Ecotoxicity of Sediments Contaminated by the Oil Spill Associated with the Tanker "Prestige" Using Juveniles of the Fish *Sparus aurata*

Carmen Morales-Caselles,[1,3] Natalia Jiménez-Tenorio,[2] M. Luisa González de Canales,[2,3] Carmen Sarasquete,[1,3] T. Ángel DelValls[2,3]

[1] Instituto de Ciencias Marinas de Andalucía CSIC, Avda. República Saharaui s/n. Puerto Real 11510, Cádiz, Spain
[2] Facultad de Ciencias del Mar y Ambientales, University of Cádiz, Avda. República Saharaui s/n Apdo, 40 Puerto Real 11510, Cádiz, Spain
[3] Unidad Asociada de Calidad Ambiental y Patología (CSIC & UCA), Spain

Abstract. In November 2002, the oil spill from the tanker *Prestige* in the Galician Coast caused an ecological catastrophe in Spain. The adverse effects associated with the contaminants bound to sediments were tested using juveniles of the fish *Sparus aurata* (seabream). The approach evaluates sediment quality by using an integrated assessment including chemical and ecotoxicological data. Sediment samples were physicochemically characterized, and the concentration of contaminants (polycyclic aromatic hydrocarbons—(PAHs) and metals) was measured. Different biomarkers of exposure (metallothioneins and ethoxyresorufin *O*-deethylase activity (EROD)) and biomarkers of effect (histopathology) were analyzed along the time. A multivariate analysis approach was used to correlate concentration of contaminants and sublethal effects measured in individuals of fish. Results show that increasing concentrations of PAHs in sediments were related to increased EROD activities and histopathological lesions. This is the first evidence showing adverse effects associated with petroleum contamination of PAHs in sediments after this spill, and it demonstrates the utility of the sublethal toxicity tests for monitoring the impact of petroleum spills.

It has long been demonstrated that sediments can adsorb persistent and toxic chemicals to levels many times higher than water column concentrations (DelValls *et al.* 2002), whereas the sediment may become sufficiently polluted to disrupt natural biological communities (Adams *et al.* 1992; Tolun *et al.* 2001).

For a better assessment of the pollution process in the marine coastal environment, several authors have proposed determinations based on chemical measurements together with laboratory toxicity tests (Chapman 1988; Luoma and Ho 1992).

Sediment toxicity bioassays are instruments used to test the ecotoxicity and bioavailability of chemical compounds in sediments to benthic organisms. In this kind of bioassay, the organisms are exposed to sediment samples collected *in situ*

and after the incubation period, a biological response is measured; this response must be sensitive, ecologically relevant, and easy to standardize (Stebbing *et al.* 1980). Bioassays provide information on the toxicity of contaminated sediments that can be neither derived from chemical analysis nor from ecological surveys performed alone (Chapman and Long 1983; Long and Chapman 1985). Interest in the effects of environmental stressors on health and disease 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). These sublethal responses have been found to be a powerful tool to evaluate sediment toxicity effects (DelValls *et al.* 1998a).

Biomarkers in fishes have been previously studied in the assessments of oil spills such as Exxon Valdez (Varanasi *et al.* 1995; Jewett *et al.* 2002), Braer (Ritchie and O'Sullivan 1994), and Sea Empress (Kirby *et al.* 1998). In the present study, histopathology was conducted as a biomarker of effect in order to measure the damage caused in the target tissues by the presence of chemicals in the sediments. Two biomarkers of exposure were selected to address the biological adverse effects associated with contaminants present in the studied sediments. The toxicity of metals was assessed by metallothionein (MT) induction, whereas ethoxyresorufin *O*-deethylase activity (EROD) represents a good marker in MFO (mixed-function oxygenase), which is the first mode of detoxification of many organic pollutants (polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls). The EROD measurement in fish is considered a monitor of pollution exposure and an indicator of potential future problems in the health of fish populations (Carballeira 2003). Furthermore, EROD induction can be documented in fish exposed to spilled petroleum despite low tissues of PAHs (George *et al.* 1995; Whyte *et al.* 2000); The reason for using metallothionein induction is because we are studying a mixture of contaminants in the environment, and metallothioneins are one of the main biomarkers. In addition, it has been proved that the induction of this biomarker is not always just related to metals (Stegeman *et al.* 1992; Muto *et al.* 1999; Van der Oost *et al.* 2003).

Correspondence to: T. Á. DelValls; *email:* angel.valls@uca.es

The composition of the oil spilled by the tanker *Prestige* was a mixture of saturated hydrocarbons, aromatic hydrocarbons, resins, and asphaltenes, with most of the PAHs being of medium to high molecular weight (Albaigés and Bayona 2003). Furthermore, it presents some trace metals such as Ni, V, Cu, Pb, and Zn (CSIC 2003; Albaigés and Bayona 2003; Prego and Cobelo-García 2003; Prego and Cobelo-García 2004). The physicochemical characteristics of this fuel show that the soluble fraction is low and the kinetics of degradation are slow under natural conditions so it is expected to be accumulated in sediments. The biological effects associated with the chemicals from the oil spill will be dependent on the nature of the ecosystem that accepts them and the organisms living in it (DelValls 2003). The first research notes about the early impact support the prediction that the acute toxicity of the weathered fuel (Mariño-Balsa 2003), very rich in high molecular weight compounds, was relatively low for the organisms tested (clams and microalgae). However, although concentrations of individual PAHs in aquatic environments are usually much lower than concentrations that are acutely toxic to aquatic organisms, sublethal effects can be produced (Albers 2003).

The results presented in this work show the status of the quality of the sediments 2 years after the accidental spill by linking sublethal responses measured in the fish exposed to oil-contaminated sediments with chemical data determined in sediments.

Materials and Methods

Approach

The present study was carried out by using sediment samples collected along different littoral areas in the North and the South of Spain. In the North, we chose sampling stations that have been affected by the oil spill in differing degrees and located along the Galician Coast (Ga1, Ga2, Ga3). Another sample was located in the South of Spain, in the Bay of Cádiz (BC) which is considered a pristine area (Riba *et al*. 2004a) and was used as the negative control reference. An artificial sample (TM) was made by mixing a toxic mud from an accidental mining spill in Spain (Aznalcóllar, April 1998) with the clean sediment and used as positive control (Riba *et al*. 2003).

Sediment samples from each station were collected with a 0.025-m² Van Veen grab and placed in a cooler until a sufficient amount of sediment was collected from a particular station (about 30 L). The contents of the cooler were homogenized with a Teflon® spoon until no color or textural differences could be detected. The samples were subsampled for physical characterization and chemical quantification. After that, sediment samples were maintained in the cooler at 4°C in the dark until used in sediment toxicity tests. Testing occurred within 2 weeks of collection. Sediment was filtered (0.5 mm) prior to the toxicity test in order to remove means interferences such as shells, predators, and other residues.

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 total organic carbon concentration and sediment grain size. Organic carbon content was determined using the method of Gaudette *et al*. (1974) with El Rayis (1985) modification. 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 and Chapman (1998b).

For trace metal analysis, the sediments were digested as described by Loring and Rantala (1992). Fe, Mn, Zn, and Cu concentrations in the extracts were determined with a Perkin–Elmer 2100 flame atomic absorption spectrophotometer. Concentrations of Hg and As were determined by means of Perkin–Elmer MHS-FIAS coupled with a Perkin–Elmer 4100 ZL spectrophotometer. The other trace metals were measured by graphite furnace atomic absorption spectrophotometry (Perkin–Elmer 4100 ZL). 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 showed a 90–110 range.

PAHs were analyzed by using gas chromatography/mass spectrometry (U.S. Environmental Protection Agency SW-846 Method 8270); 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 procedure allows agreement with the certified values in a 90–112 range.

Sediment Bioassays

Toxicity tests were carried out using juveniles of *Sparus aurata* obtained from an aquaculture farm and transported to the laboratory where the fish spent 1 month to acclimatize. *S. aurata* was selected because is a common species along the Spanish coast. Its biology is well known, having been used in previous pollution studies (DelValls *et al*. 1998a), and it is easy to acclimatize to laboratory conditions. The sea water used during the acclimatization period and the bioassay was clean marine water. A baseline of 10 randomly chosen individuals were weighed to provide data for feeding calculations. After the acclimatization period, the fish had a weight that averaged 4 ± 1 *g*.

Approximately 4 L of sediment from the negative control (BC) and the other stations (Ga1, Ga2, Ga3, TM) were placed in replicate 25-L glass tanks with clean sea water before the beginning of the experiment. After 24 h of particle settling, aeration was provided to maintain adequate oxygen concentrations (greater than 80% saturation). At the beginning of the test, another baseline group of 10 randomly chosen individuals was measured, weighed, anesthetized, and processed for biomarker responses (exposure and effect) to be used as the initial cellular control. Twelve individuals were placed in every tank after checking each tank's water quality and were fed 2 or 3 times per day with commercial food (approximately 0.2 g per fish per day of "Mar Perla T" 1.4–2.2 mm). The test was conducted over 2 months, during which time no mortality was recorded. After the exposure period, individuals from each station were anesthetized and processed for histopathological, MTs, and EROD analysis. During the experiment natural photoperiod was selected and constant temperature was maintained (19 ± 1°C). The physicochemical parameters pH, temperature, oxygen, and salinity were recorded and controlled when necessary to maintain quality control during the test. Water replacement was performed every day by renewing 33% of the water column using a peristaltic pump.

Histological Procedures

Organisms from the toxicity tests were analyzed to determine the histopathological damages in different target tissues (liver and gills). When the water was renewed, the survival rate for all tanks was determined. Fish were removed from the tanks after 56 days of exposure time and samples were collected. Fish were anesthetized with 0.1% of 99% pure 2-phenoxyethanol during 5–10 min, then weighed, measured for length, and externally examined. Target tissues (liver and gills) from all of 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 hematoxylin–eosin and hematoxylin–VOF (Gutiérrez 1967). Sections were reviewed by light microscopy (Leitz Laborlux S) and photographed (Sony DKC-CM30). Damage to the tissues was semi-quantified by detecting the frequency of the lesions in each detected alteration.

Biochemical Analysis

Fish were sampled for biochemical analysis, and after dissection, the liver was kept at −80°C prior to the homogenization. The samples were homogenized following the procedure developed by Lafontaine *et al.* (2000).

Metallothionein Concentration (MT)

Samples obtained to determine metallothionein content were centrifuged at 28,000g for 40 min. The supernatant was added to 0.9 ml of NaCl (0.9%), heated to 95°C for 4 min, and centrifuged at 10,000g for 15 min at 4°C. Supernatant was stored at −80°C prior to MT concentration determinations by Anodic Stripping Voltammetry (Olafson and Olsson 1987) using purified rabbit metallothionein (Sigma-Aldrich). Total protein determination was carried out using the methodology described by Bradford (1976). Concentrations were expressed as µg MT/mg total protein.

Mixed Function Oxidase Assay (EROD)

After homogenization of the samples, EROD samples were centrifuged at 10,000g for 30 min, and the supernatant was used for the EROD activity determination and the total protein content described by Bradford (1976). Mixed function oxygenase activity was measured using the adapted EROD assay (Gagné and Blaise 1993). Briefly, 50 µl of supernatant (homogenate 10,000g 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, was 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 520 nm (excitation) and 590 nm (emission) filters. 7-Hydroxyresorufin concentration in the samples was achieved through a standard calibration curve developed with concentrations of 7-hydroxyresorufin. Results were expressed as pmol/mg total protein.

Statistical Analysis

Analysis of variance was performed in order to determine significant differences ($p < 0.05$; $p < 0.01$) among sites in relation to the bio-markers responses; the Tukey test was used as the post-hoc comparison. Also, contamination and toxicity data were linked by factor analysis, and using principal components analysis (PCA) as the extraction procedure, which is a multivariate statistical technique to explore variable distributions (Riba *et al.* 2003). The original data set used in the analysis included two biomarkers (EROD activity and metallothionein induction), two histopathological indexes (lesions in gills (LIG), and lesions in liver (LIL)), the concentration of different contaminants (PAHs, Cd, Cr, Cu, Ni, Pb, Zn, Hg), and the geochemical matrix characteristics (including total organic carbon and grain size distributions). The objective of PCA is to derive a reduced number of new variables as linear combinations of the original variables. This provides a description of the structure of the data with the minimum loss of information.

Results

Sediment Contamination

Summarized results of total organic carbon, grain size (percent of fine grain <63 µm), concentration of metals and PAHs are shown in Table 1. Of all the stations, the negative control (BC) showed the lowest values of most of contaminants. In general, it is observed that the concentration of PAHs in the area of Galicia (Ga3>Ga2>Ga1) was higher than those measured in the toxic mud and the sediments from the Bay of Cádiz (not detected). The toxic mud, used as positive control, showed high levels of metals in comparison to the other sample sites.

These chemical data can be compared to international sediment quality guidelines (SQGs) that account for the chemical contaminants levels associated with biological effect (DelValls *et al.* 2004). In Table 1, the contaminants that exceed any SQG are highlighted. The letter that appears with the number indicates which SQG is surpassed.

Biomarker Responses

The basal level of metallothioneins measured in liver on day 0 of exposure was 20.1 µg^{-1} mg^{-1} and was lower than the levels of this biomarker after 56 days of exposure; the measures show that metallothionein levels in liver were significantly different ($p < 0.5$; $p < 0.01$) between fishes exposed to control and those exposed to other sediment (Figure 1). These differences were more significant for the station Ga3 and TM ($p < 0.01$) than for the other two stations located in the area of Galicia, Ga1, Ga2 ($p < 0.05$).

EROD activity determined in the liver of juveniles of *S. aurata* on day 0 was 0.3 pmol mg^{-1} min^{-1}. Results after 56 days of exposure are higher than basal levels and showed low values in fish exposed to sediment samples with absence or low levels of total PAHs (TM and BC, respectively) and were significantly different ($p < 0.05$) from stations affected to a different degree by the oil spill (Ga#) (Figure 1). The fish from these stations present high values of EROD activity and high levels of PAHs in their sediment (Ga1, Ga2, and Ga3). These relationships increase when the concentration of PAHs in sediments increases (Ga3 > Ga2 > Ga1). The EROD activity in liver of fish exposed to sediments from station Ga2 and Ga3 showed a significant difference ($p < 0.01$) between the control

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

	Contaminant	BC	Ga1	Ga2	Ga3	TM
	TOC	1.07	0.60	1.19	2.00	1.00
	Fines	1.04	0.06	0.03	0.01	10.1
PAHs (mg kg^{-1})	Total PAHs	n.d.	0.19	2.12	5.10 [a]	n.d.
	Fluorene	n.d.	0.08 [a,c]	0.13 [a,c]	0.35 [a,d]	n.d.
	Acenaphthene	n.d.	0.06 [a,c]	0.17 [a,d]	0.27 [a,d]	n.d.
	Naphthalene	n.d.	0.31 [a,c]	0.63 [a,d]	1.40 [a,d]	n.d.
	Phenanthrene	n.d.	0.10 [a,c]	0.15 [a,d]	1.36 [a,d,e]	n.d.
	Anthracene	n.d.	0.02	0.03	0.18 [a,c]	n.d.
	Fluoranthene	n.d.	0.12	0.18	0.10	n.d.
	Pyrene	n.d.	0.09	0.13 [c]	0.39	n.d.
	Benzo[a]anthracene	n.d.	0.05	0.09 [c]	0.20 [c]	n.d.
	Chrysene	n.d.	0.08	0.12 [c]	0.39 [a,c]	n.d.
	Benzofluoranthene	n.d.	0.11	0.18	0.06	n.d.
	Benzo[e]pyrene	n.d.	0.08	0.13	0.16	n.d.
	Benzo[a]pyrene	n.d.	0.05	0.09 [c]	0.10 [c]	n.d.
	Perilene	n.d.	0.03	0.05	0.04	n.d.
	Dibenzo[ah]anthracene	n.d.	0.01 [c]	0.02 [c]	0.02 [c]	n.d.
	Indene[123-cd]pyrene	n.d.	0.02	0.02	0.02	n.d.
	Benzo[ghi]perilene	n.d.	0.01	0.02	0.06	n.d.
Metals (mg kg^{-1})	Cd	0.92	0.16	0.05	n.d.	5.40 [a,d]
	Cr	0.10	n.d.	2.00	1.51	3.28
	Cu	6.98	12.8	0.65	1.19	210 [a,d]
	Ni	0.06	1.71	0.42	0.66	8.50
	Pb	2.28	2.73	1.14	1.26	790 [b,d,e]
	Zn	21.3	14.7	3.95	6.45	2181 [b,d,e]
	Hg	n.d.	n.d.	0.01	n.d.	5.61 [b,d,e]

[a] ERL, Effect Range-Low (NOAA 1999).
[b] ERM, Effect Range-Median (NOAA 1999).
[c] ISQG, Interim sediment quality guideline (CCM 1999).
[d] PEL, Probable effect level (CCM 1999).
[e] SQG for San Francisco Bay (Long *et al.* 1989).
Notes: Not detected is expressed by n.d.

treatment. Also, those from station Ga1 were significantly different from control treatment but with different value of the statistical *p* (0.05).

For metallothioneins, Tukey test results set the five stations in three homogeneous groups according to the differences of averages among the sites. The first group includes only BC, which is the negative control; the second group is constituted by Ga1, Ga2, and Ga3; and the third group includes TM, which is the positive control.

For EROD activity, there are three homogeneous groups set by the Tukey test results. The first group includes BC and TM, both negative and positive control, which do not present significant differences; the second group is constituted by Ga1 ($p < 0.05$) and Ga2 ($p < 0.01$); and the third group includes Ga3 ($p < 0.01$).

Histopathological Approaches

The organisms analyzed on day 0 did not present histopathological damages. Different alterations were observed in target tissues (gills and liver) of fish exposed to sediment collected after 56 days of exposure in the different stations, mainly in gills, which showed shortening of secondary lamellae, hypertrophy, and hyperplasia, necrosis, and loss of epithelial cells in Ga# and TM; fusion of the secondary lamellae above all in Ga3 and TM; and presence of edematous areas in the distal portion of lamellae in Ga#. Also, liver showed lesions: vacuolization of hepatocytes, necrosis, and decrease of the zymogen granules of the exocrine pancreas in Ga# and TM. In general, an increase of cytoplasmic basophilia was detected in the liver and exocrine pancreas of all exposed fish related to the increase of PAHs. An example of some of these lesions is shown in Figure 2.

These lesions have been previously recorded as related to contaminants bound to sediments in *S. aurata* (DelValls *et al.* 1998a) and in other fish species such as *Solea senegalensis* (Riba *et al.* 2004b, 2004c). As previously reported by DelValls *et al.* (1998a) and based on the damage observed in the different tissues, histopathological alterations were evaluated semiquantitatively in the fishes exposed to the different stations by ranking the frequency of lesions measured in a total number of 6 individuals: – (0 individuals), +/– (1 individual), + (2 individuals), ++/+ (3 individuals), ++ (4 individuals), +++/++ (5 individuals), and finally the maximum is associated with the presence of a disease in the total number of individuals, +++ (6 individuals sampled). Gills were shown to be the most damaged tissue, showing different lesions mainly in Ga#
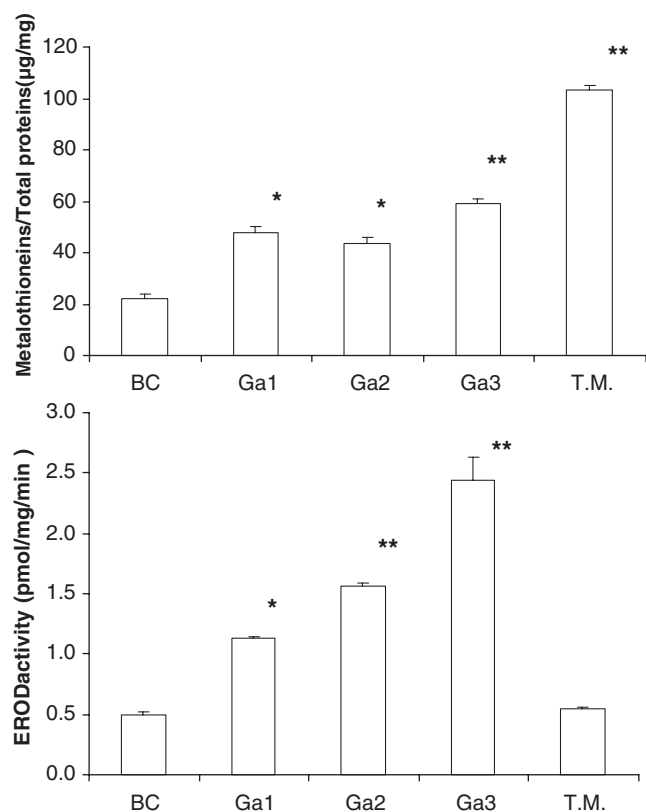
Fig. 1. Results of metallothionein concentration (mean and SD) in µg/mg of protein and EROD activity (mean and SD) in pmol/mg/min of protein in liver samples of *S. aurata* collected at 56 days of the experiment in sediments sampled in the Bay of Cádiz (BC), Galicia coast (Ga#), and toxic mud (TM) treatments. Asterisks indicate significant differences among the biomarker induction in the stations and the negative control (**$p < 001$, *$p < 005$)

and TM. An average of this semiquantitative evaluation of the frequency of the lesions measured from the different replicate results is shown in Table 2.

General indexes of lesion (lesion index in gills [LIG] and lesion index in liver [LIL]) were calculated for each tissue as an average value of the fish damage semiquantified (Figure 3).

Fish exposure to sediment samples produced lesion damage related to the increase of the concentrations of contaminants (PAHs in Ga# and high levels of metals in TM) in the sediments selected in the bioassay. The lesions identified in all the tissues analyzed were almost always present in animals exposed to sediments from stations Ga2 and Ga3. Evaluations of histology of gills and liver revealed clear significant differences ($p < 0.05$) between the negative control of toxicity and the Ga3 station and the toxic mud. The severity of the lesions detected in the tissues of fish exposed to sediments collected in Bay of Cádiz was lower than those measured in the area of Galicia. Results show the lowest indexes in the Bay of Cádiz (BC), which were significantly different ($p < 0.05$) from the values from Galicia (Ga#) and toxic mud (TM). The index of lesions measured for gills (LIG) in Galicia increase with the presence of PAHs in the sediment samples (Ga3 > Ga2 > Ga1). The LIL results show that TM has the highest index related to liver lesions.

Discussion

To link the set of data obtained, the original variables from chemical concentration and sublethal responses were analyzed by factor analysis, using PCA as the extraction procedure, which is a multivariate statistical technique (MAA) to explore variable (chemical concentration, $n = 25$; toxicity data, $n = 4$) distributions. The factor analysis was performed on the correlation matrix, and the variables were autoscaled (standardized) so as to be treated with equal importance (Riba *et al.* 2004a). The applications of MAA to the original 29 variables indicate that they can be grouped in two new factors. These factors explain 88.4% of the variance in the original data set. Negative values of sorted rotated factor loadings (negative salience) are as important as positive values (positive salience). In the present study, we selected to interpret a group of variables as those associated with a particular component where loading was 0.40 or higher (Table 3). This approximates Comreys' cutoff of 0.55 (Comreys 1973) for a good association between an original variable and a factor, and also takes into account discontinuities in the magnitudes of loadings approximating the original variables.

The first principal factor, #1, is predominant and accounts for 62.4% of the variance; it explains the toxicity of individual PAHs and combines the concentrations of PAHs (fluorene, acenaphthene, 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 sediment, the total organic carbon, all the indexes of histopathological lesions (gills, LIG; and liver, LIL), and the EROD activity. The second factor, #2, accounts for 26.1% of the variance; it explains the toxicity associated with the metals in sediment combining, with negative loading, the chemical concentrations of the metals Cd, Cr, Cu, Ni, Pb, Zn, and Hg with the grain size with all the indexes of lesions (gills, LIG; and liver, LIL) and with the induction of metallothioneins (MTs). Figure 4 shows the influence of both factors in the five different stations. Factor 1, with positive loading, is defined as the toxic responses of the fish to PAHs bound to sediments; thus, station Ga2 (0.44) and especially Ga3 (1.48) show the significant prevalence of this factor, whereas this factor does not affect station Ga1, and both controls positive (TM) and negative (BC). The definition of Factor 2 as the toxic responses of the fish to metals bound to sediment only has prevalence in the positive control (TM); the negative loading of the toxic responses and the metals concentration in sediment implies that the prevalence of these factors in the station is associated with negative factor scores. Furthermore, a linear relationship can be observed in the scores of this factor from BC to Ga3, which confirms the increase of toxicity when PAHs increase in sediments.

It is estimated that about 63,000 tons of heavy fuel oil were lost from the single-hull tanker Prestige. Although a large quantity of this fuel was collected and removed from the coast, a large amount likely settled down at the bottom of the sea covered with sediment reaching the littoral area of the Galician coast after the first months of the spill (Albaiges and Bayona 2003). In the present study, we have aimed to assess the impact of this enrichment in littoral sediments collected in different affected areas and 2 years after the oil spill using juveniles of
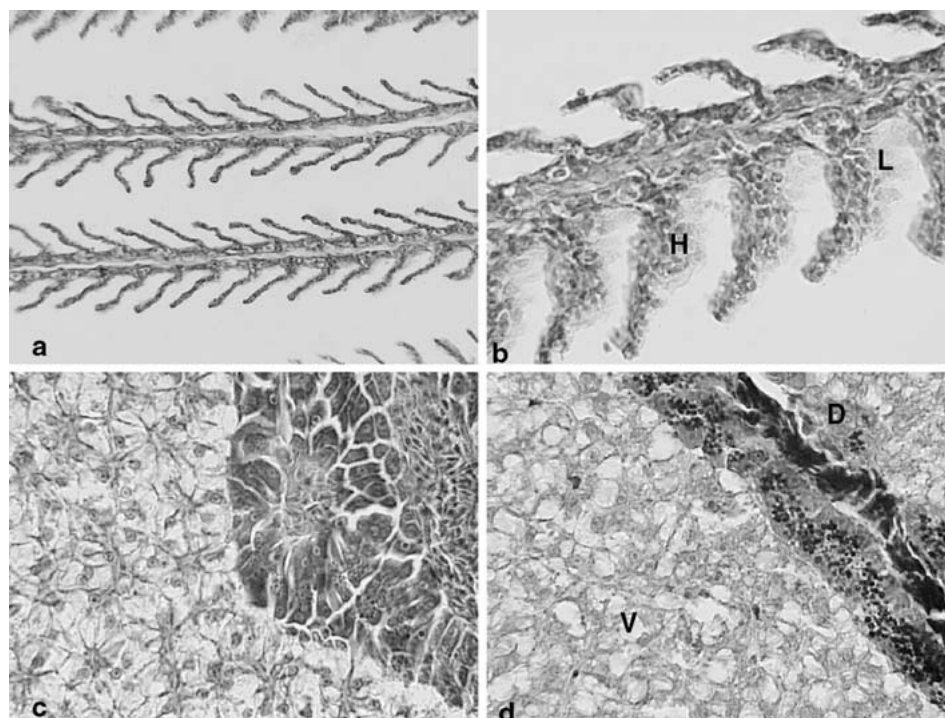
Fig. 2. Example of histological sections associated with contaminants bound to sediments used in the *Sparus aurata* sediment toxicity test. (a) Gills from fish exposed to referent sediment showing primary lamellae and secondary lamellae arising from these, parallel with them and perpendicular to the filament axis BC (H & E ×10). (b) Hypertrophy and hyperplasia of the secondary lamellae Ga3 (H & E ×25). (c) Liver from control fish showing the exocrine pancreas around the blood vessels. Parenchymatous distribution of the hepatocytes in cords around the sinusoids BC (H & E ×25). (d) Hepatocytes and exocrine pancreas alteration TM (H & VOF ×25). D: decrease of the zymogen granules; H: hypertrophy and hyperplasia; L: loss of epithelial cells; V: vacuolization of hepatocytes

Table 2. Frequency of lesions detected in microscopic abnormalities of individuals of juveniles of the fish *Sparus aurata* sampled in the Bay of Cádiz (BC), Galicia coast (Ga#) and toxic mud (TM) treatments on day 56 of exposure

Organ	Histopathology	Samples zones				
		BC	Ga1	Ga2	Ga3	TM
Gills	Hypertrophy/hyperplasia	+/−	+++	++	+++	+/++
	Fusion of secondary lamellae	+	+/++	++	+/++	+
	Shortening of secondary lamellae	+	+/++	+/++	+/++	+/++
	Edematous areas or aneurysm in distal portion of lamellae	+/++	++/++++	++/+++	+++	+/++
	Necrosis and lost of cells epithelial	+	+/++	+/++	++	++
Liver	Increase of lipid vacuoles in the hepatocytes	+/−	+	+	+	++
	Increase of cytoplasmic basophilia of hepatocytes	+/−	+	++	+	+/−
	Necrosis and decrease of the zymogen granules of exocrine pancreas	−	+/−	+	+/−	+

(0 individuals), +/− (1 individual), + (2 individuals), ++/+ (3 individuals), ++ (4 individuals), +++/++ (5 individuals) and finally the maximum is associated with the presence of a disease in the total number of individuals, +++ (6 individuals sampled).

the fish *S. aurata* by means of different sublethal endpoints such as histopathological lesions, metallothionein induction, and EROD activity.

Previous studies have shown how in water the toxicity of individual PAHs increases as molecular weight (MW) increases up MW 202 and beyond it; solubility reduces and so does lethal toxicity, but sublethal effects can result (Albers 2003). In the present study, it has been shown that the Galician sediments (mainly Ga2 and Ga3) analyzed present levels of PAHs with low MW (fluorene, acenaphthene, naphthalene, phenanthrene, anthracene), medium MW (pyrene), and high MW (benzo[a]anthracene, chrysene, benzo[a]pyrene, dibenzo[ah]anthracene), higher than some of the SQGs proposed by international agencies. Furthermore, all individual PAHs seem to have induced hepatic EROD and to produce histopathological damage; it is quite difficult to determine which of the individual PAHs is the main pollutant that has caused the biological effects (explained by factor 1 in the MAA);

however, it can be concluded that PAHs are the compounds that are producing the adverse effects to the fishes.

The significant differences of EROD induction between Ga# (Ga1, $p < 0.05$; Ga2 and Ga3, $p < 0.01$) show a strong relationship with the concentration of PAHs in the Galician sediments (Ga1, Ga2, and especially in Ga3—impacted by the oil spill—and the histopathological lesions in gills and liver, studied in the MAA. Despite differences in the induction of EROD among these Galician samples, the validity of this biomarker of contamination was shown.

twbIn the absence of fish mortality, other research on the impact of the "Sea Empress" oil spill in the UK in 1996 (Edwards and White 1999) showed the possibility of sublethal and chronic effects using a variety of techniques such as EROD activity. In these studies, there was evidence of high levels of EROD activity in the sites exposed to oil constituents in comparison with the control sites. There are other studies, carried out using biomarkers as EROD activity, that
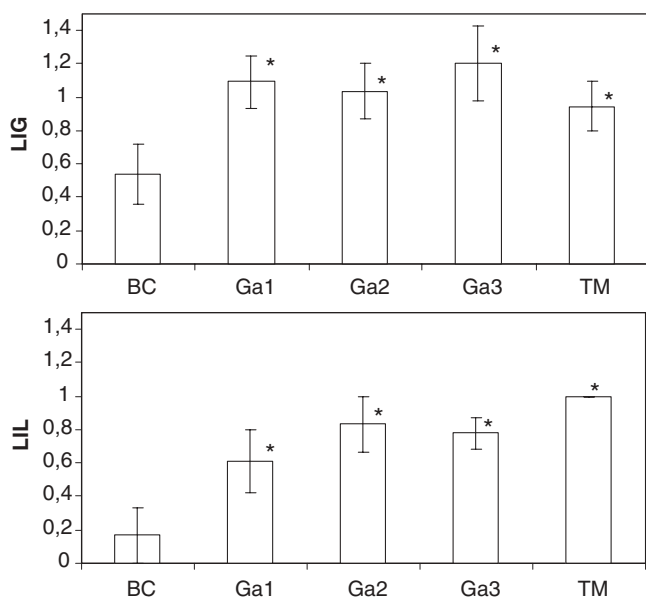
Fig. 3. General indexes of lesions (mean and SD) measured in gills (LIG) and liver (LIL) of *Sparus aurata* juveniles exposed to sediments sampled in the Bay of Cádiz (BC), Galicia coast (Ga#), and toxic mud (TM) treatments. Asterisks indicate significant differences among the index value in the stations and the negative control (*$p < 005$)

Table 3. Sorted rotated factor loadings (pattern) of 29 variables for the two principal factors resulting from the multivariate analysis of results obtained from the bioassay with juveniles of *Sparus aurata*

% Variance	Factor 1 62.4	Factor 2 26.1
TOC	0.78	—
Fines	—	−0.93
Fluorene	0.96	—
Acenaphthene	0.97	—
Naphthalene	0.97	—
Phenanthrene	0.88	—
Anthracene	0.90	—
Fluoranthene	0.63	—
Pyrene	0.95	—
Benzo[a]anthracene	0.97	—
Chrysene	0.95	—
Benzofluoranthene	0.43	—
Benzo[e]pyrene	0.93	—
Benzo[a]pyrene	0.92	—
Perilene	0.83	—
Dibenzo[ah]anthracene	0.91	—
Indene[123-cd]pyrene	0.83	—
Benzo[ghi]perilene	0.95	—
Cd	—	−0.91
Cr	—	−0.88
Cu	—	−0.95
Ni	—	−0.95
Pb	—	−0.96
Zn	—	−0.96
Hg	—	−0.96
MT	—	−0.97
EROD	0.98	—
IGG	0.79	−0.54
IGL	0.50	−0.74

support the conclusion of the persistent exposure of the organisms to hydrocarbons after 10 years of the oil spill caused by the tanker Exxon Valdez in Alaska in 1989 (Jewett *et al.* 2002), emphasizing the potential for continuing oil availability to biota. The ability of fish to metabolize many PAHs makes the use of EROD induction for biomonitoring purposes more beneficial than analytical measurements of PAH uptake, providing a sensitive chemical exposure information many years after a contamination event (Whyte *et al.* 2000). The histopathological analysis showed histomorphological alterations that have been previously reported in this organism when affected by sediment contamination caused by metals and organic compounds (DelValls *et al.* 1998a; Riba *et al.* 2004b, 2004c; Ortiz *et al.* 1999; Au 2004) such as hyperplasia and hypertrophy of gills, and alterations in hepatocytes and exocrine pancreas (*i.e.*, increased cytoplasmic basophilia and vacuolization, necrosis, loss of zymogen granules, *etc.*). An increase of cytoplasmic basophilia was generally detected in liver and exocrine pancreas of all exposed fish. This fact could be related to a decreased protein synthesis (Sarasquete and Gutiérrez 2005), and possibly related to necrotic focus. Moreover, loss of cytoplasmic hepatic glycogen is an early toxic response and may cause an apparent increase in cytoplasmic basophilia (Vethaak and Wester 1996). In general, contaminants can produce osmoregulatory, acid–base, or hemodynamic dysfunctions, and it was proposed that such symptoms are secondary to toxin interactions with specific transport steps or membrane-bound receptors (Evans 1987).

Results show that LIG is always higher than LIL in all of the stations; this could be explained by the affirmation that fish gill is a multifunctional organ sensitive to chemicals in water, because gill filaments and lamellae provide a very large sur-

face area for direct and continuous contact with contaminants in water. Fish gill and liver are highly sensitive to pollutant exposure; however, as previously indicated (Arellano *et al.* 1999), these pointed histopathological alterations are, in general, nonspecific effects, meaning that they are responsive to a variety of pollutants, and therefore only indicative of the general quality of the environment rather than specific types of pollutants (Au 2004). The increase of lipid vacuoles (small size) present in the hepatocytes can indicate an alteration of lipid metabolism or a partial change in their morphology, or in that of lysosomes (Arellano *et al.* 1999; Segner and Storch 1985). The cause–effect relationships and detailed mechanisms leading to the development of most pathological symptoms are not generally clear. Nevertheless, certain hepatic lesions in fish have been well correlated with contaminant exposure (Au 2004). Lamellar fusion of gills could be a protective effect for diminishing the amount of vulnerable gill surface area (Mallat 1985).

The comparison between chemical analysis and the different toxic response (biomarkers of exposure and of effect at different levels) is a useful tool to determine the quality of the studied sediments. The importance of the use of chronic bioassays that provide long-term information on the effects of the exposure to a toxic compound has been proved, because a compound cannot reflect a considerable lethal toxicity, but it is able to produce lesions at different levels to the organism exposed.
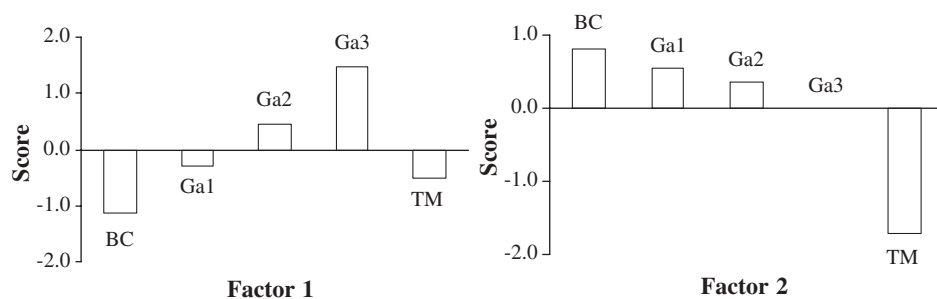
Fig. 4. Estimated factor scores for the two factors in each of the five cases. The factor scores quantify the prevalence of every factor for each station and are used to establish the description of each factor

Despite the repercussion of the spill in the biota, shown as a decrease of the abundance of the microfauna (Junoy *et al.* 2005), previous studies have shown that there was not an important toxic effect in different marine organisms (clams and microalgae) exposed to samples of the sediments and their elutriates associated with the spill caused by the tanker "Prestige" (Mariño-Balsa *et al.* 2003). The bioassay using juveniles of the fish *S. aurata* showed results sensitive enough to determine the hazard associated with this oil-contaminated sediment, displaying good correlation between the toxicity and the contaminant levels using a sublethal set of measurements including both biomarkers of exposure and effect. This study demonstrates the necessity to monitor the impact of the spill on sediment quality in the areas affected. Furthermore, it shows that a subchronic test using a sensitive and sublethal endpoint is a powerful tool to identify the risk associated with the enrichment of PAHs in affected sediments. The higher sensitivity of this bioassay compared to the acute tests previously used indicates the need to incorporate this kind of approach as part of a more complete and integrated study based on a weight-of-evidence approach, as previously recommended by some authors (Carballeira 2003).

References

Adams WJ, Kimerle RA, Barnett JW (1992) Sediment quality and aquatic life assessment. Environ Sci Technol 26:1865–1875

Albaigés J, Bayona JM (2003) La huella del fuel. Ensayos sobre el <<Prestige>>. Fundación Santiago Rey Fernández-LaTorre, pp 80–103

Albers PH (2003) Petroleum and individual polycyclic aromatic hydrocarbons. In: Hoffman DJ, Rattner BA, Burton GA, Cairns J (eds) Handbook of ecotoxicology. Lewis Publishers, Boca Raton, FI Chapt 14

Arellano JM, Storch V, Sarasquete C (1999) Histological changes and copper accumulation in liver and gills of the Solea *senegalensis*. Ecotoxicol Environ Saf 44:62–72

Au DWT (2004) The application of histo-cytopathological biomarkers in marine pollution monitoring: a review. Mar Pollut Bull 48 817–834

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

Carballeira A (2003) Considerations in the design of a monitoring program of the biological effects of the Prestige oil spill. Cienc Mar 29:123–139

Chapman PM (1988) Marine sediment toxicity tests. Chemical and biological characterization of sludges sediments dredge spoils and drilling fluids STP976 American Society for Testing and Materials, Philadelphia, Pennsylvania, pp 391–402

Chapman PM, Long ER (1983) The use of bioassays as a part of a comprehensive approach to marine pollution assessment. Mar Pollut Bull 14:81–84

Comreys AL (1973) A first course in factor analysis Academic Press, New York

CSIC (2003) Heavy metal presence in the shipwreck area of Prestige tanker and metal contents in the emulsioned fuel (in Spanish). Technical report no. 2, http://csicprestige.iim.csic.es/

DelValls TA (2003) The oil spill produced by the tanker Prestige (13/11/2002): Impact assessment of the northwest coast of the Iberian Peninsula. Cienc Mar 29:i–iii

DelValls TA, Andres A, Belzunce MJ, Buceta JL, Casado-Martinez MC, Castro R, Riba I, Viguri JR, Blasco J (2004) Chemical and ecotoxicological guidelines for managing disposal of dredged material. TrAC 23:819–828

DelValls TA, Blasco J, Sarasquete MC, Forja JM, Gómez-Parra A (1998a) Evaluation of heavy metal sediment toxicity in littoral ecosystems using juveniles of the fish *Sparus aurata*. Ecotoxicol Environ Saf 41:157–167

DelValls TA, Chapman PM (1998b) Site-specific sediment quality values for the Gulf of Cádiz (Spain) and San Francisco Bay (USA) using the sediment quality triad and multivariate analysis. Cienc Mar 24:313–336

DelValls TA, Forja JM, Gómez-Parra A (2002) Seasonality of contamination toxicity and quality values in sediments from littoral ecosystems in the Gulf of Cádiz (SW Spain). Chemosphere 46:1033–1043

Edwards R, White I (1999) The Sea Empress oil spill: Environmental impact and recovery. Proceedings of the International Oil Spill Conference 1999, Seattle, pp 97–102

El Rayis OA (1985) Re-assessment of the titration method for the determination of organic carbon in recent sediments. Rapp Comm Int Mer Medit 29:45–47

Evans DH (1987) The fish gills: Site of action and model for toxic effects of environmental pollutants. Environ Health Perspect 71:47–58

Gagné F, Blaise C (1993) Hepatic metallothionein level and mixed function oxidase activity in fingerling rainbow trout (*Oncorhynchus mykiss*) after acute exposure to pulp and paper mill effluents. Wat Res 27:1669–1682

Gaudette HE, Flight WR, Torner L, Folger DW (1974) An inexpensive titration method for the determination of organic carbon in recent sediments. J Sedim Petrol 44:249–253

George SG, Wright J, Conroy J (1995) Temporal studies of the impact of the Braer oil spill on inshore feral fish from Shetland, Scotland. Arch Environ Contam Toxicol 29:530–534

Gutiérrez M (1967) Coloración histológica para ovarios de peces crustáceos y moluscos. Inv Pesq 31:265–271

Jewett SC, Dean TA, Woodin BR, Hoberg MK, Stegeman JJ (2002) Exposure to hydrocarbons 10 years after the *Exxon Valdez* oil spill: evidence from cytochrome P4501A expression and biliary FACs in nearshore demersal fishes. Mar Environ Res 54:21–48

Junoy J, Castellanos C, Viéitez JM, de la Huz MR, Lastra M (2005) The macroinfauna of the Galician sandy beaches (NW Spain) affected by the Prestige oil-spill. Mar Pollut Bull 50:526–536

Kirby MF, Neall P, Tylor T (1998) Erod activity measured in flatfish from the area of the Sea empress oil spill. Chemosphere 38:2929–2949

Lafontaine Y, Gagné F, Blaise C, Costan G, Gagnon P, Chan HM (2000) Biomarkers in zebra mussels (*Dreissena polymorpha*) for the assessment and monitoring of water quality of the St Lawrence River (Canada). Aquat Toxicol 50:51–70

Long ER, Chapman PM (1985) A sediment quality triad: measures of sediment contamination toxicity and infaunal community composition in Puget sound. Mar Pollut Bull 16:405–415

Loring DH, Rantala RTT (1992) Methods for the geochemical analyses of marine sediments and suspended particulate matter. Earth-Sci Rev 32:235–283

Luoma SN, Ho KT (1992) The appropriate uses of marine and estuarine sediment bioassays. In: The handbook of ecotoxicology vol 1. Blackwell Scientific Publications, Cambridge, Massachusetts, pp 193–226

Malins DC, McCain BB, Brown DW, Chan SL, Myers MS, Landhal JT, Prohaska PG, Friedman AJ, Rhodes LD, Burrows DG, Gronlund WD, Hodgings HO (1984) Chemical pollutants in sediments and disease of bottom-dwelling fish in Puget Sound, Washington. Environ Sci Technol 18:705–713

Mallat J (1985) Fish gill structural changes induced by toxicants and other irritants. A statistical review. Can J Fish Aquat Sci 42:630–648

Mariño-Balsa JC, Pérez P, Estévez-Blanco P, Saco-Álvarez L, Fernández E, Beiras R (2003) Assessment of the toxicity of sediment and seawater polluted by the Prestige fuel spill using bioassays with clams (*Venerupis pollastra, Tappes decussatus* and *Venerupis rhomboideus*) and the microalga *Skeletonema costatum*. Cienc Mar 29:115–122

Muto N, Ren HW, Hwang GS, Tominaga S, Itoh N, Tanaka K (1999) Induction of two major isoforms of metallothionein in crucian carp (*Carassius cuvieri*) by air-pumping stress, dexamethasone, and metals. Comp Biochem Physiol C: Pharmacol Toxicol Endocrinol 122:75–82

Olafson RW, Olsson PE (1987) Electrochemical detection of metallothionein zinc and copper levels during an annual reproductive cycle in Rainbow trout (*Salmo gairdneri*). Fish Physiol Biochem 31:39–47

Ortiz JB, de González Canales ML, Saraquete C (1999) Quantification and histopathological alterations produced by sublethal concentrations of copper in *Fundulus heteroclitus*. Cienc Mar 25:119–143

Prego R, Cobelo-García A (2003) Zinc concentrations in the water column influenced by the oil spill in the vicinity of the *Prestige* shipwreck. Cienc Mar 29:103–108

Prego R, Cobelo-García A (2004) Cadmium, copper and lead contamination of the seawater column on the Prestige shipwreck (NE Atlantic Ocean). Anal Chim Acta 524:23–26

Riba I, Forja JM, Gómez-Parra A, DelValls TA (2004a) Sediment quality in littoral regions of the Gulf of Cádiz: a triad approach to address the influence of mining activities. Environ Pollut 132:341–353

Riba I, Casado-Martínez MC, Blasco J, DelValls TA (2004b) Bioavailability of heavy metals bound to sediments affected by a mining spill using *Solea senegalensis* and *Scrobicularia plana*. Mar Environ Res 58:395–399

Riba I, de González Canales ML, Forja JM, DelValls TA (2004c) Sediment quality in the Guadalquivir estuary: sublethal effects associated with the Aznalcóllar mining spill. Mar Pollut Bull 48:153–163

Riba I, Zitko V, Forja JM, DelValls TA (2003) Deriving sediment quality guidelines in the Guadalquivir estuary associated with the Aznalcóllar mining spill: A comparison of different approaches. Cienc Mar 29:261–264

Ritchie W, O'Sullivan M (1994) In: Ritchie W, O'Sullivan M (eds) The environmental impact of the wreck of the Braer. The Scottish Office, Edinburgh (1994) ISBN 0-7480-0900-0, p 207

Sarasquete C, Gutiérrez M (2005) New tetrachromic VOF stain (type iii-gs) for normal and pathological fish tissues. Eur J Histochem 49:105–114

Segner H, Storch V (1985) Influence of water-borne iron on the liver of *Poecilia reticulata*. Sonderdruk aus Zeitschrift für Angewandte Ichthyology Bd H1S:39–47

Stebbing ARD, Akesson B, Calabresse A, Gentile JH, Jensen A, Lloyd R (1980) The role of bioassays in marine pollution monitoring bioassay. Panel Rep Rapp P-V Reun Cons Int Explor Mer 179:322–332

Stegeman JJ, Brouwer M, Richard TDG, Förlin L, Fowler BA, Sanders BM, van Veld PA (1992) Molecular responses to environmental contamination: enzyme and protein systems as indicators of chemical exposure and effect. In: Huggett RJ, Kimerly RA, Mehrle PM Jr, Bergman HL (eds) Biomarkers: biochemical, physiological and histological markers of anthropogenic stress. Lewis Publishers, Chelsea, Michigan, pp 235–335

Stein JE, Collier TK, Reichert WL, Casillas E, Hom T, Varanashi U (1992) Bioindicators of contaminant exposure and sublethal effects: Studies with benthic fish in Puget Sound, Washington. Environ Toxicol Chem 11:701–714

Tolun LG, Okay OS, Gaines AF, Tolay M, Tüfrkçi H, Kiratli N (2001) The pollution status and the toxicity of surface sediments in Izmit Bay (Marmara Sea), Turkey. Environ Int 26:163–168

US EPA (1984) Test methods for evaluating solid waste, physical/chemical methods. Office of Solid Waste and Emergency Response. 3rd ed. US Environmental Protection Agency, Washington, DC, EPA 530/SW-846

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

Varanasi U, Collier TK, Krone CA, Krahn MM, Johnson LL, Myers MS, Chan SL (1995) Assessment of oil spill impacts on fishery resources: measurement of hydrocarbons and their metabolites, and their effects, in important species; Exxon Valdez Oil Spill State/Federal Natural Resource Damage Assessment Final Report (Subtidal Study Number 7); National Marine Fisheries Service, NOAA, Seattle, Washington, 311 pp, NTIS no. PB96-194741

Vethaak AD, Wester PW (1996) Diseases of flounder *Platichthys flesus* in Dutch coastal and estuarine waters with particular reference to environmental stress factors. Dis Aquat Org 26:99–116

Whyte JJ, Jung RE, Schmitt CJ, Tillit DE (2000) Ethoxyresorufin-O-deethylase (EROD) activity in fish as a biomarker of chemical exposure. Crit Rev Toxicol 30:347–570