

## Bioaccumulation and biochemical responses in mussels exposed to the water-accommodated fraction of the *Prestige* fuel oil

MONTSERRAT SOLÉ<sup>1</sup>, ASTRID BUET<sup>1</sup>, LAURA ORTIZ<sup>2</sup>, FRANCESC MAYNOU<sup>1</sup>, JOSEP MARIA BAYONA<sup>2</sup> and JOAN ALBAIGÉS<sup>2</sup>

<sup>1</sup>Institut de Ciències del Mar (ICM-CSIC), Pg. Marítim de la Barceloneta 37-49, 08003 Barcelona, Spain.  
E-mail: msole@icm.csic.es

<sup>2</sup>Environmental Chemistry Department (IIQAB-CSIC), Jordi Girona, 18-26.08034 Barcelona, Spain.

**SUMMARY:** The activity of the antioxidant defences catalase (CAT, EC 1.11.1.6), glutathione peroxidase (t-GPX, EC 1.11.1.9), glutathione reductase (GR, EC 1.6.4.2), phase II glutathione *S*-transferase (GST, EC 2.5.1.18) along with the NADPH-dependent cytochrome c (CYP) reductase (EC 1.6.2.4), NADH-dependent cytochrome c reductase (EC 1.6.2.2), and NADH-dependent ferricyanide ( $b_5$ ) reductase (EC 1.18.1.1) was determined in the digestive gland of mussels *Mytilus galloprovincialis* fed with *Tetraselmis* sp. pre-exposed to the water accommodated fraction of the *Prestige* oil. Mussel gills were also used for measuring acetylcholinesterase activity (AChE, EC 3.1.1.7) and lipid peroxidation (LP) as an indication of neurotoxicity and oxidative stress damage respectively. Bioaccumulation of the selected polycyclic aromatic hydrocarbons (2 to 6 rings PAHs) in mussels after 2, 4, 7 and 10 days of exposure did not show any significant trend; the 2-3 ring PAHs were best represented (51%). A significant ( $p < 0.05$ ) bioaccumulation in exposed mussels was only observed for some alkylated 2-3 ring PAHs. Biochemical antioxidant responses (CAT, t-GPX and GR) significantly increased over time, regardless of exposure, whereas NADH-dependent reductases and LP were affected, regardless of the length of exposure. However, due to the low solubility of the *Prestige* crude, the PAH levels reached in exposed mussels were not sufficient to cause a clearly associated biochemical response.

**Keywords:** *Prestige* oil spill, *Mytilus galloprovincialis*, *Tetraselmis* sp., water-accommodated fraction, biomarkers.

**RESUMEN:** BIOACUMULACIÓN Y RESPUESTAS BIOQUÍMICAS EN MEJILLONES EXPUESTOS A LA FRACCIÓN ACOMODADA EN AGUA DEL CRUDO DEL *PRESTIGE*. – La actividad enzimática de las defensas antioxidantes: catalasa (CAT, EC 1.11.1.6), glutatión peroxidasa (t-GPX, EC 1.11.1.9), glutatión reductasa (GR, EC 1.6.4.2), el enzima de fase II glutatión *S*-transferasa (GST, EC 2.5.1.18) y la actividad citocromo c reductasa NADPH-dependiente (CYP) (EC 1.6.2.4), NADH-dependiente (EC 1.6.2.2) y la ferricianida reductasa NADH-dependiente ( $b_5$ ) (EC 1.18.1.1) se determinaron en la glándula digestiva de mejillón *Mytilus galloprovincialis* alimentado con algas (*Tetraselmis* sp.) pre-expuestas a la fracción acomodada del agua de crudo del *Prestige*. En branquias de mejillón se midió la actividad acetilcolinesterasa (AChE, EC 3.1.1.7) como indicador de neurotoxicidad y la peroxidación lipídica (LP) como indicador de daño por estrés oxidativo. La bioacumulación en mejillones de determinados hidrocarburos aromáticos policíclicos (PAHs de 2 a 6 anillos) después de 2, 4, 7 y 10 días de exposición, no mostró ninguna tendencia significativa, siendo los PAHs de 2-3 anillos los mejor representados (51%). En los mejillones expuestos sólo se observó una bioacumulación significativa ( $p < 0.05$ ) en PAHs alquilados de 2-3 anillos. Las respuestas antioxidantes (CAT, t-GPX y GR) incrementaron de manera significativa con el tiempo, independientemente de la exposición, mientras que las reductasas NADH-dependientes y la LP se vieron afectadas por la exposición pero no por el tiempo de exposición. Sin embargo, debido a la baja solubilidad del crudo del *Prestige*, los niveles de PAHs alcanzados en los mejillones expuestos, no fueron lo suficientemente altos como para originar una respuesta bioquímica asociada clara.

**Palabras clave:** vertido de crudo del *Prestige*, *Mytilus galloprovincialis*, *Tetraselmis* sp., fracción acomodada en agua, biomarcadores.

## INTRODUCTION

The spill of more than 60000 tonnes of a heavy fuel (M-100) by the *Prestige* tanker in November 2002, in front of the Galician coast (NW Spain), produced one of the largest ecological disasters in the region, affecting from Galicia in Spain to Brittany in France. The oil contained a significant amount (ca. 50%) of polycyclic aromatic hydrocarbons (PAHs) some of them known to be carcinogenic and/or mutagenic to aquatic organisms (Albers, 2003). Shortly after the accident, several monitoring programs were initiated in order to assess the fate of the oil in the marine environment and its accumulation and effects on marine biota (see *Mar. Pollut. Bull.* Vol. 53).

Mussels are filter-feeding bivalves that are used widely in environmental monitoring due to their ability to bioconcentrate pollutants as well as to respond to their presence (Livingstone, 1987). Moreover, they are of high economic interest to the Galician region with an annual production of more than  $26 \times 10^4$  tonnes. Mussels, despite their low metabolism compared to fish, respond to oil compounds by means of changes in the mixed-function oxygenase (MFO) system involved in the xenobiotics metabolism, as well as in changes in conjugating enzymes and in the antioxidant response (Livingstone, 2001).

Chemical analysis of the contaminants of concern coupled with a set of sublethal exposure and/or effect biomarkers has been recommended in coastal pollution monitoring. This approach has already been applied in the region (Solé *et al.*, 1996; Porte *et al.*, 2000; 2001a; Moreira *et al.*, 2004) and recently in relation to the *Prestige* tanker wreck (Pérez-Cadahía *et al.*, 2004; Marigómez *et al.*, 2006; Martínez-Gómez *et al.*, 2006). In line with these studies, the present work integrates the characterisation of the water soluble PAHs derived from the *Prestige* fuel oil with measuring several MFO components (flavin reductases i.e. NAD(P)H cytochrome c and NADH ferricyanide reductases) as putative markers of PAH exposure in mussels (Livingstone, 1987; Solé and Livingstone, 2005). The conjugation phase II glutathione *S*-transferase (GST) was also considered since many xenobiotics undergoing phase I (MFO) reactions may be further eliminated in this way (Fitzpatrick *et al.*, 1997). The antioxidant enzyme activities catalase (CAT), total glutathione peroxidase (t-GPX) (sum of selenium

dependent and independent forms) and glutathione reductase (GR) were also included. CAT detoxifies  $H_2O_2$  to  $H_2O$  and  $O_2$ , t-GPX and some GST forms detoxify  $H_2O_2$  and organic peroxides, and GR maintains the GSSH-GSH balance towards the reduced form. Moreover, the enzymatic biomarker of neurotoxicity, acetylcholinesterase (AChE), was also considered as it is affected not only by pesticides such as carbamates and organophosphates but also by heavy metals, surfactants and complex xenobiotic mixtures including PAHs (Akcha *et al.*, 2000). AChE was determined in gills as it may be considered the first target of water-borne pollutants (Cheung *et al.*, 2004). In addition, the concentration of a main lipid peroxidation (LP) product, the free malondialdehyde, was measured in this organ as an indicator of oxidative damage.

Despite the existence of several studies that evaluate the impact of oil spillages in the field using mussels as sentinels, few have focused on the short-term effects of complex mixtures of water soluble PAHs from the oils under laboratory exposure. Furthermore, most of those controlled experiments are focused on particular highly toxic/carcinogenic oil components, such as benzo[*a*]pyrene (BaP) (Canova *et al.*, 1998; Okay *et al.*, 2000; Akcha *et al.*, 2000; Cheung *et al.*, 2004). This may be adequate for certain types of oils enriched in high molecular weight PAHs; however, this is not the case of the *Prestige* spill in which the alkylated 2-3 aromatic ring PAHs predominate (Albaigés and Bayona, 2003). These components have been reported to have, at sublethal environmentally realistic concentrations, a narcotic effect on invertebrates (Barata *et al.*, 2005), exhorting certain genotoxicity in *M. edulis* (Hamountene *et al.*, 2002) but a negligible toxic effect on fish (Seruto *et al.*, 2005). Moreover, no cytotoxic effects were observed in RTG-2 cells, no effects on the growth of *Chlorella vulgaris* nor acute or reproductive effects on *Daphnia magna* exposed to the water accommodated fraction (WAF) of the *Prestige* oil (Navas *et al.*, 2006).

The aim of this study was to evaluate, in mussels, the short-term response of several biomarkers of PAH exposure to the *Prestige* oil, by simulating the field concentrations as closely as possible. The oil water-accommodated fraction was obtained following a standardised methodology (Singer *et al.*, 2000), and both dissolved and particulated fuel oil components were administered to mussels through diet and test media using 24 h pre-exposed algae.

This environmentally realistic approach also allowed us to enhance the bioavailable PAHs present in the low water soluble *Prestige* oil.

## MATERIAL AND METHODS

### Reagents

Perdeuterated standards used as surrogates were obtained from Cambridge Isotope Laboratories (Andover, USA). A cyclohexane solution containing the 16 EPA priority PAHs at 10 mg/L each was obtained from Dr. Ehrenstorfer (Augsburg, Germany). Decafluorobiphenyl was from Merck (Hohenbrunn, Germany). Suprasolv grade methanol, hexane and dichloromethane GR for analysis was obtained from Merck (Darmstadt, Germany). Silica gel and neutral alumina were also obtained from Merck (Darmstadt, Germany), extracted with dichloromethane before use and then activated at 120°C overnight. Cytochrome c, NADH (reduced  $\beta$ -nicotinamide adenine dinucleotide), NADPH (reduced  $\beta$ -nicotinamide adenine dinucleotide phosphate), and the other reagents were also obtained from the Sigma Chemical Company.

### Water-accommodated fraction (WAF)

Fuel oil from the *Prestige* cargo provided by Repsol (A Coruña, Spain) was dissolved in filtered sea-water of 35‰ salinity at a 1:500 (w:v) ratio. The water-accommodated fraction (WAF) of the fuel oil was obtained according to the standardised procedure proposed by Singer *et al.* (2000). That is, 1 g of crude was dissolved in 0.5 L of sea water at room temperature (22-23°C) and magnetically stirred for 24 hours in a sealed bottle protected from the light, in order to minimise evaporation and degradation of the oil components.

### Experimental design

Two hundred mussels (*Mytilus galloprovincialis*) with a shell length of  $6.9 \pm 0.6$  cm were collected from a pristine area (Roses, NW Mediterranean), held in hanging nets at the laboratory and acclimated for two weeks to the experimental conditions. They were maintained in two round 50 L tanks (100 mussels in each) containing filtered sea water (temperature  $18^\circ\text{C} \pm 2$ , salinity  $37 \text{ psu} \pm 0.4$  and  $\text{pO}_2$

$100\% \pm 0.3$ ) with a photoperiod regime of 12 h light-darkness.

During the acclimation period animals were fed daily with 4 L of the algae *Tetraselmis* sp. at a density of  $1.3\text{-}2.5 \times 10^5$ . During the experiment, mussels from the exposed tank were fed with algae previously held for 15 hours in a static system of 4 L seawater flasks and at a low  $\text{O}_2$  pressure rate with a 1:10,000 crude dilution, obtained from the previously 1:500 filtered WAF (as described above). The entire 4 L of seawater containing the algae was used to feed the mussels. Mussels from the control tank were fed with 4 L of unexposed algae. The mussels were kept in flow-through water, which was only stopped during the 4 h feeding period. Time and doses used were chosen based on the work of Okay *et al.* (2000) and Pérez-Cadahía *et al.* (2004).

About 20 specimens were sampled from control and exposed tanks after 2, 4, 7 and 10 days of the crude-feeding experimental initiation period. After sampling, mussels were measured and immediately dissected. Gills and digestive glands were separately frozen in liquid  $\text{N}_2$  and stored at  $-80^\circ\text{C}$  until biochemical analysis. The rest of the tissue was homogenised and frozen at  $-20^\circ\text{C}$  for chemical analysis of PAHs. An aliquot of the original sample was placed at  $105^\circ\text{C}$  overnight to determine its moisture content.

### Chemical analysis of PAHs in the WAF and mussel tissue

#### WAF

About 800 mL of WAF was spiked with the following perdeuterated surrogates, naphthalene (272 ng), anthracene (242 ng), pyrene (144 ng) and benzo[a]pyrene (244 ng) in methanol, and then extracted 4 times with 20 mL of dichloromethane. The extracts were percolated through anhydrous sodium sulphate (2 g), cautiously evaporated to around 1 mL and fractionated by column chromatography with neutral alumina and silica gel, both with 6 g, and 5% deactivated with MilliQ water. Two fractions were obtained, the first containing the aliphatic hydrocarbons and the second PAHs, eluted with 20 mL of hexane and 50 mL of hexane : dichloromethane (80:20) respectively.

The collected extracts were rotary evaporated and analysed by gas chromatography coupled to mass spectrometry (GC-MS) using a Trace Thermo-

Electron Corporation (Austin, TX, USA) in the electron impact (EI) mode at 70 eV. Injection was performed in the splitless mode at 280°C using hexane as the solvent. The purge valve was activated 50 sec after the injection. A 30 m × 0.25 mm ID capillary column coated with 0.25 µm of ZB-5MS stationary phase was obtained from Phenomenex (Torrance, CA, USA). The column temperature was kept at 60°C for 1 min, then the temperature was programmed until 200°C at 10°C/min and finally to 320°C at 4.8°C/min, maintaining the final temperature for 10 min, start acquiring after 6 min. Transfer line and ion source temperatures were kept at 250 and 200°C respectively. Acquisition was performed in the full scan mode from 50 to 350 amu at 2 scans/sec (10 scans/peak).

Quantification of the polycyclic aromatic hydrocarbons (PAHs) was performed from the reconstructed ion chromatograms obtained from the molecular ion by the internal standard procedure by using decafluorobiphenyl and recovery correction with the corresponding surrogates.

#### *Mussel tissue*

An aliquot of the sample (5 g, wet weight) was placed in a centrifuge tube containing 5 mL of aqueous NaOH 6M and the sample was spiked with the following perdeuterated surrogates, naphthalene (272 ng), anthracene (242 ng), pyrene (144 ng) and benzo[*a*]pyrene (244 ng) in methanol. The mixture was left in darkness for 18 h at 40°C and then extracted by sonication 4 times with 10 mL hexane : dichloromethane (80:20), separating the phases by centrifugation. The extracts were passed through anhydrous sodium sulphate (2 g), cautiously evaporated to around 1 mL and fractionated and analysed for PAHs as indicated above.

#### **Biochemical analyses**

Two digestive glands (ca. 0.5-1 g wet wt.) were used for each of the 4 or more replicates made per assay. Samples were homogenised in 10 mM Tris-HCl pH 7.6 containing 0.15 M KCl and 0.5 M sucrose in a tissue:buffer ratio of 1:4 (w/v) using an electrically-driven Polytron® homogeniser. Following centrifugations at 12000 g × 30 min and 100000 g × 60 min, the supernatant obtained was the cytosol and the pellet was the microsomal fraction. The latter was further resuspended in 10 mM Tris-HCl pH 7.6 containing 20% (w/v) glycerol to give a protein concentration of

approximately 10 mg mL<sup>-1</sup>. The whole procedure was conducted either in ice or at 4°C.

Pooled gill tissue (0.05-0.1 g wet wt) corresponding to approx 2-4 individuals was also homogenised with the Polytron® homogeniser in a 100 mM Tris pH 8.0 buffer and at a tissue:buffer 1:5 (w/v) ratio. Gill measurements were carried out in the supernatant resulting from a 9000 g × 30 min centrifugation.

All assays were carried out in duplicate at 25°C, and in a final reaction volume of 1 ml (GPX, GR, reductases, AChE and LP) or 3 ml (catalase). Catalase (CAT) activity was measured by the decrease in H<sub>2</sub>O<sub>2</sub> at 240 nm ( $\epsilon = 40 \text{ M}^{-1}\text{cm}^{-1}$ ) using quartz cuvettes. Total Glutathione peroxidase (t-GPX) activity was measured by the NADPH consumption at 340 nm ( $\epsilon = 6.2 \text{ mM}^{-1}\text{cm}^{-1}$ ) during the formation of reduced glutathione by commercial glutathione reductase (GR). Glutathion *S*-transferase (GST) was measured using 1-chloro-2,4-dinitrobenzene (CDNB) as change in OD/min at 340 nm ( $\epsilon = 9.6 \text{ mM}^{-1}\text{cm}^{-1}$ ), a more detailed description of the protocols can be found in Solé *et al.*, (1996). Glutathione reductase (GR) was also measured by the NADPH consumption following the Carlberg and Mannervik protocol (1985). Acetylcholinesterase (AChE) was measured using 100 µL of the gill supernatant fraction by the Ellman method (1961). Lipid peroxidation (LP) was measured using 200 µL of the same gill fraction and absorbance was read at 586 nm versus a standard solution of 1,1,3,3-tetramethoxypropane as described in Durand *et al.* (2002).

Microsomal NAD(P)H-dependent cytochrome c and NADH-dependent ferricyanide reductase activities were measured respectively by the increase in absorbance at 550 nm (ext. coeff. 19.6 mM<sup>-1</sup> cm<sup>-1</sup>) and the decrease in absorbance at 420 nm (ext. coeff. 1.02 mM<sup>-1</sup>cm<sup>-1</sup>). Final assay conditions in a final volume of 1 mL were: 50 mM Tris-HCl pH 7.6, 1 mM KCN, 0.26 mM NAD(P)H, and 30 µM cytochrome c or 0.2 mM potassium ferricyanide. Sample volumes were: 50 µL of microsomes for NADPH and 10 µL for NADH dependent reductases (Solé and Livingstone, 2005).

Total protein was determined by Bradford's method (1976) using bovine serum albumin as standard.

#### **Statistics**

Non-parametric analyses were used for correlations and comparisons between treatments due to the

TABLE 1. – Concentration of PAHs in the WAF used for the experiment and mean mussel tissue concentration during the experiment (considering control and exposed tanks at all time exposures) and the corresponding bioconcentration factors (BCF).

PAHs (Nr. of rings)	Acronym	Water 1:500 (mg/L)	Mussel tissue <sup>b</sup> (min-max.) (ng/g d.w)	BCF <sup>a</sup>
Naphthalene (2)	N	45.7	13.3 ± 1.7 (7.7-21.2)	1.1 ± 0.1
Methylnaphthalenes (2)	N1	55.3	21.3 ± 2.5 (13.9-32.8)	1.5 ± 0.2
Dimethylnaphthalenes (2)	N2	52.7	65.4 ± 4.3 (46.2-85.7)	4.9 ± 0.4
Trimethylnaphthalenes (2)	N3	19.6	66.9 ± 7.3 (40.8-94.7)	13.5 ± 1.4
Fluorene (2)	F	2.50	12.0 ± 1.0 (6.4-14.1)	18.9 ± 1.7
Phenanthrene (3)	Ph	3.33	15.9 ± 0.6 (13.4-18.2)	19.5 ± 0.8
Methylphenanthrenes (3)	Ph1	3.28	23.1 ± 2.0 (16.6-32.4)	3.0 ± 0.2
Dimethylphenanthrenes (3)	Ph2	2.71	29.5 ± 3.1 (18.0-46.1)	2.7 ± 0.3
Trimethylphenanthrenes (3)	Ph3	n.d.	22.2 ± 1.3 (18.4-29.1)	n.c.
Anthracene (3)	A	0.40	5.5 ± 0.4 (4.0-7.8)	4.0 ± 0.4
Dibenzothiophene (3)	D	3.04	8.2 ± 0.7 (5.6-11.9)	3.9 ± 0.4
Methyldibenzothiophenes (3)	D1	1.78	10.6 ± 0.7 (7.1-12.5)	24.4 ± 1.8
Dimethyldibenzothiophenes (3)	D2	1.08	19.4 ± 1.4 (13.1-25.2)	74.3 ± 5.4
Trimethyldibenzothiophenes (3)	D3	n.d.	19.7 ± 1.3 (16.8-23.8)	n.c.
Fluoranthene (4)	Fl	0.13	8.7 ± 0.4 (7.0-10.3)	276 ± 12.5
Pyrene (4)	Py	0.28	12.8 ± 1.6 (9.9-23.8)	204 ± 25.5
Methylpyrenes (4)	Py1	0.49	7.2 ± 0.4 (5.4-8.7)	60.3 ± 3.5
Benzo[a]anthracene (4)	BaA	0.15	8.6 ± 0.4 (6.8-10.5)	231 ± 15.0
Chrysene (4)	C	0.25	7.2 ± 0.3 (5.4-8.3)	118 ± 5.9
Benzo[b]fluoranthene (5)	BbF	0.08	10.9 ± 0.4 (9.7-13.4)	556 ± 32.2
Benzo[k]fluoranthene (5)	BkF	0.02	7.2 ± 0.6 (5.8-10.1)	1578 ± 138.2
Benzo[e]pyrene (5)	BeP	0.32	10.4 ± 0.7 (8.6-13.3)	131 ± 10.7
Benzo[a]pyrene (5)	BaP	0.14	n.d.	n.d.
Perylene (5)	Per	0.05	6.4 ± 0.5 (5.0-8.3)	436 ± 73.6
Indeno[1.2.3-cd]pyrene (6)	IPy	0.04	n.d.	n.d.
Benzo[ghi]perylene (6)	BPer	0.06	n.d.	n.d.
DiBenzo[ah]anthracene (5)	DBA	0.05	n.d.	n.d.

<sup>a</sup> BCF is calculated as: concentration in mussel tissue without digestive gland (ng/g wet weight) / concentration in water (mg/L). Data expressed as mean ± SD (n=8) considering all times and exposures; <sup>b</sup> Data expressed as mean ± SD (n=8); n.d. = not detected; n.c.= not calculated

small number of replicates, which could not ensure normality or homogeneity of variances. Kruskal-Wallis two-way ANOVA was used to determine whether there were significant differences in chemical and bio-chemical markers, using exposure/control and time as factors. The significance of the Kruskal-Wallis statistic was assessed using the chi-square test (Sokal and Rohlf, 1981). The correlation between chemical and biochemical markers was assessed with Spearman's correlation coefficient ( $\rho$ , Sokal and Rohlf, 1981). The significance of the  $\rho$  statistic was assessed with a *t*-test. In both the Kruskal-Wallis two-way ANOVA and the correlation analyses the significance level was set at  $\alpha = 0.05$ .

## RESULTS AND DISCUSSION

### PAH bioaccumulation

The chemical composition of the WAF fraction (1:500) used for the experiment exposure as well as the concentration of individual PAHs and their alkyl derivatives in mussel tissue are shown in Table 1. The contribution of the different types of PAHs

throughout the experiment, according to the number of aromatic rings, is summarised in Figure 1. The presence of PAHs in control mussels may be due to their presence in the sea water used for the experiment. A previous collection of mussels from the vicinity of the water supply already showed similar PAH levels (Baumard *et al.*, 1998). However, exposed organisms showed significantly higher contents of alkylated 2-3 ring PAHs, namely N3 ( $\rho=0.873$ ,  $p=0.005$ ), Ph1 ( $\rho=0.873$ ,  $p=0.005$ ), Ph2 ( $\rho=0.764$ ,  $p=0.027$ ) and Ph3 ( $\rho=0.873$ ,  $p=0.005$ ), which are some of the most representative components of *Prestige* fuel oil. However, no significant

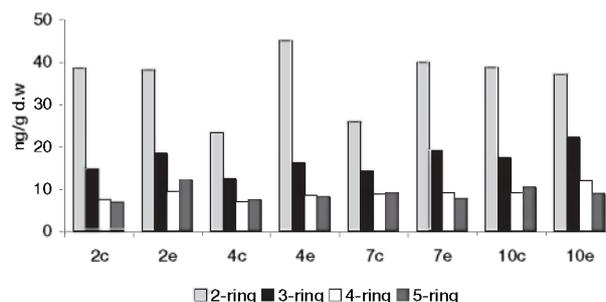


FIG. 1. – Concentration of PAHs (in relation to the number of rings) in water (upper-right graph) and in mussel tissue (lower graph): c. control; e. exposed. 2, 4, 7 and 10 days since the initiation of the

TABLE 2. – Protein yield (PY), antioxidant enzymes (CAT, t-GPX and GR) and phase II (GST) activity analysed in the cytosolic fraction of the digestive gland of *Mytilus galloprovincialis*.

Days		PY <sup>1</sup>	CAT <sup>2</sup>	t-GPX <sup>3</sup>	GR <sup>3</sup>	GST <sup>3</sup>
2	Control (n=4)	154 ± 22.2	6.7 ± 1.0	8.9 ± 1.4	4.3 ± 0.8	13.0 ± 2.2
	Exposed (n=4)	143.2 ± 11.0	4.5 ± 0.1	12.1 ± 1.0	4.5 ± 0.3	11.6 ± 1.0
4	Control (n=4)	98.0 ± 12.3	4.9 ± 0.9	15.1 ± 2.3	5.5 ± 0.8	14.4 ± 0.7
	Exposed (n=4)	126.5 ± 11.9	6.6 ± 2.4	9.9 ± 1.7	4.4 ± 0.3	17.7 ± 0.9
7	Control (n=4)	119.7 ± 20.2	6.7 ± 0.9	19.5 ± 2.4	4.9 ± 0.7	14.7 ± 2.3
	Exposed (n=4)	113.3 ± 18.0	8.5 ± 1.0	17.4 ± 3.7	5.1 ± 0.8	12.5 ± 3.1
10	Control (n=4)	115.4 ± 13.0	6.6 ± 0.4	19.7 ± 1.4	7.1 ± 0.4	11.4 ± 1.9
	Exposed (n=4)	102.3 ± 2.4	12.6 ± 1.1	23.4 ± 4.1	6.9 ± 0.6	13.8 ± 0.8

<sup>1</sup> mg protein g<sup>-1</sup> wet weight; <sup>2</sup> mmol min<sup>-1</sup> mg prot<sup>-1</sup>; <sup>3</sup> nmol min<sup>-1</sup> mg prot<sup>-1</sup>

increase over time was seen for any of the individual PAHs analysed, which probably indicates a steady state situation. Thus, the PAH bioconcentration factors (BCF) were calculated considering the concentrations in the water and in the mussel tissue (without the digestive gland) for all eight situations (Table 1). In general, the BCF values increased with the PAH ring number, except for the high molecular weight components which were not well represented in the oil WAF. In this respect, it has been reported that exposure of mussels to higher doses of BaP (2 and 50 µg/L) do not reach a steady state situation even after 15 days of exposure (Okay *et al.*, 2000).

The reason for the lack of significant bioaccumulation in the present study could be due to: (1) the composition of the WAF, which is enriched in low molecular weight compounds that can be rapidly released to the atmosphere and therefore are not available for aquatic organisms, (2) the further 1:10000 dilution of the 1:500 extract that, although it simulates the maximal solubility of the oil, may be too low to cause any significant bioaccumulation over our experimental temporal span, and (3) the 4 hour feeding period, although theoretically sufficient for the mussels to filter the entire 50 L of water, may not have been sufficient for the exposed

mussels to do so. Overall, the PAH contents in mussels in our laboratory experiment is in line with those reported for background levels of PAHs in mussels in the Mediterranean (Baumard *et al.*, 1998), those regained in the Galician coast 3 years after a major spillage (Porte *et al.*, 2001a) or even in postlarval mussel recruits from the Galician coast recently after the actual *Prestige* spillage (Labarta *et al.*, 2005).

## Biochemical markers

### Digestive gland responses

A selection of the biochemical responses in mussels, as an indication of the PAH exposure and associated with oxidative stress, was based on evidence from other laboratory and field studies. The measured biochemical responses in the two groups of mussels (control and exposed) and over time in the cytosolic and microsomal fractions of the digestive gland are given in Tables 2 and 3 respectively. Apparently, the cytosolic parameters analysed were not affected by the exposure but the antioxidant responses CAT (KW<sub>1,3</sub>=8.48, p=0.037), GR (KW<sub>1,3</sub>=11.20, p=0.011) and t-GPX (KW<sub>1,3</sub>=10.85,

TABLE 3. – Protein yield (PY) and flavin monooxygenase (MFO) components analysed in the microsomal fraction of the digestive gland of *Mytilus galloprovincialis*.

Days		PY <sup>1</sup>	NADPH cyt c red <sup>2</sup>	NADH cyt c red <sup>2</sup>	NADH ferricyanide red <sup>2</sup>
2	Control (n=5)	4.7 ± 0.8	11.5 ± 1.9	46.7 ± 2.8	403.6 ± 52.5
	Exposed (n=5)	4.0 ± 0.9	8.8 ± 1.5	57.4 ± 7.3	458.7 ± 20.7
4	Control (n=4)	3.7 ± 0.4	11.9 ± 2.6	56.3 ± 5.4	376.3 ± 41.8
	Exposed (n=4)	3.3 ± 0.1	10.7 ± 0.7	60.7 ± 5.2	403.7 ± 31.9
7	Control (n=4)	4.1 ± 0.5	13.5 ± 2.1	54.3 ± 5.4	363.0 ± 32.3
	Exposed (n=4)	3.4 ± 0.2	15.4 ± 1.3	65.7 ± 1.3	434.2 ± 29.8
10	Control (n=5)	4.2 ± 0.5	12.0 ± 1.0	48.9 ± 3.8	319.0 ± 27.4
	Exposed (n=5)	4.3 ± 0.4	16.4 ± 3.4	59.0 ± 5.3	387.9 ± 24.5

<sup>1</sup> mg protein g<sup>-1</sup> wet weight; <sup>2</sup> nmol min<sup>-1</sup> mg prot<sup>-1</sup>

$p=0.013$ ) exhibited a statistically significant increase with time.

A relationship between PAH exposure and the increase in antioxidant defences (e.g. CAT, t-GPX, GR) has been put forward (Cajaraville *et al.*, 1992; Cheung *et al.*, 2004) but mostly related to high molecular weight PAHs (e.g. BaP). Nevertheless, consistency of results has not always been achieved and depletion or no-effect on these parameters has also been reported (Solé *et al.*, 1996; Porte *et al.*, 2000; 2001b; Livingstone, 2001). Similar observations apply to the cytosolic GST activity. This phase II enzyme was enhanced in gill and digestive gland (Fitzpatrick *et al.*, 1997); digestive gland (Gowland *et al.*, 2002) and gill (Cheung *et al.*, 2004) in relation to multi-ring PAH exposure. However, the contrary has also been described in whole mussel tissue (Michel *et al.*, 1993) and gill tissue (Akcha *et al.*, 2000). Apart from tissue differences there are several isoforms of GST enzymes in mussels and using CDNB as common substrate might not be as adequate as using other particular substrates that are more specific and inducible (Fitzpatrick *et al.*, 1997).

Three reductase activities were measured in the microsomal fraction of the digestive gland and as components of the CYP-monoxygenase system (Table 3). Only the NADH dependent cyt c ( $KW_{1,3}=4.766$ ,  $p=0.029$ ) and ferricyanide ( $KW_{1,3}=4.229$ ,  $p=0.0397$ ) reductases showed a significant effect due to exposure but not over time. Not only monoxygenases are involved in the metabolism of xenobiotics, endogenous molecules such as fatty acids and sterols are also metabolised in this way. This fact could imply a change in the fatty acid profile as already pointed out in Labarta *et al.*, (2005) in relation to the *Prestige* spillage. The three reductases followed the trend observed in other molluscs including bivalves (Solé and Livingstone, 2005). That is: NADH-ferricyanide > NADH-cytochrome c > NADPH-cytochrome c reductase indicative of cytochrome *b5* reductase, cytochrome *b5* reductase and cytochrome *b5* and CYP reductase respectively. Activity levels of NADPH reductase were within the range of those observed in laboratory (Okay *et al.*, 2000) and field studies (Porte *et al.*, 2000). Induction of these reductases in relation to PAHs has been reported in some studies (Michel *et al.*, 1993; D'Adamo *et al.*, 1997), but not in others (Solé *et al.*, 1996; Porte *et al.*, 2001b).

A relative insensitivity of the MFO system (NADPH cyt c reductase form) to the *Prestige* oil

exposure is in line with observations of fish which do not respond to petroleum containing predominantly low molecular weight PAHs (Seruto *et al.*, 2005). This lack of response in our study is supported by the fact that much higher levels of encapsulated Ph, Fl and BaP, than those found in our mussels, were required to exert an effect on CAT and NADPH reductase in *M. edulis* (Krishnakumar *et al.*, 1997) or for water-borne BaP to cause CYP1A induction and oxidative damage in *M. galloprovincialis* (Canova *et al.*, 1998). In fact, dilutions lower than 1:500 of the WAF of the *Prestige* oil were necessary to induce the MFO dependent EROD activity in exposed RTG-2 cells (Navas *et al.*, 2006).

Protein yield (PY), as a general marker of increased hepatic metabolism due to xenobiotics exposure, was also considered. Although it did not indicate a significant effect of exposure conditions, in the cytosolic fraction these values were markedly higher than those obtained for other field mussels following a similar protocol procedure (personal observation). However, the microsomal fraction displayed similar PY values to mussels from other field surveys. This could explain the fact that most cytosolic determinations for specific activities in our study are lower than those reported elsewhere (Solé *et al.*, 1996; Porte *et al.*, 2001b). Other proteins could be enhanced under our experimental conditions (e.g. stress proteins) and explain the higher cytosolic total protein load.

### Gill responses

Mussels' gills are the organs that first come into contact with waterborne pollutants. Gill AChE activity has been suggested as an adequate biomarker of exposure to neurotoxic compounds in these organisms in relation to an oil spillage (Moreira *et al.*, 2004). In the present study, no difference in gill AChE activity was observed in exposed mussels in relation to the control (Table 4). Moreover, no significant time-dependence of this response was detected ( $KW_{1,3}=3.737$ ,  $p=0.291$ ). Indeed, the present PAH concentrations in mussels are similar to those found under "normal" field situations (Baumard *et al.*, 1998), so that a lack of response in the exposed specimens is not surprising. However, the gill AChE values obtained in our study are in the range of those observed in the same species under laboratory conditions (Akcha *et al.*, 2000) but lower than those observed in field mussels (40-46

TABLE 4. – Protein yield (PY), the neurotoxicity marker (AChE) and the oxidative damage marker (LP) analysed in gills of *Mytilus galloprovincialis*

Days		PY <sup>1</sup>	AChE <sup>2</sup>	LP <sup>3</sup>
2	Control (n=6)	11.4 ± 1.2	11.8 ± 2.3	32.5 ± 1.3
	Exposed (n=6)	13.4 ± 0.8	13.1 ± 1.1	39.4 ± 3.6
4	Control (n=6)	9.7 ± 1.0	13.9 ± 2.5	30.7 ± 3.1
	Exposed (n=6)	16.0 ± 1.2	9.2 ± 0.8	45.9 ± 2.6
7	Control (n=6)	10.4 ± 1.0	8.9 ± 1.4	34.5 ± 2.1
	Exposed (n=6)	11.0 ± 0.9	12.0 ± 0.9	37.8 ± 3.5
10	Control (n=6)	10.3 ± 1.2	14.6 ± 3.7	45.5 ± 3.5
	Exposed (n=6)	10.5 ± 0.4	13.6 ± 1.4	43.3 ± 2.9

<sup>1</sup> mg protein g<sup>-1</sup> wet weight; <sup>2</sup> nmol min<sup>-1</sup> mg prot<sup>-1</sup>; <sup>3</sup> nmol MDA g<sup>-1</sup> wet weight

nmol/min/mg prot) over a two year study (Bodin *et al.*, 2004) or in mussels from the Mediterranean (24–31 nmol/min/mg prot) exposed to a pollution gradient (Porte *et al.*, 2001b). Two factors could partly explain these differences: (1) the use of a protein membrane solubiliser in Bodin *et al.* (2004) and/or (2) a worse fitness condition in mussels exposed to laboratory conditions. Long (up to 30 day) acclimation periods have been recommended in mussels (D'Adamo *et al.*, 1997) to avoid the effect of the adaptation factor.

LP, as an indicator of oxidative stress damage was also evaluated in gills. Unlike AChE, the treatment had an effect of this effect marker (KW<sub>13</sub>=6.328, p=0.012). In Table 5 the Spearman

rank correlation for only those biomarkers that showed a significant relation to any of the individual PAHs is presented. Correlations between chemical body burden and biochemical responses were mostly significant for LP. In fact, LP was the only marker to significantly correlate with most individual PAHs. This suggests that the treatment had a negative effect on the mussels exposed to environmentally realistically low doses of petrogenic PAHs and that the actual defence system (not being induced under exposure) was not able to cope with any ROS formed. Nevertheless, LPO damage has mostly been associated with BaP exposure (Solé *et al.*, 1996; Porte *et al.*, 2000; Cheung *et al.*, 2004; Durand *et al.*, 2002).

TABLE 5. – Summary of Spearman rank correlation ( $\rho$ ) and significant level ( $\alpha$ ) for individual PAHs and biochemical responses. Only biomarkers displaying some significant values are considered.

PAHs	NADH cyt c reductase		CAT		t-GPX		GR		LP	
	$\rho$	$\alpha$	$\rho$	$\alpha$	$\rho$	$\alpha$	$\rho$	$\alpha$	$\rho$	$\alpha$
Naphthalene	0.426	*								
Methylnaphthalenes										
Dimethylnaphthalenes									0.477	**
Trimethylnaphthalenes									0.364	*
Fluorene									0.369	*
Phenanthrene							0.370	*	0.369	*
Methylphenanthrenes									0.389	**
Dimethylphenanthrenes			0.614	***					0.370	*
Trimethylphenanthrenes			0.539	**						
Anthracene										
Dibenzotiofene										
Methyldibenzotiofenenes									0.3873	**
Dimethyldibenzotiofenenes			0.424	*	0.418	*	0.438	*	0.4043	**
Trimethyldibenzotiofene					0.379	*			0.4281	**
Fluoranthene			0.421	*	0.416	*	0.385	*	0.5027	***
Pyrene			0.626	***	0.481	**	0.427	*	0.2952	*
Methylpyrenes									0.3980	**
Benzo[a]anthracene									0.3403	*
Crisene			0.377	*					0.3161	*
Benzo[b]fluoranthene									0.3575	*
Benzo[k]fluoranthene										
Benzo[e]pyrene									0.348	*
Benzo[a]pyrene	n.c.	n.c.	n.c.	n.c.	n.c.	n.c.	n.c.	n.c.	n.c.	n.c.
Perylene					0.475	*			0.408	**

n.c.= not calculated.

## CONCLUSION

Short-term toxicity in mussels due to environmentally realistic doses of the WAF of the *Prestige* fuel oil did not seem to be responsible for the induction of most of the digestive gland biomarkers tested. Nevertheless, a certain level of toxicity was observed in gills as increased LP levels were found. NADH reductases were also affected by the exposure, which could affect metabolism of endogenous compounds. Bioaccumulation of the *Prestige* oil PAHs and its associated effects via ingestion/filtration seem to be less likely ways of endangering the studied mussel species. The characteristics of the *Prestige* heavy oil, which has low water solubility, underline the fact that the real threat to the coastal marine organisms was mainly produced by oiling during the initial stages of the black tide episode.

## ACKNOWLEDGEMENTS

The present study was funded by the Spanish project VEM2003-20068-CO5-01 from the Spanish Ministry of Education and Science. One of us (A. Buet) received a grant from the MEC (SB2003-0198).

## REFERENCES

- Akcha, F., C., Izuel, P., Venier, H., Budzinski, T., Burgeot and J-F. Narbonne. – 2000. Enzymatic biomarker measurement and study of DNA adduct formation in benzo(a)pyrene-contaminated mussels. *Mytilus galloprovincialis*. *Aquat. Toxicol.*, 49: 269-287.
- Albaigés, J. and J.M. Bayona. – 2003. El Fuel. In: S.R. Fernández-Latorre (ed.), *La Huella del Fuel. Ensayos Sobre el Prestige*, pp. 80-103. Fundación Santiago Rey Fernández-Latorre, A Coruña.
- Albers, P.H. – 2003. Petroleum and individual polycyclic aromatic hydrocarbons. In: D.J. Hoffman, B.A. Rattner, G.A. Burton, J. Cairns (eds), *Handbook of Ecotoxicology*, pp.1-32. Lewis Publishers, New York.
- Barata, C., A. Calbet, E. Saiz, L. Ortiz and J.M. Bayona. – 2005. Predicting single and mixture toxicity of petrogenic polycyclic aromatic hydrocarbons to the copepod *Oithona davisae*. *Environ. Toxicol. Chem.*, 24: 2992-2999.
- Baumard, P., H. Budzinski, P. Garrigues, J.C. Sorbe, T. Burgeot and J. Belloco. – 1998. Concentrations of PAHs (Polycyclic aromatic hydrocarbons) in various marine organisms in relation to those in sediment and to trophic level. *Mar. Pollut. Bull.*, 36: 951-960.
- Bodin, N., T. Burgeot, J.Y. Stanisière, G. Bocquené, D. Menard, C. Minier, I. Boutet, A. Amat, Y. Chérel and H. Budzinski. – 2004. Seasonal variations of a battery of biomarkers and physiological indices for the mussel *Mytilus galloprovincialis* transplanted into the northwest Mediterranean Sea. *Comp. Biochem. Physiol.*, 138C: 411-427.
- Bradford, M. – 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.
- Cajaraville, M.P., J.A. Uranga and E. Angulo. – 1992. Comparative effects of the water accommodated fraction of three oil son mussels-3. Quantitative histochemistry of enzymes related to the detoxication metabolism. *Comp. Biochem. Physiol.*, 103C: 369-377.
- Canova, S., P. Degan, L.D. Peters, D.R. Livingstone, R. Voltan and P. Venier. – 1998. Tissue dose, DNA adducts, oxidative DNA damage and CYP1A-immunopositive proteins in mussels exposed to waterborne benzo(a)pyrene. *Mutation Res.*, 399: 17-30.
- Carlberg, I. and B. Mannervik. – 1985. Glutathione reductase. *Methods Enzymol.*, 113: 485-490.
- Cheung, C.C.C., W.H.L. Siu, B.J. Richardson, S.B. De Luca-Abbott and P.K.S. Lam. – 2004. Antioxidant responses to benzo(a)pyrene and Arochlor 1254 exposure in the green-lipped mussel, *Perna viridis*. *Environ. Pollut.*, 128: 393-403.
- D'Adamo, R., S. Pelosi, P. Trotta and G. Sansone. – 1997. Bioaccumulation and biomagnification of polycyclic aromatic hydrocarbons in aquatic organisms. *Mar. Chem.*, 56: 45-49.
- Durand, F., L.D. Peters and D.R. Livingstone. – 2002. Effect of intertidal compared to subtidal exposure on the uptake, loss and oxidative toxicity of water-born benzo(a)pyrene in the mantle and whole tissues of the mussel, *Mytilus edulis* L. *Mar. Environ. Res.*, 54: 271-274.
- Ellman, G.L., K.O. Courtney, V. Andriers Jr and R.M. Featherstone. – 1961. A new and rapid colorimetric determination of acetylcholinesterase activity. *Biochem. Pharmacol.*, 7: 88-95.
- Fitzpatrick, P.J., J. O'Halloran, D. Sheehan and A.R. Walsh. – 1997. Assessment of the glutathione S-transferase and related proteins in the gill and digestive gland of *Mytilus edulis* (L.) as potential organic pollution biomarkers. *Biomarkers*, 2: 51-56.
- Gowland, B., A. McIntosh, I. Davies, C. Moffat and L. Webster. – 2002. Implications from a field study regarding the relationship between polycyclic aromatic hydrocarbons and glutathione S-transferase activity in mussels. *Mar. Environ. Res.*, 54: 231-235.
- Hamountene, D., J.F. Payne, A. Rahimtula and K. Lee. – 2002. The use of the Comet assay to assess DNA damage in hemocytes and digestive gland cells of mussels and clams exposed to water contaminated with petroleum hydrocarbons. *Mar. Environ. Res.*, 54: 471-474.
- Krishnakumar, P.K., E. Casillas and U. Varanasi. – 1997. Cytochemical responses in the digestive tissue of *Mytilus edulis* complex exposed to microencapsulated PAHs or PCBs. *Comp. Biochem. Physiol.*, 118C: 11-18.
- Labarta, U., M.J. Fernández-Reiriz, J.L. Garrido, J.M.F. Babarro, J.M. Bayona and Albaigés, J. – 2005. Response of mussel recruits to pollution from the "Prestige" oil spill along the Galicia coast. A biochemical approach. *Mar. Ecol. Prog. Ser.*, 302: 135-145.
- Livingstone, D.R. – 1987. Seasonal responses to diesel oil and subsequent recovery of the cytochrome P-450 monooxygenase system in the common mussel, *Mytilus edulis* L., and the periwinkle, *Littorina littorea* L. *Sci. Total Environ.*, 65: 3-20.
- Livingstone, D.R. – 2001. Contaminant-stimulated reactive oxygen species production and oxidative damage in aquatic organisms. *Mar. Pollut. Bull.*, 42: 656-666.
- Marigómez, I., M. Soto, I. Cancio, A. Orbea, L. Garmendia and M.P. Cajaraville. – 2006. Cell and tissue biomarkers in mussel, and histopathology in hake and anchovy from Bay of Biscay alter the Prestige oil spill (Monitoring campaign 2003). *Mar. Pollut. Bull.*, 53: 287-304.
- Michel, X.R., P. Suteau, L.W. Robertson and J-F. Narbonne. – 1993. Effects of benzo(a)pyrene, 3,3',4,4'-tetrachlorobiphenyl and 2,2',4,4',5,5'-hexachlorobiphenyl on the xenobiotic-metabolizing enzymes in the mussel (*Mytilus galloprovincialis*). *Aquat. Toxicol.*, 27: 3535-344.
- Moreira, S.M., M. Moreira-Santos, R. Ribeiro and L. Guilhermino. – 2004. The "Coral Bulker" fuel oil spill on the North Coast of Portugal: Spatial and Temporal biomarker responses in *Mytilus galloprovincialis*. *Ecotoxicology*, 13: 619-630.
- Navas, J.M., M. Babín, S. Casado, C. Fernández and J.V. Tarazona. – 2006. The Prestige oil spill: A laboratory study about the toxicity of the water-soluble fraction of the fuel oil. *Mar. Environ. Res.*, 62: S352-S355.
- Okay, O.S., P. Donkin, L.D. Peters and D.R. Livingstone. – 2000. The role of algae (*Isochrysis galvana*) enrichment on the bioaccumulation of benzo(a)pyrene and its effects on the blue mussel *Mytilus edulis*. *Environ. Pollut.*, 110: 103-113.
- Pérez-Cadahía, B., B. Laffon, E. Páraso and J. Méndez. – 2004.

- Evaluation of PAH bioaccumulation and DNA damage in mussels (*Mytilus galloprovincialis*) exposed to spilled Prestige crude oil. *Comp. Biochem. Physiol.*, 138C: 453-460.
- Porte, C., X. Biosca, M. Solé and J. Albaigés. – 2000. The Aegean Sea oil spill on the Galician coast (NW Spain). III. The assessment of long-term sublethal effects on mussels. *Biomarkers*, 5: 436-446.
- Porte, C., X. Biosca, M. Solé and J. Albaigés. – 2001a. The integrated use of chemical analysis, cytochrome P450 and stress proteins in mussels to assess pollution along the Galician coast (NW Spain). *Environ. Pollut.*, 112: 261-268.
- Porte, C., M. Solé, V. Borghi, M. Martínez, J. Chamorro, A. Torreblanca, M. Ortiz, A. Orbea, M. Soto and M.P. Cajaraville. – 2001b. Chemical, biochemical and cellular responses in the digestive gland of the mussel *Mytilus galloprovincialis* from the Spanish Mediterranean Coast. *Biomarkers*, 6: 335-350.
- Seruto, C., Y. Sapozhnikova and D. Schlenk. – 2005. Evaluation of the relationship between biochemical endpoints of PAHs exposure and physiological endpoints of reproduction in male California Halibut (*Paralichthys californicus*) exposed to sediments from a natural oil seep. *Mar. Environ. Res.*, 60: 454-465.
- Singer, M.M., D. Aurand, G.E. Bragins, J.R. Clark, G.M. Coelho, M.L. Sowby and R.S. Tjeerdema. – 2000. Standardization of the Preparation and Quantification of water-accommodated fractions of petroleum for toxicity testing. *Mar. Pollut. Bull.*, 40: 1007-1016.
- Sokal, R.R. and F.J. Rohlf. – 1981. *Biometry*. Freeman and Co, San Francisco.
- Solé, M. and D.R. Livingstone. – 2005. Components of the cytochrome P450-dependent monooxygenase system and 'NADPH-independent benzo[a]pyrene hydroxylase' activity in a wide range of marine invertebrate species. *Comp. Biochem. Physiol.*, 141C: 20-31.
- Solé, M., C. Porte, X. Biosca, C.L. Mitchelmore, J.K. Chipman, D.R. Livingstone and J. Albaigés. – 1996. Effects of the "Aegean Sea" oil spill on biotransformation enzymes, oxidative stress and DNA-adducts in digestive gland of the mussel (*Mytilus edulis*L.). *Comp. Biochem. Physiol.*, 113C: 257-265.
- Scient. ed.: M. Gaspar.  
Received October 25, 2006. Accepted February 19, 2007.  
Published online May 24, 2007.