholinesterase activity (AChE) in head tissues, with a vital role in cholinergic neurotransmission; lactate dehydrogenase (LDH) activity in the muscle, involved in the anaerobic pathway of energy production; gill glutathione *S*-transferases (GST) activity that are involved in biotransformation and in the prevention of lipid peroxidation; catalase (CAT), superoxide dismutase (SOD), glutathione reductase (GR), and selenium dependent-glutathione peroxidase (GPx) activities in the liver as antioxidant defenses; and lipid peroxidation (LPO) levels in the liver as indicative of oxidative damage.

AChE activity was determined by the Ellman's method (Ellman et al. 1961) adapted to microplate (Guilhermino et al. 1996). Determination of LDH was based on the method of Vassault (1983) adapted to microplate (Diamantino et al. 2001). GST activity was assayed through the method of Habig et al. (1974), adapted to microplate (Frasco & Guilhermino, 2002). CAT activity was measured according to Aebi (1984). GPx was assayed according to Flohé & Günzler (1984), adapted to microplate (Lima et al. 2007). GR determination followed the method of Carlberg and Mannervik (1975), adapted to microplate (Lima et al. 2007). SOD was assayed after the method of McCord and Fridovich (1969) adapted to microplate (Lima et al. 2007). LPO was determined according to Ohkawa (1979). The protein concentration in the samples was determined, in triplicate, by the Bradford method (Bradford, 1976) adapted to microplate (Guilhermino et al. 1996).

All chemicals used were of reagent grade and purchased from Sigma, except the protein-dye assay reagent that was purchased from Bio-Rad Laboratories, Inc. All microplate determinations were carried out in a BioTek Power Wave 340 spectrophotometer. CAT and LPO levels were determined in a JASCO V600 UV-VIS spectrophotometer.

Statistical analysis

Differences in water variables and the concentrations of metals and PAHs among sampling sites and among seasons were investigated by one-way analysis of variance (ANOVA) followed by the Tukey's multiple comparison test. Differences in biological variables were analyzed by two-way ANOVA with interaction, taking the sampling site and the season as sources of variation. Planned contrasts were used for pairwise comparisons. Assumptions of normality and homogeneity of variances were checked using the Kolmogorov-Smirnov and the Levene's tests, respectively. For each variable, values more than 3.0 standard deviations distant from their group mean were considered as outliers and removed from the analysis. When required the square root transformation was applied to the data to meet ANOVA assumptions. Values are reported as mean ± standard error (s.e.). All univariate tests were performed with SPSS 16.0.

Redundancy analysis (RDA) was used to investigate patterns of biological parameters and their environmental correlates, as previously described (Guimarães et al. 2009). Chemical analyses in sediments or water parameters were used as quantitative environmental variables. Sampling sites were included as nominal variables; seasons were used as supplementary variables. A model-based type of Monte Carlo permutation test was used (ter Braak & Šmilauer, 2002). All multivariate analyses were performed with Canoco for Windows 4.5.

In all statistical tests performed, the significance level was set at 0.05.

Results

Water variables

Significant differences among sampling sites were found for salinity and conductivity, the concentrations of nitrites, nitrates, phosphates and iron, and for water hardness (Tables 1 and A.1). On average, all contaminated sites showed iron concentrations higher than those of site R2 (reference estuary of Minho River), and water hardness higher than that of R1 and R2 (Table 1). S1 (Lima estuary) and S3 (Douro estuary) had higher salinity and conductivity, relatively to R1. S2 (Cávado estuary) showed higher concentrations of nitrites, nitrates and phosphates than both R1 and R2. The concentrations of nitrates + nitrites equaled 8.9 μM in R1, 8.8 μM in R2, 9.6 μM in S1, 18.9 μM in S2, and 11.3 μM in S3. The concentrations of phosphorous equaled 0.7 μM in R1, 0.6 μM in R2, 0.9 μM in S1, 2.4 μM in S2, and 1.4 μM in S3. Significant differences among seasons were found for temperature, pH, dissolved oxygen, nitrates, ammonium, phosphates, phenol, silicate, and turbidity (Tables 1 and A.1).

Concentrations of metals and PAHs in sediments

Significant differences among sites were found for cadmium, copper, nickel, lead, zinc, vanadium, and the total sum of metals (Tables 2 and B.1). For these, the lowest concentrations were found in R2. R1 showed concentrations of metals generally comparable to those of the contaminated sites and occasionally higher. S1 showed increased concentrations of cadmium, copper, lead and zinc, compared to R2. S2 showed significantly higher concentrations of copper, lead and zinc than R2. S3 showed metal concentrations comparable to those of R2 and significantly lower than those of R1. Significant seasonal variation was found for cadmium, chromium, copper and vanadium (Tables 2 and B.1).

Significant differences among sampling sites were also found for 11 of the 16 PAHs analyzed (Tables 3 and B.2). For these, the lowest concentrations were detected in R2. The concentrations found in R1 sediments were within the range of those found in contaminated sites. Additionally, S2 showed significantly higher concentrations of acenaphethylene, fluoranthene, pyrene, benzo[*a*]anthracene, chrysene, benzo[*b*]fluoranthene, benzo[*k*]fluoranthene, benzo[*a*]pyrene, perylene, and indeno[*1,2,3-cd*]pyrene, relatively to R2. S3 showed higher concentrations of fluoranthene, pyrene, benzo[*b*] fluoranthene, benzo[*a*]pyrene, and indeno[*1,2,3-cd*] pyrene, relatively to R2 (Table 2). Significant seasonal variation was found for 12 of the 16 measured PAHs (Table A.1). PAH concentrations were usually higher during the summer and/or the autumn and lower during the winter and/or the spring.

Concentrations of metals and PAHs in fish

Concentrations of metals in fish body were significantly higher in R1, S1, S2, and S3 than in R2, but generally lower or similar to those found in sediments (Tables 2 and B.1).

Table 1. Estuarine and seasonal variation of the water parameters assessed.

	Sampling site					Season			
	R1	R2	S1	S2	S3	Winter	Spring	Summer	Autumn
T	14.74	13.23	14.08	14.18	16.93	10.19^{a}	14.91^{b}	20.74^{c}	12.69^{ab}
	(1.31)	(1.37)	(1.31)	(1.13)	(1.49)	(0.58)	(0.7)	(0.62)	(0.64)
Sal	1.72^{a}	6.48^{ab}	15.54^{b}	5.11^{a}	9.89^{b}	4.37	5.89	10.23	10.5
	(0.87)	(2.14)	(2.22)	(2.42)	(2.1)	(1.19)	(1.71)	(2.47)	(2.59)
Cond	3.36^{a}	10.84^{ab}	25.49^{b}	9.17^{a}	16.41^{b}	7.45	9.88	17.35	17.53
	(1.36)	(3.47)	(3.64)	(4.09)	(3.42)	(1.9)	(2.72)	(3.99)	(4.27)
pH	7.86	8.01	8.31	7.87	7.93	7.60^{a}	7.98^{ab}	8.25^{b}	8.16^{ab}
	(0.12)	(0.2)	(0.18)	(0.14)	(0.18)	(0.15)	(0.19)	(0.09)	(0.07)
DO	8.94	8.87	9.22	8.87	10.27	13.22^{a}	7.34^{b}	6.35^{b}	10.03^{c}
	(1.03)	(0.79)	(0.62)	(0.6)	(1.39)	(0.63)	(0.28)	(0.15)	(0.55)
NO_2^-	0.01^{a}	0.01^{a}	0.01^{a}	0.04^{ab}	0.03^{ab}	0.02	0.03	0.01	0.02
	(0.00)	(0.00)	(0.00)	(0.01)	(0.00)	(0.00)	(0.01)	(0.0)	(0.00)
NO_3^-	0.55	0.54	0.59	1.16	0.69	0.96^{ac}	1.17^{a}	0.16^{b}	0.54^{c}
	(0.16)	(0.12)	(0.12)	(0.29)	(0.12)	(0.18)	(0.17)	(0.02)	(0.07)
NH_4^+	0.33	0.47	0.21	0.44	0.28	0.15^{a}	0.79^{b}	0.23^{ab}	0.21^{ab}
	(0.14)	(0.39)	(0.06)	(0.07)	(0.04)	(0.04)	(0.3)	(0.05)	(0.04)
PO_4^{3-}	0.07^{a}	0.06^{a}	0.09^{a}	0.23^{b}	0.13^{ab}	0.19^{a}	0.11^{ab}	0.07^{b}	0.09^{ab}
	(0.01)	(0.01)	(0.02)	(0.06)	(0.01)	(0.05)	(0.02)	(0.01)	(0.01)
Fe	0.04	0.02	0.05	0.06	0.07	0.08	0.04	0.03	0.04
	(0.01)	(0)	(0.02)	(0.01)	(0.02)	(0.01)	(0.02)	(0.01)	(0.01)
C_6H_5OH	0.03	0.04	0.02	0.07	0.02	0.06^{a}	0.06^{a}	0.01^{b}	0.02^{b}
	(0.01)	(0.03)	(0.01)	(0.01)	(0.01)	(0.02)	(0.01)	(0.00)	(0.00)
SiO_2	1.42	0.85	0.77	1.42	0.9	1.66^{a}	1.50^{a}	0.10^{b}	1.04^{a}
	(0.34)	(0.17)	(0.22)	(0.36)	(0.34)	(0.35)	(0.22)	(0.01)	(0.14)
$CaCO_3$	225^{a}	528^{b}	1101^{c}	733^{abc}	903^{bc}	601	865	543	781
	(55.71)	(109)	(143)	(158)	(153)	(134)	(148)	(120)	(138)
Turb	2.83	2.17	3.67	5.50	4.00	1.73^{a}	3.20^{a}	7.60^{b}	2.00^{a}
	(1.29)	(1.11)	(1.15)	(0.89)	(1.5)	(0.73)	(0.85)	(1.3)	(0.65)
Chla	0.19	0.02	0.08	0.04	0.13	0.18	0.1	0.06	0.02
	(0.05)	(0.00)	(0.03)	(0.01)	(0.12)	(0.09)	(0.06)	(0.03)	(0.01)

Note: Values represent the mean and corresponding standard error (within brackets) of temperature (T, °C), salinity (Sal, psu), conductivity (Cond, mS/cm), pH, dissolved oxygen (DO, mg/L), nitrites (NO_2^-, mg/L), nitrates (NO_3^-, mg/L), ammonium (NH_4^+, mg/L), phosphates (PO_4^{3-}, mg/L), iron (Fe, mg/L), phenol (C_6H_5OH, mg/L), silica (SiO_2, mg/L), water hardness ($CaCO_3$, mg/L), turbidity (Turb, FTU), and chlorophyll a (Chla, mg/g dw). R1 and R2, sampling sites in the Minho estuary; S1, S2, and S3, sampling sites in the Lima, Cávado, and Douro estuaries, respectively. Different letters identify significant differences among estuaries or seasons, as indicated by the Tukey's multiple comparison test.

Table 2. Concentrations of metals (μg/g dry weight) determined in samples of sediments and fish body collected seasonally at the five sampling sites.

	Sampling site					Season			
	R1	R2	S1	S2	S3	Winter	Spring	Summer	Autumn
Sediments									
Cd	0.03[a]	0.01[a]	0.06[b]	0.03[a]	0.03[a]	0.03	0.05	0.03	0.04
	(0.01)	(0.00)	(0.02)	(0.01)	(0.01)	(0.01)	(0.02)	(0.01)	(0.01)
Cr	10.88	6.04	11.56	9.33	6.79	4.60[a]	5.39[a]	9.49[b]	16.20[c]
	(3.01)	(2.40)	(3.32)	(3.82)	(1.96)	(1.00)	(1.80)	(1.26)	(2.09)
Cu	5.15[ac]	1.49[b]	4.42[ad]	7.69[c]	2.27[d]	3.47[ab]	3.68[ab]	3.40[a]	6.27[a]
	(0.65)	(0.09)	(0.52)	(2.33)	(0.36)	(0.72)	(0.90)	(0.73)	(2.30)
Hg	0.04	< 0.04	< 0.04	0.02	0.01	< 0.04	0.02	< 0.03	< 0.05
	(0.00)			(0.00)	(0.00)		(0.01)		
Ni	10.62[a]	2.69[b]	5.81[b]	2.96[b]	3.01[b]	4.24	6.86	3.64	5.33
	(3.16)	(0.24)	(0.25)	(0.95)	(0.47)	(0.82)	(3.39)	(1.22)	(0.85)
Pb	6.19[a]	1.89[b]	6.08[a]	9.36[c]	4.51[a]	4.83	4.81	5.77	7.02
	(0.92)	(0.25)	(0.36)	(1.74)	(0.26)	(1.14)	(0.99)	(0.94)	(2.11)
Zn	34.92[ad]	8.46[be]	28.44[cde]	31.33[d]	15.33[e]	24.00	23.05	29.27	19.85
	(4.09)	(3.66)	(3.49)	(5.68)	(2.93)	(3.85)	(6.47)	(5.48)	(8.11)
V	17.58[a]	3.61[b]	8.56[b]	11.03[b]	6.37[b]	8.03[a]	7.34[a]	5.67[a]	17.57[b]
	(8.04)	(1.10)	(1.06)	(2.57)	(2.00)	(0.96)	(1.86)	(1.36)	(6.30)
Σ	85.41[a]	24.19[b]	64.93[c]	71.75[ac]	38.32[b]	49.20	51.20	57.27	72.28
	(3.38)	(3.06)	(2.67)	(3.33)	(4.55)	(5.36)	(7.47)	(5.40)	(9.31)
Fish body									
Cd	0.03[a]	0.03[a]	0.01[b]	0.03[a]	0.02[ab]	0.02	0.02	0.03	0.02
	(0.00)	(0.01)	(0.00)	(0.01)	(0.00)	(0.00)	(0.00)	(0.01)	(0.00)
Cr	0.13	0.13	0.22	0.20	0.19	0.17[a]	0.12[a]	0.10[a]	0.30[b]
	(0.08)	(0.02)	(0.04)	(0.06)	(0.06)	(0.03)	(0.04)	(0.03)	(0.03)
Cu	2.77[a]	2.01[b]	1.86[b]	2.40[ab]	2.46[ab]	2.35	2.47	2.30	2.08
	(0.21)	(0.13)	(0.12)	(0.22)	(0.19)	(0.19)	(0.3)	(0.21)	(0.11)
Hg	0.09[ab]	0.03[a]	0.08[ab]	0.25[b]	0.09[ab]	0.09	0.06	0.09	0.22
	(0.02)	(0.00)	(0.02)	(0.13)	(0.00)	(0.02)	(0.01)	(0.03)	(0.14)
Ni	0.53	0.51	0.58	0.62	0.76	0.48[a]	0.40[a]	0.52[a]	1.00[b]
	(0.04)	(0.13)	(0.13)	(0.13)	(0.33)	(0.04)	(0.06)	(0.06)	(0.2)
Pb	0.26	0.17	1.75	0.29	0.41	0.26	1.52	0.21	0.32
	(0.09)	(0.07)	(1.47)	(0.05)	(0.12)	(0.03)	(1.16)	(0.05)	(0.11)
Zn	124[ac]	101[b]	111[abc]	124[c]	130[c]	118[ab]	108[a]	126[b]	120[ab]
	(7.76)	(5.34)	(6.04)	(6.16)	(5.51)	(7.83)	(6.98)	(7.39)	(3.85)
V	< 0.75	< 0.75	< 0.75	< 0.75	< 0.75	< 0.75	< 0.75	< 0.75	< 0.75
Σ	128[a]	104[b]	115[ab]	128[a]	133.34 [a]	121.10	112.06	129.38	124.27
	(4.80)	(3.94)	(4.08)	(5.02)	(4.26)	(5.26)	(4.31)	(5.12)	(3.27)

Note: Values represent the mean and corresponding standard error (within brackets). R1 and R2, sampling sites in the Minho estuary; S1, S2, and S3, sampling sites in the Lima, Cávado, and Douro estuaries, respectively. Different letters identify significant differences among estuaries or seasons, as indicated by the Tukey's multiple comparison test. Cd, cadmium; Cr, chromium; Cu, copper; Hg, mercury; Ni, nickel; Pb, lead; Zn, zinc; V, vanadium.

However, mercury and zinc levels were approximately 2 to 13 folds and 4 to 12 folds higher in fish than in sediments, respectively (Table 5). Concentrations of PAHs in the fish body were fairly comparable among sampling sites (Tables 4 and B.3). Benzo[*a*]pyrene, perylene, indeno[*1,2,3-cd*]pyrene, dibenzo[*a,h*]anthracene, and benzo[*g,h,i*]perylene levels were below the limits of detection in all the samples analyzed. Slightly higher concentrations in fish than in sediments were found for acenaphthylene at all sites but S2, and for fluorene at all contaminated sites. For the remaining PAHs, concentrations in the fish body were similar or lower than those found in sediments (Table 5).

Biological parameters

The two-way ANOVAs indicated significant main effects of the sampling site and the season on all the biological parameters (Tables 6 and C.1). Significant effects of the interaction between these two factors were also found, indicating that these variables were differently affected by seasonal factors in fish from the different sites (Table C.1). On average, the lowest values of the Fulton's Condition Index were found in fish from R1 during the winter and spring and in fish from R2 in all seasons but the summer. Significantly higher values were found in fish from S1 and S3 in the spring (relatively to R1 and R2) and in the

Table 3. Concentrations of PAHs (ng/g dry weight) determined in samples of sediments collected seasonally at the five sampling sites.

	Sampling site					Season			
	R1	R2	S1	S2	S3	Winter	Spring	Summer	Autumn
ANY	0.30^{ab}	0.24^{ab}	0.25^{a}	0.74^{b}	0.26^{a}	0.35	0.4	0.8	0.32
	(0.06)	(0.09)	(0.03)	(0.06)	(0.03)	(0.11)	(0)	(0)	(0.09)
F	0.97	1.77	0.52	0.77	0.45	0.76	1.64	0.59	0.59
	(0.14)	(1.37)	(0.14)	(0.18)	(0.05)	(0.14)	(1.07)	(0.1)	(0.16)
P	7.04^{a}	1.36^{b}	2.19^{ab}	3.90^{ab}	2.48^{ab}	2.38	2.48	3.10	5.61
	(4.15)	(0.53)	(0.27)	(1.03)	(0.65)	(1.02)	(0.53)	(0.65)	(3.46)
A	1.42	0.12	0.50	1.01	0.32	0.37	0.35	0.72	1.26
	(1.07)	(0.02)	(0.21)	(0.2)	(0.09)	(0.19)	(0.1)	(0.26)	(0.85)
FL	8.80^{ad}	1.80^{b}	4.61^{ab}	18.30^{c}	12.38^{cd}	5.14^{a}	5.5^{ab}	9.84^{ab}	16.23^{b}
	(4.96)	(0.99)	(1.4)	(2.04)	(7.35)	(3.91)	(1.84)	(3.54)	(5.84)
PY	7.05^{ab}	1.66^{a}	3.86^{a}	14.71^{b}	10.85^{b}	4.35^{a}	4.76^{ab}	7.64^{ab}	13.75^{b}
	(3.76)	(0.9)	(0.95)	(1.64)	(6.65)	(3.2)	(1.38)	(2.68)	(5.14)
BA	3.19^{ab}	0.24^{a}	1.94^{ab}	6.16^{b}	3.22^{ab}	1.06^{a}	1.81^{ab}	5.11^{b}	3.81^{ab}
	(1.55)	(0.09)	(0.89)	(2.47)	(1.71)	(0.75)	(0.9)	(2.18)	(1.55)
C	1.98^{ab}	0.27^{a}	1.33^{ab}	3.74^{b}	2.47^{ab}	0.92^{a}	1.2^{ab}	2.66^{ab}	3.04^{b}
	(0.85)	(0.02)	(0.32)	(0.73)	(1.12)	(0.55)	(0.35)	(0.93)	(0.89)
BBF	3.34^{ab}	0.39^{a}	2.53^{ab}	6.68^{b}	5.42^{b}	1.88^{a}	2.24^{ab}	4.59^{ab}	6.28^{b}
	(1.68)	(0.13)	(0.75)	(1.83)	(3.81)	(1.29)	(1.12)	(1.87)	(2.87)
BKF	3.33^{ab}	0.18^{a}	1.53^{ab}	4.51^{b}	3.48^{ab}	1.8^{a}	1.24^{a}	2.55^{ab}	5.99^{b}
	(2.31)	(0.03)	(0.33)	(0.82)	(2.24)	(0.71)	(0.67)	(1.14)	(1.91)
BEP	5.20	0.35	2.64	5.79	8.32	2.52^{a}	1.94^{a}	3.96^{ab}	8.69^{b}
	(3.66)	(0.11)	(0.8)	(1.32)	(5.99)	(1.65)	(0.89)	(1.46)	(3.96)
BAP	2.56^{ab}	0.27^{a}	2.17^{a}	7.50^{b}	6.30^{b}	2.21^{a}	1.99^{a}	4.64^{ab}	7.13^{b}
	(1.45)	(0.08)	(0.86)	(1.63)	(3.71)	(1.73)	(1.1)	(2.1)	(2.28)
Per	4.30^{ab}	0.40^{a}	7.60^{b}	2.30^{ab}	2.23^{ab}	1.64^{a}	1.87^{a}	2.64^{ab}	9.21^{b}
	(1.59)	(0.05)	(4.94)	(0.57)	(1.39)	(0.84)	(0.9)	(0.76)	(4.56)
IN	2.46^{ab}	0.22^{a}	1.74^{ab}	5.81^{b}	5.88^{b}	1.38^{a}	2.05^{ab}	4.21^{ab}	4.74^{b}
	(0.79)	(0.09)	(0.81)	(2.01)	(3.75)	(1.19)	(1)	(1.97)	(2.23)
DBA	0.34	< 0.06	0.23	0.49	0.94	0.28^{a}	0.21^{a}	< 0.06^{a}	0.58^{b}
	(0.13)		(0.00)	(0.21)	(0.00)	(0.00)	(0.00)		(0.15)
BPE	2.34	0.16	2.26	3.67	5.30	0.96^{a}	1.37^{ab}	3.64^{b}	4.01^{b}
	(0.95)	(0.07)	(0.87)	(1.96)	(3.1)	(0.00)	(0.48)	(1.59)	(1.94)
Σ	54.62^{acd}	9.43^{b}	35.90^{c}	86.08^{d}	70.30^{d}	28.00 ^{a}	31.05 ^{a}	56.69^{ab}	91.24^{b}
	(15.34)	(3.97)	(6.57)	(12.86)	(21.37)	(8.40)	(7.21)	(11.90)	(17.96)

Note: Values represent the mean and corresponding standard error (within brackets). R1 and R2, sampling sites in the Minho estuary; S1, S2, and S3, sampling sites in the Lima, Cávado, and Douro estuaries, respectively. Different letters identify significant differences among estuaries or seasons, as indicated by the Tukey's multiple comparison test. ANY, acenaphethylene; F, fluorene; P, phenanthrene; A, anthracene; FL, fluoranthene; PY, pyrene; BA, benzo[*a*]anthracene; C, chrysene; BBF, benzo[*b*]fluoranthene; BKF, benzo[*k*]fluoranthene; BEP, benzo[*e*]pyrene; BAP, benzo[*a*]pyrene; Per, perylene; IN, indeno[*1,2,3-cd*]pyrene; DBA, dibenzo[*a,h*]anthracene; BPE, benzo[*g,h,i*]perylene.

summer (relatively to R2). Compared to fish from the reference estuary, the LSI was significantly higher in fish from S2 and S3 in all seasons and in fish from S1 in the spring and summer. Relatively to fish from the reference estuary, significantly lower (~30% less) AChE activity was found in fish from S1 in the winter, spring and summer, and in fish from S3 in the spring (Table 6). LDH activity was significantly higher in fish from S1, S2 and S3 during the winter and autumn, and in fish from S1 during the summer, compared to the reference sites. Induction of gill GST activity was found in fish from all contaminated sites during the spring and summer and in fish from S2 during the autumn, relatively to fish from R1.

CAT activity was significantly lower in fish from S3 in the winter, compared to the reference estuary. In addition, higher activity was measured in fish from S3 in the summer and in fish from all contaminated sites in the autumn. Significant differences in GR activity were mainly found in the winter with fish from the contaminated sites showing higher enzymatic levels than those from the reference estuary. SOD activity was significantly higher in fish from all contaminated estuaries in the winter and in fish from S1 and S2 in the spring and summer, compared to the reference estuary (Tables 6 and C.1). LPO levels were significantly higher in fish from S1 in the spring and summer and in fish from all contaminated sites in the autumn, relatively to fish from the reference estuary.

Trends in biological parameters and their relationship to environmental variables

Significant correlations were found between various chemicals measured in sediments (Figure 2A). Sampling

Table 4. Concentrations of PAHs (ng/g wet weight) determined in the body of fish collected seasonally at the five sampling sites.

	Sampling site					Season			
	R1	R2	S1	S2	S3	Winter	Spring	Summer	Autumn
ANY	0.53	0.45	0.48	0.45	0.45	0.68[a]	0.44[b]	0.34[b]	0.43[b]
	(0.07)	(0.03)	(0.07)	(0.14)	(0.06)	(0.06)	(0.04)	(0.03)	(0.03)
F	1.08	1.20	1.06	1.18	1.35	1.15[a]	1.59[a]	1.28[a]	0.69[b]
	(0.16)	(0.24)	(0.09)	(0.26)	(0.33)	(0.09)	(0.17)	(0.14)	(0.10)
P	2.07	2.71	2.43	2.23	2.60	1.81[ac]	4.02[b]	2.33[c]	1.47[a]
	(0.30)	(0.67)	(0.39)	(0.57)	(1.12)	(0.11)	(0.53)	(0.36)	(0.18)
A	0.17	0.20	0.20	0.19	0.52	0.09[a]	0.24[b]	0.51[c]	0.17[b]
	(0.03)	(0.05)	(0.05)	(0.04)	(0.33)	(0.01)	(0.02)	(0.24)	(0.02)
FL	1.01	1.05	1.02	1.18	1.07	0.85[a]	1.50[b]	1.00[a]	0.91[a]
	(0.07)	(0.14)	(0.11)	(0.15)	(0.39)	(0.1)	(0.19)	(0.12)	(0.03)
PY	0.95	0.99	0.97	1.03	1.16	0.68[a]	1.43[b]	1.09[ab]	0.89[a]
	(0.07)	(0.15)	(0.15)	(0.16)	(0.33)	(0.07)	(0.17)	(0.05)	(0.04)
BA	0.07	0.07	0.09	0.09	0.36	0.03	0.13	0.32	0.07
	(0)	(0.01)	(0.02)	(0.04)	(0.23)	(0.01)	(0.04)	(0.25)	(0.02)
C	0.17	0.14	0.37	0.25	0.41	0.03[a]	0.11[b]	0.29[b]	0.47[c]
	(0.10)	(0.06)	(0.17)	(0.09)	(0.26)	(0.01)	(0.03)	(0.11)	(0.12)
BBF	0.51	0.48	0.40	0.50	0.54	0.28[a]	0.52[b]	0.37[a]	0.66[b]
	(0.02)	(0.12)	(0.08)	(0.15)	(0.12)	(0.04)	(0.06)	(0.12)	(0.06)
BKF	0.20	0.42	1.65	0.31	0.29	<0.50	<0.50	0.28	0.59
	(0.08)	(0.12)	(0.00)	(0.04)	(0.11)			(0.09)	(0.26)
BEP	<0.20	0.51	<0.20	<0.20	0.12	<0.20	<0.20	<0.20	0.31
									(0.19)
BAP	<0.40	<0.40	<0.40	<0.40	<0.40	<0.40	<0.40	<0.40	<0.40
Per	<0.30	<0.30	<0.30	<0.30	<0.30	<0.30	<0.30	<0.30	<0.30
IN	<0.70	<0.70	<0.70	<0.70	<0.70	<0.70	<0.70	<0.70	<0.70
DBA	<0.60	<0.60	<0.60	<0.60	<0.60	<0.60	<0.60	<0.60	<0.60
BPE	<0.70	<0.70	<0.70	<0.70	<0.70	<0.70	<0.70	<0.70	<0.70
Σ	5.68	6.51	7.61	6.23	7.40	4.45[a]	8.39[b]	6.53[a]	5.66[a]
	(0.41)	(0.89)	(0.55)	(0.68)	(1.10)	(0.37)	(0.77)	(0.49)	(0.39)

Note: Values represent the mean and corresponding standard error (within parentheses). Different letters identify significant differences among estuaries or seasons, as indicated by the Tukey's multiple comparison test. Legend as in Table 3.

sites and chemical analyses in sediments explained 74.9% of the overall variability of biological data (Figure 2A, F-ratio = 18.0, $p = 0.001$). The canonical horizontal axis clearly discriminated the contaminated estuaries (S1, S2, and S3) from the reference estuary (R1 and R2), explaining 40.8% of the total variability (F-ratio = 75.0, $p = 0.001$). The biomarkers contributing to this discrimination were CAT, GPx, AChE, and LPO, and to a less extent LDH, the Fulton's Condition Index, and SOD. These parameters were significantly influenced by the concentrations of chromium, crysene, benzo[*g,h,i*] perylene, indeno[*1,2,3,-cd*]pyrene, dibenzo[*a,h*]anthracene, benzo[*a*]pyrene, perylene, pyrene, and lead in sediments (Figure 2A). The correlation between the biological and the environmental axes was 92.4% indicating a highly significant relationship. The vertical axis further discriminated S1 and S2 from S3, explaining 18.5% of the variability. LPO and LDH, and to a less extent AChE, CAT, LSI, and GST, were significantly correlated with this axis, and mainly influenced by cadmium, mercury, zinc, phenanthrene, fluorene, and anthracene. The correlation between the biological and the environmental axes was 80.3%. Because of the high number and degree of correlations found between chemicals in sediments and water parameters, the sampling sites and the water variables explained as much variability (76.9%, F-ratio = 19.0, $p = 0.001$, Figure 2B) as the chemical analyses in sediments. Indeed, the concentrations of nutrients, phenol, silicate and chlorophyll *a* showed high positive correlations with cadmium, mercury and fluorene, and high negative correlations with most of the PAHs. Salinity, conductivity, pH, temperature and turbidity were positively correlated with lead, copper and several PAHs in sediments (Figure 2B). The horizontal axis explained 41.0% of the variability. Its environmental component was dominated mainly by conductivity, salinity, phenol, silicate, pH and turbidity, and to a slightly less extent by temperature, nitrates, chlorophyll a, and ammonium. A high correlation (92.5%) was found between the environmental and the biological components. The vertical axis explained 19.7% of the variability. It was correlated with the concentrations of ammonium, dissolved oxygen, nitrates, pH, and silicate concentration. The correlation with the biological component was 83.0%. Overall, the ordination diagram showed that higher values of the Fulton's Condition Index found in fish from

Table 5. Accumulation factors calculated as the ratio between concentration in fish and concentration in sediments for the eight metals and the 16 polycyclic aromatic hydrocarbons determined in samples from the five sampling sites.

	R1	R2	S1	S2	S3
Metals					
Cd	1.00	3.00	0.17	1.00	0.67
Cr	0.01	0.02	0.02	0.02	0.03
Cu	0.54	1.35	0.42	0.31	1.08
Hg	2.25			12.50	9.00
Ni	0.05	0.19	0.10	0.21	0.25
Pb	0.04	0.09	0.29	0.03	0.09
Zn	3.55	11.94	3.90	3.96	8.48
V					
Σ	1.50	4.30	1.77	1.78	3.48
Polycyclic aromatic hydrocarbons					
ANY	1.77	1.88	1.92	0.61	1.73
F	1.11	0.68	2.04	1.53	3.00
P	0.29	1.99	1.11	0.57	1.05
A	0.12	1.67	0.40	0.19	1.63
FL	0.11	0.58	0.22	0.06	0.09
PY	0.13	0.60	0.25	0.07	0.11
BA	0.02	0.29	0.05	0.01	0.11
C	0.09	0.52	0.28	0.07	0.17
BBF	0.15	1.23	0.16	0.07	0.10
BKF	0.06	2.33	1.08	0.07	0.08
BEP					
BAP					
Per					
IN					
DBA					
BPE					
Σ	0.10	0.69	0.21	0.07	0.11

Note: Legend as in Tables 2 and 3.

the contaminated estuaries appear to be related to the concentrations of lipophilic contaminants, whereas LSI values were under the influence of cadmium, mercury, and zinc. AChE activity appeared to be inhibited by increasing concentrations of several metals, fluorene, and nitrates, silicate and phenol. The latter were positively correlated to each other, and negatively correlated to most PAH concentrations. LDH activity was correlated positively to PAH concentrations, and negatively to mercury, and the levels of nitrogen, silicate and phenol. GST was negatively correlated to SOD activity and LPO levels. LPO levels tended to increase with the concentrations of copper, lead, and some PAHs. They appeared to be influenced by pH, dissolved oxygen, temperature, turbidity, salinity, and conductivity. CAT and GPx activities appeared to be highly dependent on temperature and induced by several PAHs.

Discussion

Water quality

Overall, the water quality was higher in the reference than in the contaminated sites. According to the assessment criteria of nutrient levels in transitional waters (Crouzet et al. 1999), the sites can be classified as follows: R1 and R2 as fair, S1 as poor, and S2 as bad using both nitrate + nitrite and phosphate concentrations, S3 as poor using nitrate + nitrite concentrations and as bad using phosphate concentrations. The contaminated estuaries thus exhibit deterioration of water quality with eutrophication in S2. This excess in nutrients may be caused by river loads and direct discharge from industrial, agriculture and sewage sources. These results set out the need for intervention and regular monitoring to reduce nutrient concentrations and improve water quality. For an effective program, both management authorities and agents implicated in local industry, agriculture and touristic activities should be actively involved in assessing and devising solutions to manage the impacts of their practices in downstream systems.

Contamination of sediments by metals and PAHs

Metal and PAH concentrations found in this study are below the "effects range-low" determined for the estuarine environment (Long et al. 1995). They are also generally lower than those found in sediments from other moderately polluted European estuaries (Budzinski et al. 1997; Caeiro et al. 2005; Duquesne et al. 2006; Vane et al. 2007; Fonseca et al. 2011). Considering the total sum of metals, sites can be ranked in decreasing order of contamination as follows: R1 = S2 = S1 > S3 = R2.

Taking the total sum of PAHs, the rank is as follows: S2 = S3 = R1 > S1 > R2. Sediments from R1 thus showed concentrations of metals and PAHs similar to those found in contaminated sites. This contamination may originate from the presence of a small boats docking in the vicinity of the sampling site, occasional effluent discharges from a village located nearby, and/or river transportation of contaminants from upstream areas. These results highlight the importance of having more than one reference site even in estuaries considered as low impacted. The relatively low concentrations of PAHs found in S1, a site located in the vicinity of a harbor and shipping activities, suggest a low local impact of these activities. More sampling is however needed to confirm this.

Concentrations of metals and PAHs in fish

Comparison of metal concentrations in fish and sediments suggest that *P. microps* is accumulating zinc and mercury. Regarding zinc, simple accumulation factors calculated as the ratio between the concentrations in fish and in sediments indicated that bioaccumulation was greater in fish from sites with the lowest concentrations of zinc in sediments (3.6 in R1, 11.9 in R2, 3.9 in S1, 4.0 in S2, and 8.5 in S3) than in sites with higher concentrations of this metal. Regarding mercury, low bioaccumulation was found in fish from S1 (2.0) and S2 (12.5). This raises special concern because of the toxicity of mercury, capability of biomagnification in food chains when in organic forms, and the key position of *P. microps* in food webs. These findings deserve further investigation in relation to physiological and toxicological processes of the species,

Table 6. Mean and corresponding standard error (within parentheses) of the biological parameters determined in *P. microps* collected at the five sampling sites in each season.

	Winter					Spring					Summer					Autumn				
	R1	R2	S1	S2	S3	R1	R2	S1	S2	S3	R1	R2	S1	S2	S3	R1	R2	S1	S2	S3
Morphometric indices																				
K	0.70	0.96	0.83	0.71	0.94	0.95^{a}	0.91^{a}	1.14^{b}	0.87^{a}	1.27^{b}	1.45^{a}	0.79^{b}	1.32^{a}	0.89^{b}	1.13^{a}	1.14	1.16	1.24	1.09	1.25
	(0.02)	(0.09)	(0.04)	(0.06)	(0.06)	(0.06)	(0.05)	(0.1)	(0.09)	(0.16)	(0.04)	(0.04)	(0.12)	(0.08)	(0.11)	(0.03)	(0.03)	(0.02)	(0.07)	(0.02)
LSI	2.52^{ab}	2.31b	2.85^{ab}	4.11^{c}	3.04^{ac}	2.76^{ab}	2.38^{b}	4.62^{c}	3.13^{a}	4.53^{c}	2.84^{ab}	2.21^{b}	3.11^{a}	3.88^{c}	3.94^{c}	1.71^{a}	1.63^{a}	1.69^{a}	2.84^{b}	2.42^{b}
	(0.37)	(0.21)	(0.2)	(0.19)	(0.15)	(0.29)	(0.17)	(0.2)	(0.14)	(0.03)	(0.16)	(0.18)	(0.22)	(0.58)	(0.46)	(0.12)	(0.09)	(0.07)	(0.13)	(0.06)
Neurotoxicity and energy production																				
AChE	44.97^{a}	38.19^{a}	26.79^{b}	46.48^{a}	45.62^{a}	35.00^{a}	39.31^{a}	27.28^{b}	40.43^{a}	27.64^{b}	53.41^{ab}	46.95^{b}	37.33^{c}	61.08^{a}	86.82^{d}	71.66^{a}	54.07^{b}	58.35^{b}	62.06^{ab}	70.67^{a}
	(2.37)	(3.18)	(3.93)	(6.68)	(1.70)	(0.95)	(2.05)	(0.84)	(1.87)	(5.62)	(2.11)	(4.19)	(2.15)	(3.8)	(7.54)	(6.78)	(6.15)	(3.69)	(3.46)	(4.42)
LDH	203^{a}	286b	349^{c}	474^{d}	399^{cd}	95	116	126	123	141	261^{a}	268^{a}	351^{b}	277^{a}	312^{a}	187^{a}	294^{b}	320^{c}	225^{b}	366^{c}
	(13.33)	(18.61)	(8.86)	(14.03)	(4.38)	(2.48)	(2.56)	(2.76)	(3.57)	(6.76)	(17.03)	(20.79)	(23.58)	(13.34)	(54.49)	(29.47)	(23.48)	(36.12)	(11.74)	(29.50)
Biotranformation and antioxidant defenses																				
GST	64.75	73.65	72.48	58.47	68.03	31.01^{a}	57.49^{b}	52.56^{b}	55.39^{b}	56.57^{b}	18.59^{a}	38.54^{b}	44.55^{b}	31.84^{b}	67.52^{c}	63.16^{a}	63.35^{a}	67.82^{a}	81.16^{b}	69.35^{a}
	(2.83)	(6.16)	(5.08)	(2.73)	(8.14)	(2.38)	(4.02)	(1.51)	(2.28)	(8.25)	(0.62)	(3.02)	(1.38)	(0.89)	(1.69)	(4.61)	(3.63)	(3.1)	(3.25)	(6.83)
GPx	8.06	11.15	10.04	12.73	7.16	24.13	23.60	21.00	18.73	32.67	98.45^{a}	60.75^{a}	40.78^{c}	64.65^{d}	39.34^{c}	78.80^{a}	56.65^{b}	56.02^{b}	54.60^{b}	71.04^{a}
	(2.47)	(2.22)	(2.84)	(1.97)	(2.89)	(1.21)	(1.26)	(4.00)	(1.98)	(7.17)	(6.16)	(7.91)	(7.84)	(9.5)	(8.92)	(3.91)	(6.3)	(2.16)	(7.21)	(1.35)
CAT	15.04^{a}	20.83^{a}	11.94^{b}	14.08^{b}	8.02^{b}	20.60	5.37	5.36	14.01	8.63	107^{a}	116^{a}	122^{a}	87.81^{a}	269^{b}	126^{a}	101^{a}	240^{c}	193^{ac}	244^{c}
	(1.5)	(4.76)	(0.86)	(1.9)	(1.53)	(4.62)	(1.66)	(1.84)	(3.92)	(2.39)	(21.76)	(17.6)	(21.06)	(19.65)	(14.62)	(38.54)	(29.10)	(24.38)	(13.32)	(34.14)
GR	7.30^{a}	4.13^{b}	4.17^{b}	10.07^{c}	7.77^{ac}	3.85	3.87	5.02	4.51	4.92	0.97^{a}	2.23^{b}	4.31^{c}	2.21^{ab}	2.61^{ab}	7.33	5.67	7.13	6.76	7.51
	(0.70)	(1.14)	(0.14)	(0.71)	(0.60)	(0.33)	(0.66)	(0.94)	(0.69)	(0.51)	(0.41)	(0.58)	(0.29)	(0.77)	(0.56)	(0.44)	(0.87)	(1.04)	(1.08)	(1.07)
SOD	1.00^{a}	1.03^{a}	3.29^{b}	5.28^{b}	5.88^{b}	2.03^{a}	1.41^{a}	4.62^{b}	4.14^{b}	3.20^{ab}	1.74^{a}	5.00^{b}	9.08^{c}	8.36^{c}	3.97^{ab}	4.00	4.36	2.60	3.51	3.36
	(0.49)	(0.4)	(0.76)	(0.7)	(0.97)	(0.42)	(0.27)	(1.12)	(0.29)	(0.27)	(0.57)	(1.34)	(1.17)	(0.74)	(0.42)	(1.28)	(1.04)	(0.46)	(0.56)	(0.65)
Oxidative damage																				
LPO	223	310	145	412	170	222^{a}	254^{a}	462^{b}	351^{ab}	382^{ab}	291^{a}	385^{b}	508^{c}	301^{ab}	264^{ab}	165^{a}	292^{b}	753^{c}	465^{d}	453^{d}
	(24.42)	(22.9)	(13.49)	(40.03)	(22.75)	(5.67)	(27.19)	(23.46)	(28.17)	(32.81)	(26.32)	(51.04)	(19.49)	(22.01)	(37.41)	(10.38)	(20.07)	(136.23)	(25.16)	(52.41)

Note: R1 and R2, sampling sites in the Minho estuary; S1, S2, and S3, sampling sites in the Lima, Cávado, and Douro estuaries, respectively. Different letters identify significant differences among estuaries or seasons, as indicated by planned pairwise comparisons. K, Fulton's condition index; LSI, liver somatic index; AChE, acetylcholinesterase activity (nmol/min/mg protein); LDH, lactate dehydrogenase activity (μmol/min/mg protein); GST, glutathione-S-transferases activity (nmol/min/mg protein); GPx, glutathione peroxidase activity (nmol/min/mg protein); GR, glutathione reductase activity (nmol/min/mg protein); CAT, catalase (μmol/min/mg protein); SOD, superoxide dismutase activity (U/mg protein); LPO, lipid peroxidation (nmol TBARS/g tissue).

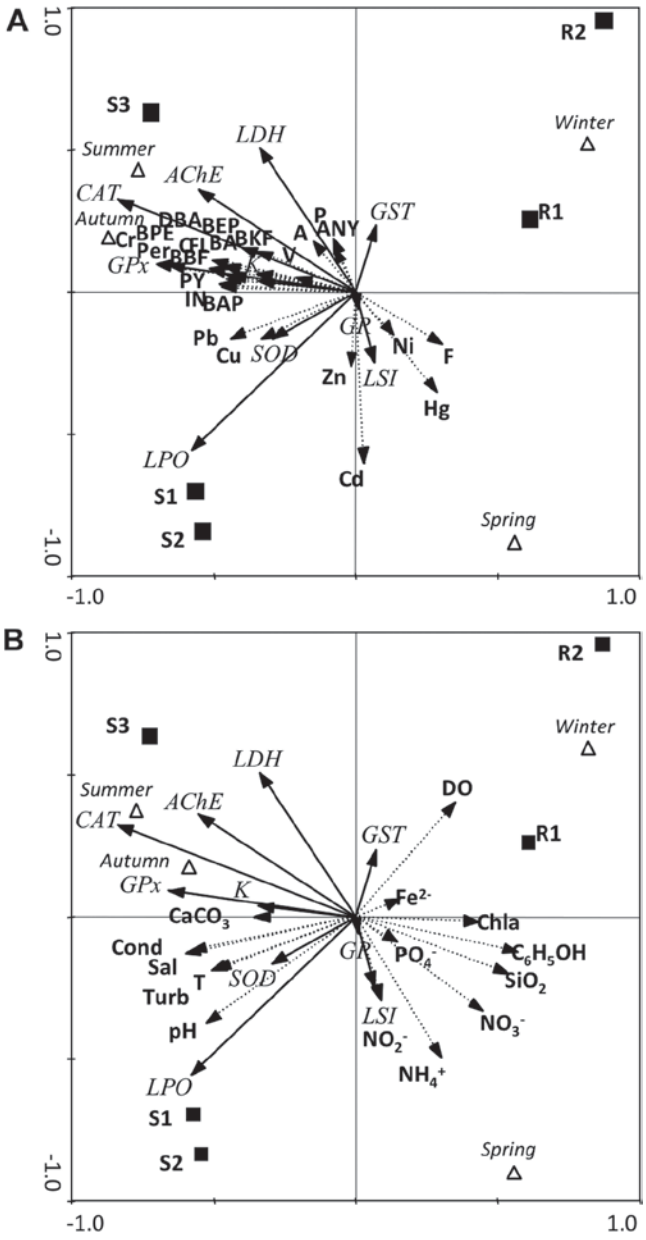

Figure 2. Ordination diagrams of RDA analyses showing the relationship between variation in the levels of environmental variables and biological parameters measured. Biological parameters are represented by straight arrows, quantitative environmental variables are represented by dotted arrows, centroids of the sampling sites are represented by black squares, and centroids of seasons by open triangles. In (A), the first and second axes significantly accounted for 40.8% and 18.5% of the total variability, respectively. In (B), the first axis significantly explained 41.0% and the second 19.7% of the variability. Legend as given in Tables 1, 2, 3 and 6

behavioral and trophic interactions, and abiotic factors that may act as relevant determinants (Garban et al. 1996; Schoon et al. 1996; Elliott et al. 2003).

Concerning PAHs, the low accumulation found in *P. microps* is in agreement with previous observations made in other fish species (van der Oost et al. 2003, and references therein). Factors that may decrease the bioavailability of PAH congeners are the presence in the aqueous phase of suspended particles with a high binding capacity, the molecular weight, and low aqueous solubility, which limits the release from sediments and may consequently reduce the uptake (Hellou et al. 1995; Haitzer et al. 1999). However, PAHs are known to be efficiently biotransformed in the fish liver and rapidly eliminated from the organism, often barely reflecting the levels in the surrounding environment (van der Oost et al. 2003). However, low accumulation of parental compounds does not preclude the induction of toxic effects. Biotransformation of PAHs generates several metabolites, some of which are highly reactive and able to cause severe toxic effects including DNA damage, endocrine disruption, and oxidative stress. The present results indicate the presence of background contamination in sediments and fish from the studied estuaries, and seasonal variation, that could act as confounding factors when assessing potential effects of oil spills. It is thus crucial to carry out further studies of these estuaries focused on the potential accumulation of such substances in this and in other keystone species.

Biological effects

Seasonal variability in the effects measured was found, thus stressing the importance of taking it into account in pollution monitoring and ecological risk assessment.

The general fish condition, as indicated by the Fulton's condition index, was similar in all estuaries but decreased in the winter. This may be related to reduced food availability and adverse environmental conditions typical of this period of the year. A similar trend was found previously for eel (*Anguilla anguilla*) populations from these estuaries (Guimarães et al. 2009). The increased LSI values found in S2 and S3 may be indicative of exposure to pollution, as the liver may increase in size to allow greater detoxication (Slooff et al. 1983). Studies carried out in polluted areas also reported increased LSI values in response to PAHs and PCBs exposure (van der Oost et al. 2003 and references therein). It is of note that PCBs are also relevant contaminants in S3 (Ferreira et al. 2004). Despite the contamination found in S1 sediments, fish from this site showed levels of all biomarkers similar to those of fish from R2, the less contaminated site. This suggests low bioavailability of contaminants in S1. The significant inhibition (approximately 30%) of AChE activity found in fish from S1 (winter and spring) and S3 (spring), relatively to fish from R2, is clearly above the 20% inhibition commonly accepted as indicative of exposure to anticholinesterase agents and the range considered as indicative of neurotoxic effects (Ludke et al. 1975). Anticholinesterase agents responsible for such inhibition may be organophosphate and carbamate insecticides used in crop fields in the vicinity of the estuaries, and/or metals and PAHs known to inhibit AChE in this species (Vieira et al. 2008, 2009). Inhibition of AChE activity was also found previously in yellow eel populations from these estuaries (Guimarães et al. 2009). The strong LDH induction found in *P. microps* from the impacted sites

suggests an increase in the anaerobic pathway to cope with energy requirements triggered by chemical stress. Increased LDH activity has been previously reported in *P. microps* following laboratory exposures to benzo[*a*] pyrene, anthracene, copper, and mercury (Vieira et al. 2008, 2009). Among the antioxidative enzymes, SOD showed the most consistent pattern of variation. A clear induction was found in S1 and S2 during most of the year. Significant SOD induction was also found in *P. microps* from sites in Ria de Aveiro and Tejo estuary contaminated by metals and PAHs (Fonseca et al. 2011). Increased SOD activity was additionally observed in laboratory exposures of *P. microps* to single PAHs and metals (Vieira et al. 2008, 2009). The pattern of variation found for CAT, GPx and GR is consistent with a bell-shaped trend of the response, which usually shows an initial activation of enzyme synthesis followed by a decrease in enzymatic activity due to the enhanced catabolic rate and/or a direct inhibitory action of toxicants on the enzyme molecules (Viarengo et al. 2007). It is also consistent with their dependence on abiotic and biotic variables that may influence biomarker responses. Seasonal changes in local concentrations of contaminants, temperature, salinity, nutrient concentrations, feeding habits and reproductive hormones may likewise account for the high variability observed.

Slight gill GST induction was found in fish from our contaminated sites mostly during the spring and summer, suggesting biotransformation induction to cope with toxicant exposure. GST enhancement was also found in laboratory exposures of *P. microps* to copper, mercury, benzo[*a*]pyrene and water accommodated fractions of a fuel oil, whereas inhibition was found following exposure to anthracene (Vieira et al. 2008, 2009). LPO levels generally agreed with the pattern of GST variation. Globally, biomarkers indicate that *P. microps* inhabiting the contaminated estuaries of Lima, Cávado and Douro rivers are at poorer health status than those from the Minho estuary. The key role of *P. microps* in the functioning of these estuarine ecosystems and the findings of the present study highlight the need for long-term monitoring of local populations of this species in relation to environmental contamination and variation of natural stressors. In addition to their importance for biodiversity conservation and sustainability of ecosystems services, these surveys will be determinant to assess potential impacts of oil and other chemical spills by providing health status information and good baseline data from both the impacted and nonimpacted systems.

Integrated data analysis

Significant relationships between biological and environmental variables were identified through RDA. The results indicated that *P. microps* from the contaminated sites exhibit toxic effects caused by exposure to multiple environmental stressors. Based on RDA, the Fulton's condition index and the LSI, the activities of the enzymes AChE, LDH, and the levels of LPO, provided a clear distinction between reference and polluted sites. These should therefore be first choice biomarkers to assess *P. microps* health status in these and other similar estuaries. According to our findings GST and SOD could also be usefully included, providing complimentary information to LPO. In particular, clear inductions of GST activity in response to oil contamination were previously found in several species after oil spills (Moreira et al. 2004; Martínez-Gómez et al. 2006; Tim-Tim et al. 2009).

RDA also showed a great number of correlations among the contaminants analyzed and strong relationships between toxicants in sediments and water quality parameters. Chemical concentrations in environmental compartments are required by regulation and most important to establish cause–effect relationships between exposure and effects. The biological information is, however, crucial to assess toxic effects and translate the outcomes of the exposure to multiple stressors, including unknown chemical contaminants. Simultaneous biological and chemical monitoring is thus strongly recommended. Water variables are of great value in monitoring studies since they influence (i) the life history and development of estuarine species, (ii) speciation, bioavailability and accumulation processes, and (iii) the organisms' responses to pollution (Bebianno et al. 2007; Guimarães et al. 2009). Moreover, they are far less expensive to measure than chemical analyses. Hence, they can be determined more frequently than biological and chemical analyses, providing relevant data on environmental quality and abiotic changes.

Conclusions

Ecological risk assessment of oil spills in estuaries under anthropogenic influence is challenging due to the presence of background pollution and natural abiotic and biotic variation which act as confounding factors. The limited knowledge on the relationships between dynamic multistressor exposure and effects on populations of keystone species causes further difficulties to this process. Here this issue was addressed by investigating the health status of *P. microps* populations of the NW Iberian Peninsula in relation to long-term exposure to chemical contamination and variation of natural stressors. Overall, the results indicate that: (i) *P. microps* populations from the contaminated estuaries of Lima, Cávado and Douro rivers have a lower health status than the population from the Minho estuary; (ii) fish are accumulating zinc (in all sites) and mercury (in S1 and S2); (iii) all the studied variables showed seasonal variability and both chemical and natural stressors influenced the biological responses measured. These findings underpin the need for more research on the combined effects of the exposure to multiple stressors in marine species. They also strengthen the utility of field approaches integrating biological, chemical and physical parameters, especially in highly dynamic

ecosystems such as estuaries. Finally, accumulation of long-term before-impact data from key species inhabiting sites with different levels of contamination is particularly important to assess ecological risk and recovery after oil and other chemical spills, helping to separate punctual from background contamination, and pollution effects from changes due to natural variability.

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Declaration of interest

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Appendix A

Water quality variables

Table A.1. Results of the statistical analyses (one-way ANOVAs) performed to investigate differences among sampling sites and among seasons in the water variables measured.

Parameter	Source of variation	*df*	*F*	*p*
T	Sampling site	4, 55	1.11	ns
	Season	3, 56	54.18	<0.001
Sal	Sampling site	4, 55	6.71	<0.001
	Season	3, 56	2.22	ns
Cond	Sampling site	4, 55	6.31	<0.001
	Season	3, 56	2.36	ns
pH	Sampling site	4, 55	1.30	ns
	Season	3, 56	4.39	0.008
DO	Sampling site	4, 55	0.41	ns
	Season	3, 56	47.24	<0.001
NO_2^-	Sampling site	4, 55	11.85	<0.001
	Season	3, 56	2.60	ns
NO_3^-	Sampling site	4, 55	2.25	ns
	Season	3, 56	11.99	<0.001
NH_4^+	Sampling site	4, 55	0.34	ns
	Season	3, 56	3.70	0.017
PO_4^{3-}	Sampling site	4, 55	5.16	0.001
	Season	3, 56	3.92	0.013
Fe	Sampling site	4, 55	2.27	ns
	Season	3, 56	2.59	ns
C_6H_5OH	Sampling site	4, 55	1.87	ns
	Season	3, 56	4.48	0.007
SiO_2	Sampling site	4, 55	1.19	ns
	Season	3, 56	10.21	<0.001
$CaCO_3$	Sampling site	4, 55	6.84	<0.001
	Season	3, 56	1.23	ns
Turb	Sampling site	4, 55	1.10	ns
	Season	3, 56	8.84	<0.001
Chla	Sampling site	4, 55	1.47	ns
	Season	3, 56	1.80	ns

Note: Legend as given in Table 1. ns, not significant.

Appendix B

Chemical analyses in sediments and fish

Table B.1. Results of the statistical analyses (one-way ANOVAs) performed to investigate differences among sampling sites and among seasons in the concentrations of metals measured in sediments and fish body.

Parameter	Source of variation	*df*	*F*	*p*
Sediments				
Cd	Sampling site	4, 55	10.77	<0.001
	Season	3, 56	4.20	0.009
Cr	Sampling site	4, 55	2.29	ns
	Season	3, 56	32.19	<0.001
Cu	Sampling site	4, 55	15.07	<0.001
	Season	3, 56	3.42	0.023
Ni	Sampling site	4, 55	17.39	<0.001
	Season	3, 56	1.88	ns
Pb	Sampling site	4, 55	27.26	<0.001
	Season	3, 56	1.89	ns
Zn	Sampling site	4, 55	17.06	<0.001
	Season	3, 56	2.50	ns
V	Sampling site	4, 55	7.11	<0.001
	Season	3, 56	8.84	<0.001
Σ	Sampling site	4, 55	27.79	<0.001
	Season	3, 56	2.30	ns
Fish body				
Cd	Sampling site	4, 55	8.84	<0.001
	Season	3, 56	1.27	ns
Cr	Sampling site	4, 55	1.17	ns
	Season	3, 56	11.42	<0.001
Cu	Sampling site	4, 55	7.72	<0.001
	Season	3, 56	1.31	ns
Hg	Sampling site	4, 55	4.51	0.003
	Season	3, 56	1.63	ns
Ni	Sampling site	4, 55	0.96	ns
	Season	3, 56	15.02	<0.001
Pb	Sampling site	4, 55	0.99	ns
	Season	3, 56	1.13	ns
Zn	Sampling site	4, 55	6.72	<0.001
	Season	3, 56	2.98	0.039
Σ	Sampling site	4, 55	7.21	<0.001
	Season	3, 56	2.55	ns

Note: Legend as given in Table 2. ns, not significant.

Table B.2. Results of the statistical analyses (one-way ANOVAs) performed to investigate differences among sampling sites and among seasons in the concentrations of PAHs measured in sediments.

Parameter	Source of variation	*df*	*F*	*p*
ANY	Sampling site	4, 55	4.14	0.005
	Season	3, 56	2.22	ns
F	Sampling site	4, 55	0.74	ns
	Season	3, 56	0.84	ns
P	Sampling site	4, 55	3.17	0.021
	Season	3, 56	1.64	ns
A	Sampling site	4, 55	2.35	ns
	Season	3, 56	1.86	ns
FL	Sampling site	4, 55	5.50	0.001
	Season	3, 56	3.80	0.015
PY	Sampling site	4, 55	5.12	0.001
	Season	3, 56	3.91	0.013
BA	Sampling site	4, 55	4.36	0.004
	Season	3, 56	3.67	0.017
C	Sampling site	4, 55	5.27	0.001
	Season	3, 56	3.85	0.014
BBF	Sampling site	4, 55	3.99	0.006
	Season	3, 56	3.62	0.019

(*Continued*)

Table B.2. (Continued).

Parameter	Source of variation	*df*	*F*	*p*
BKF	Sampling site	4, 55	3.27	0.018
	Season	3, 56	4.24	0.009
BEP	Sampling site	4, 55	2.32	ns
	Season	3, 56	5.86	0.001
BAP	Sampling site	4, 55	6.58	<0.001
	Season	3, 56	3.79	0.015
Per	Sampling site	4, 55	4.27	0.004
	Season	3, 56	5.28	0.003
IN	Sampling site	4, 55	4.87	0.002
	Season	3, 56	4.36	0.008
DBA	Sampling site	4, 55	1.10	ns
	Season	3, 56	7.30	<0.001
BPE	Sampling site	4, 55	2.49	ns
	Season	3, 56	5.39	0.002
Σ	Sampling site	4, 55	4.57	0.003
	Season	3, 56	5.76	0.002

Note: Legend as given in Table 3. ns, not significant.

Table B.3. Results of the statistical analyses (one-way ANOVAs) performed to investigate differences among sampling sites and among seasons in the concentrations of PAHs measured in fish body.

Parameter	Source of variation	*df*	*F*	*p*
ANY	Sampling site	4, 54	0.56	ns
	Season	3, 55	33.63	<0.001
F	Sampling site	4, 54	0.72	ns
	Season	3, 55	19.05	<0.001
P	Sampling site	4, 54	0.35	ns
	Season	3, 55	21.37	<0.001
A	Sampling site	4, 54	2.25	ns
	Season	3, 55	4.69	0.005
FL	Sampling site	4, 54	0.29	Ns
	Season	3, 55	10.88	<0.001
PY	Sampling site	4, 54	0.55	ns
	Season	3, 55	20.87	<0.001
BA	Sampling site	4, 54	2.36	ns
	Season	3, 55	2.16	ns
C	Sampling site	4, 54	0.67	ns
	Season	3, 55	6.63	0.001
BBF	Sampling site	4, 54	1.15	ns
	Season	3, 55	13.96	<0.001
BKF	Sampling site	4, 54	1.15	ns
	Season	3, 55	11.40	<0.001
Σ	Sampling site	4, 54	0.85	ns
	Season	3, 55	13.51	<0.001

Note: Legend as given in Table 3. ns, not significant

Appendix C

Biological parameters

Table C.1. Results of the statistical analyses (two-way ANOVAs) performed to investigate differences among sampling sites and among seasons in the biological parameters measured.

Parameter	Source of variation	*Df*	*F*	*p*
K	Sampling site	4, 107	9.71	<0.001
	Season	3, 107	22.57	<0.001
	Sampling site × Season	12, 107	5.59	<0.001
LSI	Sampling site	4, 106	23.98	<0.001
	Season	3, 106	29.28	<0.001
	Sampling site × Season	12, 106	4.82	<0.001
AChE	Sampling site	4, 108	13.98	<0.001
	Season	3, 108	55.90	<0.001
	Sampling site × Season	12, 108	4.34	<0.001
LDH	Sampling site	4, 108	22.11	<0.001
	Season	3, 108	115.70	<0.001
	Sampling site × Season	12, 108	8.27	<0.001
GST	Sampling site	4, 108	15.96	<0.001
	Season	3, 108	65.90	<0.001
	Sampling site × Season	12, 108	6.53	<0.001
GPx	Sampling site	4, 108	9.19	<0.001
	Season	3, 108	124.40	<0.001
	Sampling site × Season	12, 108	5.66	<0.001
GR	Sampling site	4, 108	4.21	<0.001
	Season	3, 108	37.10	<0.001
	Sampling site × Season	12, 108	4.18	<0.001
CAT	Sampling site	4, 108	8.02	<0.001
	Season	3, 108	114.60	<0.001
	Sampling site × Season	12, 108	5.71	<0.001
SOD	Sampling site	4, 108	11.40	<0.001
	Season	3, 108	9.97	<0.001
	Sampling site × Season	12, 108	4.98	<0.001
LPO	Sampling site	4, 108	19.50	<0.001
	Season	3, 108	14.20	<0.001
	Sampling site × Season	12, 108	8.87	<0.001

Note: Legend as given in Table 6.