Yellow eel (*Anguilla anguilla*) development in NW Portuguese estuaries with different contamination levels

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Abstract The aims of the present study were to compare the health *status* of yellow eels (*Anguilla anguilla*) developing in three estuaries of the NW Portuguese coast with different levels of pollution and their physiological responses to combined effects of environmental variation and pollution. For this, a field study was performed using a multi-parameter approach, including eels condition indexes and biomarkers, water quality variables and other environmental factors. Sixteen biological parameters were assessed, namely: hepatosomatic index (LSI), Fulton's condition index (K), lipid peroxidation (LPO), total glutathione (TG), reduced glutathione (GSH), oxidised glutathione (GSSG), GSH/GSSG, and the activity of the enzymes acetylcholinesterase (AChE), lactate dehydrogenase (LDH), sodium-potassium ATPase (Na^+/K^+-ATPase), ethoxyresorufin O-deethylase (EROD), glutathione S-transferases (GST), catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPx) and glutathione reductase (GR). Ten environmental factors were also measured in water: temperature, salinity, pH, phosphates, nitrates, nitrites, ammonium, silica, phenol and hardness. Globally, the biomarkers indicate exposure and toxic effects of pollutants on eels living in contaminated estuaries. The relationships between biological and environmental variables were assessed through redundancy analysis. K and LSI indexes, AChE and Na^+/K^+-ATPase, total glutathione levels and the antioxidant enzymes CAT, GR, and SOD where the factors most discriminating reference (Minho River estuary) from contaminated estuaries (Lima and Douro Rivers estuaries). Moreover, the most striking outcomes of pollutants exposure on biological responses were observed during winter, probably due to a joint effect of cold weather and pollution stress. Altogether, the results indicate that the development of eels in the polluted estuaries of Lima and Douro rivers is interfering with physiological functions determinant for their survival and performance. This may increase the mortality rates during the continental life-phase of the species and decrease the percentage of animals able to successfully complete their oceanic migration and, thus, reduce the contribution of each generation to the next one.

Keywords Estuarine contamination ·
Natural populations · Biomarkers of neurotoxicity ·
Osmoregulation · Oxidative stress · Energy metabolism ·
Seasonal variability · Redundancy analysis

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Introduction

The European eel, *Anguilla anguilla* L., is a catadromous fish species typical of European estuarine and freshwater basins, where it spends most of its lifetime. Juveniles reach European estuaries with about 2 years (Feunteun 2002), in a stage known as glass eel. In estuaries and river basins they develop and metamorphose into yellow eels. After spending about 8–15 years growing in continental waters they suffer a second metamorphosis (silver eel) and are

then able to initiate their oceanic migration to reproduce in the Sargasso Sea (Knights 1997; Feunteun 2002). During their continental life cycle phase, eels accumulate a considerable amount of lipid reserves, which are fundamental for the success of the long oceanic migration since during this period they do not feed (Robinet and Feunteun 2002; Ribeiro et al. 2005; Palstra et al. 2006; Pierron et al. 2007) and their digestive tract regresses (Durif et al. 2005; van Ginneken et al. 2007). In the past three decades, *A. anguilla* has suffered an intensive decline in the major part of its distribution area, and the stocks are now considered to be below safe biological limits (Moriarty and Dekker 1997; Feunteun 2002; Dekker 2004; EIFAC/ICES 2007). The species is included in the HELCOM Red list of threatened and declining species of the Baltic Sea (HELCOM 2007), in the Appendix II of the Convention on International Trade in Endangered Species of Wild Fauna and Flora previewed to enter into force until March 2009 (FAO 2007; CITES 2008), and in the Red Book of the Vertebrates of Portugal (ICN 2008). In Portugal, *A. anguilla* is considered a species of high economic value. Although the official recommendations target 220 mm as the minimum size allowed for fisheries, it is fished at all continental life stages (Baeta et al. 2005).

Over the years, the growing intensity of human activities in a considerable part of European estuaries originated an ever-increasing input of complex mixtures of contaminants, such as agriculture fertilisers, pesticides, heavy metals, organic synthetic compounds from industry, shipping and/or urban effluents from human settlings. This contamination load results in poor water quality and contaminated sediments, hastening the demise of these ecosystems (Kennish 2002). A number of laboratory and field studies have shown that eels can bioaccumulate high concentrations of environmental contaminants, depicting a relation between exposure to these chemicals and adverse effects. Several of these contaminants have been shown to cause mortality, growth delay, reproductive alterations, tumours, malformations and immunological changes, among several other negative effects (van der Oost et al. 1996b; Knights 1997; Lionetto et al. 2000; Roche et al. 2000; Peña-Llopis et al. 2001; Robinet and Feunteun 2002; Ribeiro et al. 2005; Buet et al. 2006; Gravato et al. 2006; Palstra et al. 2006; Pierron et al. 2007). In European estuaries, eels may be exposed to a variety of environmental contaminants during a crucial growth phase, when energy reserves are accumulated as fat stores, in preparation for the long migration to the spawning areas in the Sargasso Sea. Moreover, their benthic lifestyle and their high lipid content increase the risk of accumulation of hydrophobic environmental contaminants in their tissues. Additionally, during migration when fat mobilization occurs, chemicals accumulated in fat tissue may be released to the circulation and may induce both acute and chronic toxic effects (Robinet and Feunteun 2002; Ribeiro et al. 2005; Palstra et al. 2006; Pierron et al. 2007).

In the last decades, biomarkers have been successfully used as sensitive tools for the assessment of adverse effects induced by complex mixtures of pollutants in natural populations. The activity of the enzymes acetylcholinesterase (AChE), lactate dehydrogenase (LDH), ethoxyresorufin-*O*-deethylase (EROD) activity of the P450 system, and glutathione *S*-transferases (GST) have been used in Ecotoxicology mainly because they are involved in neurotransmission, energy production and detoxification, respectively, which are determinant for animal survival and performance (Cajaraville et al. 2000; Ferreira et al. 2004; Moreira et al. 2004; Cunha et al. 2005; Quintaneiro et al. 2006; Monteiro et al. 2007). In recent years, non-enzymatic and enzymatic antioxidants such as reduced (GSH) and oxidised glutathione (GSSG) and their ratio (GSH/GSSG), glutathione peroxidase (GPx), glutathione reductase (GR), superoxide dismutase (SOD), catalase (CAT), as well as lipid peroxidation (LPO), have also been widely used as environmental biomarkers (van der Oost et al. 2003; Buet et al. 2006; Gravato et al. 2007; Lima et al. 2007) since several pollutants are able to cause oxidative stress. The activity of the enzyme sodium/potassium adenosine triphosphatase (Na^+/K^+-ATPase) is also being incorporated in such studies (Buet et al. 2006) because of its involvement in osmotic regulation in teleost fish.

The aims of the present study were to compare the health status of yellow eels (*Anguilla anguilla*) developing in three estuaries of the NW Portuguese coast with different levels of pollution and their physiological responses to combined effects of environmental variation and pollution stress. For this, a field study was performed using a multi-parameter approach, including eels condition indexes and biomarkers, water quality variables and other enviromental factors.

Materials and methods

Studied estuaries

Three estuaries were selected for this study (Fig. 1): (1) the estuary of Minho River, which has been considered as relatively undisturbed and used as a reference in several studies (Cairrão et al. 2004; Castro et al. 2004b; Rodrigues et al. 2006; Monteiro et al. 2007), despite having some foci of pollution; (2) the estuary of Lima River, which receives effluents from several industries (including a paper mill) and untreated urban wastewaters, and it has an important harbour at its mouth (INAG 2000; Cairrão et al. 2004); and (3) the estuary of Douro River, which receives directly
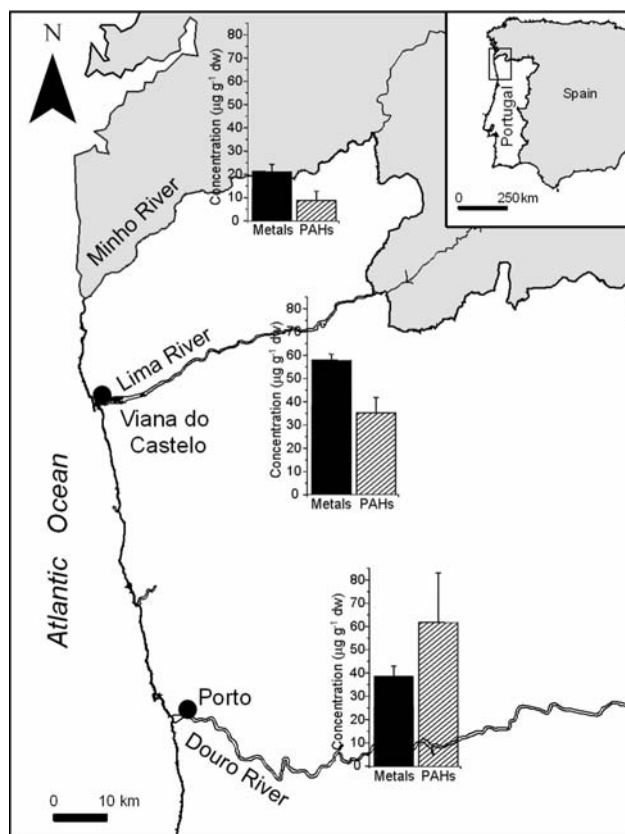
Fig. 1 Location of the studied estuaries (Minho, Lima and Douro) and chemical analyses of sediments collected at the mouth of each estuary. Total metal (metals, *black bars*) and total polycyclic aromatic hydrocarbon (PAHs, *hatched bars*) concentrations are expressed as µg g^{-1} of dry weight (dw). Significant differences among estuaries were found for both metals ($F(2, 33) = 29.1$, $P < 0.001$) and PAHs ($F(2, 33) = 4.1$, $P = 0.026$)

and/or through its tributaries domestic sewage of over one million inhabitants and effluents from urban and industrial sources, mostly untreated (Ferreira et al. 2004; Mucha et al. 2004). In the scope of a previous study performed by our team, the concentrations of eight metals (Cd, Cr, Cu, Hg, Ni, Pb, Zn, V) and 16 polycyclic aromatic hydrocarbons (PAHs; 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) were seasonally determined in sediment samples of these estuaries. The average values of total heavy metal concentrations (metals) and total PAHs in sediments revealed that heavy metals prevail in the estuary of Lima River whereas PAHs predominate in the estuary of Douro River (Fig. 1). Metals were dominated mainly by Zn, Cr, V, Pb and Cu, whereas the most common PAHs found were fluoranthene, pyrene, benzo[*e*]pyrene, benzo[*b*]fluoranthene, benzo[*a*]-pyrene, indeno[*1,2,3-cd*]pyrene, and perylene. The estuary

of Douro River is also known to be contaminated with polychlorinated biphenyls (PCBs), and dichlorodiphenyl-trichloroethane (DDT) and related compounds, and other endocrine disruptors (Ferreira et al. 2004, 2006; Almeida et al. 2007; Ribeiro et al. 2008). According to the Portuguese official statistics, European eel fishing records varied greatly among the three estuaries. For example, in 2005 nominal captures were 0.443 t in the Minho estuary, 0.004 t in the Lima estuary and 0.000 t in the Douro estuary, while in 2006 correspondent values were 0.576 t, 0.019 t and 0.000 (INE 2008).

Biological material

Twenty to forty yellow eels were seasonally collected from summer 2005 to spring 2006 at each estuary using fish basket traps. The eel catch effort was similar in the three estuaries. Captured eels were immediately transported to the laboratory and sacrificed by decapitation immediately after determination of total body length (*L*) and total body weight (*W*). The morphometric values obtained were used to determine the Fulton Condition Index [$K = ($(total body weight (g)/total body length (cm))$^3) \times 100$] (Ricker 1975). After dissection on ice, the liver was weighted and used to calculate the Hepatosomatic Index [LSI = liver weight (g)/ total body weight (g) $\times 100$] (Sloof 1982). Additionally, several organs or body parts were isolated and prepared for individual determination of biomarkers as indicated below.

Enzymatic determinations

Total brain was isolated, homogenised (Ystral GmbH Dottingen homogeniser) in 1 ml of ice-cold phosphate buffer (0.1 M, pH = 7.2), centrifuged for 3 min at 3,300g and 4°C (Sigma 3K30 centrifuge) and used for AChE determinations. AChE activity was determined by the Ellman method (Ellman et al. 1961), adapted to microplate (Guilhermino et al. 1996) using 0.50 ml of brain homogenate, in a Bio Tek Power Wave 340 microplate reader, at 412 nm, and expressed in nmol of substrate hydrolysed/min/mg of protein. Since brain tissue was used, the hydrolytic activity measured towards acetylcholine was considered as due to AChE activity.

Samples for LDH activity determinations were prepared by homogenisation of a part of the dorsal muscle in ice-cold Tris/NaCl phosphate buffer, followed by centrifugation at 6,000 rpm for 3 min in a Sigma 3K30 centrifuge at 4°C. The supernatant was then collected and used for enzymatic analysis. LDH activity was determined according to the method of Vassault (1983) adapted to microplate (Diamantino et al. 2001), by measuring the amount of pyruvate consumed, through the continuous monitoring of the decrease in optic density due to NADH oxidation at

340 nm in a Bio Tek Power Wave 340 microplate reader. The enzymatic activity was expressed in μmol of substrate hydrolysed/min/mg protein.

The first and second gill arcs from the left side were put in ice-cold phosphate buffer (0.1 M, pH = 6.5) and centrifuged (Sigma 3K30) for 30 min at 9,000g. The supernatant was collected and used for determination of GST activity by following the conjugation of GSH with 1-chloro-2,4-dinitrobenzene (CDNB) at 340 nm (Habig et al. 1974) using a Bio Tek Power Wave 340 microplate reader (Frasco and Guilhermino 2002). The enzymatic activity was expressed in nmol of substrate hydrolysed/min/mg of protein.

Another gill arc from the left side was used for assessing Na^+/K^+-ATPase activity. It was introduced in 0.100 ml of SEI buffer (150 mM Sacarose + 10 mM Na_2EDTA + 50 mM Imidazole, pH 7.3), homogenised in a sonicator (Sonics & Materials Inc), and centrifuged at 13,000 rpm (Sigma 3K30) for 3 min at 4°C. The supernatant was then collected for determination of Na^+/K^+-ATPase activity by the method described by McCormick (1993) in a Bio Tek Power Wave 340 microplate reader at 340 nm. The enzymatic activity was expressed in μmol ADP/mg protein/h.

The liver was systematically divided into three parts: (1) one part of each liver was homogenised (1:5) in 50 mM Tris–HCl, pH 7.4 containing 0.15 M KCl and used for determination of EROD activity; (2) another portion was homogenised (1:10) in 0.1 M K-phosphate buffer (pH 7.4), and part of this liver homogenate was used to determine the extent of endogenous LPO, whereas the remaining homogenate was centrifuged for 20 min at 12,000 rpm (4°C) to obtain the post-mitochondrial supernatant (PMS) which was used to assess GPx, SOD and CAT activities, TG content, and liver B(a)P-type metabolites; (3) the remaining part of each liver was homogenised in 1 ml 0.1 M K-phosphate buffer (pH 6.5) and centrifuged during 30 min at 9,000g (4°C). The supernatant was further diluted in the same buffer to a final concentration of 0.5 mg/ml for determination of GST activity.

Liver microsomes were prepared according to methods previously described (Gravato and Santos 2003) and EROD activity was quantified by fixed wavelength fluorescence detection (Jasco FP-6200 Spectrofluorometer) as described by Burke and Mayer (1974). The enzyme activity was expressed in pmol/min/mg of protein.

LPO was determined by measuring the thiobarbituric acid reactive substances (TBARS) according to Ohkawa (1979) and Bird and Draper (1984), with the adaptations described by Filho et al. (2001) and Torres et al. (2002), in a Jenway 6405 UV/VIS Spectrophotometer. The LPO level was expressed in nmol of TBARS/g wt.

GPx activity was determined by measuring the decrease in NADPH at 340 nm (Jenway 6405 UV/VIS Spectrophotometer) using H_2O_2 as substrate (Mohandas et al.

1984). GR activity, which catalyses the reduction of GSSG to GSH with the concomitant oxidation of NADPH to $NADP^+$, was assayed according to Cribb et al. (1989) by monitoring the decrease in NADPH levels at 340 nm (Jenway 6405 UV/VIS Spectrophotometer). These enzyme activities were expressed in nmol/min/mg of protein.

SOD activity was assayed according to Flohé and Ötting (1984). In brief, a xanthine–xanthine oxidase system was used to produce superoxide radicals, which subsequently reduce cytochrome c. This reaction is monitored spectrophotometrically (Bio Tek Power Wave 340 microplate reader) at 550 nm. Enzymatic activity was expressed as units per milligram of protein: one unit of SOD is defined as the amount of enzyme required to inhibit the rate of reduction of cytochrome c in an extent of 50%.

CAT activity was determined according to Clairborne (1985) and represents the H_2O_2 consumption obtained at 240 nm. The enzyme activity was expressed in nmol of substrate hydrolysed/min/mg of protein.

TG content (GSH + GSSG) and oxidized glutathione (GSSG) were determined at 412 nm, using a recycling reaction of GSH with 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) in the presence of GR excess (Tietze 1969; Baker et al. 1990). 2-Vinyl-pyridine was used to conjugate GSH for the GSSG determination (Griffith 1980). GSH was calculated by subtracting GSSG from the TG levels. GSH/GSSG ratios were expressed according to Peña-Llopis et al. (2001).

GST activity in the liver was determined according to the method described above, in a Jenway 6405 UV/VIS Spectrophotometer.

All chemicals were of reagent grade and purchased from Sigma.

Protein determinations

All the enzymatic activities were expressed as a function of the protein concentration in the samples, which was determined in triplicate by the Bradford method (1976), adapted to microplate as indicated in Guilhermino et al. (1996), using bovine γ-globulins as protein standard and a wavelength of 600 nm. A Bio Tek Power Wave 340 microplate reader was used.

Abiotic factors determination

Water temperature (T), salinity (Sal) and pH were measured in situ, in triplicate, at the mouth of each studied estuary at each sampling season, using the multiparametric sea gauge WTW multi 340i and the appropriate probes (pH Sen Tix 41 and Tetracon 325). At the same time, subsurface water samples were collected, in triplicate, with 1.5 l

polyethylene-terephthalate bottles and stored at 4°C until nutrient analysis. Concentrations of nitrites (NO_2), nitrates (NO_3), ammonium ion (NH_4^+), phosphates (PO_4), silica (SiO_2), phenol (C_6H_5OH), and water hardness ($CaCO_3$) in these samples were determined by colorimetry using a Palintest 7000 interface photometer.

Statistical analysis

All values are reported as mean ± standard error. Comparisons of abiotic factors, among sampling estuaries and among sampling seasons, were performed using the Kruskal–Wallis test. When significant differences were found, the Mann–Whitney *U*-test, with adjustment of the significance level for the number of comparisons performed according to Bonferroni method (Sokal and Rohlf 1995), was used to identify their origin. Data concerning morphometric parameters, physiological condition indexes and biomarkers activities were analysed by two-way analysis of variance (ANOVA) with interaction to test for the effect of both estuaries and seasons. When significant differences were found, Bonferroni multiple comparisons procedure was used to identify their origin. Kolmogorov–Smirnov and Barlett's tests were used to check normality of data distribution and homogeneity of variances between groups, respectively. For all the variables analysed, values more than 3.5 standard deviations distant from their group mean were considered as outliers and removed from the analyses, because they were producing departures from normality of group distributions. When appropriated, the square root transformation was applied to data, in order to fulfil normality and homogeneity of variance assumptions. Pearson's product moment coefficient (*r*) was used to assess possible linear relations between the variables assessed, both biological and environmental. Nominal environmental variables (i.e., estuary and season) were dichotomised, relatively to each category by attribution of the codes 1 and 0, and the point biserial correlation coefficient (r_{pb}) was then used to assess linear relations between these and non-dichotomous variables of interest. Significant differences among estuaries were detected for the morphometric parameters measured (see Sect. "Results") and a very high correlation coefficient was found between total body length and total body weight of eels ($r = 0.93$, $P < 0.0001$). Total body weight was thus used as a covariate in the two-way ANOVAs performed.

Redundancy analysis (RDA), an ordination method of direct gradient analysis (ter Braak and Prentice 1988), was also performed to search for patterns in the set of biological parameters and to assess relationship between biological and environmental data. This multivariate analysis also provides an overall significance test of biological parameters change. Monte Carlo permutation was used to assess

statistical significance of the canonical axes. The following nominal environmental variables (categories indicated within brackets) were used: estuary (Minho, Lima, Douro) and season (summer, autumn, winter, spring). These factors were coded, as in multiple regression, as series of dummy environmental variables, one variable for each category. In the ordination diagram, the categories of the nominal environmental variables are indicated by centroids (average scores of the samples belonging to that category). The quantitative environmental variables were the abiotic factors *T*, Sal, pH, NO_2, NO_3, NH_4^+, PO_4, SiO_2, C_6H_5OH, and $CaCO_3$. Of these, four variables (*T*, NO_2, NO_3, and $CaCO_3$) had to be removed from the final analysis because they were strongly correlated with one or more of the remaining environmental variables, producing colinearity problems. The scaling was focused on inter-species correlations and the scores of the biological variables were adjusted for the corresponding variance after RDA was carried out. In addition, an RDA was first carried out using total body weight of eels as the only explanatory variable to evaluate the need for its inclusion as a covariate in the definitive analysis. The results indicated that the first constrained axis obtained had no significant contribution to explain the overall variability of the data and so no covariate was included in the model. The significance of the second canonical axis obtained in the final analysis was tested by performing an RDA analysis on the data using environment-derived samples scores for the first axis as a covariate (ter Braak and Prentice 1988; Lepš and Šmilauer 2003).

For all statistical tests performed, the significance level was set at 0.05. All statistical analyses were carried out using SPSS for Windows v15.0 and CANOCO for Windows v4.52.

Results

Abiotic factors and chemical analyses

Statistically significant effects of the estuary on Sal ($K = 6.3$, $P = 0.032$) and $CaCO_3$ ($K = 7.5$, $P = 0.011$) were observed, with water samples from the estuaries of rivers Lima and Douro showing much higher levels of these abiotic factors than those from the estuary of Minho River (Table 1). Significant effects of the estuary on the concentration of NO_2 ($K = 7.9$, $P = 0.006$) were also found. Samples from the estuary of Douro River showed a higher concentration of NO_2 than those from the estuaries of rivers Minho and Lima. Concerning variation among seasons, statistically significant differences were only observed for water temperature ($K = 9.4$, $P = 0.002$). Water samples collected during summer and spring showed higher *T* while those collected in autumn and winter

Table 1 Variation of abiotic factors among estuaries and among seasons

	Estuary			Season			
	Minho	Lima	Douro	Summer	Autumn	Winter	Spring
T (°C)	14.83	16.90	15.35	22.03[a]	11.43[b]	11.03[b]	18.27[a]
	(2.56)	(2.97)	(2.54)	(0.99)	(0.47)	(0.48)	(0.68)
Sal (‰)	2.38[a]	9.09[b]	12.79[b]	12.70	8.77	4.77	6.10
	(1.77)	(1.96)	(3.37)	(5.83)	(2.45)	(2.40)	(3.12)
pH	7.44	7.43	7.85	7.64	7.53	7.60	7.53
	(0.27)	(0.38)	(0.17)	(0.41)	(0.58)	(0.16)	(0.21)
NO_2 (mg/l)	0.03[a]	0.02[a]	0.06[b]	0.04	0.04	0.02	0.03
	(0.01)	(0.00)	(0.01)	(0.02)	(0.01)	(0.01)	(0.01)
NO_3 (mg/l)	1.76	0.97	2.42	1.58	1.33	2.47	1.48
	(0.32)	(0.08)	(0.73)	(0.45)	(0.38)	(1.10)	(0.17)
NH_4^+ (mg/l)	0.44	0.19	0.31	0.17	0.36	0.64	0.09
	(0.35)	(0.09)	(0.12)	(0.08)	(0.18)	(0.43)	(0.02)
PO_4 (mg/l)	0.39	0.08	0.31	0.23	0.12	0.46	0.23
	(0.10)	(0.02)	(0.14)	(0.13)	(0.04)	(0.17)	(0.13)
SiO_2 (mg/l)	2.59	1.41	1.76	1.26	1.67	2.46	2.29
	(0.22)	(0.33)	(0.80)	(0.42)	(0.64)	(0.97)	(0.32)
C_6H_5OH (mg/l)	0.06	0.08	0.04	0.07	0.07	0.07	0.02
	(0.02)	(0.01)	(0.01)	(0.00)	(0.00)	(0.02)	(0.02)
$CaCO_3$ (mg/l)	120.42[a]	2,072.50[b]	2,675.00[b]	2,386.67	2,418.89	728.33	956.67
	(93.78)	(638.95)	(742.91)	(1,118.37)	(1,181.11)	(453.45)	(483.40)

Values represent the mean and the corresponding standard error in brackets of temperature (T), salinity (Sal), pH, nitrites (NO_2), nitrates (NO_3), ammonium ion (NH_4^+), phosphates (PO_4), silica (SiO_2), phenol (C_6H_5OH) and water hardness ($CaCO_3$). Different letters identify statistically significant differences (at $P < 0.05$), either among estuaries or among seasons, as indicated by the results of Kruskal–Wallis and Mann–Whitney U tests

showed lower average temperatures, around 11°C (Table 1). Apart from these, in general, the abiotic variables assessed showed similar variation patterns among estuaries and among seasons.

In addition, high correlations were observed between the abiotic factors, the concentrations of metals and PAHs ($r = 0.50$), and between the former two and the estuaries and the sampling season (Table 2). For instance, the concentration of metals showed high inverse relationships with the estuary of Minho River ($r_{pb} = -0.73$) and the concentration of SiO_2 ($r = -0.64$), and high positive correlations with the estuary of Lima River ($r_{pb} = 0.69$) and Sal ($r = 0.63$). PAHs were also negatively correlated with Minho estuary and SiO_2, and were positively correlated with Douro estuary, autumn, Sal and $CaCO_3$ (Table 2). As to T, it was positively correlated with summer ($r_{pb} = 0.80$) and negatively with autumn and winter. NO_2 and NO_3 were more relevant in Douro estuary, showing positive correlations with it and negative correlations with Lima estuary. Also, $CaCO_3$ was associated with Sal and SiO_2 concentrations.

Morphometric measures and physiological condition indexes

The total body length of yellow eels sampled ranged from 20.8 to 60.9 cm, with average values of 30.8, 31.8 and 35.6 cm for eels from the estuaries of Minho, Lima and Douro Rivers, respectively (Table 3). Total body weight ranged from 12.3 to 439.5 g, with average values of 53.2, 56.1 and 91.1 g for eels from the estuaries of Minho, Lima and Douro Rivers, respectively (Table 3). Eels collected in the estuary of Douro River were longer (Table 2, $F(2, 270) = 21.1$, $P < 0.001$) than those collected in the estuaries of Minho and Lima Rivers, particularly in the summer, as indicated by the interaction estuaries × season ($F(6, 270) = 3.7$, $P = 0.001$). Similarly, eels collected in the Douro estuary had a higher body weight ($F(2, 270) = 23.3$, $P < 0.001$) than those collected in the estuaries of rivers Minho and Lima. These differences were also particularly evident during summer, as indicated by the statistically significant interaction estuaries × season ($F(6, 270) = 5.1$, $P < 0.001$).

Table 2 Significant Pearson (*r*) and point biserial (*r*$_{pb}$) correlation coefficients, above 0.40 ($P < 0.001$), between the environmental variables assessed (metals and PAHs in sediments, water abiotic factors, and estuary and sampling season)

Environmental variables		r/r_{pb}
Metals	Minho	−0.73
	Lima	0.69
	PAHs	0.50
	Sal	0.63
	SiO$_2$	−0.64
PAHs	Minho	−0.43
	Douro	0.48
	Autumn	0.58
	Sal	0.52
	NO$_2$	0.45
	SiO$_2$	−0.69
	CaCO$_3$	0.74
T	Summer	0.80
	Autumn	−0.55
	Winter	−0.51
NO$_2$	Lima	−0.62
	Douro	0.87
	Sal	0.53
NO$_3$	Lima	−0.55
	Douro	0.56
	Winter	0.49
CaCO$_3$	Minho	−0.74
	Douro	0.49
	Sal	0.75
	SiO$_2$	−0.58

T Temperature; *Sal* salinity; *NO$_2$* nitrites; *NO$_3$* nitrates; *SiO$_2$* silica; *CaCO$_3$* water hardness; *Metals* total heavy metals; *PAHs* total polycyclic aromatic hydrocarbons

The *K* index for yellow eels from the three studied estuaries varied between 0.10 and 0.25, with mean values of 0.17 in Minho and Douro estuaries and 0.15 in the Lima River estuary (Table 3). Significant differences were observed among estuaries ($F(2, 268) = 32.1$, $P < 0.001$), with *K* in general lower in yellow eels from the estuary of Lima River compared to those from the other estuaries, especially in the winter. In addition, an effect of the interaction estuary × season on the *K* index was observed ($F(6, 268) = 10.1$, $P < 0.001$) (Table 3). The highest mean LSI value (1.77) was obtained for the Minho River population, while the lowest was determinated for Lima estuary eels (1.42). Concomitantly, eels collected in the contaminated estuaries, presented, on average, lower LSI values than eels collected in the reference estuary ($F(2, 269) = 19.4$, $P < 0.001$). Moreover, the three populations showed different patterns of temporal variation (interaction estuary × season: $F(6, 269) = 17.1$, $P < 0.0001$), with the

Table 3 Variation of weight, length, and condition indexes among estuaries and among seasons

Parameter	Estuary			Season											
				Summer			Autumn			Winter			Spring		
	Minho	Lima	Douro	Minho	Lima	Douro	Minho	Lima	Douro	Minho	Lima	Douro	Minho	Lima	Douro
L (cm)	30.8^a (0.50)	31.8^a (0.61)	35.6^b (0.80)	30.4^a (1.15)	31.7^a (1.09)	42.2^b (2.49)	32.2 (1.01)	34.5 (1.18)	35.1 (1.13)	28.4 (0.67)	27.7 (0.64)	31.8 (1.08)	31.7 (0.98)	32.8 (1.4)	35.4 (1.58)
W (g)	53.2^a (3.27)	56.1^a (4.04)	91.1^b (7.69)	49.1^a (7.85)	61.0^a (7.56)	160.2^b (26.55)	62.3 (7.04)	70.6 (9.11)	83.9 (11.69)	45.7 (4.27)	28.4 (2.49)	59.9 (6.6)	53.1 (6.17)	57.5 (7.59)	84.1 (12.38)
K	0.17^a (0.003)	0.15^b (0.002)	0.17^a (0.002)	0.16 (0.006)	0.16 (0.004)	0.18 (0.007)	0.17 (0.005)	0.15 (0.004)	0.17 (0.003)	0.19^a (0.007)	0.13^b (0.004)	0.17^a (0.002)	0.15 (0.005)	0.15 (0.004)	0.17 (0.003)
LSI	1.78^a (0.053)	1.44^b (0.037)	1.55^b (0.038)	1.40 (0.066)	1.58 (0.047)	1.39 (0.046)	1.95^a (0.062)	1.32^b (0.077)	1.66^a (0.069)	2.21^a (0.119)	1.14^b (0.033)	1.72^c (0.07)	1.53 (0.072)	1.63 (0.09)	1.28 (0.06)

Values represent the mean and the corresponding standard errors in brackets of total body length (*L*), total body weight (*W*), Fulton's condition factor *K*(*K*), and hepatosomatic index (LSI). For each studied parameter, different letters identify statistically significant differences ($P < 0.05$) among estuaries, for each sampling season as, indicated by the Bonferroni multiple comparisons test

lowest LSI values recorded in autumn and winter especially in the Lima estuary.

Biomarkers of neurotoxicity, energy production, osmoregulation, and biotransformation (Phase I and II)

Significant differences in brain AChE activity were found among eels collected in the different estuaries ($F(2, 269) = 17.8$, $P < 0.001$). Eels from the Douro estuary showed, on average, lower AChE activity than those from Minho and Lima (Fig. 2). Significant differences among seasons were also found ($F(3, 269) = 94.9$, $P < 0.001$), with the highest activities observed during the summer and the lowest during the autumn. Additionally, the interaction term revealed differences among estuaries that are dependent on the sampling season ($F(6, 269) = 16.2$, $P < 0.001$); eels from Douro estuary showed a 25% decrease in AChE activity during autumn, while those from Lima and Douro estuaries showed significant 23 and 43%

decreases, respectively, during winter, relatively to eels collected in the reference estuary during the same seasons (Fig. 2).

In our study, a significant effect of the estuary on muscle LDH activity was observed ($F(2, 266) = 40.7$, $P < 0.001$). In general, eels from Douro estuary showed a 23% increase in LDH activity compared to eels from Minho estuary. Concerning seasonal variation, LDH levels in Lima estuary were constant across sampling seasons, whereas those found in eels from Minho and Douro estuaries showed a general decreasing tendency, with the highest activities observed during summer and the lowest during spring (Fig. 2, $F(3, 266) = 35.1$, $P < 0.001$). The interaction between the two factors was also significant ($F(6, 266) = 17.3$, $P < 0.001$). Eels from Lima estuary showed a 48% decrease of LDH activity during summer compared to the activities observed for the reference estuary during the same season. In contrast, those collected in Douro estuary during autumn showed a strong increase of LDH

Fig. 2 Biomarkers of neurotoxicity, energy production, osmoregulation, and Phase I and Phase II biotransformation analysed in yellow eels collected in the estuaries of Minho, Lima and Douro rivers during four sampling seasons. *Values* represent the mean and the corresponding standard errors of acetylcholinesterase (*AChE*), lactate dehydrogenase (*LDH*), sodium/potassium ATPase (*Na$^+$/K$^+$-ATPase*), ethoxyresorufin-*O*-deethylase (*EROD*) and glutathione *S*-transferases (*GST*) activities. *Different letters* identify statistically significant differences ($P < 0.05$) among estuaries for each season as indicated by the Bonferroni multiple comparisons test
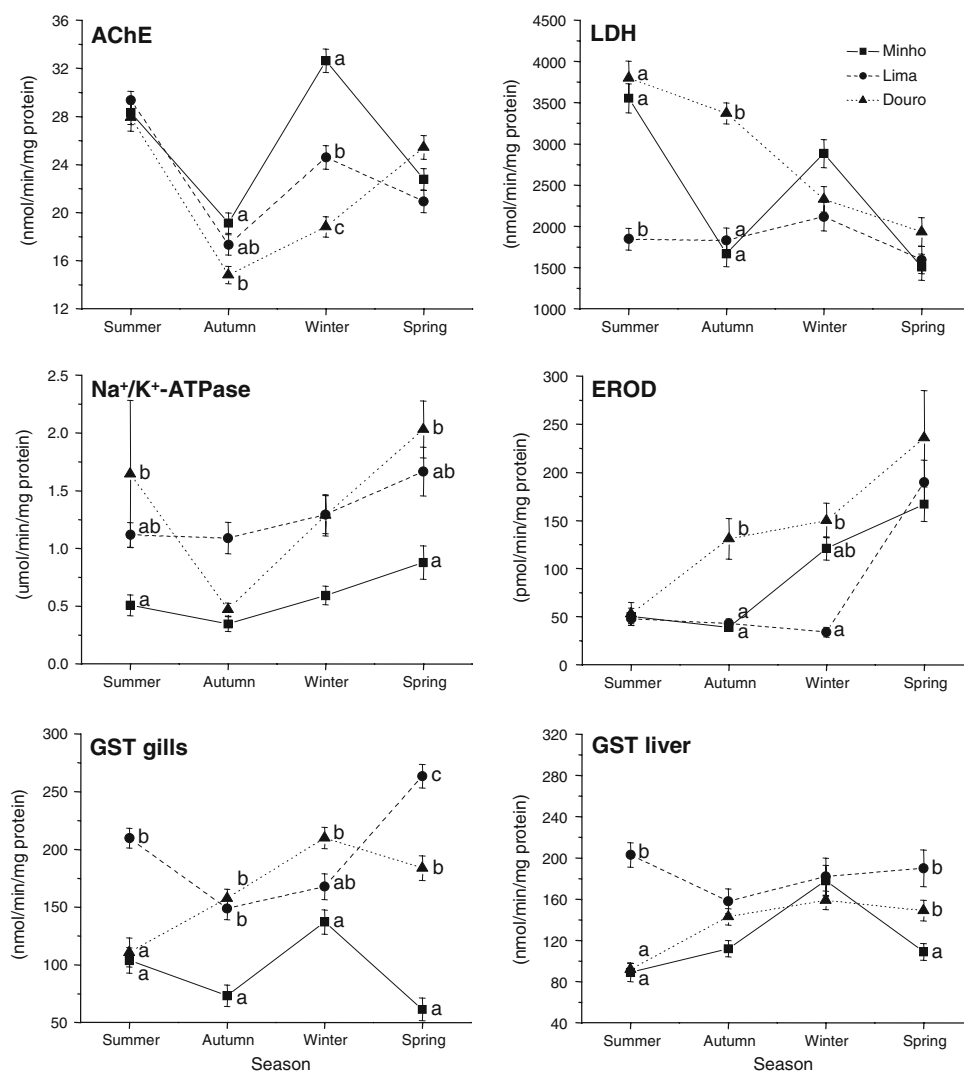
activity (about 100%) followed by a decrease to levels comparable to those of eels from Minho estuary, in winter.

During the studied year, Na^+/K^+-ATPase activity in the gills was significantly increased in Lima ($\sim 118\%$) and Douro ($\sim 107\%$) estuaries, compared to that of eels from Minho (Fig. 2, $F(2, 251) = 17.1$, $P < 0.001$). Regarding seasonal variation, a general increasing trend of Na^+/K^+-ATPase activity was observed from summer to winter ($F(3, 251) = 10.3$, $P < 0.001$); low values were found for eels collected during autumn, while high activities were observed in eels collected during spring. The differences among estuaries were also dependent on the sampling season ($F(6, 251) = 2.4$, $P < 0.026$); increased levels of Na^+/K^+-ATPase activity were found for eels collected in Lima and Douro estuaries during all seasons (Fig. 2), except for those collected in Douro estuary during autumn. The latter showed values similar to those found for eels collected in Minho estuary during the same period.

Mean values of hepatic EROD activity were different among estuaries ($F(2, 244) = 11.7$, $P < 0.001$), showing a 51% significant increase ($P < 0.01$) in eels from Douro, compared to those from Minho estuary. A general increasing trend was observed from summer through spring, with the highest values of EROD activity found in winter and spring ($F(3, 244) = 30.6$, $P < 0.001$). A significant effect of the interaction estuary × season was also observed ($F(6, 244) = 2.7$, $P < 0.015$). Eels collected in Douro estuary during autumn showed a more than two-fold increase of EROD activity, while those collected in Lima estuary during winter showed an equivalent decrease of

EROD activity, relatively to those collected in the reference estuary during the same seasons.

Phase II biotransformation, assessed as GST activity, was significantly induced in yellow eels from the contaminated estuaries in both liver ($F(2, 264) = 29.1$, $P < 0.001$) and gills ($F(2, 260) = 110.5$, $P < 0.001$), compared to those collected in Minho estuary (Fig. 3). This increase was particularly high, more than two-fold, for GST determined in gills of eels collected in Lima estuary. In respect to season, statistically significant effects were found for GST activity in both liver ($F(3, 264) = 7.6$, $P < 0.001$) and gills ($F(3, 260) = 14.9$, $P < 0.001$). In general, the lowest values of GST activity were observed in summer and autumn whereas the highest values were observed in winter and spring. Moreover, significant effects of the interaction term were observed for GST both in liver ($F(6, 264) = 6.0$, $P < 0.001$) and gills ($F(6, 260) = 18.9$, $P < 0.001$). Relatively to eels from the reference estuary, those collected during summer and spring in the Lima estuary, and in Lima and Douro estuaries, showed the highest changes of GST activity in liver and gills, respectively.

Non-enzymatic and enzymatic antioxidants

Since the levels of GSH determined in the liver generally tended to be higher than those of GSSG, the variation of TG (GSH + GSSG) across estuaries and seasons reflected mostly that of GSH. An effect of the estuary on the hepatic levels of TG and GSH was observed (Fig. 3, TG: $F(2,$

Fig. 3 Variation of oxidative stress co-factors for yellow eels collected in the estuaries of Minho, Lima and Douro rivers during four sampling seasons. *Values* represent the mean and the corresponding standard errors of liver total glutathiones (*TG*), reduced glutathione (*GSH*), oxidised glutathione (*GSSG*) and *GSH/GSSG* ratio
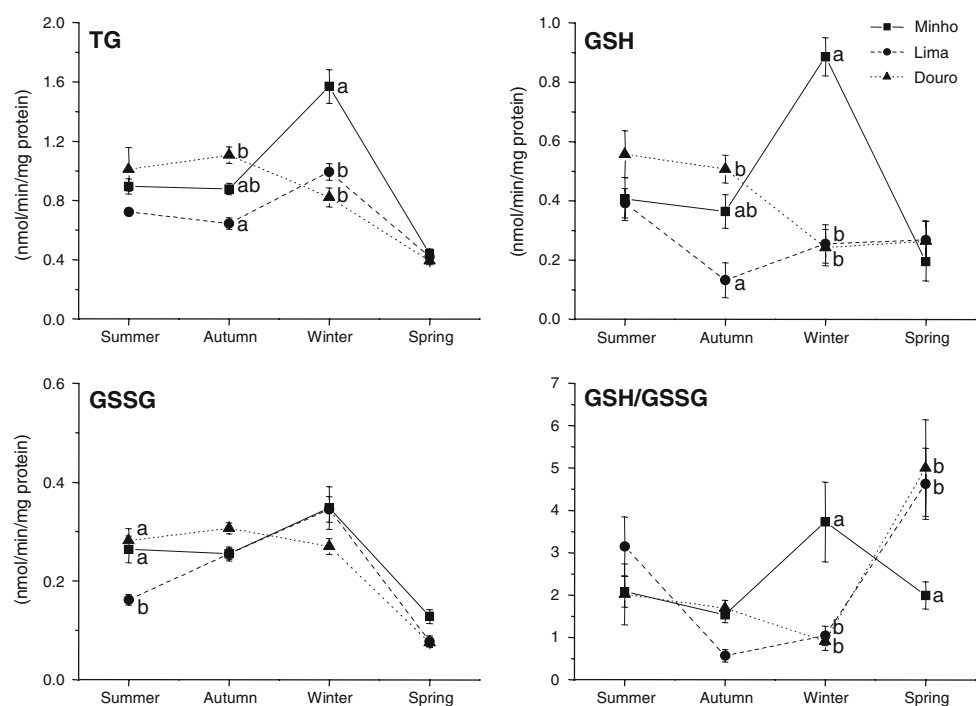
257) $= 17.7$, $P < 0.001$; GSH: $F(2, 243) = 11.0$, $P < 0.001$). Particularly, TG and GSH levels were significantly lower (~ 27 and 40%, respectively) in eels from the Lima estuary, compared to those from the reference and the Douro estuaries. Regarding seasonal variation, significant differences were also observed (TG: $F(2, 257) = 67.0$, $P < 0.001$; GSH: $F(3, 243) = 7.9$, $P < 0.001$), with the highest levels of glutathione recorded during winter and the lowest during spring. Significant effects of the interaction estuary × season were found as well (TG: $F(6, 257) = 67.0$, $P < 0.001$; GSH: $F(6, 243) = 12.3$, $P < 0.001$). Yellow eels collected in Lima and Douro estuaries during winter showed reduced levels (37 and 48%, respectively) of TG, whereas GSH levels were particularly low in Lima estuary during autumn and in Lima and Douro estuaries during winter. In contrast, no relevant overall differences of both hepatic GSSG levels and the ratio of reduced to oxidised glutathione (GSH/GSSG) were detected among eels from the different estuaries (Fig. 3). Significant effects of the sampling season were, however, observed (GSSG: $F(3, 250) = 75.5$, $P < 0.001$; GSH/GSSG: $F(3, 241) = 11.5$, $P < 0.001$). Levels of GSSG showed an increasing trend from summer to winter, and then strongly decreased, attaining the lowest values in spring. As to GSH/GSSG values, they decreased from summer to autumn, increasing thereafter and reaching their maximum in spring. Significant effects of the interaction term were also observed for both antioxidant parameters (GSSG: $F(6, 250) = 6.3$, $P < 0.001$; GSH/GSSG: $F(6, 241) = 6.3$, $P < 0.001$), indicating that differences among estuaries were dependent on the sampling season. While values of GSSG were lower in eels collected during summer in the estuary of Lima River than in the estuary of Minho River, diminished values of GSH/GSSG were recorded in eels collected in the contaminated estuaries during winter, and an augmented ratio was found in eels collected in the estuary of Douro River during spring.

In general, no relevant hepatic induction of antioxidant enzymes was observed in Lima and Douro estuaries (Fig. 4). On the contrary, a significant effect of estuary was observed for CAT activity ($F(2, 252) = 8.2$, $P < 0.001$), which was even inhibited in eels from Lima estuary compared to those from the estuary of Minho River, although not for SOD, GPx, and GR. A high seasonal variation was also observed for these enzymatic activities (CAT: $F(3, 252) = 157.5$, $P < 0.001$; SOD: $F(3, 235) = 40.3$, $P < 0.001$; GPx: $F(3, 259) = 621.3$, $P < 0.001$; GR: $F(3, 266) = 72.8$, $P < 0.001$). In general, CAT, SOD, GPx, and GR activities were higher in summer, showing decreases thereafter, and reaching very low levels during winter and spring. Differences among estuaries were mainly found in relation to the sampling season for CAT ($F(6, 252) = 7.6$, $P < 0.001$), SOD ($F(6, 235) = 9.3$, $P < 0.001$) and GR

($F(6, 266) = 3.6$, $P < 0.002$), indicating mostly an inhibition of these antioxidant enzymes in Lima estuary during summer. In addition, liver lipid peroxidation was significantly different among estuaries ($F(2, 259) = 16.9$, $P < 0.001$), increasing ($\sim 17\%$) in eels collected in Lima estuary (Fig. 4), compared to eels from Minho estuary. LPO levels also showed strong seasonal variation ($F(3, 259) = 32$, $P < 0.001$), with the highest values recorded during spring and the lowest during summer. The interaction estuary × season indicated that eels from different estuaries showed different patterns of seasonal variation ($F(6, 259) = 18.3$, $P < 0.001$). Whereas eels from Minho estuary showed an increase of LPO from summer to autumn then decreasing from autumn to winter and rising again thereafter, those from Lima estuary showed an augmentation from summer to autumn, with levels significantly higher than the reference estuary exhibited during winter, when strong glutathione depletion was found, decreasing only thereafter.

Effects of environmental parameters on physiological condition indexes and biomarkers

The relationship between biological and environmental parameters was studied using a redundancy analysis. The results show that environmental variables explained 44.4% of the overall data variability (Fig. 5, F-ratio $= 19.6$, $P = 0.0020$, Monte Carlo permutation). The first pair of axes (horizontal) explained 23.7% of the total data variability (F-ratio $= 101.2$, $P = 0.0020$) and showed a strong positive correlation with the activities of liver antioxidative enzymes GPx, GR, CAT. This axis was also correlated with the K condition factor and brain AChE activity, though to a slightly less extent (Fig. 5). The environmental axis is a seasonal gradient, which was positively correlated with the concentration of phenol and negatively with the concentration of silica (Table 4). The correlation between these two axes was 87.0% indicating a highly significant relationship. The second constrained axis (vertical) explained 7.8% of the total data variability (F-ratio $= 32.4$, $P = 0.0020$) and was correlated mainly with the hepatic levels of TG and GSSG, with LSI and, to a less extent, with gill Na^+/K^+-ATPase activity. The environmental axis clearly separated the reference estuary (Minho) from the contaminated ones (Lima and Douro), suggesting the occurrence of a contamination gradient, being positively correlated with the concentration of ammonia and negatively with salinity (Table 4). The correlation for this canonical axis was 79.8% also indicating a highly significant relationship. The analysis of the ordination diagram indicate that CAT, GR, and SOD anti-oxidant enzymes tend to show their highest activities in yellow eels from

Fig. 4 Variation of anti-oxidative enzymes and lipid peroxidation for yellow eels collected in the estuaries of Minho, Lima and Douro rivers during four sampling seasons. *Values* represent the mean and the corresponding standard errors of the activities of the enzymes catalase (*CAT*), glutathione peroxidase (*GPx*), glutathione reductase (*GR*) and superoxide dismutase (*SOD*), and the hepatic levels of lipid peroxidation (*LPO*). *Different letters* identify statistically significant differences ($P < 0.05$) among estuaries for each season as indicated by the Bonferroni multiple comparisons test
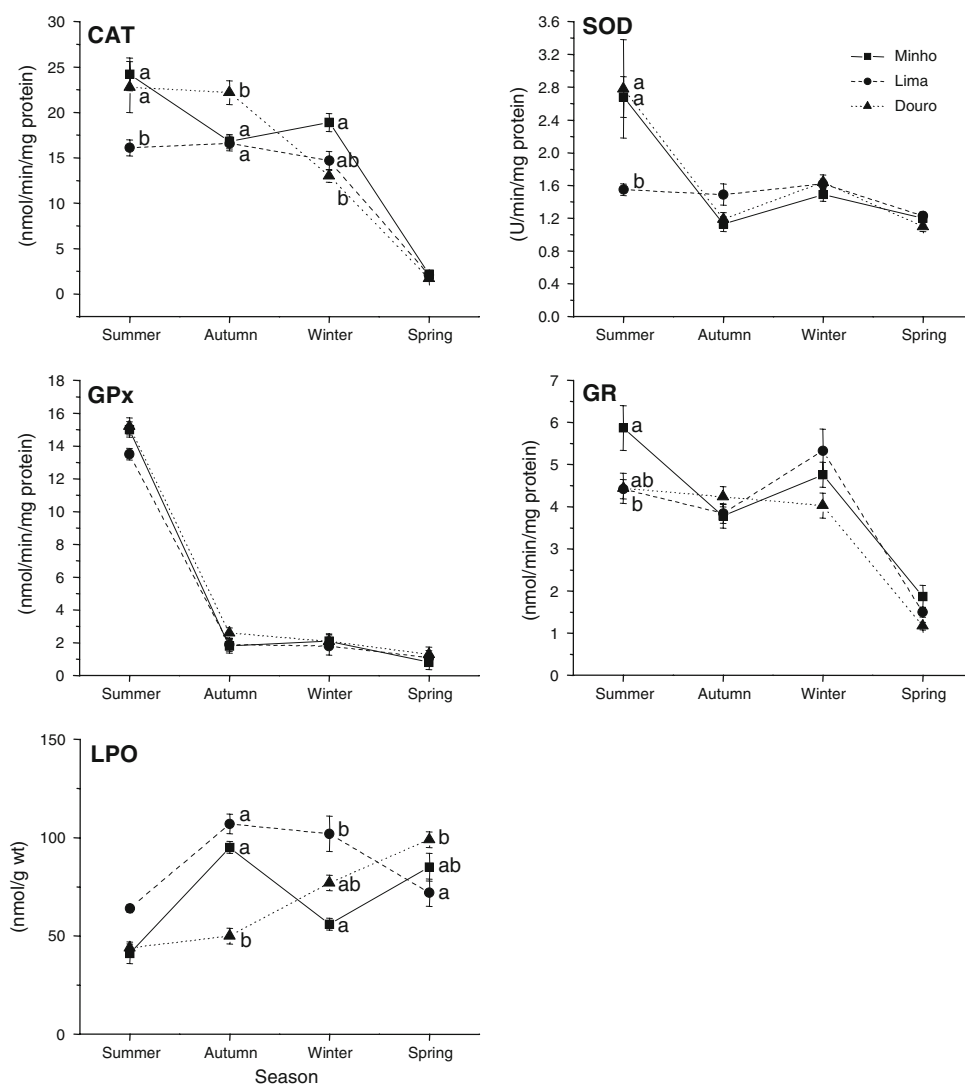
Minho estuary during summer, and their lowest activities in eels from Lima and Douro estuaries, especially in winter (Fig. 5). This trend is accompanied by a very similar tendency for TG and GSSG, and for *K* and LSI indexes (Fig. 5). In contrast, GPx showed its higher activity in eels collected during summer, and similar activity among estuaries. Furthermore, AChE attained its lowest activity in eels from Lima and Douro estuaries in autumn and winter. Na^+/K^+-ATPase activity was particularly increased in eels from Lima estuary, during summer (Fig. 5), contributing to discriminate the two contaminated estuaries. Concerning abiotic factors, the *K* condition index and the enzymes CAT and GR tend to increase with increasing water phenol concentrations, whereas the activities of AChE and SOD seem to be influenced by silica. Moreover, Na^+/K^+-ATPase activity appears to be under the influence of a balance between salinity and ammonia levels (Table 4, Fig. 5).

Discussion

Eels are long living species and therefore they are good models to assess long term effects induced by pollution. In the present study, an integrated approach including morphometric parameters, condition indexes, biomarkers and environmental variables was used to compare *A. anguilla* populations from three estuaries with different levels of environmental contamination.

The values obtained for both *K* and LSI in the studied estuaries are in the same range of those found in yellow eels of similar body length from other European estuarine areas, such as the River Havel system in Germany (Simon 2007), the Rhône Delta in southern France (Roche et al. 2000; Ribeiro et al. 2005) and the Amsterdam area (van der Oost et al. 1996a). According to *K* index that has been used as an indicator of health condition (Ricker 1975), eels from
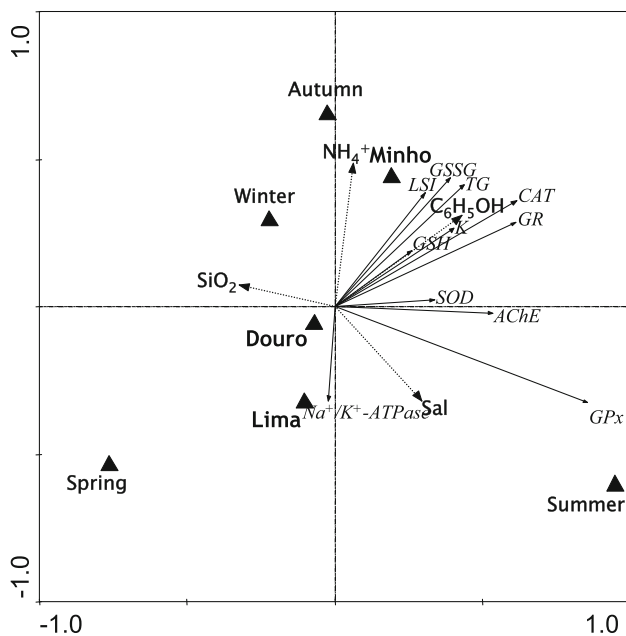
Fig. 5 Redundancy analysis ordination diagram displaying the scores for biological variables (*straight arrows*), quantitative environmental variables showing correlations above 0.30 with the environmental axes (*dotted arrows*), and nominal environmental variables (*triangles*): first axis is *horizontal*, second axis *vertical*. Only biological variables that have at least more than 8% (derived from the eigenvalue for the second canonical axis) of their variability explained by environmental variables are shown. The diagram explains 44.4% of the total variance in biological data, the first two eigenvalues being 0.273 and 0.078, respectively. The third and fourth eigenvalues are 0.033 and 0.025, respectively. The biological variables are: Fulton's condition factor K (K), hepatosomatic index (*LSI*), acetylcholinesterase (*AChE*), sodium/potassium ATPase (Na^+/K^+-*ATPase*), catalase (*CAT*), glutathione peroxidase (*GPx*), glutathione reductase (*GR*) and superoxide dismutase (*SOD*) activities, total glutathione (*TG*), reduced glutathione (*GSH*) and oxidised glutathione (*GSSG*) levels. The quantitative environmental variables are: salinity (*Sal*), ammonium ion (NH_4^+), silica (SiO_2) and phenol (C_6H_5OH). The nominal environmental variables are: the studied estuaries (*Minho*, *Lima* and *Douro*) and the sampling seasons (*summer*, *autumn*, *winter* and *spring*)

Lima estuary show a poorer health condition than those from Minho and Douro estuaries, particularly in winter (Table 3). This may be related to food availability and other unfavourable environmental conditions as suggested in other studies (Ricker 1975; Hoque et al. 1998; Lloret et al. 2002). The lower LSI found in Lima and Douro eels relatively to Minho animals may indicate poor habitat quality and/or low food available forcing the animals to metabolize liver stored lipids and reducing liver weight (Hoque et al. 1998). However, it should be noted that several studies report increased or decreased LSI values in response to different environmental stressors. An increased LSI may occur due to accumulation of parasites, liver damage or exposure to pollution (e.g., some metals, PAHs and PCBs), as the liver increases in size due to the presence

Table 4 Biological responses in eel populations along a pollution gradient, from Fig. 5: canonical and intraset correlations of environmental variables with the first two axes of redundancy analysis

Axis variable	Canonical coefficients		Correlation coefficients	
	1	2	1	2
Minho	0.00	0.00	0.20	0.45
Lima	0.03	−0.50	−0.11	−0.37
Douro	−0.56	−1.10	−0.08	−0.07
Summer	0.76	−0.26	0.83	−0.53
Autumn	0.53	0.95	−0.02	0.67
Winter	−0.60	0.31	−0.19	0.25
Spring	0.00	0.00	−0.63	−0.45
Sal	1.37	1.08	0.30	−0.32
NH_4^+	0.92	0.57	0.06	0.49
SiO_2	0.97	0.35	−0.33	0.07
C_6H_5OH	−0.38	−0.54	0.43	0.31

The environmental variables are: the studied estuaries (Minho, Lima and Douro), the sampling seasons (summer, autumn, winter and spring), and the abiotic factors salinity (Sal), ammonia (NH_4^+), silica (SiO_2), and phenol (C_6H_5OH)

of parasites, to allow greater detoxification or as a consequence of toxicants accumulation (Slooff et al. 1983; van der Oost et al. 2003).

Inhibition of AChE activity above 20% has been considered indicative of exposure to anticholinesterase agents (Ludke et al. 1975). Lima and Douro eels showed significant inhibition of AChE activity in autumn and winter (23–43%) relatively to the reference estuary, indicating the presence of anticholinesterase agents at these periods of the year. These agents may be heavy metals, PAHs, organophosphate and carbamate pesticides and/or detergents that have been found to be cholinesterase inhibitors (Payne et al. 1996; Guilhermino et al. 1998; Buet et al. 2006). In fact, metals and PAHs have been found in sediments of Lima and Douro estuaries at concentrations high enough to cause AChE inhibition (ppm range). In addition, anticholinesterase insecticides might also arrive to estuaries by runoff of agriculture fields and detergents from domestic and industrial effluents. Moderate levels of AChE inhibition may result in alterations of nervous and neuromuscular functions (Payne et al. 1996) interfering with several physiological functions and development. They may also increase the mortality rate since, for e.g., animals may have difficulties in escaping from predators or in avoiding unfavourable environmental conditions.

The strong induction of LDH activity observed in eels collected in Douro estuary during the autumn suggests a change to anaerobic metabolism during this period. Increases in anaerobic metabolism have been found to be a rapid response caused by chemical stress, when organisms need additional energy to cope with pollutants exposure,

such as PAHs (Cohen et al. 2001) and metals (De Coen et al. 2001; Diamantino et al. 2001).

The general induction of Na$^+$/K$^+$-ATPase observed in eels collected in Lima and Douro estuaries during all seasons may be related to the higher salinity found in contaminated estuaries relatively to the reference one and/or to effects of metals and lipophilic contaminants on the activity of this enzyme involved in osmoregulation. For instance, an increase of Na$^+$/K$^+$-ATPase activity with increasing concentrations of several PAHs and PCBs has been reported in yellow eels from polluted sites of the Rhône Delta (Buet et al. 2006). In addition, despite the inhibitory effect of some metals on this enzyme activity (Watson and Beamish 1981; Stagg et al. 1992a; Lionetto et al. 2000), compensatory mechanisms may increase the amount of enzyme present through the synthesis of new Na$^+$/K$^+$-ATPase and/or enhanced chloride cell turnover (Wendelaar Bonga 1997) leading to an increased activity or to its maintenance at pre-exposure levels (Sheppard and Simkiss 1978; Watson and Beamish 1980; Stagg et al. 1992a, b).

EROD activity in fish liver has been considered a very sensitive biomarker for the assessment of contamination by organic compounds (Livingstone et al. 1993). Several laboratory and field studies have reported increases in EROD activity in many fish species after exposure to organic pollutants such as PAHs, and PCBs (van der Oost et al. 2003) which are relevant contaminants in Douro estuary, among other organic pollutants (Ferreira et al. 2004, 2006; Almeida et al. 2007; Ribeiro et al. 2008). The increased EROD activities found in eels from this estuary, particularly in the autumn, are thus in good agreement with the presence of these agents in the estuary and also with the induced liver EROD activity found in mullets from this estuary (Ferreira et al. 2004, 2006). Furthermore, liver GST that is also involved in the detoxification of both PCBs and PAHs (van der Oost et al. 1996a; Buet et al. 2006) was also found to be in general increased in eels from Douro estuary. Concerning the inhibition of EROD activity observed in the Lima estuary during winter, it is noteworthy that in vitro experiments revealed that very low concentrations of heavy metals, such as copper and mercury, added to liver microsomes of sea bass treated with β-naphthoflavone or benzo[a]pyrene (B[a]P) significantly decreased EROD activity (Viarengo et al. 1997).

The general decrease of GT, GSH, and GSH/GSSG observed in eels from Lima and Douro during autumn and winter is consistent with the types of contaminants present in these estuaries. GSH is considered an important antioxidant agent involved in the protection of cell membranes from lipid peroxidation by scavenging reactive oxygen species (ROS), yielding glutathione disulphide (GSSG). Additionally, it acts also as cofactor of enzymes catalyzing the detoxification and excretion of several toxic compounds, as GPx and GST (Peña-Llopis et al. 2001). Field studies have shown a decrease in glutathione content in fish species, including eel, from sites polluted with PAHs and PCBs (van der Oost et al. 1996a, 2003). It is also known that several metals show high affinity to this molecule either originating the spontaneous formation of complexes with the thiol group of GSH or promoting its oxidation (Christie and Costa 1984). Moreover, laboratory experiments have shown that copper induces a significant depletion of TG, GSH, and GSH/GSSG content in eels pre-exposed to PAH-like compounds (Gravato et al. 2006). Overall, the enzymatic antioxidant activity observed in eels from Lima and Douro estuaries during winter seems to be insufficient to face pollution stress. This may contribute to the glutathione depletion observed, through both ineffective removal of ROS and recycling of GSH. Such a situation may also be an indirect consequence of exposure to several metals, as the latter can both stimulate the formation of ROS and inhibit the normal pathways by which they are removed (Christie and Costa 1984).

In this study responses of different nature and intensity were observed in the biological parameters assessed. For instance, high seasonal variability was generally found, which may be attributed to both natural seasonal fluctuations on the biological parameters examined and seasonal fluctuations of environmental factors, including contamination. Also, eel metabolism is known to be dependent of temporal variations of biotic (e.g., age, nutritional state, maturity, reproduction) and abiotic factors (Knights 1997). While interpretation of changes on a biomarker-by-biomarker basis was useful to detect significant differences among eels from different estuaries, it provided more limited information on the global significance of biological responses and their relationship with abiotic factors. Therefore, data were also analysed through a redundancy analysis which showed that condition indexes, glutathione levels, and the activities of the enzymes AChE, Na$^+$/K$^+$-ATPase, CAT, GR, and SOD were the biological parameters that contributed most to discriminate among estuaries. AChE and Na$^+$/K$^+$-ATPase activities were also found to be responsive biomarkers in field studies with yellow eels performed in the Rhône Delta (Buet et al. 2006).

Interestingly, CAT and SOD, which showed different responses to several environmental contaminants in previous studies with eels questioning their value as biomarkers (van der Oost et al. 1996a, 2003), were found to be relevant in the present study. On the contrary, despite being widely used as a sensitive biomarker for organic contaminants (Livingstone et al. 1993; van der Oost et al. 1996a, 2003), EROD activity was not a discriminating biomarker in our study, also in good agreement with the work of Buet et al. (2006). In relation to these apparently contradictory

findings, it was previously found by other authors that the daily oral administration of B[a]P to the aerolated grouper (*Epinephelus areolatus*) induced hepatic EROD activity after 1 week, with maximum levels after 2 weeks; however, after 4 weeks, EROD activity returned to background levels, despite the daily administration of B[a]P (Wu et al. 2003).

Here and contrary to what was expected, LPO was not a major discriminating factor. In fact, although inhibition of antioxidant enzymes and diminished glutathione levels were observed in contaminated estuaries during winter, there was not an increase in lipid peroxidation as found in other studies (Livingstone et al. 1993; van der Oost et al. 2003). According to field transplantation (Cossu et al. 2000) and laboratory experiments (Gravato et al. 2005) with mussels this relationship between antioxidant enzymes and cytotoxic damage through lipid peroxidation was observed after short exposures to toxic compounds inducing severe reductions of antioxidant defences, but not for exposures inducing moderate decreases. Furthermore, Regoli and Principato (1995) also working with mussels have suggested that these concomitant alterations, mostly obtained in laboratory exposures, may represent only the initial phase of a biological response, which would be followed by some compensatory adaptive mechanisms probably more frequent in field conditions.

Concerning the relationship between biological parameters and abiotic factors, salinity was found to be associated to Na$^+$/K$^+$ ATPase as expected due to the osmoregulatory role of this enzyme. The inverse correlation between this enzyme and ammonium levels is probably due to the capability of this ion to replace K$^+$ in the molecule of Na$^+$/K$^+$ ATPase (Towle and Holleland 1987; Sherwood et al. 2005). GPx responses appear to be uniquely associated with temperature, which was highly positively correlated with summer and negatively with autumn and winter. These results suggest that temperature variations may act as a confounding factor when assessing the response of GPx to pollution.

Regarding temporal variation, RDA shows that a high amount of total variability in the biological parameters was explained by sampling season. Similar results were found in other studies carried out in the NW Portuguese coast concerning the effects of environmental pollution on natural populations (Quintaneiro et al. 2006; Monteiro et al. 2007). Despite this variability, the second axis of RDA clearly distinguished polluted estuaries from the reference one, additionally confirming autumn and winter as difficult seasons for eels in good agreement with other studies (Oliver et al. 1979; Lemly 1993, 1996). Lemly (1996) even described the winter stress syndrome (WSS), a condition of metabolic distress in warm-water fish, which may develop when external stressors, such as parasitism or chemical

pollution, and cold temperatures are simultaneously present. According to this work, these conditions may reduce fish activity and food intake, in a way that organisms are not able to respond to stressors with increased feeding to cope with additional energetic demand due to stress exposure. Stored body lipids will then be depleted in an attempt to overcome these adverse circumstances and, in consequence, body condition will drop and fish may die (Lemly 1996). WSS has thus been considered a serious threat because it occurs when compensatory capacity could be at its lowest point and stressors levels that would normally be tolerated become lethal as water temperature decreases and food intake drops. Interestingly, eels have been described as essentially warm-water species, which become quiescent at temperatures below 8–10°C lying buried in sediments; these habits result in growth rates that are very dependent of temperature (Knights 1997). In the present study, average temperature during autumn and winter was close to the range indicated as triggering both quiescence in eels and WSS. Furthermore, several important changes compatible to a combined effect of cold and chemical stress were observed in the contaminated estuaries of Lima and Douro Rivers during the autumn and winter: (1) low *K* and LSI indexes suggesting that lipid storages are decreased in these populations; (2) neurotoxic effects due to AChE inhibition which may impair feeding as found in studies performed with other fish species (Castro et al. 2004a); and (3) lower antioxidant capacity increasing the vulnerability of eels to oxidative stressors. Overall, these changes may increase the mortality in the population during cold seasons or increase the accumulation of pollutants in already vulnerable eels.

Several biomarkers show variations with age and developmental stage. This may be particularly relevant in long living species, such as eels, when assessing the effects of pollution since these natural fluctuations may act as confounding factors. For example, juveniles may not have completely developed systems and aged animals may have deficient liver and kidney functions interfering with detoxification and excretion capabilities. In addition, for continuous exposures, the accumulation of several environmental contaminants is age dependent. Since in eels age is only accurately determined by otolith analysis that was not performed in the present study, one can not exclude a possible contribution of this factor to biomarkers variability. Another factor that may be important for some biomarkers is gender. During the macroscopic observation performed, it was not possible to distinguish differentiated gonads in any of the animals. However, considering the length range found (20.8–60.9 cm) it is possible that some of the animals already had differentiated gonads since they can start to develop at a body length of about 20–22 cm (Colombo and Grandi 1996).

Therefore, and as a microscopic study of gonads differentiation was not performed, differences among animals associated to gender and gonads development can not be excluded. In addition, during the yellow eel stage fat is accumulated as animals develop and, thus, differences due to this factor may also exist. The inclusion of body weight in the statistical analysis provided some correction to this factor.

In conclusion, the multi-parameter approach was useful to distinguish among estuaries with different histories and levels of contamination. RDA provided an integrated analysis of the parameters assessed indicating that: (1) K and LSI indexes, glutathione levels, and the activities of the enzymes ChE, Na^+/K^+-ATPase, CAT, GR, and SOD were the biological parameters most contributing to discriminate among estuaries; (2) sampling season, temperature, salinity, silica, ammonium and phenol, were the environmental factors playing a major influence on the biological responses observed and (3) autumn and winter are key seasons for the effects of contaminants on the health of yellow eels. The overall analysis of data indicated, therefore, that eels developing in the contaminated estuaries of Lima and Douro rivers are affected by pollution that is causing alterations in cholinergic transmission, osmoregulation and anti-oxidant defences. This may contribute to increase mortality during the continental life phase and reduce the probability of survival to the oceanic migration due to a poor health status. In addition, because both estuaries are contaminated with several persistent organic pollutants, some of which are well known endocrine disruptors, yellow eels developing in these areas probably accumulate considerable amounts of these chemicals in their fat tissue. The mobilization of fat during periods of food shortage (e.g., autumn and winter) may release into blood circulation these compounds, thus, increasing the exposure levels and possibly causing toxic effects that might include interferences with sexual development. This may also occur during the long oceanic migration to the Sargasso Sea and during the final phase of gonads development in the reproduction area when stored fat is also used (Van Ginneken and Van den Thillart 2000). All these processes may contribute to decrease the contribution of each generation to the next one. Therefore, in the continuation of this study, it will be important to investigate the bioaccumulation of environmental contaminants in relation to age, the effects of persistent organic pollutants commonly found in continental waters in growth, gonad development and maturation, and the contribution of toxic effects to mortality rate in exposed populations of eels. Integrated approaches including in situ experiments, laboratory assays and studies in natural populations (also using reproductive endpoints) may be of great value to address these issues.

References

Almeida CM, Serôdio P, Florêncio MH et al (2007) New strategies to screen for endocrine-disrupting chemicals in the Portuguese marine environment utilizing large volume injectioncapillary gas chromatography–mass spectrometry combined with retention time locking libraries (LVI-GC-MS-RTL). Anal Bioanal Chem 387:2569–2583

Baeta F, Pinheiro A, Corte-Real M et al (2005) Are the fisheries in the Tagus estuary sustainable? Fish Res 76:243–251

Baker MA, Cerniglia GJ, Zaman A (1990) Microtiter plate assay for the measurement of glutathione and glutathione disulfide in large numbers of biological samples. Anal Biochem 190:360–365

Bird RP, Draper AH (1984) Comparative studies on different methods of malondihaldehyde determination. Methods Enzymol 90:105–110

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254

Buet A, Banas D, Vollaire Y et al (2006) Biomarker responses in European eel (*Anguilla anguilla*) exposed to persistent organic pollutants. A field study in the Vaccares lagoon (Camargue, France). Chemosphere 65:1846–1858

Burke MD, Mayer RT (1974) Ethoxyresorufin: direct fluorimetric assay of a microsomal-*o*-deethylation which is preferentially inducible by 3-methylcholanlrene. Drug Metab Dispos 2:583–588

Cairrão E, Couderchet M, Soares AMVM et al (2004) Glutathione-*S*-transferase activity of *Fucus* spp. as a biomarker of environmental contamination. Aquat Toxicol 70:277–286

Cajaraville MP, Bebianno MJ, Blasco J et al (2000) The use of biomarkers to assess the impact of pollution in coastal environments of the Iberian Peninsula: a practical approach. Sci Total Environ 247:295–311

Castro BB, Sobral O, Guilhermino L et al (2004a) An in situ bioassay integrating individual and biochemical responses using small fish species. Ecotoxicology 13:667–681

Castro M, Santos MM, Monteiro NM et al (2004b) Measuring lysosomal stability as an effective tool for marine coastal environmental monitoring. Mar Environ Res 58:741–745

Christie NT, Costa M (1984) In vitro assessment of the toxicity of metal compounds. IV. Disposition of metals in cells: interactions with membranes, glutathione, metallothioneins and DNA. Biol Trace Elem Res 6:139–158

CITES (2008) Convention on international trade in endangered species of wild fauna and flora: apendices I, II, and III. United Nations Environmental Programme, International Environment House, Geneva

Clairborne A (1985) Catalase activity. In: Greenwald RA (ed) CRC handbook of methods in oxygen radical research. CRC Press, Boca Raton, pp 283–284

Cohen A, Nugegoda D, Gagnon MM (2001) Metabolic responses of fish following exposure to two different oil spill remediation techniques. Ecotoxicol Environ Saf 48:306–310

Colombo G, Grandi G (1996) Histological study of the development and sex differentiation of the gonad in the European eel. J Fish Biol 48:493–512

Cossu C, Doyotte A, Babut M et al (2000) Antioxidant biomarkers in freshwater bivalves, Unio tumidus, in response to different contamination profiles of aquatic sediments. Ecotoxicol Environ Saf 5:106–121

Cribb AE, Leeder JS, Spielberg SP (1989) Use of a microplate reader in an assay of glutathione reductase using 5, 5′-dithiobis(2-nitrobenzoic acid). Anal Biochem 183:195–196

Cunha I, Garcia LM, Guilhermino L (2005) Sea-urchin (Paracentrotus lividus) glutathione S-transferases and cholinesterase activities as biomarkers of environmental contamination. J Environ Monit 7:288–294

De Coen WM, Janssen CR, Segner H (2001) The use of biomarkers in Daphnia magna toxicity testing V. In vivo alterations in the carbohydrate metabolism of Daphnia magna exposed to sublethal concentrations of mercury and lindane. Ecotoxicol Environ Saf 48:223–234

Dekker W (2004) Slipping through our hands—population dynamics of the European eel. University of Amsterdam, Amsterdam, p 186

Diamantino TC, Almeida E, Soares AMVM et al (2001) Lactate dehydrogenase activity as an effect criterion in toxicity tests with Daphnia magna straus. Chemosphere 45:553–560

Durif C, Dufour S, Elie P (2005) The silvering process of Anguilla anguilla: a new classification from the yellow resident to the silver migrating stage. J Fish Biol 66:1025–1043

EIFAC/ICES (2007) Report of the 2007 session of the joint EIFAC/ICES working group on eels. In: EIFAC Occasional Paper No 38/ICES CM 2007/ACFM:23. European Inland Fisheries Advisory Commission/International Council for the Exploration of the Sea, Bordeaux/Copenhagen, p 526

Ellman GL, Courtney KD, Andres V Jr et al (1961) A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem Pharmacol 7:88–95

FAO (2007) Report of the second FAO ad hoc expert advisory panel for the assessment of proposals to amend appendices I and II of CITES concerning commercially-exploited aquatic species. In: FAO Fisheries Report No 833, FAO, Rome, p 133

Ferreira M, Antunes P, Gil O et al (2004) Organochlorine contaminants in flounder (Platichthys flesus) and mullet (Mugil cephalus) from Douro estuary, and their use as sentinel species for environmental monitoring. Aquat Toxicol 69:347–357

Ferreira M, Moradas-Ferreira P, Reis-Henriques MA (2006) The effect of long-term depuration on phase I and phase II biotransformation in mullets (Mugil cephalus) chronically exposed to pollutants in River Douro Estuary, Portugal. Mar Environ Res 61:326–338

Feunteun EE (2002) Management and restoration of European eel population (Anguilla anguilla): an impossible bargain. Ecol Eng 18:575–591

Filho DW, Tribess T, Gáspari C et al (2001) Seasonal changes in antioxidant defenses of the digestive gland of the brown mussel (Perna perna). Aquaculture 203:149–158

Flohé L, Ötting F (1984) Superoxide dismutase assays. Methods Enzymol 105:93–104

Frasco MF, Guilhermino L (2002) Effects of dimethoate and beta-naphthoflavone on selected biomarkers of Poecilia reticulata. Fish Physiol Biochem 26:149–156

Gravato C, Santos MA (2003) Genotoxicity biomarkers' association with B(a)P biotransformation in Dicentrarchus labrax L. Ecotoxicol Environ Saf 55:352–358

Gravato C, Oliveira M, Santos MA (2005) Oxidative stress and genotoxic responses to resin acids in Mediterranean mussels. Ecotoxicol Environ Saf 61:221–229

Gravato C, Teles M, Oliveira M et al (2006) Oxidative stress, liver biotransformation and genotoxic effects induced by copper in Anguilla anguilla L.—the influence of pre-exposure to beta-naphthoflavone. Chemosphere 65:1821–1830

Gravato C, Faria M, Alves A et al (2007) Biomonitoring studies performed with European eel populations from the estuaries of Minho, Lima and Douro Rivers (NW Portugal). In: Kim YJ, Platt U (eds) Advanced environmental monitoring. Springer, New York, pp 390–401

Griffith OW (1980) Determination of glutathione and glutathione disulfide using glutathione reductase and 2-vinyl-pyridine. Anal Biochem 106:207–212

Guilhermino L, Lopes MC, Carvalho AP et al (1996) Acetylcholinesterase activity of Daphnia magna Straus. Bull Environ Contam Toxicol 57:979–985

Guilhermino L, Barros P, Silva MC et al (1998) Should the use of inhibition of cholinesterase as a specific biomarker for organophosphate and carbamate pesticides be questioned? Biomarkers 3:157–163

Habig WH, Pabst MJ, Jakoby WB (1974) Glutathione S-transferases. The first enzymatic step in mercapturic acid formation. J Biol Chem 249:7130–7139

HELCOM (2007) HELCOM Red list of threatened and declining species of lampreys and fish of the Baltic Sea. In: Baltic Sea environmental proceedings, p 40

Hoque MT, Yusoff FM, Law AT et al (1998) Effect of hydrogen sulphide on liver somatic index and Fulton's condition factor in Mystus nemerus. J Fish Biol 52:23–30

ICN (2008) Livro vermelho dos vertebrados de Portugal—Peixes dulciaquícolas e migradores, anfíbios, répteis, aves e mamíferos, 3rd edn. Assírio & Alvim, Lisboa

INAG (2000) Planos das bacias Hidrográficas dos rios Luso-Espanhois—Síntese. Caracterização e Diagnóstico. Instituto da Água, Direcção de Serviços de Recursos Hídricos, Divisão de Recursos Subterrâneos, p 398

INE (2008) Estatísticas da Pesca 2005 e 2006. INE—Instituto Nacional de Estatística, I.P. Dados não publicados, Lisboa

Kennish MJ (2002) Environmental threats and environmental future of estuaries. Environ Conserv 29:78–107

Knights B (1997) Risk assessment and management of contamination of eels (Anguilla spp.) by persistent xenobiotic organochlorine compounds. Chem Ecol 13:171–212

Lemly AD (1993) Metabolic stress during winter increases the toxicity of selenium to fish. Aquat Toxicol 27:133–158

Lemly AD (1996) Winter stress syndrome: an important consideration for hazard assessment of aquatic pollutants. Ecotoxicol Environ Saf 34:223–227

Lepš J, Šmilauer P (2003) Multivariate analysis of ecological data using CANOCO. Cambridge University Press, New York

Lima I, Moreira SM, Osten JR et al (2007) Biochemical responses of the marine mussel Mytilus galloprovincialis to petrochemical environmental contamination along the North-western coast of Portugal. Chemosphere 66:1230–1242

Lionetto MG, Giordano ME, Vilella S et al (2000) Inhibition of eel enzymatic activities by cadmium. Aquat Toxicol 48:561–571

Livingstone DR, Lemaire P, Matthews A et al (1993) Pro-oxidant, antioxidant and 7-ethoxyresofurin O-deethylase (EROD) activity responses in liver of Dab (Limanda limanda) exposed to sediment contaminated with hydrocarbons and other chemicals. Mar Pollut Bull 26:602–606

Lloret J, Gil de Sola L, Souplet A et al (2002) Effects of large-scale habitat variability on condition of demersal exploited fish in the north-western Mediterranean. ICES J Mar Sci 59:1215–1227

Ludke J, Hill E, Dieter M (1975) Cholinesterase (ChE) response and related mortality among birds fed ChE inhibitors. Arch Environ Contam Toxicol 3:1–21

McCormick SD (1993) Methods for non-lethal gill biopsy and measurement of Na$^+$, K$^+$-ATPase activity. Can J Fish Aquat Sci 50:656–658

Mohandas J, Marshall JJ, Duggins GG et al (1984) Differential distribution of glutathione and glutathione related enzymes in rabbit kidney: possible implications in analgesic neuropathy. Cancer Res 44:5086–5091

Monteiro M, Quintaneiro C, Nogueira AJ et al (2007) Impact of chemical exposure on the fish *Pomatoschistus microps* Kroyer (1838) in estuaries of the Portuguese Northwest coast. Chemosphere 66:514–522

Moreira SM, Moreira-Santos M, Ribeiro R et al (2004) The 'Coral Bulker' fuel oil spill on the North coast of Portugal: spatial and temporal biomarker responses in *Mytilus galloprovincialis*. Ecotoxicology 13:619–630

Moriarty C, Dekker W (1997) Management of the European eel. Fish Bull 15:1–110

Mucha AP, Bordalo AA, Vasconcelos MT (2004) Sediment quality in the Douro river estuary based on trace metal contents, macrobenthic community and elutriate sediment toxicity test (ESTT). J Environ Monit 6:585–592

Ohkawa H (1979) Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Anal Biochem 95:351–358

Oliver JD, Holeton GF, Chua KE (1979) Overwinter mortality of fingerling smallmouth bass in relation to size, relative energy stores, and environmental temperature. Trans Am Fish Soc 108:130–136

Palstra AP, van Ginneken VJT, Murk AJ et al (2006) Are dioxin-like contaminants responsible for the eel (*Anguilla anguilla*) drama? Naturwissenschaften 93:145–148

Payne JF, Mathieu A, Melvin W et al (1996) Acetylcholinesterase, an old biomarker with a newfuture? Field trials in association with two urban rivers and a paper mill in Newfoundland. Mar Pollut Bull 32:225–231

Peña-Llopis S, Pena JB, Sancho E et al (2001) Glutathione-dependent resistence of the European eel *Anguilla anguilla* to the herbicide molinate. Chemosphere 45:671–681

Pierron F, Baudrimont M, Bossy A et al (2007) Impairment of lipid storage by cadmium in the European eel (*Anguilla anguilla*). Aquat Toxicol 81:304–311

Quintaneiro C, Monteiro M, Pastorinho R et al (2006) Environmental pollution and natural populations: a biomarkers case study from the Iberian Atlantic coast. Mar Pollut Bull 52:1406–1413

Regoli R, Principato G (1995) Glutathione, glutathione-dependent and antioxidant enzymes in mussel, *Mytilus galloprovincialis*, exposed to metals under field and laboratory conditions: implications for the use of biochemical biomarkers. Aquat Toxicol 31:143–164

Ribeiro CAO, Vollaire Y, Sanchez-Chardi A et al (2005) Bioaccumulation and the effects of organochlorine pesticides, PAH and heavy metals in the eel (*Anguilla anguilla*) at the Camargue nature reserve, France. Aquat Toxicol 74:53–69

Ribeiro C, Tiritan M, Rocha E et al (2008) Seasonal and spatial distribution of several endocrine-disrupting compounds in the Douro river estuary, Portugal. In: Arch Environ Contam Toxicol

Ricker WE (1975) Computation and interpretation of biological statistics of fish populations. Bull Fish Res Board Can 191:1–382

Robinet TT, Feunteun EE (2002) Sublethal effects of exposure to chemical compounds: a cause for the decline in Atlantic eels? Ecotoxicology 11:265–277

Roche H, Buet A, Jonot O et al (2000) Organochlorine residues in european eel (*Anguilla anguilla*), crucian carp (*Carassius carassius*) and catfish (*Ictalurus nebulosus*) from Vaccares lagoon (French National Nature Reserve of Camargue)—effects on some physiological parameters. Aquat Toxicol 48:443–459

Rodrigues P, Reis-Henriques MA, Campos J et al (2006) Urogenital papilla feminization in male *Pomatoschistus minutus* from two estuaries in northwestern Iberian Peninsula. Mar Environ Res 62(Suppl):S258–S262

Sheppard K, Simkiss K (1978) The effects of heavy metal ions on Ca2$^+$ ATPase extracted from fish gills. Comp Biochem Physiol B 61:69–72

Sherwood L, Klandorf H, Yancey PH (2005) Animal physiology: from genes to organisms, 1st edn. Thomson Brooks/Cole, Belmont

Simon J (2007) Age, growth, and condition of European eel (*Anguilla anguilla*) from six lakes in the River Havel system (Germany). ICES J Mar Sci 64:1414–1422

Sloof W (1982) Skeletal anomalies in fish from polluted surface waters. Aquat Toxicol 2:157–173

Slooff W, van Kreijl CF, Baars AJ (1983) Relative liver weights and xenobiotic-metabolizing enzymes of fish from polluted surface waters in the Netherlands. Aquat Toxicol 4:10–14

Sokal RR, Rohlf FJ (1995) Biometry: the principles and practice of statistics in biological research, 3rd edn. W. H. Freeman and Company, New York

Stagg RM, Goksoyr A, Rodger G (1992a) Changes in branchial Na$^+$, K$^+$-ATPase, metallothionein and P450 1A1 in dab *Limanda limanda* in the Germain Bight: indicators of sediment contamination? Mar Ecol Prog Ser 91:105–115

Stagg RM, Rusin J, Brown F (1992b) Na$^+$, K$^+$-ATPase activity in the gills of the flounder (*Platichthys flesus*) in relation to mercury contamination in the Firth of Forth. Mar Environ Res 33:255–266

ter Braak CJF, Prentice IC (1988) A theory of gradient analysis. Adv Ecol Res 18:271–317

Tietze F (1969) Enzymic method for quantitative determination of nanogram amounts of total and oxidized glutathione. Anal Biochem 27:502–522

Torres MA, Testa CP, Gáspari C et al (2002) Oxidative stress in the mussel *Mytella guyanensis* from polluted mangroves on Santa Catarina Island, Brazil. Mar Pollut Bull 44:923–932

Towle DW, Holleland T (1987) Ammonium ion substitutes for K$^+$ in ATP-dependent Na$^+$ transport by basolateral membrane vesicles. Am J Physiol 252:R479–R489

van der Oost R, Goksoyr A, Celander M et al (1996a) Biomonitoring of aquatic pollution with feral eel (*Anguilla anguilla*) II. Biomarkers: pollution-induced biochemical responses. Aquat Toxicol 36:189–222

van der Oost R, Opperhuizen A, Satumalay K et al (1996b) Biomonitoring aquatic pollution with feral eel (*Anguilla anguilla*). I. Bioaccumulation: biota-sediment ratios of PCBs, OCPs, PCDDs abd PCDFs. Aquat Toxicol 35:21–46

van der Oost R, Beyer J, Vermeulen NPE (2003) Fish bioaccumulation and biomarkers in environmental risk assessment: a review. Environ Toxicol Pharmacol 13:57–149

Van Ginneken V, Van den Thillart GE (2000) Eel fat stores are enough to reach the Sargasso. Nature 403:156–157

van Ginneken V, Durif C, Balm SP et al (2007) Silvering of European eel (*Anguilla anguilla* L.): seasonal changes of morphological and metabolic parameters. Anim Biol 57:63–77

Vassault A (1983) Lactate dehydrogenase. In: Bergmeyer MO (ed) Methods of enzymatic analysis enzymes: oxireductases, transferases. Academic Press, New York, pp 118–126

Viarengo A, Betella E, Fabbri R et al (1997) Heavy metal inhibition of EROD activity in liver microsomes from the bass *Dicentrarchus labrax* exposed to organic xenobiotics: role of GSH in the reduction of heavy metal effects. Mar Environ Res 44:1–11

Watson TA, Beamish FWH (1980) Effects of zinc on branchial ATPase activity in vivo in rainbow trout, *Salmo gairdneri*. Comp Biochem Physiol C 66:77–82

Watson TA, Beamish FWH (1981) The effects of zinc on branchial adenosine triphosphatase enzymes in vitro from rainbow trout, *Salmo gairdneri*. Comp Biochem Physiol C 68:167–173

Wendelaar Bonga SE (1997) The stress response in fish. Physiol Rev 77:591–625

Wu RSS, Pollino CA, Au DWT et al (2003) Evaluation of biomarkers of exposure and effect in juvenile areolated grouper (*Epinephelus areolatus*) on foodborne exposure to benzo[*a*]pyrene. Environ Toxicol Chem 22:1568–1573