Validating a multi-biomarker approach with the shanny Lipophrys pholis to monitor oil spills in European marine ecosystems

M.M. Santos a,*,1, M. Solè b,1, D. Lima a, B. Hambach b, A.M. Ferreira c, M.A. Reis-Henriques a

a CIMAR/CIIMAR – Interdisciplinary Centre of Marine and Environmental Research, Laboratory of Environmental Toxicology, University of Porto, Rua dos Bragas 177, 4050-123 Porto, Portugal
b Institut de Ciències del Mar (ICM-CSIC),Pg. Marítim de la Barceloneta, 37-49 08003 Barcelona, Spain
c IPIMAR – Institute for Fisheries and Sea Research, Avenida Brasilia, 1449-006 Lisbon, Portugal

A R T I C L E   I N F O
Article history:
Received 12 May 2010
Received in revised form 21 July 2010
Accepted 28 July 2010
Available online 24 August 2010

Keywords:
Hydrocarbon
Fish
Water Framework Directive
Biomarkers
Oil spill
EROD

A B S T R A C T
Oil spills are an important source of polycyclic aromatic hydrocarbons (PAHs) in the aquatic environment. Intertidal communities are particularly sensitive since most organisms from these ecosystems are sessile or present reduced mobility. Hence, it is important to validate the use of resident species as sentinels to characterize the impact of oil spills on the rocky shores and the improvement during the restoration process. Recently the advantages of using the shanny Lipophrys pholis in pollution monitoring within the northwestern Atlantic coast has been pointed out. Therefore, with the aim of further validating the use of L. pholis in pollution monitoring associated with petrogenic hydrocarbon contamination, a multi-biomarker approach study was carried out 1 week after a moderate oil spill from the waste treatment plant (WTP) of the major Portuguese refinery in the north of Portugal (Petrogal). Fish collected at 2 km from the accident displayed a significant induction of ethoxyresorufin-O-deethylase activity (EROD) and fluorescent aromatic compounds (FACs) in bile (up to a 5-fold induction) in comparison with the pre-spill scenario, and a 15% induction in erythrocytic nuclear abnormalities (ENA), a biomarker of genotoxicity. In contrast, no significant differences were recorded in the reference site. In order to better characterize the time-course accumulation of FACs in bile after a PAH insult, laboratory exposure of L. pholis to benzo[a]pyrene (BaP) was performed. A clear dose–response accumulation of BaP metabolites was observed that closely reflected nominal exposure concentrations already after 3 d. Overall, the findings of the present study highlight the potential of L. pholis in pollution monitoring dealing not only with chronic contamination, but also with oil spill accidents of a moderate scale. Taking into consideration that EROD and FACs determinations in L. pholis are cost effective, rapid and easy to use, they offer a great potential to be incorporated into risk assessment of PAHs in the scope of national monitoring programs and the European Water Policy legislation.

© 2010 Elsevier Ltd. All rights reserved.

1. Introduction

The presence of petrogenic hydrocarbons in marine ecosystems is a ubiquitous phenomenon due to its generalized use (Hannam et al., 2010). In addition to the chronic hydrocarbon contamination of many aquatic ecosystems, mostly in the vicinity of urban and industrialized areas due to point and non-point sources, oil spills are another important source of contamination (Viñas et al., 2009). Long lasting effects in marine organisms have been reported after oil spills, which will depend on several factors such as the habitat sensitivity, the amount and nature of the oil spilled, the hydrographic and geomorphologic characteristics of the affected areas. Intertidal rocky shores are usually among the most impacted ecosystems, since the majority of the marine organisms inhabiting these areas are sessile or show a reduced mobility, thus making them a prime target of oil aggregates or diffuse pollution. Since oil spills are likely to affect different phyla and distinct trophic levels within the intertidal ecosystem, it is important to use and validate species from different groups as sentinels in order to evaluate the impact on rocky shores and follow up their improvement/amelioration during the restoration process. Furthermore, it is of major importance from a legal point of view, the development of adequate monitoring tools, which can be used to demonstrate the ecological loss in the process of claims and compensations after spills. In the past, the use of fish as sentinel organisms in monitoring programs associated with hydrocarbon contamination has been shown to be an adequate methodology (Huggett et al., 2006; Lima et al., 2008). In fact, the utility of the shanny Lipophrys pholis in...
pollution monitoring within the northwestern Atlantic coast rocky shores has been recently evidenced (Lima et al., 2008; Solé et al., 2008a; Ferreira et al., 2009). The major advantage of this species, in relation to other intertidal rocky shore fish species, lies on the fact that they are abundant and easy to catch, ubiquitous, its ecology and behavior has been intensively studied, (Faria et al., 1996; Monteiro et al., 2005), and it has a restricted home range. Hence, this species is representative of local environmental conditions and pollutants exposure (Faria et al., 1996; Monteiro et al., 2005). In a previous study (Lima et al., 2008) with adult L. pholis, a clear correlation between the distance to polycyclic aromatic hydrocarbons (PAHs) sources and the response of several biomarkers such as ethoxyresorufin-O-deethylase activity (EROD) and bilirubin fluorescent aromatic compounds (FACs) was found. Hence, this data supports the use of L. pholis in monitoring PAH contamination.

In February 2007, a moderate scale spill from the waste treatment plant (WTP) of the major Portuguese refinery, located in the north of Portugal (Petrogal), occurred. While no information on the amount of hydrocarbons spilled to the vicinity coast was available, it was possible to detect traces of spilled oil in the sand in approximately 3–5 km of coastline nearby. Therefore, with the aim of further validating the use of L. pholis in pollution monitoring regarding PAH contamination, fish were collected 1 week after the accident at approximately 2 km north of the refinery and also in a control reference site. In order to get more information into the time-course responses of selected biomarkers after a PAHs insult, controlled laboratory experiments were undertaken with L. pholis, and the findings compared with the field observations.

2. Material and methods

2.1. Field studies

2.1.1. Study area

Animals were collected at the site of Cabo do Mundo, located at approximately 2 km north from the Petrogal WTP, where the spill took place. This site was selected because we had former baseline data on biomarker responses in L. pholis, and PAHs levels in mussels, for over 1 year before the accident. Hence, direct comparison with the pre-spill situation during the winter period could be made. This is an important aspect to consider since some biomarkers, such as ethoxyresorufin-O-deethylase activity (EROD), are season dependent (Santos et al., unpublished data). In parallel, a control location (Vila Praia de Âncora) where baseline data was also available was selected for comparison purposes. A global position system (GPS) was used to determine the coordinates of the sampling sites, which are briefly described below:

Cabo do Mundo (C. Mundo), N 41, 22401; W 008, 71667: this site is located 2 km north of an important oil refinery and 4.6 km north of Leixões harbour, the biggest commercial harbour in the North of Portugal. It is also in the vicinity of a small water house (Joane), which has been reported to be contaminated both with industrial and urban effluents (Lima et al., 2008).

Vila Praia de Âncora (V. P. Ânc.), N 41, 79726; W 008, 87306: located 3 km south of a small urbanized area under the same name. No human settlement exists near this sampling site.

2.1.2. Sampling and tissue handling

In February 2007, 1 week after the accident, specimens of L. pholis were collected with hand-nets in rocky pools that emerged during ebb tides. Approximately 15 individuals were sampled at each site. Typically, juveniles of this species suffer a shift in their pattern of microhabitat occupation and feeding behavior when they are about 7 cm, and they migrate to bigger rocky pools, located in a lower part of the intertidal zone. By that time, the onset of reproduction takes place and animals of about 8 cm start to present mature ovaries and testis (Monteiro et al., 2005). Thus, in order to avoid using animals at different maturation stages, only adults were selected for the present study (between 8 and 15 cm). After collection, animals were transported alive to the laboratory in a refrigerated and aerated container. This approach was validated in previous studies with L. pholis (Lima et al., 2008).

Once in the laboratory, fish were anaesthetized in saline water and ice and immediately killed by decapitation, body length and weight were determined. After dissection, liver was weighted and immediately frozen in liquid nitrogen and stored at –80 °C. Enzymatic assay was performed no later than 2 months after collection. The gallbladder was removed, frozen in liquid nitrogen and stored at –80 °C until analyses. Gonads were weighted, sexed and the maturation condition was determined and the presence of food in the stomach annotated. Mussels from the same locations were also collected in February 2007 and used to determine tissue levels of PAHs.

2.1.3. Biochemical measurement

Each liver was homogenized independently in a 1:3 (weight:volume) ratio in ice-cold buffer (50 mM Tris–HCl, pH 7.4, containing 0.15 M KCl). Microsomes were prepared in 50 mM Tris–HCl buffer containing 1 mM NaEDTA, pH 7.4, 1 mM dithiothreitol, 20% (v/v) glycerol, and were obtained by centrifugation of the 9000g supernatant at 36 000g for 90 min in a SIGMA 3K30 centrifuge. The obtained pellet was resuspended, washed in the above mentioned buffer, and spun down at 36 000g for 120 min (Fent and Bucheli, 1994). Microsomes resuspended in EDTA free buffer were stored at –80 °C until use. CYP1A1-dependent EROD activity was determined essentially as described in (Burke and Mayer, 1974), with some modifications. A detailed description can be found in Lima et al. (2008). Fluorescence was determined using a BIORAD SFM25 fluorimeter at excitation/emission wavelengths of 530 and 585 nm. Microsomal proteins were measured by the method of Lowry et al. (1951), using bovine serum albumin as standard. Liver EROD activity is reported in pmol min⁻¹ mg protein⁻¹.

2.1.4. Fluorescent aromatic compounds (FACs) analysis

Fluorescent aromatic compounds (FACs) in the bile were determined by Fixed Wavelength Fluorescence (FF). Five microliters of bile were diluted in 5 ml of ethanol 48% and were centrifuged for 5 min at 1800g, 4 °C. The supernatant was then used for FF determination at the excitation/emission wavelength pairs 260/380, 290/335, 341/383 and 380/430 nm, denoted FF260/380, FF290/335, FF341/383 and FF380/430, respectively. Phenanthrene type metabolites are detected at FF260/380. At FF290/335, mainly naphthalene type of metabolites, typically associated with petroleum products are detected, and benzo[a]pyrene type of metabolites are more efficiently detected at FF380/430. At FF341/383, mainly pyrene-derived metabolites are detected (Lima et al., 2008). Measurements were performed on a BIORAD SFM25 fluorimeter. To allow samples comparisons, a calibration curve was made for each metabolite, using the following standards: 1-hydroxyphenanthrene (10, 5, 2.5, 0.625 and 0.156 μg L⁻¹), phenanthrene (400, 100, 25, and 2.5 μg L⁻¹), naphthalene (1000, 750, 375 and 75 μg L⁻¹) and B(a)P (5, 1, 0.3 and 0.1 μg L⁻¹) (Lima et al., 2008). Biliverdin content was also determined to normalize FACs concentration, but since no differences between normalized and non-normalized data was seen, only non-normalized data is shown.

2.1.5. Erythrocytic nuclear abnormalities

Genotoxic damage was evaluated using the erythrocytic nuclear abnormalities (ENA) assay. Briefly, the ENA test was performed according to Carrasco et al. (1990) and Pacheco and Santos
its ranged from 0.2 to 1.0 ng g$^{-1}$ per 10 min, slides were once more allowed to air-dry and were further stained with 5% Giemsa for 45 min. Four thousand erythrocytes per fish were scored for the presence of ENA under a 1000× magnification lens. Slides were coded and scored blindly by the same observer, with two replicate slides per fish. ENA were classified into one of the following categories: (1) micronuclei, small (<1/3 of the main nucleus) non-refractive, circular or ovoid chromatin bodies, showing the same pattern as the main nucleus (Al-Sabti and Metcalfe, 1995); (2) cells with two nuclei were considered as binuclei; (3) nuclei with two lobes were classified as segmented nuclei; (4) nuclei with a central and unilateral constriction were classified as kidney shaped nuclei. The final results were expressed as the sum for all individual lesions per 1000 erythrocytes.

2.1.6. Polycyclic aromatic hydrocarbons determination in mussels

Approximately 100 mussels of the species Mytilus galloprovincialis (2–3 cm size) per site were homogenized, and the pool used for PAHs determinations. In a former study (Lima et al., 2008) we determined that a pool of 100 mussels was representative of PAHs tissue accumulation in the field. Mussel tissue was Soxhlet extracted with acetone/hexane (1:1) for 24 h and after addition of pre-deuterated surrogate PAH standards from Supelco. Isolation and purification clean-up was completed by silica gel/alumina columns (1:1). Extracted PAHs were separated with dichloromethane/hexane (9:1 and 4:1) and measured by gas chromatography mass spectrometry (GC–MS), using a Trace GC Ultra connected to a 5% phenyl–95% methylsilicone column (15 m × 0.32 mm) at a column temperature of 40 °C for 2 min pre-incubation, the reaction was started adding 50 μL of sample (diluted or undiluted) and after 2 min pre-incubation, the reaction was started adding 50 μL of the substrate. A 5-fold dilution of the sample was necessary for AChE determination but not for any of the other esterases. Determination of esterase activities was done using the principle of Ellman et al. (1961) with appropriate modifications for microplate.

Reading was performed in triplicate at 405 nm in a microplate reader (TECAN Infinite200) during 5 min at 25 °C. Activity was expressed in nmol min$^{-1}$ mg prot$^{-1}$.

LP was determined in muscle using 200 μL of the same supernatant homogenate, as for esterases, and mixed with 650 μL of 1-methyl-2-phenyldimethyl in acetoni-tol (1:3) and 150 μL of 37% HCl. This mixture was incubated at 65 °C for 60 min, the reaction was stopped in ice and further centrifuged at 13 000 rpm × 10 min to precipitate proteins, following a modification of Shaw et al. (2004) protocol. Absorbance was read at 586 nm versus a standard solution of 1,1,3,3-tetramethoxypropane treated similarly. LP content was expressed as mmol MDA (malondialdehyde) g$^{-1}$ wet weight.

2.3. Statistical analyses

In order to evaluate differences among groups, a one-way analysis of variance (ANOVA) was performed, followed by the multiple comparison test Fisher LSD. When data did not fit ANOVA assumptions of normality and homogeneity of variance, a non-parametric ANOVA Kruskal Wallis followed by multiple comparisons of means ranks was applied. The Man-Witney U test was used for a direct comparison between two groups. All tests were performed using the software Statistica 7.0.

3. Results

3.1. Field studies

Fig. 1 reports on EROD activity in L. pholis from the impacted site (Cabó do Mundo) and the reference site (Vila Praia de Ancora), during the pre-spill period (January 2006) and 1 week after the
spill (February 2007). A 5-fold induction in EROD activity was observed in Cabo do Mundo after the spill, if compared with the pre-spill period ($p < 0.001$, Kruskal Wallis non-parametric ANOVA followed by multiple comparisons of means ranks). No significant differences were recorded in the reference site at both sampling periods.

Similarly, the comparison of FACs concentrations in *L. pholis* bile between the pre-spill situation and 1 week after the spill shows an approximately 2-fold increase for naphthalene and B[a]P type metabolites and a 3-fold increase for phenanthrene and 1HO-pyrene type metabolites in Cabo Mundo (Fig. 2). This increase reached statistical significance ($p < 0.05$, Kruskal Wallis non-parametric ANOVA followed by multiple comparisons of means ranks) for phenanthrene, naphthalene and 1HO-pyrene type metabolites.
On the contrary, the reference site showed similar levels for all FACs tested between 2006 and 2007. These results are in agreement with an increase in the total PAHs detected in mussels collected at Cabo do Mundo in 2007, if compared with 2006 (an approximately 3-fold increase), whereas the same range of PAHs levels were recorded in the reference site, Vila Praia de Âncora, between 2006 and 2007 (Fig. 3). In 2006, the levels of FACs in fish bile (i.e., phenanthrene and 1HO-pyrene type metabolites) and total PAHs in mussel tissues were already elevated in Cabo do Mundo if compared with the control site.

The percentage of ENA in *L. pholis* from Cabo do Mundo in January 2006 (49‰) were already higher (although it did not reach significance) than the levels recorded in the control site Vila Praia de Âncora (30‰) (Fig. 4). After the spill, a further 15% increase in the percentage of ENA was observed in Cabo do Mundo although differences were not significant in comparison with the 2006 sampling period due to high sample variability (*p* > 0.05, Kruskal Wallis non-parametric ANOVA followed by multiple comparison of means ranks). In 2007, a significant elevation of ENA was observed in Cabo do Mundo in comparison with the control site.

### 3.2. Laboratory exposure to B[a]P

#### 3.2.1. B[a]P equivalents accumulation

Fig. 5 displays the accumulation of B[a]P equivalents in *L. pholis* bile 3 and 8 d after laboratory exposure to B[a]P. Fish exposed to the lowest concentration (0.1 µg L\(^{-1}\)) showed already a 10-fold B[a]P increase in comparison to controls after 3 d exposure. A 10-fold increase in B[a]P levels in bile was also observed between the 0.1 and the 1 µg L\(^{-1}\) exposed groups, which reflects the difference in the nominal concentrations between both treatment groups. An approximately 7-fold increase in B[a]P equivalents was observed between the 1 and the 10 µg L\(^{-1}\) exposed groups. Overall, a correlation coefficient of *r* = 0.99, *p* < 0.01, was observed between nominal concentrations of the parent compounds and B[a]P metabolites in fish bile. These differences were significant for the two highest B[a]P exposure levels in comparison with control (*p* < 0.05, Kruskal Wallis non-parametric ANOVA followed by multiple comparison of means ranks). However, the non-parametric ANOVA could not detect significant differences between control treatments and B[a]P at 0.1 µg L\(^{-1}\), despite a 10-fold induction in the B[a]P exposed animals. The use of the Man-Witney U test for a direct comparison between solvent control and B[a]P at 0.1 µg L\(^{-1}\) clearly indicate a significant accumulation (*p* < 0.001) at the lowest B[a]P exposure if compared with controls (Fig. 5B). No significant differences between 3 and 8 d were observed in any of the treatments, which indicate that the FACs accumulation in bile is quick, and that after 3 d the equilibrium had been reached. Despite a clear accumulation of B[a]P metabolites in fish bile, the determination of B[a]P levels in *L. pholis* liver showed a lack of bioaccumulation of the parent compound (data not shown) which suggests its rapid elimination in *L. pholis*.

#### 3.2.2. Erythrocytic nuclear abnormalities

Fig. 6 displays the levels of ENA at the end of the exposure period (8 d) in fish from the controls and B[a]P treatment at the highest concentration tested (10 µg L\(^{-1}\)). Despite a slight trend towards an increase in ENA values between control and the other conditions, differences did not reach significance (*p* > 0.05, Kruskal Wallis non-parametric ANOVA followed by multiple comparisons of means ranks).

---

**Fig. 4.** Average erythrocytic nuclear abnormalities frequency (ENA 1000 erythrocytes\(^{-1}\)) in *L. pholis* erythrocytes in Cabo do Mundo and Vila Praia de Âncora in January 2006 and February 2007. Values are mean ± SE (*n* = 9).

**Fig. 5.** Concentration of B[a]P equivalents in *L. pholis* bile after 3 and 8 d of B[a]P exposure under laboratory conditions (A – all treatment groups; B – solvent control versus B[a]P at 0.1 µg L\(^{-1}\)). Values are mean ± SE (*n* = 12).

**Fig. 6.** Average erythrocytic nuclear abnormalities frequency (ENA 1000 erythrocytes\(^{-1}\)) in *L. pholis* erythrocytes in solvent control and B[a]P at 10 µg L\(^{-1}\) at the end of exposure. Values are mean ± SE (*n* = 12).
study indicates that the spill led to the contamination of several kilometers of the coastline in the proximity of the oil refinery, with a clear impact in a local representative species. Records from local newspapers indicate that problems arising from the refinery WTP are recurrent, and thus our data highlights the need for improving their operational procedures.

Laboratory studies with B[a]P further supported the adequacy of *L. pholis* as sentinel and the selected exposure biomarkers to follow up petrogenic exposure. After 3–d, a clear dose–response accumulation of B[a]P metabolites was already evident in bile. Since no differences were observed between both sampling periods (3 and 8 d), we can conclude that for the range of B[a]P concentrations used in the laboratory studies, B[a]P metabolites in bile quickly reach an equilibrium. Even at the lowest exposure, a 10-fold increase in B[a]P metabolites was observed, if compared to control fish. Furthermore, at environmental relevant levels (i.e., B[a]P at 0.1–1 μg L⁻¹), a 10-fold increase in bile B[a]P metabolites was observed between the 0.1 and the 1 μg L⁻¹ treatments, thus reflecting the normal water concentrations. Hence, not only B[a]P metabolites in *L. pholis* bile increased quickly after exposure, they also reflected very closely the actual B[a]P exposure concentrations. Taking into account that *L. pholis* has a wide geographical distribution (from Mauritania to Norway including the Azores Islands and into the Mediterranean), their strong homing behavior, their easiness to catch, and the fact that FACs levels in bile are quickly, easily determined and cost effective, it becomes clear that FACs levels in *L. pholis* bile have a great potential to be included in monitoring programs dealing with PAH contamination.

Several polycyclic aromatic hydrocarbons, in particular B[a]P, have been shown to be genotoxic. In fact, a previous study using *L. pholis* in environmental monitoring reported higher levels of DNA adducts in specimens collected in a site affected by the Sea Empress oil spill, in comparison with the reference sites (Lyons et al., 1997; Harvey et al., 1999). Therefore, during the present oil spill accident, genotoxicity occurrence in fish was evaluated using the presence of ENA. This technique is one of the most well accepted approaches to evaluate exposure to genotoxic agents (Çavas and Ergene-Gozukara, 2005; Micael et al., 2007). For most marine species from relatively clean areas, the normal background ENA levels are in the range of 3–20% (Oliveira et al., 2007; Van Ngan et al., 2007), which is similar to the observed levels in *L. pholis* erythrocytes along the Portuguese coast. The background level of ENA in Cabo do Mundo in 2006 was already twice the recorded in the reference site, thus indicating that in the pre-spill period fish were already under chronic levels of genotoxic agents. After the spill, although a further increase in ENA was observed in fish collected in Cabo do Mundo, this increase did not reach significance in comparison with the pre-spill period. This could be due to the fact that ENA was already induced in these animals, and in order to have a significant induction above the observed levels, exposure to higher concentrations of genotoxic agents are required. Alternatively, it could be associated with the mechanisms of ENA formation in erythrocytes. ENA are formed in cells undergoing mitosis. That is, when fish from Cabo do Mundo were sampled 1 week after the accident, a fair part of the erythrocytes could have already been formed during the pre-spill period. The laboratory exposures with B[a]P seems to support this second hypothesis. Although B[a]P has been described as a model carcinogen (Tsuiji and Walle, 2007), exposure of *L. pholis* for 8 d did not increase ENA above the background levels of control animals. In fact, the absolute ENA levels observed for the highest B[a]P concentration was approximately half of that recorded in field animals from the impacted area in 2007. Nevertheless, laboratory data from other fish species indicates that ENA induction after a genotoxic insult usually takes place between 3 and 7 d (Çavas and Ergene-Gozukara, 2005). Hence, future studies should investigate in detail the kinetics of

### Table 1

<table>
<thead>
<tr>
<th>Day</th>
<th>AChE</th>
<th>BChE</th>
<th>PrChE</th>
<th>LP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>3</td>
<td>36.49±3.30</td>
<td>21.11±0.29</td>
<td>16.96±10.59</td>
</tr>
<tr>
<td>Sol. control</td>
<td>3</td>
<td>41.91±2.44</td>
<td>2.34±0.23</td>
<td>19.76±1.44</td>
</tr>
<tr>
<td>8</td>
<td>3</td>
<td>45.04±2.44</td>
<td>2.69±0.21</td>
<td>21.49±2.11</td>
</tr>
<tr>
<td>B[a]P 0.1</td>
<td>3</td>
<td>41.35±3.30</td>
<td>2.17±0.25</td>
<td>19.15±1.59</td>
</tr>
<tr>
<td>B[a]P 1</td>
<td>3</td>
<td>38.39±1.93</td>
<td>2.63±0.29</td>
<td>21.35±1.39</td>
</tr>
<tr>
<td>B[a]P 10</td>
<td>3</td>
<td>38.95±1.70</td>
<td>2.20±0.22</td>
<td>17.82±1.33</td>
</tr>
<tr>
<td>8</td>
<td>3</td>
<td>49.63±3.06</td>
<td>2.28±0.26</td>
<td>23.53±2.96</td>
</tr>
<tr>
<td>690 M.M. Santos et al. / Chemosphere 81 (2010) 685–691</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### 3.2.3. Cholinesterase activities and lipid peroxidation levels

Table 1 displays the effects of B[a]P exposure in *L. pholis* muscle cholinesterases activity and lipid peroxidation levels at both sampling periods. No significant differences were observed due to treatment and/or length of exposure (p > 0.05, one-way ANOVA).
ENA formation in *L. pholis* in relation to exposure to model carcinogens, in order to further validate the application of this effect biomarker in routine monitoring programs.

During the laboratory exposures, in addition to the determination of FACs and ENA, we also evaluated the effects of B[alpha]P on *L. pholis* muscle cholinesterases activity and LP levels. While AChE is clearly a neurotoxic marker, pseudocholinesterases (PtChE and BuChE) physiological role is less clearly understood. The lack of enhanced LP levels in muscle tissue of exposed animals and of AChE inhibition suggest that B[alpha]P action is through other mechanisms. In fact, ChE activities and LP levels are similar to those observed in a field study using *L. pholis* as sentinel (Solé et al., 2008a). Similarly, lack of a neurotoxic response to B[alpha]P (measured as brain AChE) was seen in *Sparus aurata* (Cunha et al., 2007), or in LP and AChE in juveniles of *Solea senegalensis* exposed to Prestige fuel oil for a short period (Solé et al., 2008b).

The European commission aims at reaching a good water quality status of all European water bodies by 2015. In order to achieve this, the Water Framework Directive and the European Marine Strategy are key legal instruments that should be adopted by all member states. Both legal instruments set the need to consider both chemical and ecological status. To date, the use of biomarker responses in key sentinel species as early warning signals in the context of these legal instruments has played a limited role. Nevertheless, the need for instruments which can anticipate an impact at higher levels of biological organization (i.e., ecological level) has been identified (Hagger et al., 2008; Sanchez and Porcher, 2009). Hence, the validation of biological responses in key sentinel species within European waters is an important step in the implementation of these directives. The findings of the present study highlight the potential of *L. pholis* in pollution monitoring dealing not only with chronic PAH contamination, but also with oil spill accidents even those of a moderate scale. Taking in consideration that EROD activity for a short period (Solé et al., 2008b).

**Acknowledgements**

The present study was funded by and Interreg III B “Atlantic Area” project EROCIPS and a GRICES-CSIC agreement (Ref. 2005P10020). We would like to acknowledge the comments of two anonymous reviewers that help us improve the manuscript.

**References**


Burke, M.D., Mayer, R.T., 1974. Ethoxyresorufin – direct fluorimetric assay of a microsomal o-dealkylation which is preferentially inducible by 3-methylcholanthrene. Drug Metab. Dispos. 2, 583–588.


