Monitoring biomarkers in fish (Lepidorhombus boscii and Callionymus lyra) from the northern Iberian shelf after the Prestige oil spill

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Abstract

Hepatic biomarker responses were measured in two demersal fish species (Lepidorhombus boscii and Callionymus lyra) from the northern Iberian shelf associated with the massive Prestige oil spill (POS), five months after the accident. The biomarkers selected were 7-ethoxyresorufin-O-deethylase (EROD), glutathione-S-transferase (GST), glutathione reductase (GR), catalase (CAT), and DNA integrity. Interspecies differences and spatial variations in biomarker responses were observed along the shelf. GST, GR and CAT activities were significantly elevated in L. boscii in the most oil impacted area (Finisterre) and positively correlated (p < 0.05) with POS tar aggregate densities. The lack of previous data from the area together with the existence of chronic background pollution of the shelf implies that the observed biomarker responses cannot be solely attributed to the petroleum hydrocarbon components of the spilled oil. This first biological effect assessment showed that L. boscii is a potentially suitable target species to be used in future biomonitoring programmes along the northern Iberian shelf.

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1. Introduction

The Prestige oil tanker suffered an accident off the Galician coast on 13 November 2002. After being towed along an erratic route (14–18 November 2002), it broke into two pieces, 130 miles west of the southern coast of Galicia and sank on the continental slope of the Galician shelf (Spain) at a depth of 3700 m. The oil tanker was carrying 77,000 tons of crude oil (type M-100). The decision of the authorities to move the damaged tanker away from the coast, prior to its sinking, caused a wide spread spill. An estimated 19,000 tonnes leaked during the towing operation and about 14,000 remained in the tanks of the Prestige at the time of the accident. In all, about 60,000 tonnes of crude oil were leaked over an area of 30,000 km² (Sánchez et al., 2006) and during the months following the accident successive oil slicks drifted from Galician waters onto the Cantabrian shelf, affecting approximately 2600 km of the north Iberian coastline.

Immediately after the shipwreck the Spanish authorities initiated a monitoring programme in order to evaluate the presence of petroleum-derived compounds in sediments, water and biota. One month after the accident, tar aggregates on the bottom were found in different areas of the northern Iberian shelf. Maximum densities of 300 kg km⁻² at depths of around 150 m were estimated in an area of the Galician Shelf (see Fig. 2 in Sánchez et al., 2006). Five months later, the ecotoxicity assessment on marine biota using biomakers was incorporated.

At a biochemical level, exposure to environmental pollutants may induce a variety of enzymes involved in the...
biotransformation system of lipophilic pollutants, including (among many other compounds), some polychlorinated biphenyls (PCBs) and PAHs. This primary and highly specific but reversible effect has its origin at the subcellular level where it may act as the starting point for a sequence of functional alterations. These alterations can reach higher levels of biological organization where they may alter vital functions that affect the survival of organisms and damage the population and communities (De Maagd and Vethaak, 1998).

Enzymatic and genotoxic biomarkers (for oil, PAHs and other contaminants) in fish have been well established in pollution monitoring and risk assessments (OSPAR, 2003; Van der Oost et al., 2003). In fish, most of the oxidative phase I biotransformation is catalysed by the cytochrome P450-dependent microsomal monoxygenase enzymes. The activity of 7-ethoxyresorufin-O-deethylase (EROD) appears to be the most sensitive catalytic probe for determining the inductive response of the cytochrome P450I1A1 (CYP1A1) (Goksøyr and Förlin, 1992). Numerous field studies have demonstrated the suitability of EROD activity in episodes of PAH pollution (e.g. Devaux et al., 1998; Lee and Anderson, 2005) and this biomarker was successfully used to monitor oil exposure related to the Erika and Exxon-Valdez oil spillages (ICES, 2001; Jewett et al., 2002). Chronic exposure to PAHs also produces an increase in oxygen-derived free radicals, generating oxidative stress in organisms (Di Giulio et al., 1993). Glutathione-S-transferases (GSTs) catalyse the conjugation of electrophilic compounds (or phase I metabolites) with glutathione (GSH) and are one of the main enzymes involved in xenobiotic phase II metabolism. In some field studies, significant alterations have been observed on the effect of PAHs and halogenated xenobiotics on fish hepatic GST activity (Vigano et al., 1995; Van der Oost et al., 2003). Catalases (CATs) are hematin-containing enzymes that facilitate the removal of hydrogen peroxide (H$_2$O$_2$), which is metabolised to molecular oxygen (O$_2$) and water. Glutathione reductase (GR) activity maintains the homeostasis of reduced (GSH) and oxidized (GSSG) glutathione under oxidative stress conditions (Winston and Di Giulio, 1991). The genotoxic effects in fish that dwell on bottoms contaminated by PAHs have also been examined in several field studies (Pietrapiana et al., 2002; Van der Oost et al., 2003).

Until the Prestige oil spill (POS), no studies using biomarkers of contaminant exposure or effects in fish had been made in the northern Iberian shelf. The aims of this study were first to investigate a set of biomarkers in fish after the POS to assess toxicological damage, and second to explore the applicability of the selected biomarkers in Lepidorhombus boscii (four-spot megrim) and Callinymus lyra (dragonet) in future biomonitoring programmes along the north Iberian shelf.

The choice of L. boscii and C. lyra as target species was based on monitoring criteria including their wide distribution, close contact with sediment and relative abundance in catches. Previous studies carried out off the French Atlantic coast and in the North Sea have shown the suitability of several flatfish species, such as Limanda limanda, Platichthys flesus and Callinymus lyra as target species (Galgani et al., 1991; Burgeot and Galgani, 1994; OSPAR, 2003). However, except for C. lyra, none of the three demersal flatfish species mentioned occur in sufficient abundance on the northern Iberian shelf. C. lyra dwells on sandy bottoms, at depths less than 150 m (Sánchez et al., 2002), and is a common species on the Galician and Cantabrian shelf. L. boscii is a flatfish of high commercial value in Spain and it is widely distributed along the northern Iberian shelf. It dwells on muddy bottoms on the middle and outer shelf at depths of 100–300 m and it has low migratory capacity (Sánchez et al., 1998). Previous studies carried out in the NW Mediterranean Sea have demonstrated that L. boscii is a very sensitive species to PAH exposure (Pietrapiana et al., 2002). The simultaneous use of both species allowed us to obtain information from different shelf environments since C. lyra can be considered representative for the inner shelf (70–120 m) whereas L. boscii represents the middle and outer shelf (120–350 m).

2. Material and methods

2.1. Sampling procedure

Fish sampling along the northern Iberian shelf was performed within the framework of a multidisciplinary survey which took place between April 15 and May 2, 2003 (Sánchez et al., 2006). For the purpose of our study the continental shelf was divided into seven geographical areas: Galicia S, Finisterre, Galicia N, Asturias W, Asturias E, Cantabria and Basque Country and a total of 39 stations were sampled (Fig. 1).

In order to collect individuals of the target species a set of sampling stations was fixed in each area. The aim was to collect for each species a random sample of between 10 and 20 individuals of each sex per station as recommended by OSPAR (2003). This aim was only partially achieved due to scarcity of fish at some areas. Specimens were caught by 30 min bottom trawls (baca 44/60 otter trawl gear). The fish were killed by severing their spinal cord, and afterwards sexed and weighed. The length of the individuals was recorded and the liver removed. The liver samples were stored in liquid nitrogen until further analyses. To reduce the possible effect of fish size and reproductive status on biomarker responses (OSPAR, 2003) L. boscii specimens were randomly sampled within the size range of 18–20 cm (age class 2) and the C. lyra specimens in the range of 19–21 cm (age class 2), consisted only of non-mature specimens.

During the survey, the quantity and distribution of macroscopic heavy oil residues on the bottom shelf was quantified at each sampling station using a 3.5 m beam-trawl gear, originally designed to sample of benthic communities in deep areas (Serrano et al., 2006). The beam trawls lasted for 15 min with a mean speed of 2.5 knots. The mean area
swept at each sampling station was 3307 ± 192 m². Taking into account the chronic pollution shown by PAHs in shelf sediments (OSPAR, 2000; IEO, 2003; Franco et al., 2006) and the presence of tar aggregates because of the POS on the bottom shelf in winter 2002 we assumed that Finisterre was the area of the greatest impact by the POS, followed by Galicia N and Basque Country and one of lesser impact, namely Asturias W.

2.2. Enzymatic biomarkers

Ethoxyresorufin-O-deethylation (EROD), glutathione-S-transferase (GST), glutathione reductase (GR) and catalase (CAT) activities were assayed in both species from all sampling stations. Microsomes were prepared according to Förlin and Andersson (1985). All steps in microsome preparations were performed at 4 °C. In brief, the livers were immersed in ice-cooled KCl buffer and then homogenized in 0.1 M phosphate buffer, pH 7.4, containing 1 mM dithiothreitol, 1 mM EDTA and 150 mM KCl, 25% w/v. Homogenates were centrifuged at 10,000 g for 20 min and the resulting supernatant was centrifuged at 100,000 g for 60 min. The supernatant, containing cytosol, was stored at −80 °C. The microsomal pellet was resuspended (1:1 liver w/v) in Tris–HCl buffer pH 7.4, containing 1 mM DTT, 0.1 mM EDTA and 20% glycerol. Resuspended microsomes were stored at −80 °C for subsequent assays.

EROD activity was assayed in microsomes according to the method of Eggens and Galgani (1992), a modified version of the fluorimetric method described by Burke and Mayer (1974), adapted to a microplate reader. Final concentrations in the well (350 µl) were 100 mM phosphate buffer pH 7.4, 2 µM 7-ethoxyresorufin and 0.25 mM NADPH. The progressive increase in fluorescence was monitored (excitation wavelength 535, emission wavelength 585).

GST and antioxidant activities (GR and CAT) were measured spectrophotometrically in the cytosolic fraction. GST activity was determined according to Habig et al. (1974) by following the conjugation of reduced glutathione with 1-chloro-2,4-dinitrobenzene at 340 nm (extinction coefficient: ε = 9.6 mM⁻¹ cm⁻¹). GR was measured according to Ramos-Martínez et al. (1983) by following the decrease in absorbance at 340 nm (ε = 6.22 mM⁻¹ cm⁻¹) due to the oxidation of NADPH. The following cuvette concentrations were used: 100 mM Na–phosphate buffer pH 7.1 mM GSSG and 60 µM NADPH. CAT activity was measured according to Livingstone et al. (1992), by the decrease in absorbance at 240 nm due to H₂O₂ consumption (extinction coefficient: ε = 0.04 mM⁻¹ cm⁻¹). Final concentrations in the cuvette were 50 mM Na–phosphate buffer pH 7 and 50 mM H₂O₂. All these enzymatic assays were performed at an incubation temperature of 25 °C and the enzymatic activities were normalized to either microsomal or cytosolic protein content, analysed by using the method described by Lowry et al. (1951) with bovine serum albumin as standard.

2.3. DNA integrity

DNA integrity was established in terms of single-strand breaks or labile sites in alkali, using the alkaline elution method (Kohn et al., 1981) with minor modifications for hepatic tissue, as described in the manual for the biomarkers recommended for the MEDPOL biomonitoring programme (UNEP/RAMOG, 1999). DNA single strand breaks or weak points in the alkali are identified by measuring the rate at which single-strand DNA passes through a membrane filter of known porosity under alkaline denaturing conditions. Filter holders with 0.22 µm pore size filters (GVWP, 25 mm diameter, Millipore Corp., USA) were used. The eluted DNA and that remaining on the filter were measured according to Stout and Becker (1982), using bisbenzimide (Hoescht, Germany) as fluorescent stain. Fluorescence was read at 450 nm with an excitation of 360 nm using a spectrofluorometer. The results are expressed as the elution rate constant “k” (m⁻¹) = −ln(y)/v, where “y” is the fraction of DNA retained on
the filter after the elution of the volume “v”. When an increase of single-strand breaks in the DNA of liver cells occurs, high values of the elution rate constant (k) are expected and indicate a lower DNA integrity.

Due to limited available tissue (the same livers were also used for other analyses), the assessment of the DNA integrity in L. boscii could only be carried out at 15 sampling stations (Nos. 2, 14, 17, 24, 26, 36, 53, 74, 80, 83, 95, 104, 109, 110 and 116) and in the case of C. lyra at 7 stations (Nos. 10, 23, 59, 60, 66, 96 and 107) (Fig. 1).

2.4. Statistical analyses

Data were ln transformed and their normality evaluated by the Shapiro–Wilk test. Sample sizes were too small in this study to allow a sex-specific analysis. To test differences among the mean values, one-way ANOVAs were carried out with the pooled data for the two sexes of each biomarker in both species, using the geographical area as a fixed factor. Because the requirement of equality of group variances was not fulfilled in all the considered groups (Levene’s test) and because the sampling effort was unequal within the sampling areas, the ANOVA were applied using a significance level of \( p = 0.01 \) (Pietrapiana et al., 2002). When significant differences were detected the Tukey’s b-test was applied for a pair-wise comparison. Correlation analyses were performed using standardised data of the sampling stations in order to test the influence of sampling depth on enzymatic responses. Using standardised mean data from different areas, correlation analyses (Pearson correlation coefficient) were applied to test relationships between biological responses and fuel oil deposits. The significance level used was \( p = 0.05 \) (Sokal and Rohlf, 1981).

The analyses were carried out using the SPSS statistical package (SPSS v.11.0).

3. Results and discussion

3.1. Fuel oil on the bottom shelf

The presence of tar aggregates on the bottom sediments of the continental shelf provided an estimation of the degree of the impact of the POS and contributed to a better definition of the oiled and non-oiled areas. Mean tar aggregate densities (at each sampling stations) in the different areas are shown in Tables 1 and 3. Macroscopic heavy oil residues were found at 50% of the sampling stations where they appeared as tar aggregates with diameters ranging from 2 to 12 cm, smaller than those observed in previous surveys (Serrano et al., 2006). Maximum concentrations were found at depths between 120 and 200 m in Finisterre, followed by Galicia N and sampling station number 116 in the Basque Country (89.33 kg km\(^{-2}\)). Except for the station number 15 (0.36 kg km\(^{-2}\)) tar aggregates were not found at any sampling station in Galicia S and Asturias W. The chromatographic analysis of these oil residues evidenced they were all originated by the POS (Albaigés, personal communication).

3.2. Biomarkers in L. boscii

The results of EROD, GST, GR and CAT enzymatic activities and DNA integrity in L. boscii specimens of different genders are presented in Table 1. No apparent differences between the sexes could be found in biomarker responses in the different areas. The differences between

### Table 1

Mean biomarkers values and Standard Error (SE) for male and female L. boscii at each area in April 2003

<table>
<thead>
<tr>
<th>Area</th>
<th>Fuel(^{a})</th>
<th>Sex</th>
<th>EROD(^{b}) ± SE</th>
<th>GST(^{c}) ± SE</th>
<th>GR(^{d}) ± SE</th>
<th>CAT(^{d}) ± SE</th>
<th>K(^{e}) ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Galicia S</td>
<td>0.09 ± 0.09 (4)</td>
<td>Male</td>
<td>21.2 ± 3.3 (8)</td>
<td>139.8 ± 23.7 (9)</td>
<td>12.1 ± 1.2 (9)</td>
<td>0.37 ± 0.06 (9)</td>
<td>0.11 ± 0.05 (3)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Female</td>
<td>26.7 ± 4.8 (11)</td>
<td>101.2 ± 15.1 (13)</td>
<td>13.2 ± 2.4 (13)</td>
<td>0.36 ± 0.05 (13)</td>
<td>0.08 ± 0.03 (4)</td>
</tr>
<tr>
<td>Finisterre</td>
<td>80.10 ± 38.10 (6)</td>
<td>Male</td>
<td>19.9 ± 1.8 (18)</td>
<td>151.5 ± 7.7 (18)</td>
<td>17.8 ± 1.5 (18)</td>
<td>0.57 ± 0.04 (18)</td>
<td>0.08 ± 0.01 (12)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Female</td>
<td>28.9 ± 4.0 (17)</td>
<td>143.5 ± 15.46 (17)</td>
<td>15.3 ± 1.3 (17)</td>
<td>0.53 ± 0.06 (17)</td>
<td>0.10 ± 0.02 (6)</td>
</tr>
<tr>
<td>Galicia N</td>
<td>10.61 ± 7.53 (6)</td>
<td>Male</td>
<td>35.1 ± 3.8 (22)</td>
<td>143.8 ± 8.4 (25)</td>
<td>14.1 ± 0.9 (25)</td>
<td>0.40 ± 0.04 (25)</td>
<td>0.10 ± 0.01 (8)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Female</td>
<td>29.0 ± 3.5 (17)</td>
<td>106.8 ± 9.2 (17)</td>
<td>14.0 ± 1.8 (17)</td>
<td>0.36 ± 0.04 (17)</td>
<td>0.07 ± 0.01 (4)</td>
</tr>
<tr>
<td>Asturias W</td>
<td>0.0 ± 0.0 (4)</td>
<td>Male</td>
<td>35.5 ± 7.6 (13)</td>
<td>105.7 ± 15.0 (13)</td>
<td>9.8 ± 0.9 (13)</td>
<td>0.30 ± 0.03 (13)</td>
<td>0.09 ± 0.01 (8)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Female</td>
<td>24.7 ± 4.1 (11)</td>
<td>100.2 ± 14.1 (11)</td>
<td>10.4 ± 0.7 (11)</td>
<td>0.36 ± 0.04 (11)</td>
<td>0.07 ± 0.01 (4)</td>
</tr>
<tr>
<td>Asturias E</td>
<td>11.11 ± 6.86 (4)</td>
<td>Male</td>
<td>41.8 ± 4.6 (13)</td>
<td>129.9 ± 10.6 (13)</td>
<td>10.8 ± 1.0 (13)</td>
<td>0.33 ± 0.03 (13)</td>
<td>0.09 ± 0.01 (9)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Female</td>
<td>54.4 ± 8.6 (12)</td>
<td>111.2 ± 9.3 (12)</td>
<td>11.8 ± 1.6 (12)</td>
<td>0.44 ± 0.04 (12)</td>
<td>0.13 ± 0.04 (3)</td>
</tr>
<tr>
<td>Cantabria</td>
<td>1.42 ± 0.91 (4)</td>
<td>Male</td>
<td>56.5 ± 10.8 (12)</td>
<td>126.9 ± 8.8 (12)</td>
<td>10.8 ± 0.8 (12)</td>
<td>0.39 ± 0.03 (12)</td>
<td>0.07 (1)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Female</td>
<td>42.0 ± 7.1 (9)</td>
<td>104.2 ± 7.7 (10)</td>
<td>10.6 ± 1.4 (10)</td>
<td>0.46 ± 0.08 (10)</td>
<td>0.09 ± 0.04 (15)</td>
</tr>
<tr>
<td>Basque C</td>
<td>45.419 ± 43.91 (2)</td>
<td>Male</td>
<td>25.8 ± 2.4 (7)</td>
<td>86.4 ± 11.3 (8)</td>
<td>16.7 ± 2.7 (8)</td>
<td>0.42 ± 0.04 (8)</td>
<td>0.07 ± 0.01 (3)</td>
</tr>
</tbody>
</table>

\(^{a}\) kg m\(^{-2}\).

\(^{b}\) pmol min\(^{-1}\) mg prot\(^{-1}\).

\(^{c}\) nmol min\(^{-1}\) mg prot\(^{-1}\).

\(^{d}\) mmol min\(^{-1}\) mg prot\(^{-1}\).

\(^{e}\) Elution rate constant.

The mean densities and SE of the POS-associated tar aggregates are also given. Sampling size is given in parentheses.
areas were tested by using the data gathered from each biomarker in both sexes (one-way ANOVAs) and the results are displayed in Table 2.

EROD activity showed significant differences between specimens collected from different areas ($p = 0.000$). Significantly higher values were observed in specimens from Cantabria and Asturias E (50.3 and 47.8 pmol min$^{-1}$ mg prot$^{-1}$, respectively) than in those from Galicia S, Finisterre and the Basque Country (24.4, 24.3 and 27.6 pmol min$^{-1}$ mg prot$^{-1}$, respectively). Two significant homogeneous subsets were established ($p = 0.01$) (Fig. 2).

For GST activities, significant differences between areas were also found, which could be divided into two homogeneous subsets of significance ($p = 0.002$) (Fig. 2). These differences were found in the most impacted area, Finisterre (147.6 nmol min$^{-1}$ mg prot$^{-1}$), in comparison with those measured in the Basque Country and Asturias W (92.6 and 103.2 nmol min$^{-1}$ mg prot$^{-1}$, respectively).

Important differences in antioxidant enzyme activities (GR and CAT) between areas were also found and once again, two homogeneous subsets of significance ($p = 0.000$) were established (Fig. 2). GR activity was significantly higher in Finisterre area (16.6 nmol min$^{-1}$ mg prot$^{-1}$) than in Asturias W, Cantabria and Asturias E (10.0, 10.8 and 11.32 nmol min$^{-1}$ mg prot$^{-1}$, respectively). Similarly, CAT values were significantly higher in Finisterre (0.426 mmol min$^{-1}$ mg prot$^{-1}$) than in Asturias W and Galicia S areas (0.278 and 0.290 mmol min$^{-1}$ mg prot$^{-1}$, respectively). Results concerning DNA integrity in L. boscii did not show any significant differences between areas ($k = 0.101$) (Fig. 2). The higher values of the elution constant were found in Asturias E.

One of the key findings in this study was the significant positive correlations between GR activities and tar aggregate data ($R = 0.869; p = 0.011$) and between CAT activities and tar aggregate data ($R = 0.814; p = 0.026$) (Fig. 3). No significant correlations were detected between the biomarker responses and the depths of the sampling stations (data not shown).

### 3.3. Biomarkers in C. lyra

The results of EROD, GST, GR and CAT activities and DNA integrity ($k$) in C. lyra in male and female fish are presented in Table 3 and Fig. 4. Similar to L. boscii, no apparent differences between the sexes could be found in biomarker responses in the different areas. The results of the one-way ANOVAs using the combined data for the two sexes are given in Table 2. The biomarker data obtained from Cantabria were excluded due to the low sample size obtained in this area ($n = 4$).

Differences in EROD activities were found when data from Finisterre (200.3 pmol min$^{-1}$ mg prot$^{-1}$), considered the most heavily impacted area, were compared with those from Asturias E (54.3 pmol min$^{-1}$ mg prot$^{-1}$). However, they were not statistically significant ($p = 0.161$).

The highest mean GST activity was found in Galicia N (181.6 nmol min$^{-1}$ mg prot$^{-1}$), which was considered as the second most impacted area by the POS, and the lowest in Asturias W (151.1 nmol min$^{-1}$ mg prot$^{-1}$) although once again, they were not statistically significant ($p = 0.706$). CAT and GR activities did not present any differences which can be associated with the POS. The highest mean GR activity was measured in Asturias W (23.7 ± 2.0 nmol min$^{-1}$ mg prot$^{-1}$) followed by Galicia N (23.6 ± 2.3 nmol min$^{-1}$ mg prot$^{-1}$), while the highest CAT activities were observed in Asturias E (6.1 ± 1.1 mmol min$^{-1}$ mg prot$^{-1}$) followed by Galicia N (6.0 ± 1.0 mmol min$^{-1}$ mg prot$^{-1}$).

The assessment of genotoxic damage in C. lyra was conducted only in four geographical areas. Similar to GST activity, the lowest DNA integrity was found in Galicia N ($k = 0.118$) and the highest in Asturias W ($k = 0.070$). However, such differences were not statistically significant. When the tar aggregates derived from the POS and biomarker data were studied no significant relationship could be established.

### 3.4. Ecotoxicity assessment

The Quality Status Report for Region IV of the North-East Atlantic (OSPAR, 2000) and recent studies (IEO, 2003; Franco et al., 2006) show that the concentrations of heavy metals, PAHs and organochlorinated compounds in the sediments of the northern Iberian shelf are relatively low in the offshore shelf but show clearly higher values in certain hot spots of the inner shelf, especially in southern Galicia, Pontevedra estuary, A Coruña, Gijón, Bilbao and San Sebastián (Fig. 1). In general, the concentrations are higher on the Cantabrian than on the Galician shelf due to greater anthropogenic pressure. All these results indicate the existence of chronic pollution in sediments
prior to the POS in certain zones of the shelf that must be taken into consideration when interpreting the present results.

EROD activity showed clear spatial variation in both species, which represent different shelf environments (Fig. 4). Generally, inter-species variations were observed for all enzymatic activities examined, especially EROD activity. Mean EROD activity in *L. boscii* was twofold higher in Cantabria and Asturias E than in Galicia S and Finisterre, the last one being presumably the area with the greatest impact by the POS. However, regarding *C. lyra*, the mean EROD activity was fourfold higher in Finisterre than in Asturias E and Cantabria areas, although the differences were not significant, partly due to small sample sizes. In most of the studies described in the literature (Eggens et al., 1996; Kirby et al., 1999; Jewett et al., 2002) the degree of enzyme induction was assessed by comparing the biomarker levels with those observed in reference areas or in a control group. In our study we could not consider any area as a reference a priori due to the wide dispersion of the POS along the continental shelf and coast. There was the possibility that the whole continental shelf area could have been impacted. In studies carried out using *C. lyra* in the Seine Bay, one of the most polluted areas of the French Atlantic coast, EROD values between 2 and 16 times higher than those detected in specimens from a reference area were recorded (Galgani et al., 1991; Burgeot et al., 1993; Burgeot and Galgani, 1994). In our study the maximum EROD value found in this species was 12 times the minimum. Data on EROD measurements in *L. boscii* are not available in the literature, but activities between 3 and 10 times the minimum value measured here have been
recorded for other flatfish species experimentally exposed to PAHs (Peters et al., 1992; Vethaak et al., 1996) as well as in field studies along pollution gradients (Stagg et al., 1995; Kirby et al., 1999; Jewett et al., 2002).

In March 2003, total hydrocarbon concentrations in the water column were higher on the eastern Cantabrian shelf than on the Galician shelf, which seems to correspond with the oil trajectories and the later arrival of the spilled fuel-oil to this area (González et al., 2006). However, the impact of terrestrial input sources close to some of the coastal stations and the prevailing chronic pollution on the Cantabrian shelf cannot be underestimated. All these findings may explain the high values of EROD activity observed in *L. boscii* in Asturias E and Cantabria compared to Finisterre or Galicia N.

Although increased GST activities have only been observed in a limited number of fish species, its use as biomarker of exposure to organic xenobiotics has been tested in several laboratory and field studies (reviewed by Van der Oost et al., 2003). In our study, GST enzymatic activities were less variable than that of EROD among the areas. It is noteworthy that highest mean GST activities in both species were also found in Finisterre and Galicia N, the greater impact areas by the POS.

Increases in hepatic CAT activity values have been observed in numerous field studies and in fish exposed to PAH-containing sediments. However, other laboratory studies reported no significant responses after exposure to organic environmental pollutants (all these studies reviewed by Van der Oost et al., 2003). Regarding GR activity, various laboratory studies have reported induction of hepatic activity in fish exposed to a variety of organic pollutants, including PAHs and halogenated xenobiotics (Rodríguez-Ariza et al., 1993; Van der Oost et al., 2003). In our study, CAT activity values were one order of magnitude higher in *C. lyra* than in *L. boscii*. And significantly higher values of the GR and CAT activities were detected

Fig. 3. Relationship between antioxidant responses in the liver of *L. boscii* and tar aggregates concentrations on bottom shelf (*R*² = Pearson’s correlation coefficient). Values are expressed as mean ± standard error by geographical areas.

**Table 3**

Mean biomarkers values and standard error (SE) for male and female *C. lyra* at each area in April 2003

<table>
<thead>
<tr>
<th>Area</th>
<th>Fuel a</th>
<th>Sex</th>
<th>EROD b ± SE</th>
<th>GST c ± SE</th>
<th>GR d ± SE</th>
<th>CAT d ± SE</th>
<th>K e ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Galicia S</td>
<td>0.0 ± 0.0 (2)</td>
<td>Male</td>
<td>190.6 ± 37.3 (5)</td>
<td>197.7 ± 13.2 (5)</td>
<td>20.9 ± 1.8 (5)</td>
<td>6.60 ± 0.57 (5)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Female</td>
<td>93.8 ± 39.7 (6)</td>
<td>161.6 ± 23.8 (6)</td>
<td>18.0 ± 2.1 (6)</td>
<td>3.53 ± 0.95 (6)</td>
<td></td>
</tr>
<tr>
<td>Finisterre</td>
<td>0.1 ± 0.1 (3)</td>
<td>Male</td>
<td>289.4 ± 126.3 (8)</td>
<td>149.0 ± 22.5 (9)</td>
<td>22.5 ± 2.8 (9)</td>
<td>6.94 ± 0.68 (9)</td>
<td>0.08 ± 0.01 (7)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Female</td>
<td>111.2 ± 32.4 (8)</td>
<td>158.4 ± 15.9 (8)</td>
<td>22.7 ± 3.6 (8)</td>
<td>4.62 ± 0.76 (8)</td>
<td>0.09 ± 0.02 (5)</td>
</tr>
<tr>
<td>Galicia N</td>
<td>17.57 ± 14.12 (2)</td>
<td>Male</td>
<td>164.6 ± 52.2 (6)</td>
<td>234.0 ± 40.4 (6)</td>
<td>27.8 ± 3.6 (6)</td>
<td>7.99 ± 1.52 (6)</td>
<td>0.08 ± 0.04 (3)</td>
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<tr>
<td></td>
<td></td>
<td>Female</td>
<td>63.9 ± 9.2 (6)</td>
<td>129.2 ± 13.0 (6)</td>
<td>19.3 ± 1.4 (6)</td>
<td>4.00 ± 0.64 (6)</td>
<td>0.13 ± 0.01 (8)</td>
</tr>
<tr>
<td>Asturias W</td>
<td>0.0 ± 0.0 (2)</td>
<td>Male</td>
<td>173.6 ± 28.6 (10)</td>
<td>157.6 ± 23.1 (10)</td>
<td>23.9 ± 2.4 (10)</td>
<td>4.54 ± 0.58 (10)</td>
<td>0.07 ± 0.01 (6)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Female</td>
<td>53.2 ± 20.2 (2)</td>
<td>118.6 ± 12.2 (2)</td>
<td>22.7 ± 0.3 (2)</td>
<td>3.66 ± 0.81 (2)</td>
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<tr>
<td>Asturias E</td>
<td>2.62 ± 2.02 (2)</td>
<td>Male</td>
<td>63.8 ± 8.7 (9)</td>
<td>183.6 ± 28.7 (9)</td>
<td>22.9 ± 1.4 (9)</td>
<td>6.95 ± 1.28 (9)</td>
<td>0.10 ± 0.01 (8)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Female</td>
<td>25.7 ± 3.0 (3)</td>
<td>134.0 ± 6.6 (3)</td>
<td>20.0 ± 4.9 (3)</td>
<td>3.61 ± 1.20 (3)</td>
<td>0.11 (1)</td>
</tr>
<tr>
<td>Cantabria</td>
<td>7.257 (1)</td>
<td>Male</td>
<td>39.5 ± 6.6 (2)</td>
<td>116.5 ± 35.2 (2)</td>
<td>24.2 ± 5.2 (2)</td>
<td>5.77 ± 0.82 (2)</td>
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<tr>
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<td></td>
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<td>57.3 ± 28.8 (2)</td>
<td>112.5 ± 0.8 (3)</td>
<td>16.4 ± 2.8 (3)</td>
<td>3.74 ± 1.14 (3)</td>
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</table>

The mean densities and SE of the POS-associated tar aggregates are also given. Sampling size is given in parentheses.

a kg km⁻²
b pmol min⁻¹ mg prot⁻¹
c nmol min⁻¹ mg prot⁻¹
d mmol min⁻¹ mg prot⁻¹
e Elution rate constant.
in Finisterre, but only in *L. boscii*. The high values of GST, GR and CAT activities found in *L. boscii* in the greater impact areas of the POS (Finisterre and Galicia N) are in conflict with the low pollutant concentrations measured in offshore sediments (from the mid and outer continental shelf). Therefore, these results indicate an increase in oxidative stress in *L. boscii* associated with the spillage. This is supported by the significant positive linear correlations found between antioxidant activities in *L. boscii* and tar aggregate data. However, we should be careful when interpreting the significance of this observation. The uptake of most organic xenobiotics directly from water, as demonstrated in a wide variety of marine organisms, takes place by means of a passive diffusion process through semi-permeable membranes such as gills, or through the skin. Another important way in which these pollutants may enter the fish involves the absorption of lipophilic compounds from sediments and foodstuffs through the stomach-gut membrane (Spacie and Hamelink, 1985). During this survey, no fuel remains were found in the stomachs of the species studied nor were the specimens stained by oil. Nevertheless, the hypothesis of oil being carried to the bottom of the continental shelf by means of sea-snow after the POS is supported by the fact that small fuel drops were observed on individuals as well as on suprabenthic nets during the sampling of benthic communities in the same survey (Frutos and Parra, 2004), and by the oil found in the exoskeleton and in the gut of several zooplankton species (Varela et al., 2006). Although tar aggregates do not represent the bioavailable fraction of PAHs for fish, their quantities and distribution on the bottom shelf can be regarded as an indicator of the relative magnitude of exposure of fish to bioavailable compounds derived from the POS in the different areas.

Fig. 4. EROD, GST, GR and CAT activities and DNA damage values in the liver of *C. Lyra*. Values are expressed as mean ± standard error. Sampling size: *n* = 12 (Galicia S, Galicia N, Asturias W, and Asturias E); *n* = 18 (Finisterre) and *n* = 6 (Cantabria).
In general, low integrity of DNA is associated with unfavourable environmental conditions and/or with the physiological status of organisms (Everaarts, 1995). In our study, evidence for a decreased DNA integrity was found in C. lyra specimens collected from Galicia N area but high values of the elution constant \((k)\) were also found in Asturias E in both species. Though clear differences in DNA integrity between areas were found, the low number of individual fish analysed for most stations does not permit a useful comparison between areas.

The unambiguous biomarker responses observed in L. bosci (EROD and GST activities) and in C. lyra (CAT and GST activities) in Cantabria and Asturias E areas seem to be more associated to the chronic pollution on this part of the continental shelf rather than to the POS. In the Basque Country area, where high quantities of tar aggregates deriving from the POS were also found, the results were not straightforward, partly due to the total absence of C. lyra in this area. GR and CAT activities measured in L. bosci collected from the Basque Country area implied an antioxidant response comparable with that observed in Galicia N, but the levels of EROD and GST were lower. The Basque Country area is one of the most polluted areas of the Cantabrian shelf. It is therefore likely that chronic metallic and organic pollution previously present in this area may be interacting with the biotransformation enzymes (Gallagher and Di Giulio, 1989; Lemaire-Gony et al., 1995).

4. Conclusion

In conclusion, the selection of biomarkers applied in two demersal fish species allowed us to identify different biological responses between geographical areas of the north Iberian shelf. The study showed that biomarker responses from Finisterre, Galicia North, Asturias East and Cantabrian areas were generally higher than those from Asturias W offshore. The results showed that C. lyra and L. boscii can be considered as promising target species to be used in biomonitoring programmes along the north Iberian shelf. However, the use of C. lyra was compromised in this study by its low abundance in the catches. These findings suggest that the observed increase in oxidative stress in L. boscii may be the result of exposure to hydrocarbons from the spilled oil. However, the lack of pre-POS biomarker data and the chronic pollution existing along the shelf do not allow us to attribute the biomarker responses observed in fish populations directly and/or exclusively to the POS. Further research is required to establish the significance of these findings and to fully determine the potential effect that POS and chronic contaminant exposure are causing on the shelf ecosystems.

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