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The *Aegean Sea* oil spill on the Galician Coast (NW Spain). III: The assessment of long-term sublethal effects on mussels

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Several biomarkers of exposure to organic pollutants, namely the cytochrome P450 system, glutathione S-transferases, superoxide dismutase, DT-diaphorase and lipid peroxidation, were measured on mussels collected in five locations along the Galician Coast (NW Spain), 6, 9 and 12 months after the *Aegean Sea* oil spill. Among the studied biomarkers, a significant induction of the cytochrome P450 content and lipid peroxidation, determined as tissue concentration of malondialdehyde equivalents, was detected in mussels collected near the wreck point 6 months after the spillage. Thereafter, no significant differences between reference and polluted sites were detected. Nevertheless, the data suggest the existence of oxidative stress in mussel populations during the September–December samplings. A significant elevation of superoxide dismutase activity was detected in September—9 months after the accident—and this elevation was particularly evident in those stations located closest to the wreck point. Lipid peroxidation increased throughout the year and despite the existence of a strong seasonal effect, the whole data set was correlated with total PAH body burden ($R = 0.56$).

Keywords: mussels, Galicia (Spain), *Aegean Sea* oil spill, biomarkers, cytochrome P450 system, lipid peroxidation.

Introduction

The *Aegean Sea* oil spill occurred off La Coru a Harbour, on the Galician Coast (NW Spain) in early December 1992. The ship was transporting 79 000 tonnes of a relatively light crude oil (North Sea) that were emptied into the sea and partially burned. Soon after the accident, cleaning operations were undertaken and 1 month later it was estimated that the combination of fire, weather conditions and cleaning operations had reduced the oil to 10% of the volume of original crude oil that leaked. However, about 200 km of coastline were affected and a large mortality of marine organisms in the bivalve farms nearby was observed. As one of the main commercial activities in the area is shellfish production (more than 200 ktn per year), a monitoring programme on chemical analysis of petrogenic pollutants present in bivalve populations was established (Porte *et al.* 2000a), and 6 months after the accident the assessment of long-term sublethal effects on mussels was undertaken. In this respect, some biochemical responses or biomarkers related to the exposure of mussels to oil components were determined periodically and the results obtained are reported in the present study.

The biomarker approach is understood as a sublethal biological response that gives a measure of the organisms' exposure to xenobiotics (Peakall and Shugart

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1998). This approach has been applied in the survey of several oil spillages using fish (Payne *et al*. 1984, Lindström-Seppä 1988, George *et al*. 1995) or bivalves (Yawetz *et al*. 1992, Solé *et al*. 1996, Peters *et al*. 1999) as sentinel organisms but, to the best of our knowledge, the assessment of long-term effects, has received little attention.

Among the markers used in field studies, the cytochrome P450 system components, glutathione S-transferase activities (GST), antioxidant enzyme activities and stress proteins have been recognized as indicators of exposure to/effect of pollutants (Livingstone 1988, Nasci *et al*. 1989, Livingstone *et al*. 1995, Peters *et al*. 1999, Porte *et al*. 1999, Porte *et al*. 2000b). However, results are often controversial and no single marker has emerged as a unique parameter, so the use of a battery of them has been found to provide a better picture of the biological sublethal effects.

In bivalves, biomarker responses to petrogenic hydrocarbons have usually been determined in the digestive gland, which is the major organ for storage and biotransformation (Livingstone 1991). The measurements carried out in the present study consisted of: (a) cytochrome P450-dependent monooxygenase system components (total cytochrome P450 and NADPH-cytochrome c reductase); (b) glutathione S-transferase (GST), as a measure of Phase II reactions; (c) the antioxidant enzymes, superoxide dismutase (SOD) and DT-diaphorase; and (d) lipid peroxidation, as the saturation of the protective defences (Phase II and antioxidant enzymes) can result in membrane oxidative damage, which was measured in terms of thiobarbituric acid reactive substances. This suite of biomarkers was then selected to obtain information on the spatial and temporal impact of the *Aegean Sea* oil spill in mussel populations from the Galician coast potentially exposed to petrogenic contaminants.

Materials and methods

Sample handling

Adult mussels (*Mytilus edulis*) of mixed sex were collected from five indigenous populations situated along the Galician coast in June, September and December 1993, thus 6, 9 and 12 months after the *Aegean Sea* oil spill. A further control was also performed 34 months after the accident in October 1995 (Porte *et al*. 2000b). Characteristic of the sampling sites were as follows: Mera, situated closer to the accident site: Lorbé, in an aquaculture area; Cari o and Pontedeume, situated in affected areas and near industrial and urban settlements, and Meirás, considered as a reference point, located far from the spill (figure 1).

Mussel samples were wrapped in clean aluminium foil and stored at –20°C for chemical analysis, details of which are reported elsewhere (Porte *et al*. 2000a). For biochemical measurements, digestive glands were immediately dissected, frozen in liquid nitrogen and stored at –80°C, until analysis.

Biochemical analyses

Pooled digestive glands of four to six mussels were used for each replicate sample, and six replicates were prepared per site. Subcellular samples were prepared at 4°C by differential centrifugation as described by Livingstone (1988). Frozen tissues were homogenized using an electrically-driven Polytron homogenizer in a 1:4 (tissue weight:buffer volume) ratio. The homogenization buffer was 10 mM Tris–HCl pH 7.6, containing 0.15 M KCl and 0.5 M sucrose. Cytosolic and microsomal fractions were obtained after differential centrifugation at 500 g × 15 min, 10 000 g × 45 min and 100 000 g × 90 min. The microsomal pellet was resuspended in 10 mM Tris–HCl pH 7.6, 20% w/v glycerol at protein concentrations of approximately 10 mg ml^{-1}. Biochemical measurements were carried out either immediately (cytosolic fractions), or after overnight storage in liquid nitrogen (microsomes).

All assays were carried out in duplicate. Cytochrome P450 components and activities were measured on microsomes as follows. Total cytochrome P450 and '418-peak' (putative denatured cytochrome P450) contents were assayed by the carbon-monoxide difference spectrum of sodium dithionite reduced

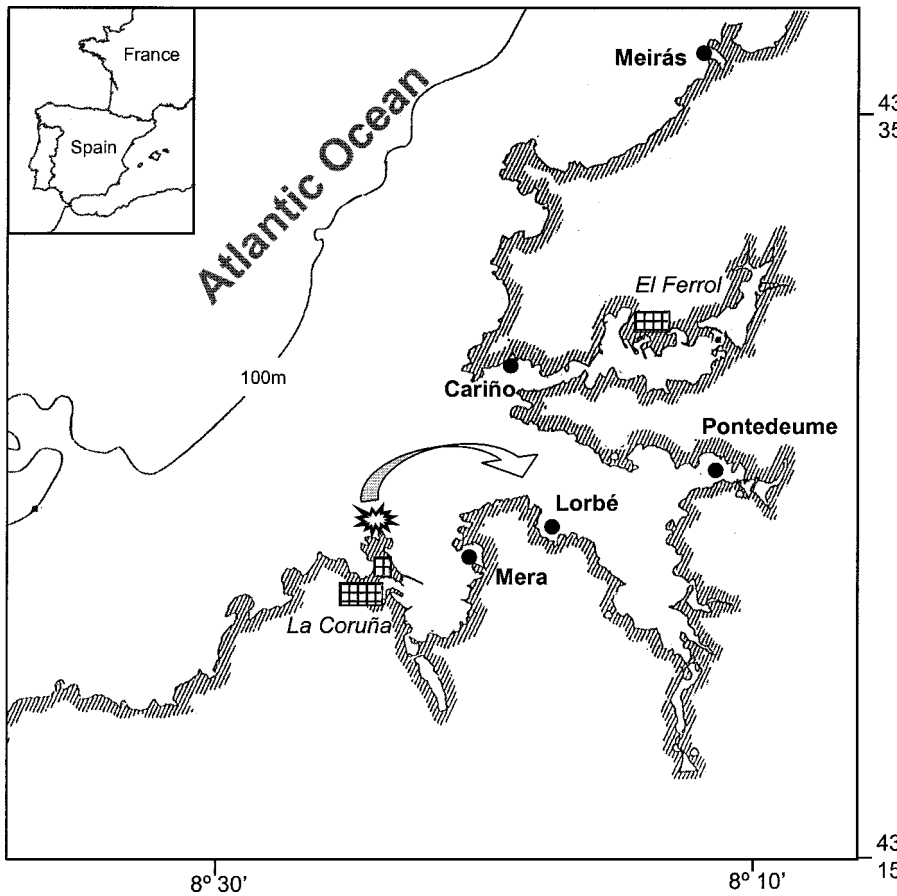

Figure 1. Map of the area of study showing the grounding site of the Aegean Sea (*), the movement of the oil slick and the sampling stations.

samples as described by Livingstone (1988), using an extinction coefficient of 91 $mM^{-1}\,cm^{-1}$ for cytochrome P450. For details in the determination of NADPH-cytochrome c(P450) reductase and DT-diaphorase activity see Livingstone (1988) and Livingstone *et al*. (1990).

GST and SOD activities were assayed spectrophotometrically in the cytosolic fraction after passage through a Sephadex G-25 column to remove the small molecular weight fraction (< 10 kDa) which may interfere with the SOD assay (Livingstone *et al*. 1992). GST activity was determined using 1-chloro-2,4-dinitrobenzene (CDNB) as substrate, the final reaction mixture containing 1 mM CDNB and 1 mM reduced glutathione (Habig *et al*. 1974). SOD activities were measured as described by Livingstone *et al*. (1992) by inhibition of the reduction of cytochrome c by the superoxide anion radical (O_2^-) generated by hypoxanthine/xanthine oxidase (one unit of SOD activity is defined as the amount of sample causing 50% inhibition under the standard conditions of the assay). The substrate conditions were 40 mM K_2HPO_4/KH_2PO_4 pH 7.8, 0.1 mM EDTA, 50 μM hypoxanthine, 1.8 mU xanthine oxidase, 10 μM cytochrome c.

Lipid peroxidation was determined in malonaldehyde equivalents by reaction with thiobarbituric acid as described by Livingstone *et al*. (1990). Protein was determined by the method of Lowry *et al*. (1951) using bovine serum albumin as standard.

Statistical treatment

The biochemical results are presented as mean±SEM ($n = 6$). Differences between groups of values were tested by multivariance one-way ANOVA analysis, $p < 0.05$ was accepted as statistically significant.

Results

Levels of cytochrome P450 and NADPH cytochrome c(P450) reductase activity in mussels collected in the area of the accident are presented in figure 2. Spatial and temporal changes caused by the oil spill could only be assessed by comparing the activities between polluted and reference sites at each sampling time. Although a rather large coastal area was affected immediately after the spill, detailed chemical analysis of mussel tissues revealed Meirás as an adequate reference site 6 months later, when the biomarker survey started (Porte *et al*. 2000a). Total cytochrome P450 content was significantly elevated at the place of the accident (Mera) at the time of the first sampling but in subsequent samplings (9 and 12 months after the spill), no site dependent differences were observed (figure 2(A)). No differences between sites were recorded for NADPH-cytochrome c reductase activity during the whole sampling period (figure 2(B)). Nevertheless, temporal changes were evident for both cytochrome P450 and reductase activity; the highest values were detected in June (6 months after the accident) and the lowest in December.

The activity of cytosolic GST measured with CDNB as substrate is given in figure 2(C). The activity of this enzyme ranged from 55.9 to 86.6 nmol min^{-1} mg^{-1} protein, and 6 months after the accident, mussels from Lorbé and Pontedeume showed activities lower than those from the reference site ($P < 0.05$). In subsequent samplings, no differences among sampling sites were observed. No clear temporal trend was either recorded for this enzymatic activity.

Antioxidant enzyme activities are shown in figure 3. As previously observed for cytochrome P450, a strong temporal effect was detected for DT-diaphorase activity, the lowest activity recorded 12 months after the accident. Differences between polluted and reference sites were only observed for DT-diaphorase activity which was significantly at a low level in relation to the reference site in mussels from Lorbé and Pontedeume, 6 and 9 months after the spill, respectively. On the other hand, SOD activity did not show significant differences among sampling sites. The highest activities were recorded 9 months after the spill, this increase being only significant in mussels from Mera, Lorbé and Pontedeume, the stations located closer to the spill.

Levels of lipid peroxides in mussel digestive gland were enhanced in the accident site ($P < 0.05$ in comparison to Meirás) 6 months after the spillage, and these levels increased over time in mussels from all the sampling sites (figure 4).

Discussion

A previous study has shown that mussel concentrations of petrogenic hydrocarbons decreased sharply in all the stations 6 months after the *Aegean Sea* oil spill, and reached background values at the end of the year, in the range of 50–100 μg g^{-1} dry wt of aliphatic hydrocarbons and 100–300 ng g^{-1} dry wt of polycyclic aromatic hydrocarbons (Porte *et al*. 2000a). These values were similar to those previously found in the region by Soler *et al*. (1989), and to those detected 3 years after the accident in a control survey (Porte *et al*. 2000b). Chemical analysis of mussel tissue and the use of some classical (fossil) markers gave evidence of an incidental increase of petrogenic hydrocarbons in mussels during the autumn–winter (9–12 months after the spill), probably due to resuspension of sediments by the rough weather. Tissue levels of total aliphatic and aromatic hydrocarbons during this period are summarized in table 1.

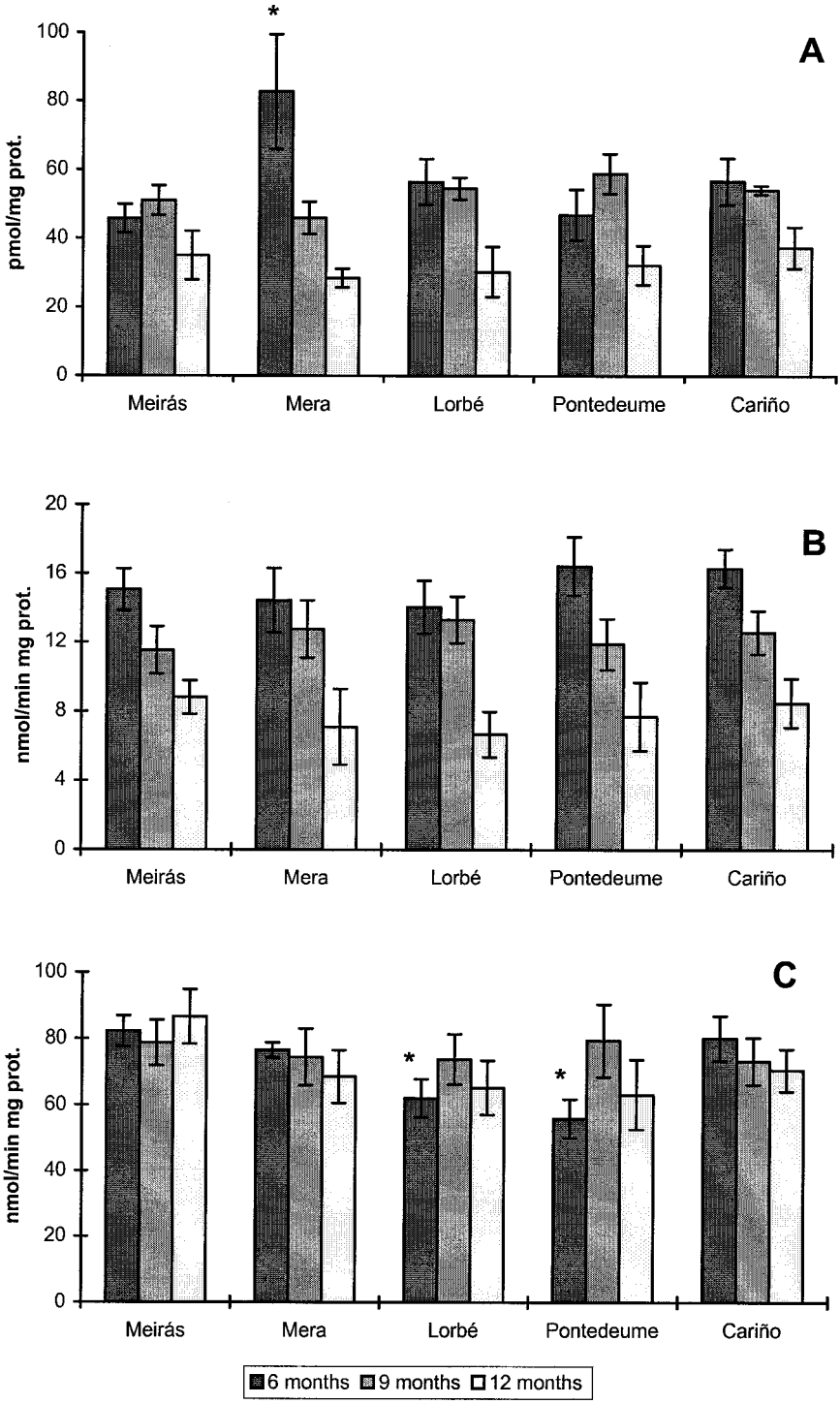

Figure 2. Cytochrome P450 content (A), NADPH cytochrome (P450) reductase (B) and glutathione S-transferase activity (C) in digestive gland of mussels (*Mytilus edulis*) collected along the Galician Coast 6, 9 and 12 months after the *Aegean Sea* oil spill. Values are means±SEM ($n = 6$); * $p < 0.05$ compared with Meirás.

Table 1. Temporal evolution of concentrations of unresolved aliphatic hydrocarbons (UCM) and polycyclic aromatic hydrocarbons (PAHs) in different bivalve samples.

	Months after the spill									
	UCM (in μg g^{-1} dw)					PAHs (in ng g^{-1} dw)				
	3	6	9	12	34	3	6	9	12	34
Mytilus edulis										
Meirás	86	35	61	45	4	123	47	55	106	58
Mera	407	47	103	116	34	1350	307	225	564	539
Lorbé	616	128	84	123	93	1205	296	170	768	181
Pontedeume	717	120	132	125	102	1250	216	183	406	357
Cari o	594	116	389	94	95	754	131	615	267	505
Tapes semidecussata										
Pontedeume	90	64	31	165	165	170	100	59	140	267

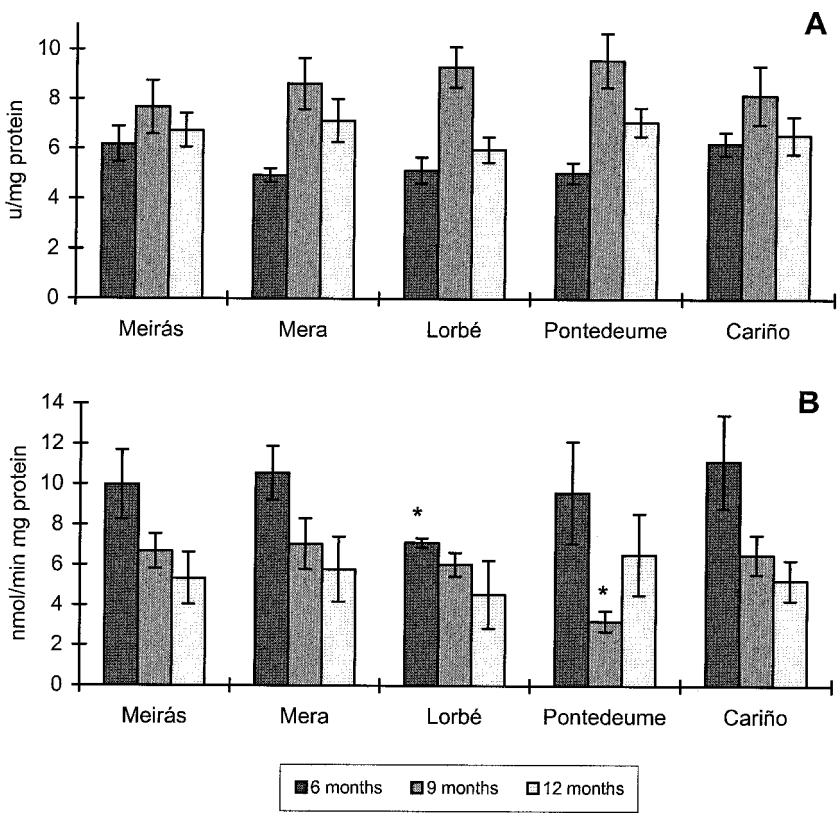

Figure 3. Superoxide dismutase (A) and DT-diaphorase (B) activities in digestive gland of *Mytilus edulis* collected after the *Aegean Sea* oil spill. Values are means±SEM ($n = 6$); * $p < 0.05$ compared with Meirás.

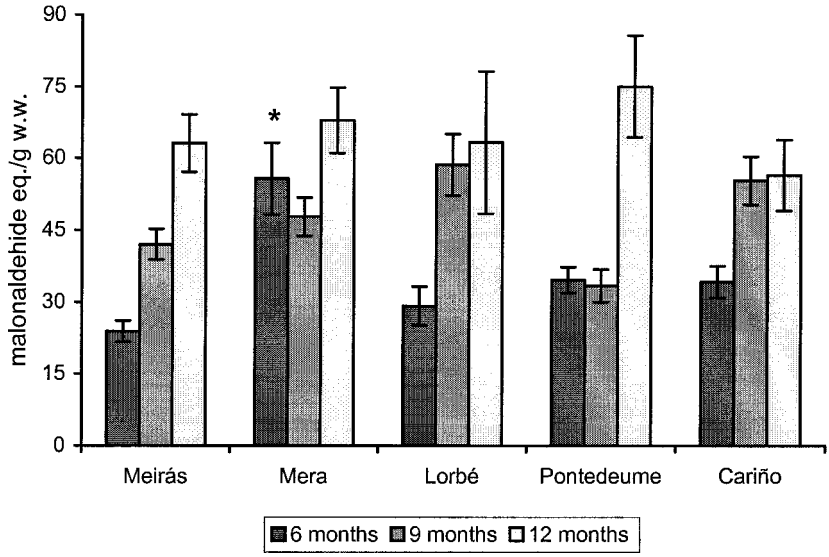

Figure 4. Lipid peroxidation in digestive gland of *Mytilus edulis* collected after the *Aegean Sea* oil spill. Results in malonaldehyde equivalents per gram weight. Values are means±SEM ($n = 6$); * $p < 0.05$ compared with Meirás.

Induction of cytochrome P450 system, Phase II enzymes and antioxidant defences have often been attributed to PAH exposure, although many other contaminants (e.g. PCBs, dioxines, organotin compounds, etc.) may have an effect on these enzymatic systems. The use of the cytochrome P450 system in molluscs as a marker of oil exposure is supported by previous laboratory and field studies. In laboratory experiments, elevation of molluscan cytochrome P450 content/activities occurred after exposure to diesel oil (Livingstone 1988). Similar results were reported for the cockle *Cerastoderma edule* exposed to petrogenic compounds (Moore *et al.* 1987), and for *M. galloprovincialis* after waterborne exposure to benzo[*a*]pyrene (Michel *et al.* 1993, Canova *et al.* 1998). In the field, levels of cytochrome P450 increased in the bivalve *Donax trunculus* sampled 2 months after an oil spill (Yawetz *et al.* 1992), and in *M. edulis* sampled 25 and 130 days after the *Sea Empress* oil spill in Wales (Peters *et al.* 1999).

In the present work, a maximal 10-fold PAH gradient was observed 6 months after the accident between mussels sampled nearer the spillage (Mera) and those from the reference site (table 1). This gradient seemed to be high enough to induce sublethal responses in exposed mussels even 6 months after the spill, when levels of PAHs in their tissues have almost reached background levels for the region. Elevations of total cytochrome P450 content and other biochemical responses, such as CYP1A-immunodetected protein, lipid peroxidation and DNA adducts formation were recorded at this time (Solé *et al.* 1996). Nevertheless, in further samplings (9 and 12 months after the spill), no significant differences were observed among sampling sites, and these findings question the usefulness of total cytochrome P450 as a biomarker of exposure in mussels inhabiting moderately and chronically polluted environments. Contradictory results have certainly been reported regarding cytochrome P450 induction in field studies. While some studies

have pointed out a good response of the system to PAH exposure (Porte *et al*. 1991, Michel *et al*. 1994, Livingstone *et al*. 1995), others have detected little or no response (Nasci *et al*. 1989, Narbonne *et al*. 1991, Porte *et al*. 2000). The lack of response of NADPH-cytochrome c reductase activity (figure 2(B)) is consistent with other field observations (Livingstone 1988, Nasci *et al*. 1989, Porte *et al*. 1991, Solé *et al*. 1995), although marked increases in this activity had been seen in *M. edulis* after diesel oil exposure (Livingstone 1988).

Besides this, levels of both cytochrome P450 and reductase activity steadily decreased over the year in all the sampling sites. This decrease suggests the existence of a seasonal effect which was also evident for DT-diaphorase (figure 3(B)), whose synthesis is regulated by the same Ah-gen battery as cytochrome P450. Seasonal dependence on the cytochrome P450 components has been reported for *Mytilus edulis* (Kirchin *et al*. 1992), and this fact can mask the response to pollutants (Livingstone 1987, Nasci *et al*. 1989, Narbonne *et al*. 1991). This may be the case of the present study, where a statistically significant decrease in cytochrome P450 was recorded in December (12 months after the spill) in mussels from all the stations, except those from the reference site (Meirás). The decrease was particularly evident in mussels from Mera, the station closest to the wreck point. Thus, the data may suggest the concurrence of both a seasonal trend but also a strong decrease in P450-inducing agents.

Concerning Phase II enzyme activities, multiple forms of GST have been characterized in digestive gland of *Mytilus edulis* (Fitzpatrick *et al*. 1997) and its use as markers of PAH exposure has been proposed (Sheehan *et al*. 1991). However, the lack of elevated GST activity in PAH-exposed mussels detected in the present work (figure 2(C)) is consistent with other field studies (Yawetz *et al*. 1992, Livingstone *et al*. 1995, Looise *et al*. 1996, Fitzpatrick *et al*. 1997).

Stimulation of reactive oxygen species (ROS production) is seen to be a PAH mediated mechanism of toxicity in mussels (Livingstone *et al*. 1992). For that reason, antioxidant responses (SOD, DT-diaphorase) and oxidative damage (lipid peroxidation) were also included in the study as possible biomarkers of pollutant impact (Winston and Di Giulio 1991). Correlations between several antioxidant enzymes and PAH tissue levels have been variable (Porte *et al*. 1991, Livingstone *et al*. 1995, Solé *et al*. 1995), and were not observed in the present study. This fact may be related to the transient nature of antioxidant enzyme responses to pollutants in aquatic organisms, for example, DT-diaphorase activity in *Mytilus edulis* was elevated after 6 but not 19 days of exposure to benzo[*a*]pyrene (Livingstone *et al*. 1990). Nonetheless, a significant elevation (cf Méiras) in oxidative damage was detected at the tanker wreck site 6 months after the accident (figure 4), and lipid peroxidation was related to total PAHs body burden ($R = 0.64$). Thereafter, no site-related differences were seen, though lipid peroxidation increased throughout the year, and the whole data set supported a lineal regression model with a positive correlation with total PAHs ($R = 0.56$), and 4–6 ring PAHs ($R = 0.60$).

Results on oxidative stress might also be affected by the existence of a seasonal effect. Viarengo *et al*. (1991) reported a reduction of the antioxidant defence systems and an enhanced susceptibility of mussels to oxidative stress during winter, and the same trend was evident in the present study (figures 3 and 4). On the other hand, the increase in tissue PAH levels observed in December (table 1) may also lead to increased lipid peroxidation. Hence, both pollution and seasonal effects appear to be superimposed in terms of oxidative stress, and this can certainly have a

negative effect on the health of mussel populations long after the accident, when chemical monitoring programmes report that petrogenic hydrocarbons have already reached background levels in the affected area.

In addition, it is worth mentioning the increase in SOD activity observed in autumn, 9 months after the spill (figure 3(A)). This increase was statistically significant in mussels from Mera, Lorbé and Pontedeume, the stations closest to the wreck point, and suggests an increased oxidative stress in those organisms, despite the fact that hydrocarbons and PAH residues in mussel tissues were lower than in the previous sampling (table 1). Seasonal variation in SOD activity has also been reported, but this activity was low throughout the autumn–winter period, increasing in spring to a maximum in June (Viarengo *et al*. 1991). Hence, the high SOD activities detected in autumn–winter in the present work, further suggests the existence of pollutant-mediated oxidative stress in the area closest to the accident. The same trend was observed in clams—*Tapes semidecusatta*—collected in the area, which showed a strong increase in SOD activity in autumn–winter, with values ranging from 16.3–18.7 in September (9 months after the spill) to 20.3–23.6 units mg^{-1} protein in December, compared with 5.9–6.6 units mg^{-1} protein in June. These 'delayed' responses in antioxidant enzymes may well reflect the existence in the environment, and particularly in the sediment compartment, of oxidized hydrocarbons formed through chemical and biochemical reactions which are not quantifiable by gas chromatography techniques (Malins 1980).

Three years after the spill, another survey carried out in the area was unable to detect the spilled oil in mussel tissue, but proved the existence of a pollution gradient among sampling sites due to chronic exposure to petrogenic and pyrolytic hydrocarbons, as a result of urban and industrial activities (Porte *et al*. 2000b). This pollution gradient resulted in a clear induction of stress-70 proteins in gills, but no response of the cytochrome P450 system was detected.

In summary, although a rapid decrease in tissue levels of petrogenic hydrocarbons was observed in mussels, some of the studied biomarkers (cytochrome P450, lipid peroxidation) still showed evidence of exposure to/effects of petrogenic hydrocarbons (or other pollutants) 6 months after the *Aegean Sea* oil spill. Over time, the existence of oxidative damage in mussels was indicated. The results highlight once again the need for interdisciplinary research to assess the quality and functioning of coastal habitats.

Acknowledgements

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