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TOXICITY RANKING OF ESTUARINE SEDIMENTS ON THE BASIS OF *SPARUS AURATA* BIOMARKERS

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Abstract—*Sparus aurata* biomarkers were used to rank sediments from the Sado River estuary (Portugal) according to their toxicity. Initially, the activities of liver ethoxyresorufin-*O*-deethylase, liver and gill glutathione *S*-transferases, muscle lactate dehydrogenase, and brain acetylcholinesterase were tested in a laboratory bioassay with the reference compound benzo[*a*]pyrene. Enzymatic activities were determined in different tissues of fish exposed for 48, 96, or 240 h to three concentrations of benzo[*a*]pyrene (25, 50, and 100 μg/L). Induction of liver ethoxyresorufin-*O*-deethylase was observed at all the exposure periods and concentrations, suggesting a continuous response of this system to toxicant exposure. Induction of liver glutathione *S*-transferases activity was only observed after 240 h of exposure, whereas gill glutathione *S*-transferases activity was significantly inhibited at all the exposure periods, suggesting a direct or indirect effect of the toxicant on these enzymes. Inhibition of lactate dehydrogenases activity was only observed after 96 h of exposure to 25 μg/L of benzo[*a*]pyrene. No significant effects were observed on acetylcholinesterase activity, suggesting that cholinergic function of *S. aurata* is not affected by benzo[*a*]pyrene. In a second phase, fish were exposed for 240 h to sediments collected at five sites of the Sado River estuary, and the same biomarkers were analyzed. For all the enzymes assayed, significant differences among sites were found. In this study, the battery of biomarkers used allowed to discrimination among sites with different types of contamination, levels of contamination, or both, after multivariate data analysis. Discrimination of sites was similar to the ranking provided by a more complex and parallel study (including chemical analysis of sediments, macrobenthic community analysis, amphipod mortality toxicity tests, and sea urchin abnormality embryo assays), suggesting its suitability to evaluate the toxicity of estuarine sediments.

Keywords—Estuarine sediments Toxicity ranking Biomarkers *Sparus aurata* Multivariate analysis

INTRODUCTION

Estuaries, as transitional river–marine environments, are widely recognized as one of the most threatened components of the coastal environment. The estuary of the Sado River is located on the west coast of Portugal (Fig. 1). Part of it is a nature reserve; therefore, it is protected by National and European legislation. Despite its classification, the Sado River estuary is subject to considerable anthropogenic pressure, including both organic and inorganic contamination from several sources. The river's basin has an area of about 7,692 km^2 and a human population of nearly 300,000 inhabitants [1]. The estuary has an area of approximately 240 km^2, being heavily industrialized in its northern margin near the city of Setúbal. About 1,280 industrial units directly drain into the river without any pretreatment. From those, 765 units are related to agriculture and animal production, 55 to mining (mainly iron, copper, and manganese) and quarrying, and 415 to other industries, including oil refineries, machinery production, building and repairing of ships, cement, glass, rubber, plastics, paints and varnishes, pulp, paper, leather products, and textile production [2].

In estuaries, sediments are an important compartment of consideration in ecotoxicological studies, mainly because of the role that they might have in the integration and amplification of the concentrations of anthropogenic chemicals. A considerable amount of estuarine contaminants are adsorbed to particulate matter and eventually settle to the bottom, where they can deleteriously affect the sediment-associated biological community. The degree to which a receiving body is affected is usually assessed by the analysis of sediments from the area of concern. In coastal zone management programs, the use of sediment quality values or guidelines (SQGs) alone might be sufficient for decision making, but in some situations, multiple lines of evidence developed from sediment chemistry, toxicity, and benthic community assessment should be used to support sediment management decisions [3]. Despite the already existing tools, methods for identification, estimation, comparative assessment, and management of risk posed by contaminants are still needed.

Enzymatic biomarkers are sensitive ''early warning'' tools for the assessment of biological effects induced by environmental contamination. The activities of the enzymes acetylcholinesterase (AChE), glutathione *S*-transferases (GST), lactate dehydrogenase (LDH), and P4501A (ethoxyresorufin-*O*-deethylase [EROD]) have been widely used as environmental biomarkers. Induction in fish liver of P4501A has been associated with exposure to several hydrophobic organic com-

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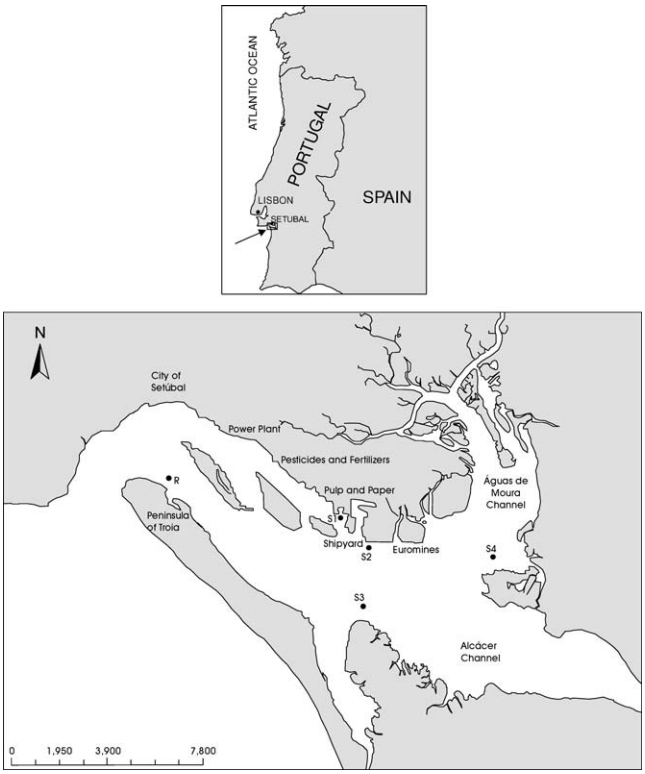

Fig. 1. Map of Sado River estuary, Portugal, showing the location of the sampling sites (R, S1, S2, S3, and S4) and the main sources of pollution.

pounds, such as some polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), dioxins, benzofurans, pesticides, and various drugs. Glutathione *S*-transferases are a well-known family of isoenzymes involved in the detoxification of both xenobiotics and endogenous substances with electrophilic centers by catalyzing their conjugation with glutathione. They also play an important role in lipid peroxidation processes. Activity of GSTs has been found to be induced by certain xenobiotics such as PAHs, PCBs, and some pharmaceuticals. Acetylcholinesterase has a determinant role in the transmission of nervous impulses across cholinergic synapses of both vertebrates and invertebrates, being responsible for the degradation of the neurotransmitter acetylcholine. Because of its sensitivity to both organophosphorous and carbamate pesticides, it has been used as a specific biomarker for these compounds. However, AChE is also sensitive to other environmental contaminants, such as some metals, surfactants, petroleum-derived products, and undetermined components of complex mixtures of pollutants [4–7]. The interconversion of pyruvate to lactate in glycolysis is catalyzed by LDH. In hypoxia or under chemical stress, animals might need additional energy in a short period of time, increasing the use of the anaerobic pathway for energy production [8] that can be detected by an increase of LDH activity. However, toxicants can also induce a decrease of LDH activity, for example, by binding to the enzymatic molecule or by blocking enzyme synthesis [9].

Gilthead seabream (*Sparus aurata*) is an economically valuable species that inhabits Atlantic and Mediterranean coastal waters, estuaries, and lagoons, including the Sado River estuary. It is intensively and extensively cultured in marine and estuarine waters, and it can be found in both pristine and contaminated sites. Furthermore, it is easy to maintain in laboratory conditions, and it is a suitable test organism. For these reasons, it has been selected for use in this study.

The main objective of this work was to perform a toxicity ranking of estuarine sediments collected in different sites of the Sado River estuary (Portugal) with a battery of *S. aurata* biomarkers. Furthermore, the ranking of sediments provided by the battery of biomarkers was compared with the ranking provided by a more complex approach, including acute toxicity tests, macrozoobenthic communities, and chemical analysis performed at the same sites [10]. In a first phase of the study, a laboratory bioassay with *S. aurata* and the reference compound benzo[*a*]pyrene (B*a*P) was performed to validate the methodology and to determine baseline values and time–concentration responses of the enzymes P4501A, GST, LDH, and AChE.

MATERIALS AND METHODS

Study area

The Sado River estuary (Fig. 1) is characterized by a north channel with weak residual currents, flow, and shear stress that enhance accumulation of sediments and settlement of locally introduced pollutants. The southern channel, separated from the north channel by sandbanks, is highly dynamic, and tides are mainly responsible for water circulation. Geometric characteristics distinguish the outer estuary (our study area) from the inner estuary, corresponding to a narrow channel (Alcácer channel). The inner part of the outer estuary (entrances of Águas de Moura and Alcácer channels) is quite shallow with large tidal flats.

Location and brief characterization of field stations

The locations of field stations in the Sado River estuary are presented in Figure 1. The characteristics of these field stations were described exhaustively by Caeiro et al. [11] with the use of samples collected during summer 2003. Here (Table 1), we present a summary of this characterization, which is based on sediment organic load, hydrodynamics, benthic biotope index (BI_{bio}) [12], sediment quality guideline quotient (SQG-Q) [13] for metals and pesticides, amphipod (*Gammarus locusta*) mortality tests with whole sediments [14], and sea urchin (*Paracentrotus lividus*) embryo abnormality bioassay with sediments [15]. The occurrence of macrobenthic communities from physical and chemical variables is predicted by BI_{bio}. The potential of adverse effects on the biota is classified by SQG-Q—in this case, either metals (SQG-QM) or pesticides (SQG-QP). Field station code correspondence between this study and the one performed by Caeiro et al. [11] is R = S116, S1 = S40, S2 = S43, S3 = S102, and S4 = S157.

The field station code R represents the reference station located in the south estuary channel near the Troia peninsula. The sediment can be considered a reference sediment with low organic load; the area has high hydrodynamics and no direct effluent disposal.

Field station S1 is located in the north channel of the estuary where most industries are located. It receives both industrial (near the outlet of a pulp mill and paper factory and near a harbor) and urban effluents. The hydrodynamics is low, and the sediments have a high organic load.

Field station S2 is located in the north channel, in front of a shipyard and near a mining industry, also receiving urban effluents. Station S2 is not far from station S1, and also has low hydrodynamics and sediment with a high organic load.

Table 1. Tabular matrix classifying the sampling sites at the Sado Rive estuary, Portugal (R, S1, S2, S3, and S4), on the basis of the sediment quality triad, lines of evidence, standard quality guideline quotient for metals (SQG-QM), standard quality guideline quotient for pesticides (SQG-QP), and benthic biotic index (BI_{bio}) (from Caeiro [10])

Sampling sites	Toxicity[a]		Chemistry		In situ alterations	Overall risk assessment
	Amphipod mortality	Sea urchin larva	SQG-QM	SQG-QP	BI_{bio}	
R	Not toxic	Not toxic	Low	Low	Marine and transition	Low
S1	Not toxic	Not toxic	Medium	Medium	Estuarine and enriched	Moderate
S2	High	High	Medium	Medium	Estuarine impoverished	High
S3	Moderate	Not toxic	Medium	Medium	Estuarine and enriched	Moderate
S4	Not toxic	Not toxic	Medium	Low	Estuarine impoverished	Moderate

[a] Not toxic = not statistically different from reference area ($p \geq 0.1$); moderate = moderate toxicity, stations statistically different from reference ($0.001 < p < 0.1$); high < high toxicity, stations statistically different from reference ($p \leq 0.001$).

Field station S3 is located in the south channel at the mouth of Alcácer channel. It has low hydrodynamics, and the sediment has a medium to high organic load. This station is not close to any industry but might be diffusely contaminated by pesticides from rice fields.

Field station S4 is located at the entrance of Águas de Moura channel. Domestic effluents, aquaculture farms, and small-scale industries drain to that channel, producing a diffuse contamination. Sediment has a high organic load and the hydrodynamics are low.

These stations were judged according to lines of evidence through a ranking scheme applied for a weight of evidence approach categorization (Table 1). Some legs of the sediment quality triad were assigned more weight than others, on the basis of expert knowledge of sediment assessment, estuary behavior, and interpretation of computed factors from factor analysis. Other variables (e.g., driving forces and pressures of each station, not displayed) were also integrated for tabular analysis of overall judgment [10]. These driving forces and pressures are potential pollutants that were defined for each station on the basis of literature and expert knowledge, including biological oxygen demand, chemical oxygen demand, acids and bases, PCBs, pesticides, tributyltin, metals, PAHs, sulfides, fat oil and grease, hydrocarbons, and pathogens.

Fish used in the experiments

Juvenile *S. aurata* (5.6 ± 0.4 cm long, 3.95 ± 0.83 g weight) was obtained from a commercial fish farm (Ria Mãe, Setúbal, Portugal). Fish were transported to the laboratory in aerated seawater and acclimated for two weeks in 60-L tanks. Salinity and temperature were maintained at 33 to 34‰ and 20°C, respectively. Animals were fed daily with commercial fish food after the third day at the laboratory.

Bioassay with BaP

In the bioassay with B*a*P, a total of 225 fish (15 fish × five B*a*P concentrations × three exposure periods) were used. They were exposed in groups of 15 fish to five different treatments: a saltwater control, a saltwater control with acetone (0.008 ppt of acetone), and three different concentrations of B*a*P in saltwater (25, 50, and 100 μg/L). These concentrations were selected according to preliminary acute toxicity tests. The B*a*P solvent was acetone at a concentration in experimental tanks with B*a*P never higher than 0.01‰. Experiments were performed at 20°C and 33 to 34‰ of salinity in 40-L tanks with filtered seawater and 15 animals each. Test media were renewed every 2 d. Animals were fed with commercial fish food. At 48, 96, and 240 h of exposure, 15 animals of each concentration and controls were sacrificed by decapitation, coded, and frozen at −80°C until biochemical assays.

Bioassays with estuarine sediments

At each location, three sediment replicates were collected with a Van Veen grab, and a composite sediment sample was formed. Experiments were performed at 18°C and 34‰ salinity for 10 d (240 h) in 11-L tanks with filtered seawater. Tanks had an area of 0.24 × 0.34 m and were filled 20 mm high with sediment from the various field stations. Three tanks were prepared per field station (five field sites) with eight fish each (24 fish per station). Seawater was recirculated and permanently aerated; one fourth of its volume was renewed every day. Animals were fed daily with commercial fish food. After 240 h of exposure, eight fish from each replicate were sacrificed, coded, and frozen at −80°C until biochemical assays.

Enzymatic assays

Excised tissues (brain, liver, gill, and muscle) were homogenized in the appropriate buffers and centrifuged, and supernatants were stored at −80°C until analysis.

Ethoxyresorufin-*O*-deethylase (EROD) activity was measured in hepatic tissue after being rinsed in 0.15 M KCl and homogenized (Ystral D-79282, Ballrechten-Dottingen, Germany) in resuspension buffer (50 mM Tris, 1 mM dithiothreitol, 1 mM ethylendiaminetetraacetic acid, 20% glycerol [v/v], pH 7.4). Homogenates were centrifuged at 9,000 *g* for 30 min at 4°C, and EROD activities were determined in postmitochondrial supernatant (S9 fraction) spectrophotometrically at 570 nm according to Hodson et al. [16], with the use of 7-ethoxyresorufin (7-ER) as substrate and reduced nicotinamide adenine dinucleotide phosphate (NADPH) as cofactor. Supernatant protein was adjusted to 4 mg/ml. A volume of 0.1 ml of supernatant to 0.194 ml of 7ER/EROD and 6 μl of NADPH was used. The 7ER/EROD solution was 2 mM in 7-ER in EROD buffer. The NADPH solution was 25 mg/ml. The EROD buffer was 0.1 M in Tris and 0.1 M in NaCl (pH 8.0) in distilled water.

Glutathione-*S*-transferase activity was determined in liver and gill tissues in S9 fractions at 314 nm [17] with 1-chloro-2,4-dinitrobenzene (CDNB) as substrate. Samples were homogenized in 0.1 M phosphate buffer (pH 6.5) and centrifuged at 9,000 *g* for 30 min. Supernatant protein was adjusted to 0.3 mg/ml. A volume of 0.1 ml of supernatant to 0.2 ml of reaction solution was used. The reaction solution contained 10 mM of CDNB and 60 mM of glutathione in phosphate buffer.

Brain AChE activity was assayed at 414 nm with acetylthiocholine as substrate according to the method of Ellman et

al. [18] and adapted to microplate [19] with the use of 0.50 ml of homogenate. Samples were homogenized in 0.1 M phosphate buffer (pH 7.2) and centrifuged at 4,637 *g* for 10 min at 4°C. Supernatant protein was adjusted to 0.25 mg/ml.

Lactate dehydrogenase activity was assayed in homogenates of fish white dorsal muscle (from the left side) at 340 nm with pyruvate as the substrate according to the method of Vassault [20] and adapted to microplate [8]. Samples were homogenized in Tris-NaCl buffer (0.08 mM Tris, 0.2 mM NaCl, pH 7.2) and centrifuged at 6,000 *g* for 3 min at 4°C. Volumes of 0.032 ml of supernatant, 0.2 ml of Tris/NaCl/NADH solution, and 0.032 ml of Tris/NaCl/piruvate solution were used. Tris/NaCl/NADH solution contained 0.23 mM NADH in Tris/NaCl buffer. Tris/NaCl/pyruvate solution contained 13.4 mM pyruvate in Tris/NaCl buffer. Tris/NaCl buffer was 0.1 M in tris(hydroxymethyl)-amino methane and 0.25 M in NaCl.

Spectrophotometric measurements were performed in a Labsystems Multiscan EX microplate reader (Helsinki, Finland). Enzymatic activities were expressed as units of activity (U) per mg of protein. Each unit of activity corresponded to 1 nmol of substrate hydrolyzed per minute in the case of AChE and GST, 1 μmol in the case of LDH, and 1 pmol in the case of EROD. Enzymatic activities were assayed at 25°C and corrected for nonenzymatic activity. Protein concentration of supernatants was determined by the method of Bradford [21] adapted to microplates with bovine γ-globulin as standard.

Statistical analysis

Enzymatic activities were reported as the mean ± standard error of the mean. Data were checked for normality and homoscedasticity before being analyzed by analysis of variance (ANOVA) and transformed if necessary.

In the bioassay with B*a*P, a one-way ANOVA was used to compare different treatments and the pos hoc Dunnett test was used to discriminate statistically significant differences in enzymatic activities between each treatment and the control group. In the bioassay with field sediments, a two-way nested ANOVA was used to compare different treatments and differences within tanks in each treatment, followed by the post hoc Dunnett test to determine statistically significantly different enzymatic activities from those of the reference station. Nonparametric Kruskal–Wallis one-way analysis, followed by Dunn's post hoc test, was used to analyze LDH data because of the lack of variance homogeneity of data. The coefficient of variation was used to determine inter- and intra-assay variability. Field data enzymatic activities were analyzed with a multivariate statistical approach. Factor analysis with the principal components analysis factor extraction procedure (Varimax normalized rotation procedure) was used. Varimax is an orthogonal rotation method that minimizes the number of variables that have high loadings in each factor, simplifying data interpretation ([22]; http://www.statsoft.com/textbook/stathome.html). A cluster analysis (single linkage rule, Euclidean distance measure) of different sampling stations, on the basis of the average enzymatic activity values for each field station, was also conducted and followed by a multidimensional scaling (MDS) analysis conducted on the matrix obtained. The purpose of factor analysis was to discover simple patterns in the relationships among variables. In particular, it seeks to discover whether the observed variables can be explained largely or entirely in terms of a much smaller number of variables, called factors. Factors represent the common variance of variables; thus, this is a correlation-focused approach seeking to reproduce the intercorrelation among variables. A visual representation of the patterns of similarity among field stations is provided by MDS. It is important to note that in MDS, axes (dimensions) are, in themselves, meaningless, and the orientation of the picture is arbitrary. Stress is a measure of distortion and imperfection of the representation, zero being the more perfect representation. All the enzymes were considered for multivariate analysis except EROD because of a lack of induction of this enzyme by the sediments from all the field stations. For the comparison of the enzymatic activity values of the control group of the B*a*P exposure assay to the reference animals in the field, a Student's *t* test was used when assumptions were met and a Mann–Whitney nonparametric *U* test for those variables lacking homogeneity of variance (P450 and LDH). Parametric analyses were performed with the software package Statistica (version 6.0, Statsoft, Tulsa, OK, USA); nonparametric analyses were performed with the software package SigmaStat, (version 3.0, SPSS, Chicago, IL, USA).

RESULTS

This study was performed in two steps. In the first step, a bioassay was performed to validate the methodology and to determine baseline values and time–concentration responses of the enzymes P4501A, GST, LDH, and AChE in *S. aurata*. Benzo[*a*]pyrene was used as the test substance because it has been considered as a reference compound. In the second step, the toxicity ranking of sediments from the Sado River estuary was performed with the use of a battery of biomarkers determined after 240 h of fish exposure to sediments collected in sites with different types and levels of environmental contamination.

Bioassay with BaP

In the bioassay with B*a*P, from the 15 animals exposed to each treatment, only three died in the concentration of 50 μg B*a*P/L after 240 h of exposure. No significant differences in EROD, GST, and AChE activities were found between seawater and acetone control groups. However, significant effects of acetone on LDH activity ($t = 52$, $df = 115$, $f < 0.001$) were found both at 48 and 240 h but not at 96 h (Fig. 2). This should be taken in consideration in data analysis interpretation. Therefore, in all the enzymatic analyses, each B*a*P treatment was compared with the control group.

In fish not exposed to chemical stress, EROD activity is usually very low. However, fish exposed to water or sediments from polluted sites can have induced EROD activity if inducers (e.g., PAHs, PCBs) are present. In this study, fish maintained in saltwater or in saltwater with 0.008 ppb of acetone presented only vestigial values of EROD activity (<1 U/mg protein). However, a significant induction of EROD activity was found at all the B*a*P concentrations tested in all the exposure periods (Fig. 2A). The lowest observed effect concentration (LOEC) was 25 μg/L for all the exposure periods (one-way ANOVA, 48 h: $F = 121.96$, $df = 3, 57$, $p < 0.001$; 96 h: $F = 89.166$, $df = 3, 54$, $p < 0.001$; 240 h: $F = 117{,}55$, $df = 3, 57$, $p < 0.001$).

Glutathione-*S*-transferase has been widely used as an environmental biomarker. It can be induced or inhibited in fish exposed to environmental contaminants, depending of the type of chemicals present. Usually, it is measured in the liver, despite its presence in other tissues. Here, GST activity was

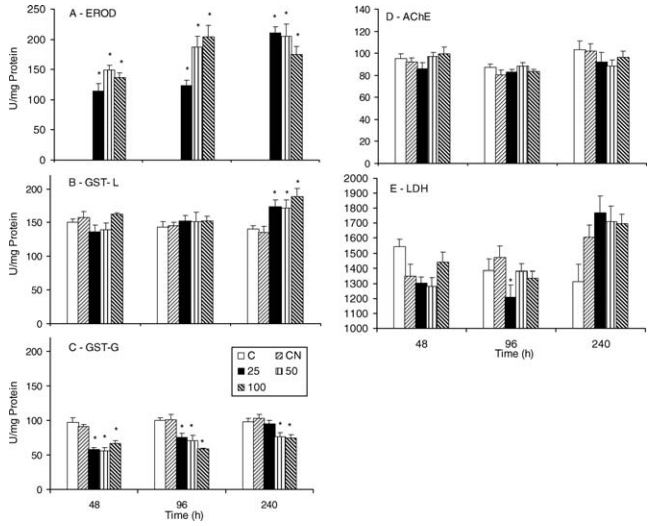

Fig. 2. Activities of liver (**A**) ethoxyresorufin-*O*-deethylase (EROD), glutathione-*S*-transferase of (**B**) liver (GST-L) and (**C**) gill (GST-G), (**D**) brain acetylcholinesterase (AChE), and (**E**) muscle lactate dehydrogenase (LDH) (order was changed to agree with letter sequence) of *Sparus aurata* exposed to seawater (C, Control), seawater with acetone (AC), and seawater with three concentrations of benzo[*a*]pyrene (25, 50, and 100 μg/L) for 48, 96, and 240 h. The values correspond to the mean and standard error of the mean of 15 animals per treatment. An asterisk (*) indicates a value significantly different from the respective acetone control group for each exposure time ($p < 0.05$). Values of EROD activity on control groups were below the detection limit of the technique.

quantified in the liver and also in gills because these could be a first line of defense against chemical exposure. Mean liver GST activity in the acetone control group was 145.0 ± 10.7 U/mg protein (Fig. 2B). After 240 h of exposure to B*a*P, liver GST values were significantly higher in fish exposed to B*a*P, the LOEC being 25 μg/L (one-way ANOVA, 48 h: $F = 2.03$, $df = 3, 45$, $p > 0.05$; 96 h: $F = 0.082$, $df = 3, 43$, $p > 0.05$; 240 h: $F = 2.821$, $df = 3, 43$, $p < 0.05$). Percentages of liver GST induction relative to the acetone control were 27.7, 26.8, and 38.8% after 240 h of exposure to 25, 50 and 100 μg B*a*P/L, respectively. In gills, an opposite effect of B*a*P was observed (Fig. 2C), with lower activities in treated fish at all concentrations and times of exposure, with the exception of those exposed to 25 μg B*a*P/L for 240 h (one-way ANOVA, 48 h: $F = 15.25$, $df = 3, 50$, $p < 0.001$; 96 h: $F = 6.431$, $df = 3, 48$, $p < 0.001$; 240 h: $F = 5.756$, $df = 3, 49$, $p < 0.05$). Percentages of inhibition of gill GST relative to the acetone control were 36.2, 38.2, and 34.7% after 48 h of exposure and 25.2, 30.0, and 41.9% after 96 h of exposure to 25, 50, and 100 μg B*a*P/L, respectively, and gill GST was 25.3 and 27.2% after 240 h of exposure to 50 and 100 μg B*a*P/L, respectively.

One of the most widely used biomarkers is AChE. It is strongly inhibited by organophosphate and carbamate insecticides, some metals, some detergents and surfactants, and complex mixtures of pollutants such as fuel oils. It has been used as a biomarker of neurotoxicity because this enzyme has a determinant function in cholinergic synapses of both vertebrates and invertebrates. The mean of brain AChE determined in this study in the acetone control group was 91.5 ± 10.9 U/mg protein (Fig. 2D). No significant effects of B*a*P on the activity of this enzyme were found at any time of exposure (one-way ANOVA, 48 h: $F = 1.097$, $df = 3, 52$, $p > 0.05$; 96 h: $F = 0.757$, $df = 3, 53$, $p > 0.05$; 240 h: $F = 0{,}598$, $df = 3, 48$, $p > 0.05$).

Lactate dehydrogenase is a cytoplasmatic enzyme present in both animal and vegetal cells. Despite the wide use of LDH activity in toxicology and clinic biochemistry, its use in ecotoxicology is relatively recent. Here, it can be used in three different approaches: as an indication of tissue damage, if quantified in the blood; as an indication of cell membrane disruption, if used in cytotoxicity assays; and as an indication of possible alterations in the anaerobic energy production pathway, if determined in muscle or other suitable tissues. In this study, it was quantified in the muscle. Therefore, an increase of activity in exposed animals will suggest an increase of the use of the anaerobic pathway in an attempt to obtain a rapid increase of energy to face chemical stress, whereas a decrease of LDH might suggest an increase of the aerobic pathway. In *S. aurata*, mean muscle LDH activity in the acetone control group was 1,475.2 ± 129.8 U/mg protein (Fig. 2E). No significant alterations of LDH activity were found in exposed fish (one-way ANOVA, 48 h: $F = 2.168$, $df = 3, 49$, $p > 0.05$; 240 h: $F = 0.914$, $df = 3, 47$, $p > 0.05$), except in animals exposed to 25 μg B*a*P/L for 96 h (one-way ANOVA: $F = 2.696$, $df = 3, 54$, $p < 0.05$) in which an inhibition of 17.9% was observed.

Benzo[*a*]pyrene caused an induction of liver EROD activity, an induction of liver GST activity, and a decrease of gill GST activity; had no effects on AChE activity; and, in general, did not cause alterations on LDH activity in *S. aurata*.

Bioassays with sediments

In this bioassay, fish were exposed to sediments collected in the Sado River estuary for 240 h in laboratory conditions as previous explained, and the enzymatic biomarkers (EROD, AChE, LDH, gill and liver GST activity) were used as effect criteria. In a parallel study with a more complex approach [10], subsamples of the same sediments were classified as presenting low toxicity (R, reference), moderate toxicity (S1, S3, S4), and high toxicity (S2; Table 1). Therefore, sampled sites presented different levels of contamination as well as differences in the type of chemical contaminants present [10].

The results of enzymatic activities determined in fish exposed to reference (R) and to contaminated sediments (S1–S4) are shown in Figure 3. The mean of EROD activity in fish exposed to sediment from the reference station (R) was 4.1 ± 3.3 U/mg protein (Fig. 3A). This value is about fourfold higher than the EROD activity level (vestigial activity) determined in the control group of the B*a*P bioassay ($U = 22.5$; $p = 0.000$), suggesting the presence of EROD activity inducers at this site, although in low amounts. Significant differences among fish exposed to different sediments were found ($F = 9.241$, $df = 4, 105$, $p < 0.001$), whereas no significant differences were observed among tanks within each treatment ($F = 0.688$, $df = 10, 105$, $p > 0.05$). No significant differences in EROD activity were found among fish exposed to sediments S1 and S2 relative to R, despite a slight induction found in fish exposed to S1 sediments (33.7%), whereas a significant decrease in relation to the reference fish was observed in fish exposed to S3 (63.5%) and S4 (46.5%) sediments.

Mean liver GST activity in fish exposed to sediment from R was 141.0 ± 21.9 U/mg protein (Fig. 3B). This value is similar ($t = -1.78$, $df = 34$; $p > 0.05$) to the activity determined in the control group of the B*a*P bioassay (144.7 ± 5.3 U/mg protein). Significant differences in liver GST activity were found among fish exposed to sediments from different field stations ($F = 12.007$, $df = 4, 81$, $p < 0.001$), whereas

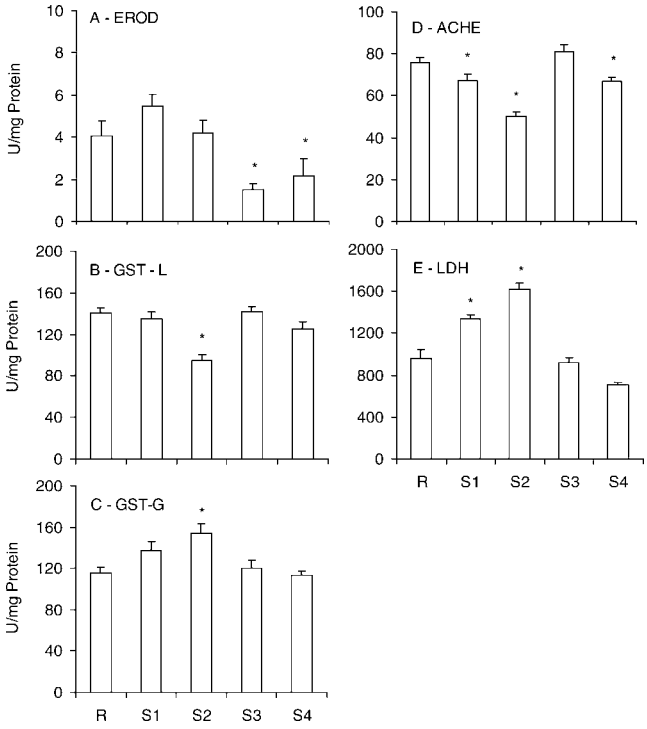

Fig. 3. Activities of (**A**) liver ethoxyresorufin-*O*-deethylase (EROD), glutathione-*S*-transferase of (**B**) liver (GST-L) and (**C**) gills (GST-G), (**D**) brain acetylcholinesterase (AChE), and (**E**) muscle lactate dehydrogenase (LDH) (order was changed to agree with letter sequence) of *Sparus aurata* exposed for 240 h to sediments collected at five field stations of the Sado River estuary, Portugal (R, S1, S2, S3, and S4). Twenty-four fish per treatment were used. The values are the mean and standard error of the mean of three tanks (with eight fish per tank). An asterisk (*) indicates a value significantly different from the reference site value at ($p < 0.05$).

no significant differences were observed among tanks within each treatment ($F = 0.898$, $df = 10, 81$, $p > 0.05$). Fish exposed to S2 sediment had significantly lower liver GST activity (32.5%) than fish exposed to R sediment. Gill GST activity of the group exposed to R sediment was 115.4 ± 26.5 U/mg protein (Fig. 3C). This value is not significantly different ($t = 2.04$, $df = 33$; $p > 0.05$) to gill GST activity determined in the control group of the B*a*P bioassay (98.7 ± 1.5 U/mg protein). Significant differences in gill GST activity among different treatments were found ($F = 6.265$, $df = 4, 79$, $p < 0.005$), with fish exposed to S2 sediment presenting significantly higher gill GST activity (33.7%) relative to fish exposed to R sediments. No significant differences were observed among the three tanks within each treatment ($F = 1.566$, $df = 10, 79$, $p > 0.05$).

Mean brain AChE activity in fish exposed to reference sediment was 75.9 ± 11.3 U/mg protein (Fig. 3D), a value 20.4% lower than the corresponding activity found in the control group of the B*a*P bioassay (95.3 ± 8.2 U/mg protein), but not statistically significant different ($t = -4.48$, $df = 33$; $p > 0.05$). Significant differences in AChE activity among different treatments were found ($F = 26.726$, $df = 4, 94$, $p < 0.001$), with fish exposed to S1, S2, and S4 sediments showing inhibition of AChE activity at the end of the test period (11.5, 33.6, and 12.3%, respectively). No significant differences were observed among tanks within each treatment ($F = 1.343$, $df = 10, 94$, $p > 0.05$).

Mean muscle LDH activity of fish exposed to R sediments was 957.29 ± 77.75 U/mg protein (Fig. 3E), which is lower than the activity determined in the control group of the B*a*P bioassay (1,412.3 ± 118.7 U/mg protein) but not statistically significant different ($U = 7.0$, $p > 0.05$). Significant differences in LDH activity among different treatments were found ($H = 66.759$, $df = 4, 93$, $p < 0.001$), with fish exposed to sediments from S1 and S2, having 39.9% and 69.5% of induction, respectively, relative to R sediments.

Table 2. Coefficient of variation (*CV*) observed on *Sparus aurata* samples after being exposed to field sediments of various stations at the Sado River estuary, Portugal[a]

	CV (%)				
	AChE	GST-G	GST-L	P450	LDH
Reference					
*CV*1	14.2	22.9	13.5	76.6	30.4
*CV*2	6.4	13.3	6.2	11.3	18.8
Station 1					
*CV*1	20.5	22.2	20.4	55.1	13.0
*CV*2	12.1	11.6	4.5	3.9	6.3
Station 2					
*CV*1	14.5	25.3	21.8	70.5	13.9
*CV*2	6.3	16.1	8.6	31.0	2.6
Station 3					
*CV*1	18.2	26.5	17.2	110.8	23.2
*CV*2	12.2	6.9	7.3	16.4	10.9
Station 4					
*CV*1	15.0	20.0	20.6	172.8	15.3
*CV*2	1.8	4.4	10.1	11.0	8.9

[a] *CV*1 = *CV* among every fish of the same treatment ($n = 24$); *CV*2 = *CV* among different tanks within the same treatment ($n = 3$); AChE = brain acetylcholinesterase activity; GST-G = gill glutathione-*S*-transferases activity; GST-L = liver glutathione-*S*-transferases activity; P450 = liver ethoxyresorufin-*O*-deethylase activity; LDH = muscle lactate dehydrogenase activity.

The coefficients of variation among all fish (24 in total) of each treatment and among the three tanks of each treatment was determined for the five enzymes assayed (Table 2).

Considering the integrated analysis of the enzymatic activities of fish exposed to field sediments, factor analysis computed tree factors that explained 87.1% of the total variance. The first factor accounts for 46.8% of the variance and combines gill GST with muscle LDH activity. The second factor explains 22.0% of the variance and is mainly determined by liver GST activity. The third factor explains 18.3% of the variance and is mainly determined by brain AChE activity. Rotated factor loadings resulting from factor analysis are presented in Table 3.

Ordination of field stations through MDS on the basis of the biomarkers analyzed is presented in Figure 4. Fish exposed

Table 3. Rotated factor loadings from factor analysis (Varimax normalized factor rotation)

Variable[a]	Factor 1	Factor 2	Factor 3
AChE	0.138	0.191	−0.941
GST-G	−0.921	−0.157	0.002
GST-L	0.144	0.971	−0.179
LDH	−0.752	−0.066	0.461

[a] AChE = brain acetylcholinesterase activity; GST-G = gill glutathione-*S*-transferases activity; GST-L = liver glutathione-*S*-transferases activity; LDH = muscle lactate dehydrogenase activity.

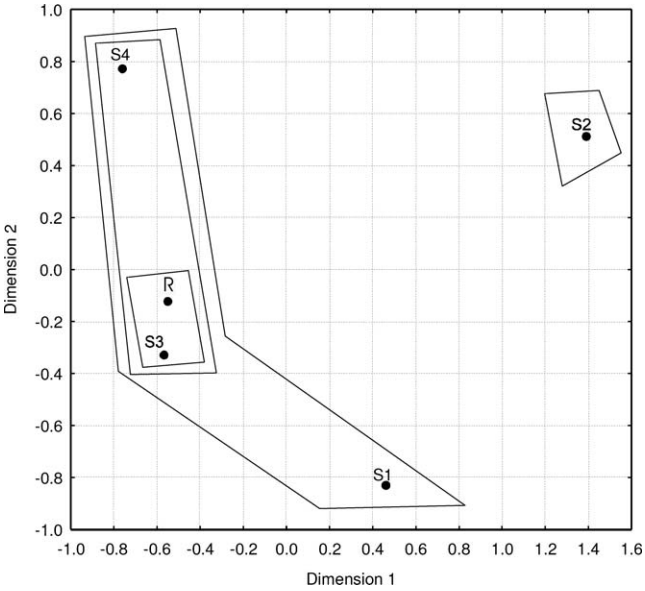

Fig. 4. Multidimensional scaling ordination of the field stations, Portugal (R, S1, S2, S3, and S4), on the basis of the enzymatic activities determined in *Sparus aurata* after 240 h of exposure to sediments (stress = 0.000).

to S2 sediment appear clearly differentiated from the other groups. Fish exposed to S3 sediment are the closest to R sediment, followed by fish exposed to S4 sediment, and, finally, by fish exposed to S1 sediment. Therefore, sediments can be ranked according their increasing toxicity as R = S3 (low toxicity) < S4 (moderate toxicity) < S1 (moderate toxicity) < S2 (high toxicity).

DISCUSSION

Sediment contamination is frequently assessed by chemical analysis. However, chemical analysis is not indicative of the effects of contaminants on the biota. Therefore, methods based on biological parameters should also be used. Recently, other lines of evidence have been developed to complement chemical analysis, such as benthic community structure and toxicity analysis [3]. Community structure analysis is an ecologically relevant approach. However, when effects have already been induced at this level, it might be difficult to repair the damages caused. In this context, biochemical biomarkers could be very useful because they allow the detection of effects early in time, before higher biological organization levels being affected. However, considering their potential use in ecological risk assessment, it is important to know how relevant the changes detected in such parameters are. Therefore, in the last years, efforts have been made toward finding relationships between biomarkers and ecologically relevant parameters, such as energy allocation, growth, reproduction, and survival.

BaP bioassay

In the first phase of this study, brain AChE, muscle LDH, gill and liver GST, and liver EROD activities were determined in control fish and in vivo exposed to B*a*P fish. In control groups, the EROD activity determined (vestigial activity) compares with values reported in the literature for the same species (<1 pmol min^{-1} [mg protein]$^{-1}$) [23]. Brain AChE activity in control fish (95.3 ± 8.2 and 91.5 ± 10.9 U/mg protein in the control and in the acetone control groups, respectively) was higher than that described by Romani et al. [24] for *S. aurata* (52.2 ± 14.1 U/mg protein), but in the same order of magnitude as the value determined in *Lateolabrax japonicus* (between 70 and 90 nmol min^{-1} [mg protein]$^{-1}$) [25] and in *Mullus barbatus* (between 80 and 120 nmol min^{-1} [mg protein]$^{-1}$) [26]. Liver GST activity of control groups (144.7 ± 5.3 and 145.8 ± 10.7 U/mg protein in the control and acetone control, respectively) was in the broad range of values (30–340 nmol min^{-1} [mg protein]$^{-1}$) described in the literature for *S. aurata* liver [23,27]. Gill GST baseline values observed (98.7 ± 1.5 and 98.5 ± 6.0 U/mg protein in the control and acetone control, respectively) are lower than in liver, being on the same order of magnitude as that determined in *Gambusia yucatan*a gills [28]. White muscle LDH activity determined in this study in the control groups (1,475.2 ± 129.8 U/mg protein in the control and acetone control, respectively) is lower than that reported by Antognelli et al. [29] for the same species. However, they are in the range reported for the black bream, *Acanthopagrus butcheri* (between 700 and 4,000 U/mg protein) [30], and higher than the corresponding values determined in the Australian sea bass, *Macquaria novemaculeata* (~600 U/mg protein) [31].

The P450 system is known to have a determinant role in the biotransformation of B*a*P in vertebrates. In this study, EROD activity was significantly induced in fish exposed to B*a*P during all the exposure periods tested, suggesting its involvement in the biotransformation of this xenobiotic by *S. aurata*. These results are in good agreement with the findings of previously performed studies on the effects of this chemical in *S. aurata* [32]. Induced EROD activity levels found in this study (100–200 U/mg protein) are higher that those observed in *Scophthalmus maximus* after waterborne exposure to B*a*P [33] and lower than those determined in *Limanda limanda* 8 d after oral administration of 2 or 50 mg B*a*P/kg body weight [34]. In fish exposed to 25 μg/L of B*a*P for 48 h (114.3 ± 11.9 pmol min^{-1} [mg protein]$^{-1}$), the EROD activity determined is similar to the corresponding activity determined in *Orysias latipes* 48 h after an intraperitoneal administration of 20 μg B*a*P/g of body weight (100.4 ± 11.3 pmol min^{-1} [mg protein]$^{-1}$) [35]. The values are also similar to those found in *S. maximus* (145.1 ± 14.4 pmol min^{-1} [mg protein]$^{-1}$) 3 d after intraperitoneal injection of 5 mg B*a*P/kg body weight [36]. In contrast, no effect on EROD activity was observed in *S. maximus* after 14 d of waterborne exposure to B*a*P (0.12–0.7 μg/L) [36]; however, B*a*P concentrations used in that study were much lower than those tested by us (25–100 μg/L).

Liver GST activity was not significantly induced in fish exposed to B*a*P after 48 and 96 h of exposure, suggesting that in short-term exposures, and at least for the concentrations tested, liver GST conjugation might not be an important pathway for B*a*P detoxification. A significant induction was found after 240 h of exposure, suggesting that in medium-term exposures, this pathway could be important. Other authors have described a late liver GST induction after exposure to B*a*P. For example, in *L. japonicus* exposed to 2 and 20 μg/L of B*a*P, an initial inhibition of liver GST was observed at day 6, no effect was found at day 12, whereas an induction was found day 18 [25]. Contrary to liver GST, B*a*P was found to cause a significant inhibition of gill GST at all the concentrations tested in all exposure periods tested. Inhibition of gill GST (~50%) was also reported for *G. yucatana* after 24 h of exposure to carbofuran [28]. Reactive oxygen species are known to alter gene expression by activating transcription factors that bind to antioxidant response elements. However, various thiol-

reactive metals have been shown to activate transcription factors (nuclear factor kappa B [NF-κB] and activator protein 1 [AP-1]) but then disrupt their binding to its promoter in vivo [37], which could explain the GST down-regulation observed by B*a*P in *S. aurata* gills. Nuclear factor kappa B controls a number of antioxidant enzymes, including GSTP1-1 in mammals [38], and the GSTτ promoter region contains NF-κB and AP-1 response elements [39]. Down-regulation of various GST isoforms by Cr(II) has been observed in *Fundulus heteroclitus* [40]. The possible role of gill GST as a first line of defense to chemical injury is an interesting question that deserves further investigation. The measurement of biotransformation enzymes in tissues in direct contact with the environment, such as gills and intestine, seems pertinent, and fish gill GST might be an interesting biomarker. However, because very few studies have been performed on the relation of gill GST activity to chemical exposure, more research should be carried out before this parameter is used as an environmental biomarker.

Muscle LDH activity was significantly inhibited after 96 h of exposure to 25 μg/L B*a*P. Inhibition of LDH has been attributed to stress that causes a cortisol-dependent decrease in activity, and, as suggested in a study with *Clarias batrachus* [41], stress can cause an initial increase of anaerobic energy production that leads to the accumulation of lactic acid. As accumulation of lactic acid occurs, the reversal reaction (that is the conversion of lactate to pyruvate which is also catalyzed by LDH) is increasingly promoted. When a new equilibrium is reached, the enzymatic activity considerably decreases. Inhibition of LDH activity was also observed in *Salmo salar* gills after 48 h of exposure to the water accommodation fraction (WAF) of Bass Strait (Australia) crude oil, to the WAF + crude oil dispersant [42] in *M. novemaculeata* muscle after 4 d of exposure to the WAF of a fuel oil [31], and in *Daphnia magna* after exposure to zinc [8].

No significant effect of B*a*P on brain AChE activity was observed, indicating that this xenobiotic does not interfere with neurotransmission processes mediated by acetylcholine, which is in good agreement with the results of a study with *L. japonicus* exposed to 2 and 20 mg of B*a*P for 6, 12, and 18 d [25].

Bioassay with sediments

In the second phase of this study, the toxicity of sediments from the Sado River estuary was evaluated with the use of liver EROD, liver and gill GST, muscle LDH, and brain AChE activities as biomarkers in *S. aurata*. Fish exposed to sediment from the reference site (R) presented an EROD activity 20% higher than the control group of the B*a*P bioassay, suggesting the presence of P450 inducers at this site. Here, an AChE depression of 20% was found in fish exposed to R sediments relative to the value determined in control fish from the laboratory assay. Because inhibition is at a diagnostic level and no statistically significant differences between the two groups of fish were found, it is difficult to conclude the presence of AChE inhibitors. However, in our opinion, their presence cannot be excluded. No significant differences in the activities of liver GST, gill GST, and LDH were found between fish exposed to sediments from station R and the control group of the B*a*P bioassay. Therefore, as a whole, these results suggest a low contamination of the reference site.

Comparing the enzymatic activities of fish exposed to sediments from different sites of the Sado River estuary (R and S1–S4), fish exposed to S3 and S4 sediments displayed a significantly lower EROD activity than those exposed to R sediment, suggesting the presence of P450A1 inhibitors. Depression of P4501A activity, through direct inhibition of the enzyme or gene expression, after exposure to some environmental contaminants has been previously reported [43]. Station S3 could be affected by pesticides used in rice fields. Because some pesticides (e.g., some organophosphate insecticides) are inhibitors of enzymes of the P450 system, the inhibition found in fish exposed to sediments from this site could be due to the presence of these types of agents. Station S4 is under the influence of small-scale industry, aquaculture, and urban effluents. Therefore, P450 inhibitors could also be present.

Among the sediments tested, only those collected at S2 caused effects on GST, inducing gill GST and inhibiting liver GST. Various hypotheses could be forwarded to explain this differential response. First, it is possible that gill GST transforms parental compounds in metabolites that could be responsible for liver GST inhibition. Second, the decrease of liver GST could be due to depletion of liver GSH, or inhibition of its syntheses; consequently, GST activity would decrease in the liver. Third, different GST isoforms with distinct sensibilities to the chemicals present in the sediment could exist in the two tissues. Various environmental contaminants and mixtures are known to inhibit GST both in vertebrates and invertebrates (e.g.,, tributyltin in rat liver [44], dieldrin and malathion in *S. aurata* liver [45], polluted harbor water in *Diplodus annularis* liver [46], and the WAF of fuel 4 fuel oil in *P. lividus* intestine [47]). Cadmium was also shown to induce a time-dependent depression (35%) of the GSH detoxification pathway, which is catalyzed by GST, in mice liver after 15 d of exposure [48]. Lead was also shown to inhibit GST activity in the rat lenticular system to 15 to 40% on the first month and up to 55% of control activity after three months of exposure, with concomitant depletion of GSH [49]. A very strong depletion of GSH levels was found to occur in HepG2 human cells after 24 h of exposure to several organochlorine insecticides, namely, endosulfan, DDT, and dieldrin [50]. Station S2 is particularly contaminated with lead, zinc, copper, heptachlor, 1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethane (*p,p′*-DDD), *p,p′*-DDT, endrin, isodrin, and endosulfan [51]. Therefore, some of these compounds or their combined effects might have contributed to the GST trends observed. The pattern of gill GST induction versus liver GST inhibition observed in the bioassay with sediments is opposite to the enzymatic behavior found in the B*a*P bioassay and might suggest that the response of the enzyme in both gills and liver could change according to the toxicants.

Fish exposed to sediments from S1, S2, and S4 presented inhibition of AChE activity in relation to those exposed to reference sediment. Stations S1 to S4 are contaminated with copper and zinc, and S1 is also contaminated with mercury [11]. These metals, as well as mining effluents heavily contaminated with several metals, have been found to inhibit the activity of cholinesterases of several species [4,52–54]. In addition, other AChE inhibitors (such as organophosphate and carbamate pesticides) might be present, particularly at S3, where these compounds are used in rice fields.

Fish exposed to sediments from S1 and S2 presented significantly higher LDH activity compared with those exposed to sediments from R, suggesting an increase of the use of the anaerobic energy production pathway in animals exposed to sediments from these sites to face chemical stress. A similar suggestion has been performed in previous studies performed

with several species [55]. Under chemical stress, additional energy might be required, for example, for tissue reparation and regeneration and to maintain the redox state that could be altered by some contaminants [56].

The results of factor analysis (Table 2) show an association of gill GST and LDH activities with the first factor, being those the variables mostly contributing to discriminate different field stations. Both enzymes were induced in fish exposed to sediments from S2, a station known to have highly toxic sediments (Table 1). Liver GST activity was best correlated to factor 2, being inhibited in fish exposed to sediments from S2. Finally, factor 3 was mostly associated with AChE activity, an enzyme that was significantly inhibited in fish exposed to sediments from S1, S2, and S4. Ordination of field station sediments through MDS on the basis of the biomarkers analyzed (Fig. 4) indicates that S3 sediment is the most similar to R, followed by S4 and, finally, by S1. Station S2 sediment is clearly different from those of the other field stations. Therefore, sediments can be ranked according to their increase in toxicity as follows: R = S3 (low toxicity) < S4 (moderate toxicity) < S1 (moderated toxicity) < S2 (high toxicity).

These results show that the classification of the field stations through the integrated analysis of the enzymes assayed is very close to the sediment ranking obtained with a more complex analysis of the sediments [51]. The only exception is that S3 in this study is classified as low toxicity (R), whereas in the study of Caeiro et al. [51], it was classified as moderate toxicity (Table 1). The study performed by Caeiro et al. [51] included chemical analysis of sediments (pesticides, metals, and metalloids), macrobenthic community analysis, and toxicity tests in two species (mortality in amphipods and abnormality of sea urchin embryos). The methodology used in this study is simpler, more cost effective, and provided a very close ranking of the sites. Therefore, it could be a very useful preliminary approach.

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