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Kinetic of Biomarker Responses in Juveniles of the Fish *Sparus aurata* Exposed to Contaminated Sediments

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Abstract Sediments in the National Park of the Atlantic Islands (Galicia, Spain) were affected by the spill of the tanker *Prestige* (November, 2002) and still present high levels of Polycyclic aromatic hydrocarbons. The adverse effects associated with the contaminants in sediments were tested using a chronic bioassay, exposing juveniles of the fish *Sparus aurata* (seabream). A toxicokinetic approach is proposed to evaluate sediment quality by linking chemical and ecotoxicological data along the time. Sediment samples were physicochemically characterized and the concentration of contaminants (Polycyclic aromatic hydrocarbons – PAHs – and metals) was measured. Fishes were exposed to contaminated sediments, and samples from different tissues were collected every 15 days throughout the 60 days that lasted the experiment. A biomarker of exposure (ethoxyresorufin *O*-deethylase activity – EROD activity) and a biomarker of effect (histopathology) were analyzed during the exposure period. Results show a relationship between the biomarkers and the concentrations in sediments of polycyclic aromatic hydrocarbons—PAHs. Besides, the toxicokinetic approach links biomarkers response providing information about the relationship between the detoxification process and the damages observed in the different tissues. The frequency of the histological damage is highest when the EROD activity slightly decreases in accordance with the mechanism of detoxification of this enzymatic system against PAHs and other organic contaminants.

Keywords EROD activity · Histopathology · PAHs · Prestige · Oil spill

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1 Introduction

The heavy fuel oil spill from the tanker Prestige on November 2002 affected more than 1,000 km of coast, from the North of Portugal up to the South-east

of France, being the Galician Coast the most damaged. The composition of this fuel was a mixture of saturated hydrocarbons, aromatic hydrocarbons, resins and asphaltenes, being most of the Polycyclic aromatic hydrocarbons—PAHs—of an intermedium-high molecular weight (Albaigés & Bayona, 2003; Blanco, Prego, Azpíroz, Fernández-Domínguez, 2006). The formation of the emulsions and the generation of tars induced processes of sedimentation, so that 1 year after the accident the marine sediments reached PAHs concentrations which were 10 times higher than those registered before the spill (IEO, 2003).

The use of biomarkers in fish which are indicative of PAHs exposure may provide an early warning of potential ecosystem degradation, contaminant bioavailability, and the defence responses of exposed organisms (Goksøyr et al., 1996; Goksøyr & Förlin, 1992; Reynolds et al., 2003). Interest in the effects of environmental stressors on health and alterations in fish and other marine organisms has increased in recent years, and in particular, histological and cellular alterations have been observed in marine fish from polluted coastal waters and estuaries (Malins et al., 1984; Stein et al., 1992). The capacity of many pollutants to alter different cells, tissues or organs has led to design histopathological techniques in order to evaluate the effects of contaminants (Lowe, 1988; Sarasquete, Muñoz-Cueto, Arellano, & González de Canales, 1997). On the other hand, the relation between contaminated environments and fish alterations has been proved by different authors (Ortiz, González de Canales, & Sarasquete, 2003; Husoy, Myers, & Goksoyr, 1996; Martín-Díaz, Tuberty, McKenney, Sales, & DelValls, 2005; Myers, Willis, Husoy, Goksoyr, & Collier, 1995; Ortiz, González de Canales, Sarasquete, 1999; Sarasquete et al., 2002).

The cytochromes P-450-1A (CYP1A) are of special interest in ecotoxicology, due to their role in the biotransformation and bioactivation of different organic xenobiotics (dioxins, PAHs, PCBs). The complex CYP1A turns by monooxygenation, determined lipophilic xenobiotics, in more water-soluble metabolites, helping its detoxification. The EROD measurement in fish is considered a monitoring instrument of pollution exposure and an indicator of potential future problems in the health of fish populations (Carballeira, 2003).

According to other authors (Moore & Simpson, 1992; Pacheco & Santos, 2002), the information provided by each biomarker individually is of limited relevance, as there is a considerable likelihood of misinterpretation; thus, biomarkers are best used as selected batteries of tests rather than individually. Furthermore, the study of the behaviour of various biomarkers along the time (toxicokinetic approach) may lead to a substantial improvement in the knowledge of integrated fish toxic response (Pacheco & Santos, 2002).

In the present study a bioassay using the fish *Sparus aurata* was conducted by exposing the individuals to environmental sediment samples collected in areas affected by the *Prestige* oil spill (November 2002) in the National Park of the Atlantic Islands two years after the *Prestige* oil spill. The main objectives of this study were: (1) to characterize the metals and PAHs contamination in sediments from the selected areas in the Galician Coast and compare them to a pristine area in the Gulf of Cádiz; (2) to determine the sediment toxicity through the study of the two biomarkers selected along the time; (3) to determine and compare the sediment quality of the different areas of the study by linking the contamination data and the biological effects, establishing a mechanism of detoxification and proposing a toxicokinetic approach.

2 Material and Methods

2.1 Approach

The area selected to carry out this study was the "Cíes" islands located in the national park of the Atlantic Islands which has a high ecological value. These islands played an important role during the *Prestige* oil spill, since they operated as a natural barrier against the entry of fuel in the "Rías Bajas" (Galician Southern coast). Three stations (Ga1, Ga2 and Ga3), whose sediments were affected in different degree by the oil spill of the tanker Prestige, were selected in the internal face of the Archipelago (Figure 1). Another sample was located in the South of Spain, in the Bay of Cádiz (BC) which is considered a clean area (Riba, Forja, Gómez-Parra, & DelValls, 2004b) and was used as the reference station.

Sediment samples from each of the stations were collected with a 0.025 m^2 Van Veen grab and were homogenized with a Teflon® spoon until no colour or textural differences could be detected. The samples

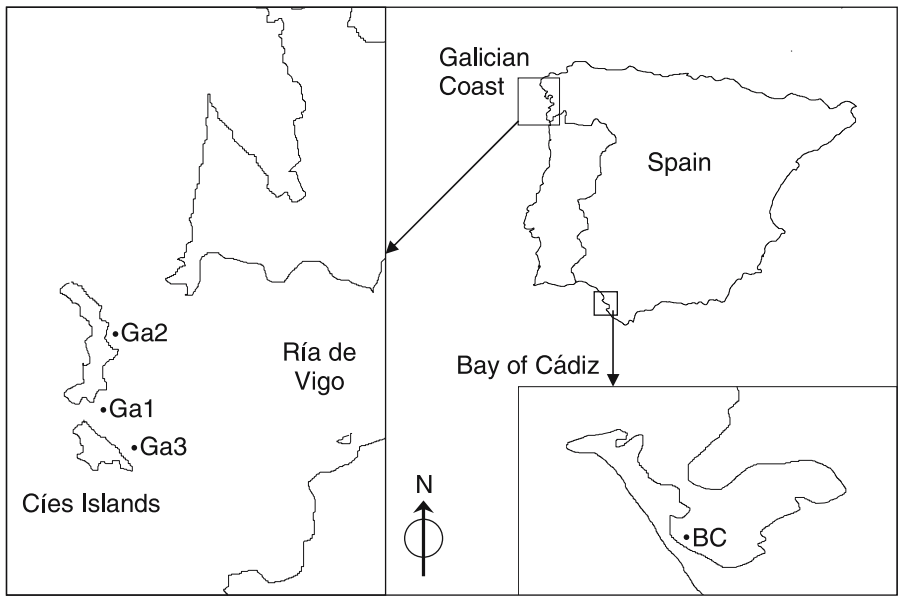

Figure 1 Map of the locations of the area selected to perform the study. *Ga1*, *Ga2* and *Ga3* are located in the Atlantic Islands in the Galician Coast affected by the oil spill related to the *Prestige* tanker (November, 2002), whereas the reference station (*BC*) is located in the Bay of Cádiz in the South of Spain (not affected by oil spills).

were subsampled for physical characterization and chemical quantification. After that, sediment samples were maintained at 4 °C in the dark until use in sediment toxicity tests (no more than 2 weeks). Sediment was filtered (1 mm) prior to the toxicity test in order to remove means interferences as shells, predators and other residues.

2.2 Chemical analysis

Sediment aliquots from each station were dried at room temperature prior to chemical analysis and then gently homogenized. Geochemical matrix characteristics were studied analyzing organic carbon (TOC) concentration and sediment grain size. For sediment grain size an aliquot of wet sediment was analyzed using a laser particle size Fristch (model Analysette 22) following the method reported by DelValls, Blasco, Sarasquete, Forja, and Gómez-Parra (1998). Organic carbon content was determined using the method of Gaudette, Flight, Torner, and Folger (1974) with El Rayis (1985) modification.

Sediments were digested for trace metal analysis, as described by Loring and Rantala (1992). Zn, and Cu concentrations in the extracts were determined with a Perkin-Elmer 2100 flame atomic absorption spectrophotometer. Cd, Cr, Ni and Pb were measured by graphite furnace atomic absorption spectrophotometry (Perkin-Elmer 4100 ZL), while concentrations of Hg were determined by means of Perkin-Elmer MHS-FIAS coupled with a Perkin-Elmer 4100 ZL spectrophotometer. Results are expressed as mg kg^{-1} dry sediment. The analytical procedures were checked using reference material (MESS-1 NRC and CRM 277 BCR) and comply with the certified values in over a 90%.

Polycyclic aromatic hydrocarbons (PAHs) were analyzed by using a gas chromatography equipped with an electron capture detector (ECD) (U.S. Environmental Protection Agency SW-846 Method 8270) (US EPA, 1984); briefly, dried samples were soxhlet extracted with n-hexane for 18 h, and the extracts were isolated by column chromatography on Florisil-alumino-silica. PAHs were eluted and their fractions were dried in a rotatory evaporator and redissolved in isooctane. Aromatic fractions were analyzed on a Hewlett-Packard (HP) 5890 Series II gas chromatograph coupled with HP 5970 mass spectrometer. Chromatographic resolution was achieved with a 30 m×0.250 mm DB-5 capillary column, which has a 0.25 μm film thickness, with helium as carrier gas. Quality control was carried out using NRC-CNRC HS-6 sediment reference material. The analytical procedures comply with the certified values in over a 90%.

2.3 Sediment bioassay

Juveniles of *S. aurata* were obtained in an aquaculture farm and were transported to the laboratory where the

fish spent one month to acclimatize before the bioassay. *S. aurata* was selected because is a common specie in the Spanish coast, its biology is well known, has been used in previous pollution studies (DelValls et al., 1998) and is easy to acclimatize to laboratory conditions. Sediment (approximately 4 l) from the negative control (BC) and the stations Ga1, Ga2, and Ga3 were placed in replicate in 25-l glass tanks with clean sea water before the beginning of the experiment. After 24 h of particle setting, aeration was provided to maintain adequate oxygen concentrations (higher than 80% saturation). A baseline group of 10 randomly chosen individuals were measured, weighed, anaesthetized, and processed for biomarkers responses (exposure and effect) to be used as the initial cellular control. After checking the tanks water quality, twelve individuals (with a weight averaged 4±1 g) were placed in every tank and were fed two or three times per day. The test was conducted during 2 months, no mortality was recorded, and every 15 days six individuals from each station were anaesthetized and processed for histopathological and EROD analysis. During the experiment natural photoperiod was selected and temperature was maintained constant (19±1 °C). Physicochemical parameters (ammonia, pH, temperature, oxygen and salinity) were recorded and controlled when necessary to maintain quality control during the test. Water replacement was performed every day to avoid increasing levels of ammonia, and the survival rate for all tanks was determined.

2.4 Histological procedures

Individual of the fish *S. aurata* proceeding from the toxicity tests were analyzed to determine the histopathological damages in gills. Fish were removed from the tanks at 15, 30, 45 and 60 days of exposure time and samples were collected. Fish were anaesthetized with 0.1% 2-phenoxyethanol 99% during 5–10 min; then weighed, measured in length and externally examined. Liver and gills from all the organisms were obtained by dissection and then fixed in phosphate buffered 10% formaldehyde (pH 7.2) for 24 h and embedded in paraffin. The histological sections were stained with Haematoxylin–Eosin and Haematoxylin–VOF (Gutiérrez, 1967). Sections were reviewed by light microscopy Leitz Laborlux S and photographed (Sony DKC-CM30).

General indexes of histological lesions were calculated for each tissue (lesion index in gills [LIG] and lesion index in liver [LIL]) as an average value of the fish damage semi_quantified as previously reported (Morales-Caselles, Jiménez-Tenorio, González de Canales, Sarasquete, DelValls, 2006; Riba, Casado-Martínez, Blasco, DelValls, 2004a; Riba et al., 2004b; Riba, González de Canales, Forja, & DelValls, 2004c). The semiquantification was performed by ranking the frequency of lesions measured in a total number of six individuals: − (zero individuals), +/− (one individual), + (two individuals), ++/+ (three individuals), ++ (four individuals), +++/++ (five individuals) and finally the maximum is associated with the presence of alterations in the total number of individuals, +++ (six individuals sampled).

2.5 Biochemical analysis

Fish were sampled for biochemical analysis, and after dissection, livers were kept at −80 °C prior to the homogenization. The samples were homogenized following the procedure developed by Lafontaine et al. (2000). After homogenization of the samples, EROD samples were centrifuged at 10,000×g for 30 min, and the supernatant was used for the EROD activity determination and the total protein content described by Bradford (1976). EROD assay was performed following the methodology described by Gagné and Blaise (1993). Briefly, 50 µl of supernatant (homogenate 10,000×g for 30 min), 10 µM 7-ethoxyresorufin and 10 mM reduced NADPH in 100 mM KH_2PO_4 buffer (pH 7.4). The reaction was started by the addition of NADPH, being allowed to proceed for 60 min at 30 °C, and stopped by the addition of 100 µl of 0.1 M NaOH. The 7-hydroxyresorufin was determined fluorometrically using 535 nm (excitation) and 580 nm (emission) filters. 7-Hydroxyresorufin concentration in the samples was achieved through an standard calibration curve developed with concentrations of 7-hydroxyresorufin. Results were expressed as picomoles per milligram Total protein (Martín-Díaz, 2004).

3 Results and Discussion

Table I shows the summarized results of total organic carbon, grain size (% of fine grain <63 µm),

Table I Values of total organic carbon (% dry weight), fines (% dry weight) and the concentration of contaminants (PAHs and metals) in sediment samples (concentrations are expressed in mg kg^{-1} dry weight)

Contaminant		BC	Ga1	Ga2	Ga3
PAHs (mg kg^{-1})	TOC	1.07	0.60	1.19	2.00
	Fines (<63 μm)	1.04	0.06	0.03	0.01
	Total PAHs	ND	0.19	2.12	5.10
	Fluorene	ND	0.08	0.13	0.35
	Acenafphthene	ND	0.06	0.17	0.27
	Naphthalene	ND	0.31	0.63	1.40
	Phenanthrene	ND	0.10	0.15	1.36
	Anthracene	ND	0.02	0.03	0.18
	Fluoranthene	ND	0.12	0.18	0.10
	Pyrene	ND	0.09	0.13	0.39
	Benzo[a]anthracene	ND	0.05	0.09	0.20
	Chrysene	ND	0.08	0.12	0.39
	Benzofluoranthene	ND	0.11	0.18	0.06
	Benzo[e]pyrene	ND	0.08	0.13	0.16
	Benzo[a]pyrene	ND	0.05	0.09	0.10
	Perilene	ND	0.03	0.05	0.04
	Dibenzo[ah]anthracene	ND	0.01	0.02	0.02
	Indene[123-cd]pyrene	ND	0.02	0.02	0.02
	Benzo[ghi]perilene	ND	0.01	0.02	0.06
Metals (mg kg^{-1})	Cd	0.92	0.16	0.05	ND
	Cr	0.10	ND	2.00	1.51
	Cu	6.98	12.8	0.65	1.19
	Ni	0.06	1.71	0.42	0.66
	Pb	2.28	2.73	1.14	1.26
	Zn	21.3	14.7	3.95	6.45
	Hg	ND	ND	0.01	ND

Not detected is expressed by n.d. Table adapted from Morales-Caselles et al. (2006).

concentration of metals (Cd, Cr, Cu, Ni, Pb, Zn, Hg) and PAHs (Fluorene, Acenafphthene, Naphthalene, Phenanthrene, Anthracene, Fluoranthene, Pyrene, Benzo[a]anthracene, Chrysene, Benzofluoranthene, Benzo[e]pyrene, Benzo[a]pyrene, Perilene, Dibenzo [ah]anthracene, Indene[123-cd]pyrene, Benzo[ghi] perilene) in the different sediment used in the test (Riba et al., 2004a). Sediments from the reference station (BC) show low values of metals while PAHs were not detected. The highest values of PAHs have been measured in sediments from the station Ga3 (5.10 mg kg^{-1} dry weight) followed by the station Ga2 (2.12 mg kg^{-1} dry weight) and Ga1 (0.19 mg kg^{-1} dry weight). The concentration of metals in sediments from the stations located in Galicia is similar to those measured in the reference station (BC). Previous studies pointed out the possible amount of some metals concentration such as Ni, V, Cu, Pb and Zn (Albaigés & Bayona, 2003; CSIC, 2003; Prego & Cobelo-García, 2003, 2004) from the oil spill although they were not observed at high levels in our study.

Figure 2 shows the values of the EROD activity measured in liver samples of the *S. aurata* exposed to sediments treatments throughout 60 days. In general, EROD activity increases with the presence of PAHs in the sediment samples (Ga3>Ga2>Ga1>BC). Several studies agree that the use of EROD induction in fish is particularly well suited for detection of PAH exposure, because parent compounds may often not be detected in tissues (Whyte, Jung, Schmitt, & Tillit, 2000).

The study of the behaviour of EROD activity during the exposure period for Ga3 shows that EROD activity increases significantly at the beginning of the experiments until day number 15 (2.4 pmol/mg/min of protein) and maximum levels are reached (2.9 pmol/mg/min of protein) on day 30. The measures of this biomarker in the liver of the organisms exposed to sediments from Ga1 and Ga2 show a lower increase than in the case of exposure to sediment in Ga3 and reach the maximum later than Ga3, the day 45 (about 1.7 pmol/mg/min of protein for both curves). In the course of the experiment the

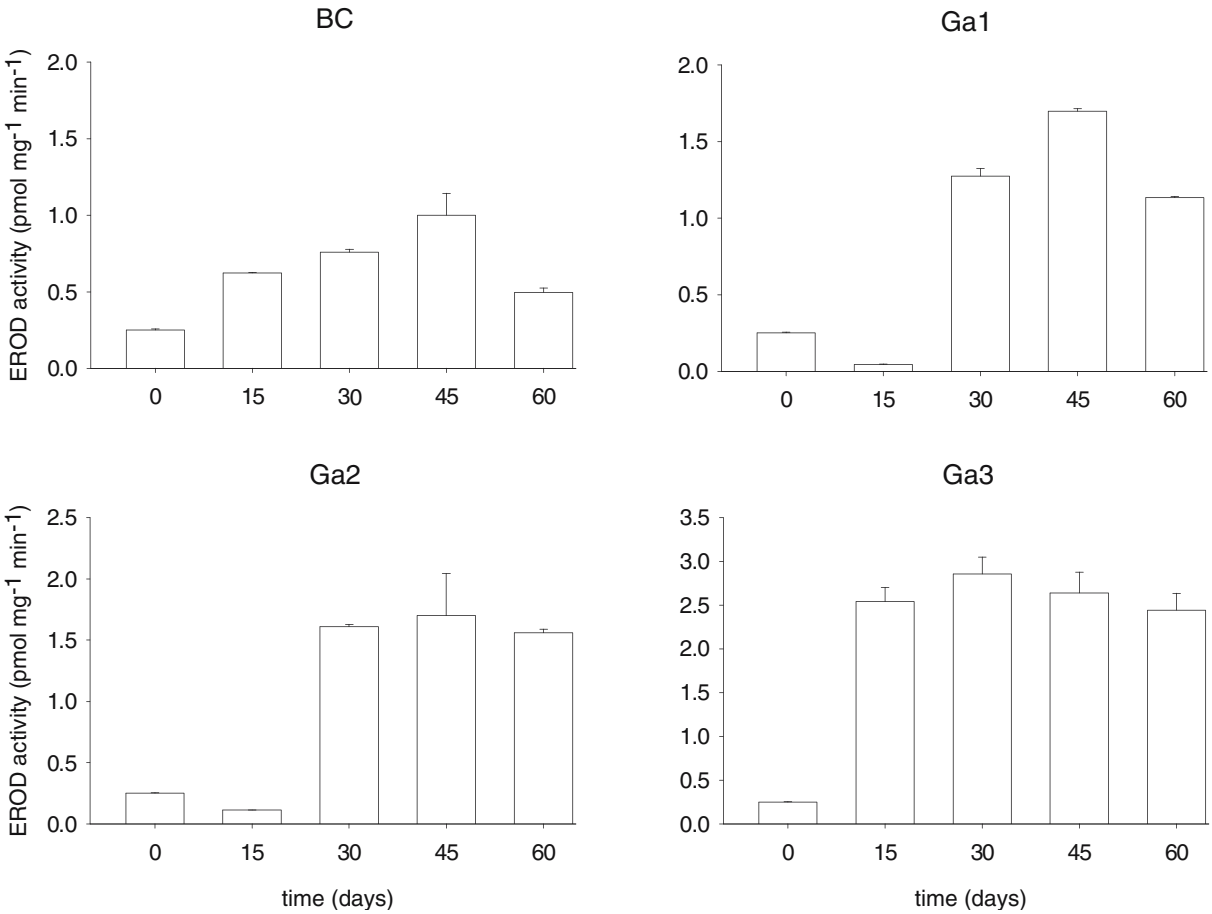

Figure 2 EROD activity in picomoles per milligram per minute of protein in liver samples of *S. aurata* exposed to sediments from Galicia (Ga#) and control (*BC*) during the 60 days of bioassay.

EROD activity for Ga3 – which is the station with the greatest amount of PAHs in their sediments, 5.1 mg kg^{-1} dry weight – is always higher than the EROD activity for Ga1 and Ga2. These sites (Ga1 and Ga2) show a similar behaviour along the time. For all the stations it is shown a slight decrease of the induction of this biomarker of exposure after the day 30 for Ga3 and after day 45 for the other three stations (including the reference station).

The histological alterations observed in target tissues (gill and liver) of fish exposed to sediment collected along the 60 days in the different stations were mainly in gills, which showed shortening of secondary lamellae, presence of edematous areas in distal portion of lamellae, hypertrophy and hyperplasy, necrosis and lost of cells epithelial in the organisms exposed to the Galician sediments; fusion of the secondary was detected specially in organisms exposed to sediments from Ga3. Liver showed lesions such as vacuolization of hepatocytes, necrosis and decrease of the zymogen granules of the exocrine pancreas in the organisms exposed to the Galician sediments. In general, an increase of cytoplasmic basophilia was detected in the liver and exocrine pancreas of all exposed fish that seems related to the increase of PAHs.

In Figures 3 and 4 the summarized results of the histopathological alteration are shown as the index of lesions. The index for gills (LIG) increases with the presence of PAHs in the sediment samples (Ga3> Ga2>Ga1>BC) and, in general, LIG increases along the time of exposure (Figure 3). The value of LIG is maximum the day 60 of the experiment and the highest frequency corresponds to the damages observed in the gills of the organisms exposed to sediment from Ga3 (LIG=2.6), followed by Ga2

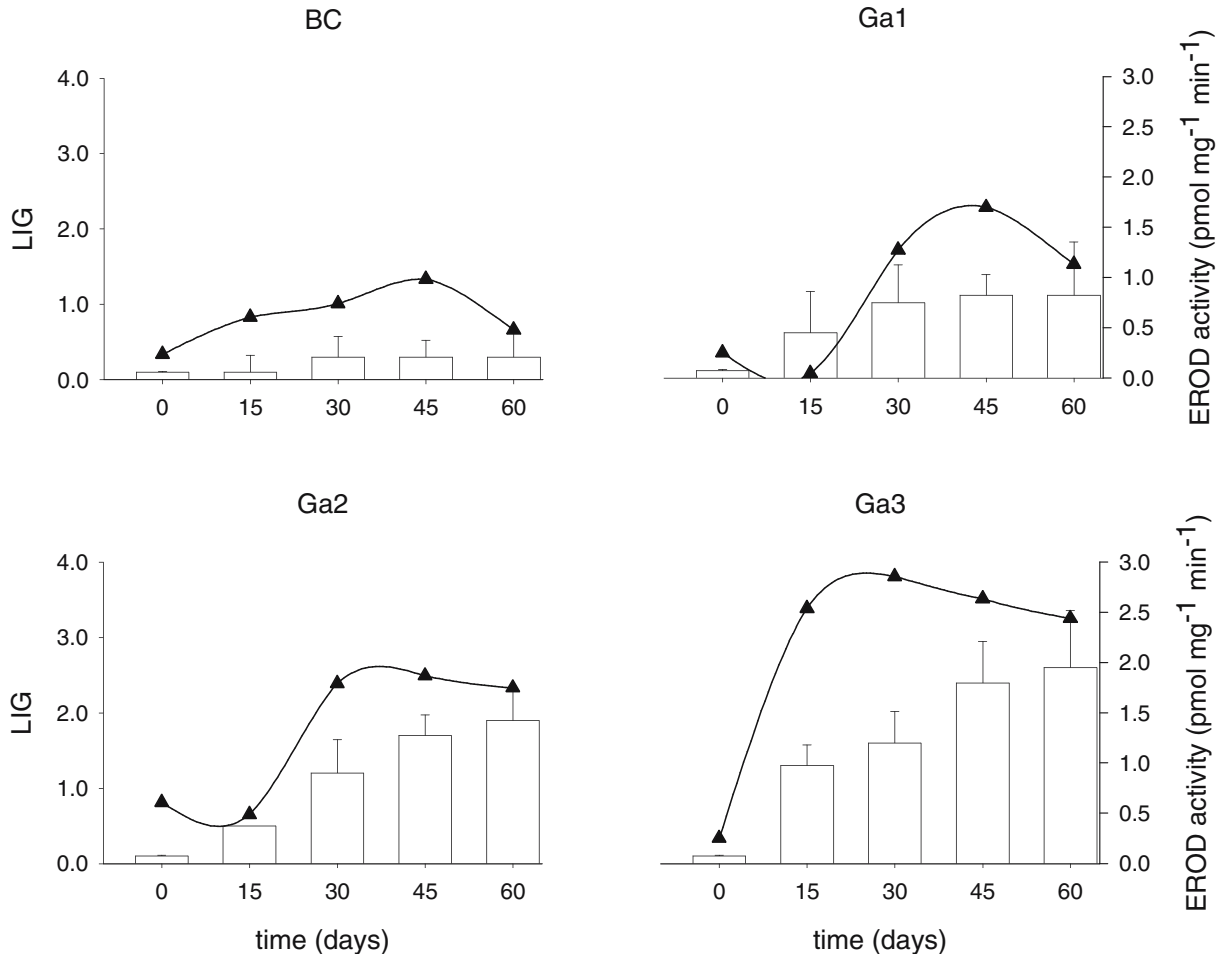

Figure 3 The General Index of Lesions measured in the fish *Sparus aurata* for gills (*LIG*) along the period of exposure to the sediments are represented by *bars*. EROD activity in picomoles per milligram per minute of protein in liver samples of *S. aurata* along the duration of the experiment for the control (*BC*) and Galician (Ga#) sites is represented by *curves*.

(LIG=1.9) and Ga1 (LIG=1.1). A similar behaviour can be observed for the index of lesions determined in liver (LIL), where Ga3 presents the highest value (Ga3: LIL=1.3; Ga2: LIL=1.1; Ga1: LIL=0.8; all of them evaluated at the end of the exposure period, after 60 days). The values of LIG were higher than LIL throughout the whole bioassay for all the stations. Gill is a multifunctional organ sensitive to chemicals in water, since gill filaments and lamellae provide a very large surface area for direct and continuous contact with contaminants in water.

The EROD activity is used as a biomarker of exposure to lipophilic organic contaminants and measures the enzymatic activity of the phase I catalyzed by the complex CYP1A; the complex CYP1A transforms some lipophilic xenobiotics in metabolites more water soluble, so that they are easier to excrete. However, some of these new compounds are highly reactive and more toxic than the original contaminant, and they might interact with biological macromolecules (Parkinson, 1995) producing lesions. In the fishes exposed to sediments from the station Ga3, it seems that there is a first phase where EROD activity is induced (days 0–30) while there are some histopathological damages. When the activity reaches a maximum and begins to decrease (days 30–60), the histopathological alterations continue increasing and the frequency of the lesions is higher. This mechanism of induction of histopathological damages when hepatic EROD decreases can be shown in the three stations affected by the *Prestige* oil spill, although it is produced faster and with higher intensity and frequency in the organisms exposed to sediments from Ga3, which also shows the highest PAHs concentra-

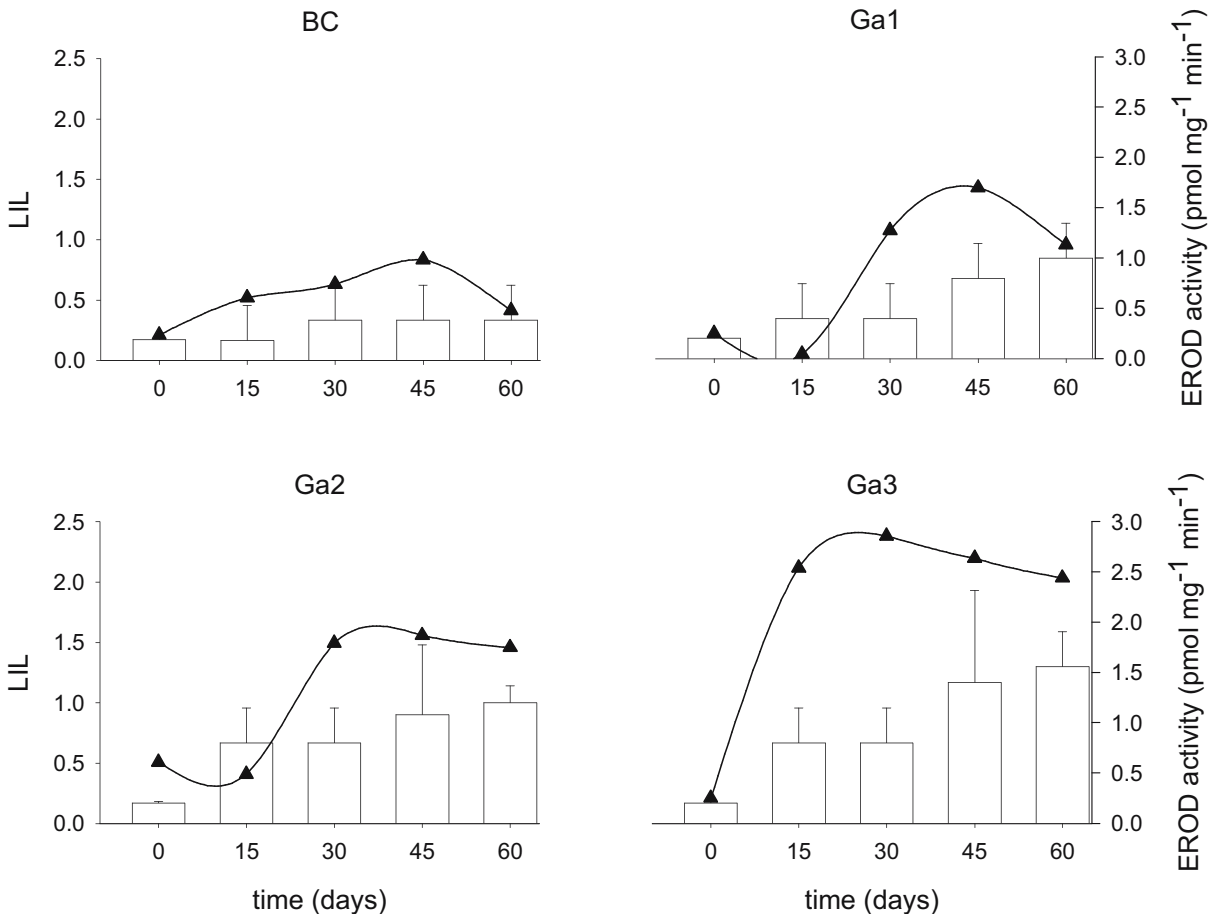

Figure 4 The General Index of Lesions measured in the fish *Sparus aurata* for liver (*LIL*) along the period of exposure to the sediments are represented by *bars*. EROD activity in picomoles per milligram per minute of protein in liver samples of *S. aurata* along the duration of the experiment for the control (*BC*) and Galician (Ga#) sites is represented by *curves*.

tion in sediment. This behaviour could be related to the production of toxic metabolites as secondary products in the detoxification process where the EROD activates. It seems that when EROD activity stabilizes or disappears from the cells, the tissues get more defenceless to organic compounds (in this case PAHs), and histopathological damages are caused with more intensity and frequency.

4 Conclusions

This study shows that the comparison between chemical analysis and the different toxic responses (biomarkers of exposure and effect) is a useful tool to determine the quality of the studied sediments that were affected by the oil spill. The results obtained demonstrate that PAHs analyzed in sediments from Galicia were the chemicals responsible for the measured adverse effects (biomarkers of exposure and effect). The toxicokinetic approach used in this study proposes a mechanism that can explain the histopathological damage associated with the exposure of fish to environmental samples contaminated by PAHs from an oil spill (Prestige, 2002). It gives us the possibility to compare entire curves of behaviour instead of numerical data (endpoint). Besides, it permits to understand better the kinetic of the toxicity based on the role of a detoxification system such as the CYP1A complex. It has been proved the importance of the use of chronic bioassays which provide long-term information of the effects of the exposure to a toxic compound analyzed in environmental samples.

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