Ecotoxicology
DOI 10.1007/s10646-009-0450-y

Integrated assessment of water quality of the Costa da Morte (Galicia, NW Spain) by means of mussel chemical, biochemical and physiological parameters

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Accepted: 30 November 2009

Abstract The aim of this study was to assess environmental quality at some of the sites most severely affected by the *Prestige* oil spill off 2 years after the spillage (April and November 2004). For this purpose analyses of polycyclic aromatic hydrocarbons (PAH) and several biochemical (antioxidant enzymes catalase, glutathione reductase, glutathione peroxidase, superoxide dismutase and DT-diaphorase and lipid peroxidation) and physiological [scope for growth (SFG)] biomarkers were determined on wild mussel populations (*Mytilus galloprovincialis*) collected at four points along the Costa da Morte and compared with those of a reference site not affected by the oil spill. Results showed that PAH contents had markedly decreased 17 months after the accident, although they were higher in April than in November, when they showed values similar to background levels reported for this area. Nevertheless, the predominance of chrysene on PAH profiles, similarly to findings obtained immediately after the spill, indicated the *Prestige* as their main source. In spite of the low PAH levels recorded, antioxidant activity levels (explained through the integrated antioxidant response-IAR) were higher in the Costa da Morte than at the reference site either in April and November. In April IAR seems to be related to PAH levels found 3 months after the accident (February 2003), suggesting the persistence in the environment of oxidative stress-producing components from the spill. However, evidence of oxidative stress was not reflected at physiological level by scope for growth, with only very slight differences being observed between values from the reference site and those from Costa da Morte sites. In conclusion, although 2 years after the spill PAHs bioaccumulated by mussels from the Costa da Morte had decreased to background levels, biochemical parameters showed signals of oxidative stress in mussels from this area. However, SFG reflected a good health status for the mussel populations studied and did not reveal evidence of physiological disturbance either 17 or 24 months after the *Prestige* spill.

Keywords *Mytilus galloprovincialis* · PAHs · Antioxidant enzymes · Lipid peroxidation · Scope for growth · *Prestige* oil spill

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Introduction

Mussels are commonly used as bioindicators in the assessment of marine environmental quality throughout the world due to their wide geographical distribution, sessile character and capacity to pump large volumes of water and concentrate many contaminants in their tissues (Widdows and Donkin 1992). In this way, they are capable of resisting high levels of chemical contamination and of bioconcentrating xenobiotics to more than a thousand times background levels, which can facilitate chemical analysis. The usefulness of mussels as bioindicators is greatly increased when chemical analyses are integrated with data on the biological effects of pollutants (Bayne et al. 1988; Cajaraville et al. 2000; Devier et al. 2005). In this context, both laboratory and field studies have demonstrated that different biochemical, cellular and physiological indexes may be useful in marine organisms as biomarkers of

Published online: 22 December 2009

exposure or damage due to chemical pollution (Lam and Gray 2003).

Oxidative stress is a common pathway of toxicity induced by several classes of pollutants (Winston and Di Giulio 1991) by which production of reactive oxygen species (ROS) is enhanced. ROS can be highly toxic to aquatic organisms as they often produce cellular damage such as lipid peroxidation (LPO) in membranes, altered pyridine nucleotide redox status and DNA damage (Lemaire and Livingstone 1993). Protection against the toxicity of oxyradicals towards cellular targets is afforded by a complex defence system consisting of both low-molecular-weight scavengers and antioxidant enzymes. Enzymatic activities for the detoxification of ROS and the degree of LPO have been proposed as biomarkers of oxidative stress in mussels exposed to different types of pollutants (Viarengo et al. 1991; Solé et al. 1996; Porte et al. 2000a; Manduzio et al. 2004; Tsangaris et al. 2007).

A further biological index whose use in integrated monitoring studies is currently being considered is scope for growth (SFG), this being a measure of growth potential, a biological process that is highly sensitive to pollutants. The estimation of energy available for growth (somatic and gonadal) from the physiological determination of energy acquisition and consumption has been used in studies evaluating the quality of coastal marine ecosystems (Widdows et al. 1995, 1997, 2002; Cotou et al. 2002; Halldörsson et al. 2005). These studies have demonstrated the correlation between SFG and the accumulation of pollutants in tissues, and the study by Crowe et al. (2004) has also shown the existence of a correlation between SFG values and biodiversity indices in benthic communities, leading us to consider that SFG is a good indicator of pollution not only in individuals but also in populations (Widdows and Staff 2006).

After the accident involving the tanker *Prestige* in November 2002, that washed up the coastline of Galicia (NW Spain), numerous studies were carried out to determine the accumulation and distribution of polycyclic aromatic hydrocarbons (PAHs) in water, sediments and biota (Carro et al. 2006; Franco et al. 2006; González et al. 2006; Viñas et al. 2009) as well as along the north-west Portuguese coast (Ferreira et al. 2003). In most of these studies mussels were used as bioindicators both to determine the change over time and distribution of accumulated hydrocarbon levels (Nieto et al. 2006; Soriano et al. 2006) and to study their biological effects (Cajaraville et al. 2006; Marigómez et al. 2006; Orbea and Cajaraville 2006; Orbea et al. 2006). However, there have been fewer integrated studies involving chemical appraisals and biological determinations performed on the same sample of mussels. The data presented in this manuscript are the first to integrate physiological indicators such as SFG with biochemical indicators such as antioxidant enzyme and LPO levels as well as chemical data on the bioaccumulation of PAHs in wild mussels from the Galician coast affected by the *Prestige* oil spill.

One of the problems presented by combined studies that assess environmental quality on the basis of both chemical and biological criteria is precisely the integration of the results obtained in order to provide a correct and faithful interpretation of the results. In this regard Beliaeff and Burgeot (2002) have defined the Integrated Biomarker Response (IBR) index, which uses star plots to display the responses obtained from a battery of biomarkers. More recently, Dagnino et al. (2007) describe a system for integrating biomarker results in mussels which called "Expert System", based on the responses of different biomarkers in control and polluted mussels. The present study uses the index defined by Beliaeff and Burgeot that we have called the Integrated Antioxidant Response (IAR) to integrate the responses given by the different enzymatic antioxidant activities.

The purpose of this study was to assess the environmental quality of a stretch of the Galician coast, the Costa da Morte, through the use of chemical (PAH accumulation), biochemical (antioxidant enzymes and LPO) and physiological (SFG) determinations on the same sample of wild mussels. The selected biomarkers are indicators of both exposure (antioxidant activities catalase-CAT, glutathione reductase-GR, NAD(P)H DT-diaphorase-DTD, glutathione peroxidase-GPX and superoxide dismutase-SOD) and effects (LPO and SFG). The area studied corresponds to the part of the Galician coast that was most severely affected by the oil spill from the tanker *Prestige*, sampling being performed more than a year after the disaster and at two clearly differentiated times of year: April and November.

Materials and methods

Sampling

Sampling was carried out in April and November 2004, i.e. 17 and 24 months after the *Prestige* oil spill. Mussels (*Mytilus galloprovincialis*) were collected (Fig. 1) at five sites along the Galician coast from which four of them (Corrubedo, Punta Insua, Muxía and Caión) were highly impacted by the spill, whilst the fifth (Santa María de Oia) was not affected by the spillage and was used as a clean reference. These sites also coincide with those used in the IEO's Marine Pollution Monitoring Program, which means that data on PAH accumulation in wild mussels is available from before the spill.

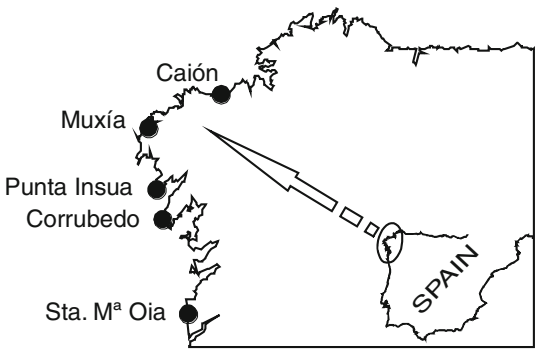

Fig. 1 Map of the Galician coast (Spain) showing the location of the five sampling sites studied

For PAH determinations 50 mussels of a standard size (4 cm shell length) were pooled as a composite sample representative of each location and sampling period. Mussels were stored at −20°C until analysis. Once the samples were defrosted, mussel meat was homogenised and freeze-dried. For biochemical analysis, 35 mussels of the same size were collected at each sampling site. Gills were immediately removed and pooled in 7 samples, which were stored in liquid nitrogen prior to analysis. For SFG, at least 20 live mussels of the same size were packed in insulated boxes and transported cool and air-exposed via express delivery to the laboratory. Physiological determinations were individually carried out on 10 individuals.

PAH content in mussel tissues

Two standard solutions containing the 13 EPA PAHs analysed in this study (10 ng/µl in acetonitrile) were purchased from Dr. Ehrenstorfer (Augsburg, Germany). LiChrosolv grade methanol was obtained from Merck (Darmstadt, Germany). HPLC grade hexane, acetonitrile and acetone were obtained from Lab-Scan (Dublin, Ireland). MP Alumina B-Super I for column chromatography was purchased from MP EcoChrom (Eschwege, Germany).

Freeze-dried mussel tissues from each sample were Soxhlet extracted with a 1:3 acetone:hexane mixture for 8 h and analysed by high performance liquid chromatography (HPLC) as described elsewhere (Soriano et al. 2006). In brief, samples to be analysed by HPLC were submitted to a clean-up step by column chromatography on deactivated alumina (10% water) and hexane elution. 13 PAHs (phenanthrene, anthracene, fluoranthene, pyrene, benz[*a*]anthracene, chrysene, benzo[*e*]pyrene, benzo[*b*]fluoranthene, benzo[*k*]fluoranthene, benzo[*a*]pyrene, benzo[*ghi*]perylene, dibenz[*ah*]anthracene and indeno[*1,2,3-cd*]pyrene) were determined by HPLC (HP 1100 apparatus, Agilent Technologies, Palo Alto, CA, USA) coupled with a wavelength programmable fluorescence detector (HP 1036, Agilent Technologies, Palo Alto, CA, USA), using a Vydac 201 TP column (Grace Vydac, Hesperia, CA, USA), eluted with a methanol:water gradient.

A multilevel calibration curve produced from the certified solutions supplied by Dr. Ehrenstorfer was used for quantification. The analytical method was subjected to external quality control process, by participation in intercalibration exercises organised by Quality Assurance of Information for Marine Environmental Monitoring in Europe (QUASIMEME II 2003, 2004).

Biochemical parameters

Gills were homogenised in K-phosphate buffer 100 mM, pH 7.6 containing 0.15 M KCl, 1 mM DTT, 1 mM EDTA and 3 µg/ml leupeptine. Cytosolic fractions were obtained after centrifugation at $600g\times$ 15 min, $13.000g\times$ 20 min and $100.000g\times$ 60 min, while microsomal fractions were obtained by resuspension of the resulting pellet in 50 mM Tris–HCl pH 7.6 containing 20% (w/v) glycerol, 1 mM DTT and 1 mM EDTA. The entire procedure was conducted at 4°C. All enzyme determinations were carried out on cytosolic fractions at 25°C and expressed by protein content determined following the method described by Lowry et al. (1951).

Catalase (CAT) activity was measured according to Claiborne (1985) by the decrease in absorbance at 240 nm by H_2O_2 consumption ($\varepsilon = -0.04$ mM^{-1} cm^{-1}) and expressed as µmol of H_2O_2 degraded min^{-1} mg^{-1} protein. Glutathione reductase (GR) activity was measured according to Ramos-Martínez et al. (1983) by following the decrease in absorbance at 340 mn ($\varepsilon = -6.22$ mM^{-1} cm^{-1}) due to the oxidation of NADPH and expressed as nmol of NADPH oxidised min^{-1} mg^{-1} protein. NAD(P)H DT-diaphorase (DTD) activity was measured as the dicumarol-inhibitable portion of NAD(P)H dependent DCPIP reductase activity (Benson et al. 1980). Reduction of DCPIP was monitored by measuring the decrease in absorbance at 600 nm ($\varepsilon = 21$ cm^{-1} mM^{-1}) and was expressed as nmol DCPIP reduced min^{-1} mg^{-1} protein. Glutathione peroxidase Se-dependent (GPX) activity was measured monitoring the formation of GSSG in a coupled enzyme system with glutathione reductase (Livingstone et al. 1992) at 340 nm ($\varepsilon = -6.22$ mM^{-1} cm^{-1}) and expressed as nmol of NADPH oxidised min^{-1} mg^{-1} protein. Superoxide dismutase (SOD) activity was determined by measuring the reduction of cytochrome c by O_2^- generated by the xanthine oxidase/hypoxanthine system at 550 nm (McCord and Fridovich 1969). Activity was expressed as Units min^{-1} mg^{-1} protein, with one unit of SOD being defined as the amount of enzyme that inhibits the reduction of cytochrome c by 50%.

Lipid peroxidation (LPO) was quantified on microsomal fractions of gills as thiobarbituric acid reactive substances (TBARS) following Buege and Aust (1978). The malondialdehyde (MDA) formed was estimated at 535 nm using a standard of malonaldehyde bis-(dimethylacetal) and lipid peroxidation was expressed as nmol of MDA formed mg^{-1} protein.

Physiological parameters

The mussels were acclimatised after transport for 24 h in tanks under controlled temperature and feeding conditions (15°C and the microalgae *Isochrysis galbana*, clone T-ISO as food). Physiological measurements were taken the day after the mussels arrived at the laboratory, to which end they were kept individually under standardised conditions (filtered 35 psu SW, 15°C, and a standard algal ration of *I. galbana*, 1 mg organic matter AFDW l^{-1}) (Widdows et al. 2002) in a flow-through system.

Clearance rates (CR) were calculated from the difference in food concentrations at the inflow and outflow points of the flow-through system. The flow rate was adjusted in order to maintain a difference between inflow and outflow concentrations of less than 40% of the inflow. Ingestion rates (IR) were obtained from the clearance and food concentration rates, expressed as units of organic matter per litre. This was done by filtering inflow samples through previously rinsed, ashed and weighed Whatman GF/C filters. The filters were then rinsed with a 0.5 M ammonium formate solution, dried for 24 h at 100°C and ashed at 450°C for 1 h. The difference between dry weight and ashed weight was taken as organic weight.

Absorption rates (AR) were obtained by multiplying the IRs by the absorption efficiency (AE), the latter being calculated according to the method described by Conover (1966) from the organic content of faeces and food.

The energy consumption rates, namely the excretion rate (ER) and the respiration rate (RR), were determined in sealed glass respirometers each containing a single mussel with filtered sea water at 15°C. Oxygen concentrations were measured using a YSI 52 DO instrument connected to a YSI 5905 self-stirring BOD probe. Ammonia concentrations were measured using the indophenol blue method as described by Solorzano (1969).

Physiological rates were standardised for a specimen of 1 g flesh dry weight using the allometric exponent $b = 0.67$, which relates the variation in physiological rates to animal size (weight) (Bayne and Newell 1983). Physiological rates were converted to energy equivalents (J g^{-1} h^{-1}) in order to calculate their energy balance, using the energy equivalents referred to in Widdows and Johnson (1988). SFG was calculated by means of the expression: $I = F + E + R + P$ thence $P(\mathrm{SFG}) = (I - F) - E - R = (I \times AE)$, where I is the energy ingested, F the energy lost in faeces, AE the absorption efficiency, R the energy consumed in respiration, E the energy eliminated during excretion and P the energy available for somatic and gonadal growth (SFG).

Statistical analysis

The variation of each biomarker was tested by one-way analysis of variance (ANOVA). When ANOVA was significant ($P < 0.05$), post hoc pair-wise comparisons between locations were made using the SNK test to determine which values differed significantly ($P < 0.05$). The homogeneity of variances was checked by means of the Levene test. The antioxidant responses offered by the enzymatic activities (CAT, GR, GPX, DTD and SOD) evaluated were integrated in the IAR (Integrated Antioxidant Response), based on the IBR (Integrated Biomarker Response) described in Beliaeff and Burgeot (2002). We used this index in order to determine the antioxidant response and/or the oxidative stress to which each site was subjected. Previously, Pearson correlation analysis, based upon the mean for each variable at each sampling site, was performed to determine the strength of the relationship between antioxidant activities in order to check the existence of cooperative enzyme behaviour which allowed us to use the IAR index.

Results

PAH contents in mussel tissues

Table 1 shows total PAH levels (sum of 13 parent PAHs) measured on whole soft tissues of mussels collected from the sampling sites in April and November 2004. It also contains the PAH levels observed before (October 2000)

Table 1 Total concentration of 13 parent PAHs (μg kg^{-1} dry weight) on whole soft tissues of wild mussels from the Galician coast at the indicated dates

Sites	Oct-00[a]	Feb-03[a]	Jun-03[a]	Nov-03[a]	Apr-04	Nov-04
Sta M[a] Oia	59	69	17	29	25	21
Corrubedo	101	7,782	1,455	430	179	48
Punta Insua	54	4,131	456	342	100	60
Muxía	63	3,548	82	214	119	108
Caion	86	3,059	692	625	255	117

Sum of phenanthrene, anthracene, fluoranthene, pyrene, benz*[a]*anthracene, chrysene, benzo*[b]*fluoranthene, benzo*[k]*fluoranthene, benzo*[e]*pyrene, benzo*[a]*pyrene, benzo*[ghi]*perylene, dibenz*[ah]*anthracene, indeno[1,2,3-cd]pyrene

[a] Data obtained from Table 1 of Soriano et al. (2006)

and after (February, June and November 2003) the *Prestige* oil spill, obtained from Soriano et al. (2006). After 4 months the accident (February 2003) the highest PAH levels were found in the area selected for this study, between Corrubedo and Caión (from 3,000 to 7,780 μg kg^{-1} dry weight). Although PAH levels had markedly decreased 17 months after the accident, in April 2004, they were still somewhat higher than before the accident, with Caión and Corrubedo showing the highest PAH content (255 and 179 μg kg^{-1} dw). Furthermore, PAH concentrations at the four Costa da Morte sampling sites continued to decrease from April to November 2004, when they showed very similar levels to those observed before the oil spill occurred. Mussels from Santa María de Oia were unaffected by the spill, there being no substantial difference in their PAH content as measured before and after the accident; the values were also the lowest of those recorded for all the sampling sites and dates.

Profiles of the distribution of the 13 PAHs at the sampling sites in April and November, including the data obtained before and after the accident, are shown in Fig. 2. In April, mussels collected from the Costa da Morte showed similar PAH profiles to those obtained immediately after the spill occurred, with a clear predominance of chrysene, whereas there had been no predominance of any single PAH at the same sites prior to the event. In November, PAH profiles were still dominated by chrysene at Corrubedo, Punta Insua and Caión and by chrysene and

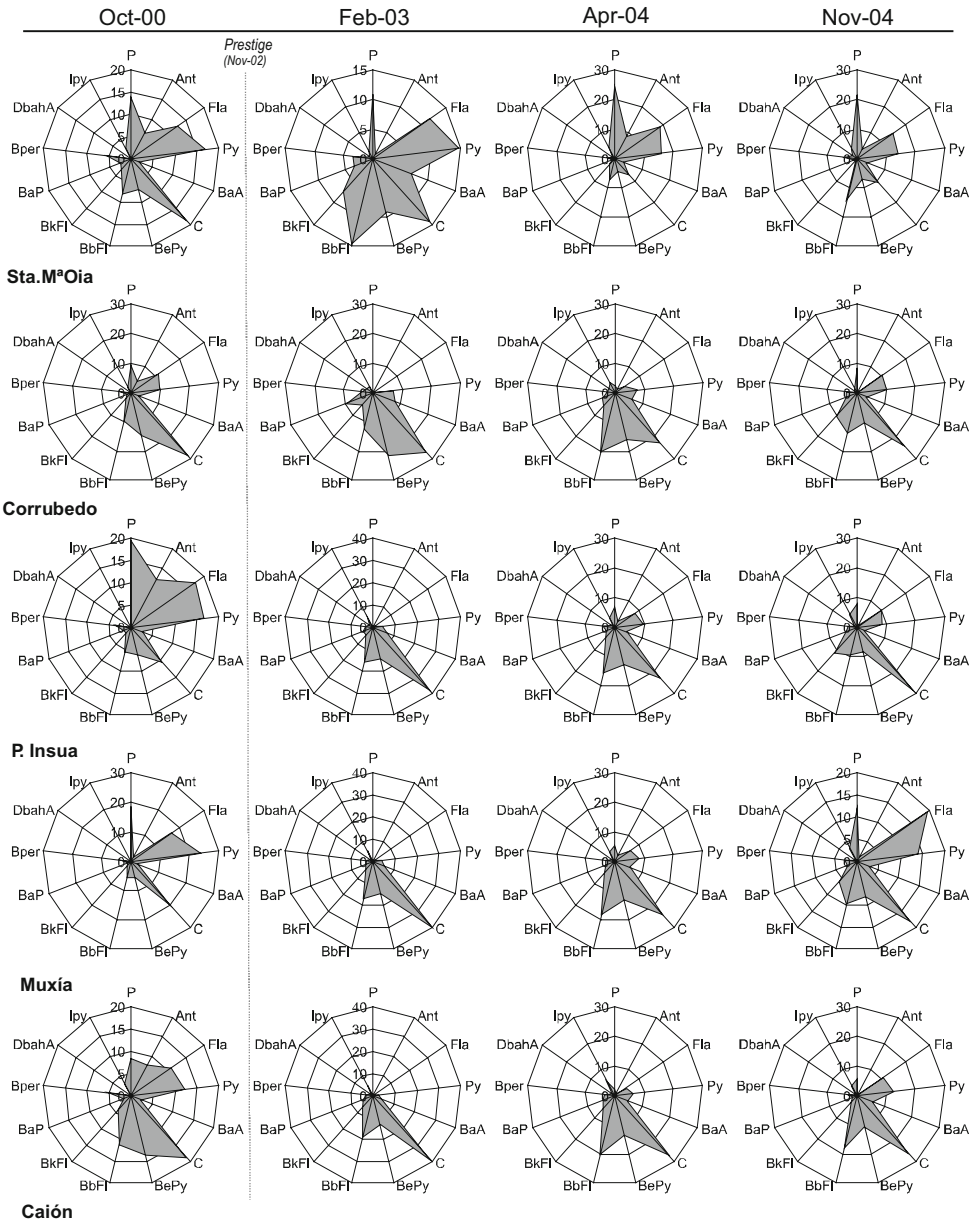

Fig. 2 Radial representation of the percentages of 13 parent PAH distribution (P: phenanthrene, Ant: anthracene, Fla: fluoranthene, Py: pyrene, BaA: benz[*a*]anthracene,C: chrysene, BePy: benzo[*e*]pyrene, BbFl: benzo[*b*]fluoranthene, BkFl: benzo[*k*]fluoranthene, BaP: benzo[*a*]pyrene, Bper: benzo[*ghi*]perylene, DbahA: dibenz[*ah*]anthracene, Ipy: indeno[*1,2,3-cd*]pyrene) in mussels from the study sites before the accident (October 2000), 3 months after the accident (February 2003) [obtained from Soriano et al. (2006)] and in April and November 2004

fluoranthene at Muxía. At Santa María de Oia, PAH profiles were similar in April and November, with a predominance of phenanthrene, fluoranthene and pyrene. PAH profiles at this site were therefore clearly different from those found at the most severely affected sites.

To sum up, a year after the disaster PAH levels obtained from the area hardest hit by the oil spill, namely the Costa da Morte, had decreased dramatically, and they continued to fall until 2 years after the oil reached the coast, by which time they had returned to values similar to those observed prior to the spill. Nevertheless, even though the levels of PAHs were low, the profile of their distribution in 2004 samples was similar to that obtained immediately following the spill.

Biochemical parameters

Results of the statistical analysis (ANOVA) carried out on both the biochemical and the physiological parameters are shown in Table 2. Levels of antioxidant enzyme activities and the IAR obtained in April and November are shown in Table 3.

In April the mean values of the IAR index ranged from 0.7 to 12.6 in the cases of Santa María de Oia and Corrubedo, respectively. At Corrubedo and Muxía the IAR values were significantly higher than the values of the reference station ($P < 0.05$). These SNK test results of the IAR values seem to be related to the mussel PAH contents observed just after the accident in February 2003 (Table 1) in the sense that higher IAR data were obtained at the same stations where higher PAHs levels were observed. From the five antioxidant activities measured that constitutes the IAR, this is particularly true in the case of the GR and DTD activities. In this way, the four sampling sites along the Costa da Morte showed the highest levels of GR activity and the SNK test shows a similar profile between these activities and the PAHs contents after the spill. Similarly, DTD enzymatic activity was significantly higher at Corrubedo and Muxía than at the reference station, and CAT and GPX activities were also higher at the former sites than at the latter, although without reaching the threshold of statistical significance ($P > 0.05$) Furthermore, at Corrubedo (the highest mussel PAH content in 2003) all the enzymatic antioxidant activities determined (CAT, GR, DTD, GPX, SOD) were significantly higher than those found at the reference site. The CAT SNK test results, on the contrary as described for the other activities, seem to be related to 2004 mussel PAH contents when the highest PAH levels found in Corrubedo and Caión (Table 1) are corresponding with higher CAT activities.

In November the pattern shown by the IAR index was noticeably different, since only the value obtained at Caión (11.5) was significantly higher than that for Santa María de Oia (3.0); although the IAR index at Muxía (5.1) was much higher than that of the reference station, this difference was not statistically significant. At Caión all the antioxidant activities evaluated were higher than at the reference site, although only CAT and DTD were significantly higher ($P < 0.05$). Similarly, at Muxía activity levels of the antioxidant enzymes CAT, GR, DTD and SOD were higher than at Santa María de Oia, although this difference was only statistically significant in the case of DTD. In contrast to April results, in this survey seems to be a relation between antioxidant activities, mainly DTD, and the PAH accumulation registered in 2004.

Some enzymes displayed a seasonal pattern, this being the case of CAT, GR and, more clearly, SOD, whose activities were significantly higher ($P < 0.05$) in April (spring) than in November (autumn).

Positive correlations were found between the different antioxidant responses evaluated (Table 4). Thus, in April DTD was significantly correlated to GPX ($r = 0.960$, $P = 0.009$), and DTD was also closely correlated to GR and SOD ($r_{GR} = 0.857$, $P = 0.064$; $r_{SOD} = 0.828$, $P = 0.084$), and SOD to GPX ($r = 0.867$, $P = 0.057$). In November, SOD was significantly correlated to CAT ($r_{CAT} = 0.961$, $P = 0.009$), with the degree of its correlation to GR and DTD falling just short of statistical significance ($r_{GR} = 0.816$, $P = 0.092$; $r_{DTD} = 0.810$, $P = 0.097$), whilst CAT was correlated to GR ($r = 0.872$) with a significance level of 0.054.

LPO values in the gills of the organisms sampled in April (Table 5) showed no significant differences between any of the various sampling sites. On the other hand, in November LPO values at Punta Insua and Muxía were significantly higher than those at the reference site.

Physiological biomarkers

Results of physiological components of the energy balance of mussels in April and November are presented in Table 6 and Fig. 3. On each of the two occasions the SFG value was higher at the reference site, Santa María de Oia. In April this difference was significant when compared to the other sites ($P < 0.05$). In spite of this level of significance, the variation in SFG was little more than 14% of the value obtained at the reference site. In November, however, the SFG value for the reference site was similar ($P > 0.05$) to that obtained at any of the sampling sites located along the Costa da Morte (Table 6).

This difference in SFG values between sampling sites depends to a great extent on the variation in ingestion rate, and thus in absorption rate. The results of the SNK test performed on SFG values coincide with those obtained for the ingestion rate. On the other hand, the variation in metabolic rates between the different sampling sites is in

Table 2 ANOVA table for biochemical and physiological variables measured

	April					November				
	SS	*df*	MS	*F*	*P*	SS	*df*	MS	*F*	*P*
CAT										
BG	807	4	201.7	1.39	0.262	1,518	4	379.4	7.86	0.000*
WG	4,358	30	145.3			1,447	30	48.2		
Total	5,165	34				2,965	34			
GR										
BG	3,041	4	760.3	5.89	0.001*	1,497	4	374.2	3.14	0.028*
WG	3,872	30	129.1			3,570	30	119.0		
Total	6,914	34				5,067	34			
DTD										
BG	461	4	115.3	6.81	0.001*	478	4	119.5	7.30	0.001*
WG	508	30	16.9			377	23	16.4		
Total	970	34				854	27			
GPX										
BG	162	4	40.5	2.59	0.057	55	4	13.9	1.20	0.332
WG	469	30	15.6			348	30	11.6		
Total	632	34				403	34			
SOD										
BG	3,862	4	965.5	8.53	0.000*	361	4	90.3	4.56	0.005*
WG	3,396	30	113.2			594	30	19.8		
Total	7,258	34				955	34			
IAR										
BG	669	4	167	31.47	0.000*	493	4	123	20.44	0.000*
WG	159	30	5			181	30	6		
Total	828	34				673	34			
LPO										
BG	1	4	0.3	2.24	0.089	5	4	1.4	6.77	0.001*
WG	4	30	0.1			6	30	0.2		
Total	6	34				11	34			
IR										
BG	9,697	4	2424.2	11.17	0.000*	4,301	4	1075.3	2.78	0.038*
WG	9,766	45	217.0			17,400	45	386.7		
Total	19,462	49				21,701	49			
AR										
BG	4,592	4	1148.1	5.81	0.001*	7,043	4	1760.8	6.26	0.000*
WG	8,887	45	197.5			12,654	45	281.2		
Total	13,479	49				19,697	49			
ER										
BG	2	4	0.5	12.29	0.000*	1	4	0.2	7.64	0.000*
WG	2	45	0.0			1	45	0.0		
Total	4	49				2	49			
RR										
BG	61	4	15.4	17.01	0.000*	6	4	1.6	3.32	0.018*
WG	41	45	0.9			21	45	0.5		
Total	102	49				27	49			
SFG										
BG	2,051	4	512.7	4.07	0.007*	1,894	4	473.5	4.29	0.005*
WG	5,671	45	126.0			4,968	45	110.4		
Total	7,722	49				6,862	49			

BG between groups, *WG* whithin groups, *SS* sum of square, *DF* degrees of freedom, *MS* mean square, *F F* value; *P* significance level. * Significant values ($P < 0.05$)

Table 3 Mean values (±standard error) of antioxidant enzymes catalase (CAT), glutathione reductase (GR), DT-diaphorase (DTD), glutathione peroxidase (GPX) and superoxide dismutase (SOD) and integrated antioxidant response (IAR) in gills of wild mussels from the Galician coast

Sites	CAT[1]		GR[2]		DTD[2]		GPX[2]		SOD[3]		IAR	
	April	November	April	November	April	November	April	November	April	November	April	November
Sta Mª Oia	49.7 ± 1.5^{a}	40.9 ± 1.7^{b}	30.6 ± 2.1^{a}	37.9 ± 3.1^{bc}	10.7 ± 1.8^{a}	11.1 ± 1.9^{a}	11.3 ± 0.6^{a}	12.4 ± 1.0^{a}	40.4 ± 3.9^{a}	16.4 ± 2.2^{ab}	0.7 ± 0.3^{a}	3.0 ± 0.9^{ab}
Corrubedo	63.8 ± 5.8^{b}	34.9 ± 1.6^{a}	61.9 ± 5.0^{c}	28.5 ± 0.9^{a}	21.0 ± 2.0^{c}	13.9 ± 1.5^{a}	17.9 ± 1.2^{b}	13.8 ± 1.6^{a}	61.4 ± 5.1^{b}	13.9 ± 1.3^{a}	12.6 ± 1.3^{c}	2.0 ± 0.9^{a}
Punta Insua	50.6 ± 2.0^{a}	39.5 ± 1.5^{ab}	50.2 ± 3.8^{b}	31.4 ± 2.3^{ab}	12.4 ± 1.5^{a}	11.2 ± 0.7^{a}	11.5 ± 0.7^{a}	10.4 ± 0.8^{a}	32.1 ± 2.5^{a}	11.8 ± 0.7^{a}	1.9 ± 0.7^{ab}	1.0 ± 0.5^{a}
Muxía	56.1 ± 4.3^{ab}	43.0 ± 2.5^{b}	42.9 ± 3.6^{b}	38.9 ± 2.9^{c}	16.5 ± 0.9^{b}	18.9 ± 0.9^{b}	15.1 ± 1.7^{ab}	11.6 ± 1.4^{a}	39.5 ± 3.9^{a}	16.7 ± 1.7^{ab}	3.9 ± 1.0^{b}	5.1 ± 1.1^{b}
Caión	63.6 ± 5.0^{b}	52.0 ± 1.8^{c}	42.3 ± 4.1^{b}	43.4 ± 2.9^{c}	13.2 ± 0.8^{ab}	21.6 ± 1.4^{b}	11.8 ± 2.1^{a}	13.5 ± 1.4^{a}	29.8 ± 2.6^{a}	21.4 ± 2.0^{b}	1.6 ± 0.7^{ab}	11.5 ± 1.1^{c}

[1] μmol min^{-1} mg $prot^{-1}$; [2] nmol min^{-1} mg $prot^{-1}$; [3] U min^{-1} mg $prot^{-1}$

Data with the same superscript indicated that they did not differ significantly at the 95% level (SNK test done after a significant ANOVA test was performed at each site)

no way related to the variation in SFG. Furthermore, the physiological rate that from a quantitative point of view has the greatest influence on calculating SFG values is the ingestion rate. In terms of total energy acquired (Fig. 3), the greatest proportion is used for growth, SFG, with slight differences depending on the time of year.

In spring, this fraction accounts for almost 70% of the ingested energy, but is somewhat less in autumn, when it falls to 62%. The energy fraction used in respiration is 7% in spring and 5% in autumn. Energy losses due to faeces are higher in autumn, at 32%, as opposed to 23% in spring. There is therefore a clear seasonal variation in physiological rates, regardless of whether they concern energy acquisition (CR, IR and AR) or energy loss (ER and RR), with all of them showing significantly higher values in spring than in autumn ($P < 0.05$). Consequently, SFG values obtained by integrating such physiological parameters also depend on the time of year, and were approximately 40% higher in April than those for November ($P < 0.05$) in all sampling sites, with the exception of Punta Insua, where the SFG values for both times of year were similar ($P > 0.05$), this being due to the fact that the clearance rates, and therefore the ingestion and absorption rates, were also similar. However, the metabolic rates (ER and RR) of the mussels gathered at this site (Punta Insua) follow the same seasonal pattern as the rest, i.e. with significantly higher values being obtained in April.

Discussion

Of the sites along the Galician coast selected for this study, only Santa María de Oia, located just north of Portugal, was not affected by the *Prestige* oil spill. The remaining four sites, located along the Costa da Morte, were severely affected by the spill, with a PAH content in mussel tissue of 7,780 μg kg^{-1} dw at Corrubedo and of between 3,000 and 4,000 μg kg^{-1} dw at the other three sites (Soriano et al. 2006). PAH levels in mussel tissue showed a considerable decrease 17 months after the spill occurred, in April 2004 (at Corrubedo, for example, the concentration of PAHs fell from 7,780 to 179 μg kg^{-1} dw), although these levels were still slightly higher than those obtained in November of the same year. At that time, precisely 2 years after the oil spill occurred, PAH levels (40–120 μg kg^{-1} dw) were similar to those recorded in other areas of Galicia situated far from possible anthropogenic influences (Viñas 2002). This recovery time is similar to that observed after the accident involving the oil tanker *Aegean Sea*, when it was concluded that 1 year after the spill PAH values in mussels at the most severely affected areas had returned to background levels (Porte et al. 2000b). On the contrary, after the *Exxon Valdez*

Table 4 Pearson correlation analysis between antioxidant activities evaluated in mussels collected from Costa da Morte after 2 years of the *Prestige* oil spill (April and November 2004)

	April				November			
	CAT	GR	DTD	GPX	CAT	GR	DTD	GPX
GR	0.276 (0.653)				0.872* (0.054)			
DTD	0.368 (0.542)	0.857* (0.064)			0.756 (0.139)	0.496 (0.395)		
GPX	0.233 (0.706)	0.701 (0.187)	0.960*** (0.009)		0.425 (0.476)	0.059 (0.925)	0.389 (0.518)	
SOD	−0.004 (0.995)	0.650 (0.235)	0.828* (0.084)	0.867* (0.057)	0.961*** (0.009)	0.816* (0.092)	0.810* (0.097)	0.550 (0.337)

Correlation coefficients are significant at the *0.1, **0.05 or ***0.01 (significance level shown between brackets). $n = 5$

Table 5 Mean values (±standard error) of lipid peroxidation (LPO) (nmol min^{-1} mg $prot^{-1}$) in gills of wild mussels from the Galician coast

Sites	LPO	
	April	November
Sta M[a] Oia	1.5 ± 0.1[a]	1.8 ± 0.1[a]
Corrubedo	1.4 ± 0.1[a]	1.7 ± 0.2[a]
Punta Insua	1.9 ± 0.2[a]	2.5 ± 0.2[b]
Muxía	1.6 ± 0.1[a]	2.4 ± 0.2[b]
Caion	1.4 ± 0.1[a]	1.4 ± 0.1[a]

Data with the same superscript did not differ significantly (SNK test, ANOVA *F*-test)

disaster, Carls et al. (2001) described that out of a total of 23 sampling stations for mussel (*Mytilus trossulus)*, only 9 had returned to pre-spill PAH levels, whilst in another 10 PAH levels 3 years after the spill were significantly higher than those considered to be background values, rising as high as 8,100 μg kg^{-1} dw at some sites. The extremely low water temperatures may slow down the PAH degradation and dissolution processes, thereby increasing the temporal persistence of these pollutants remained in the marine environment. On the other hand, 3 years after the *Erika* disaster, PAH levels in mussels from the areas most severely affected by the oil spill had decreased considerably (Tronczynski et al. 2004), although C-PAH levels were still higher than before the spill, indicating that the purification process was not yet complete. The ability to return to background levels differs from one disaster to another since not only the characteristics of the spill but also the environmental and geographical conditions of the affected areas will condition the rate at which PAHs are eliminated from mussel tissue. Other studies performed on the Galician coast after the *Prestige* disaster (Nieto et al. 2006) indicate that the process of returning to prior PAH levels in mussels is a rapid one, taking about 1 year, a finding which coincides with the data from the present study and can be related to the high dynamics of the Galician coast. It should be pointed out that the time when the final samples were taken in this study, November 2004, corresponds to a favourable time of year for the accumulation of PAHs, due to the resuspension of sediments that may still contain hydrocarbons and the high lipid content of mussel tissues as a result of the process of gametogenesis. In spite of these factors, the levels found at this time can be considered to be background levels (Viñas 2002), and are also appreciably lower than those described as background levels at other points along the European coastline (Granby and Spliid 1995; Baumard et al. 1998, 1999).

Before the spill, PAH profiles at all the sites studied, with the exception of Corrubedo, contained the whole set of 3–6 ring PAHs, indicating that these sites were unaffected by oil pollution at the time. However, at Corrubedo, which showed relatively high PAH levels before the spill occurred (101 μg kg^{-1} dw), there was a predominance of chrysene, possibly indicating a previous petrogenic input at this site prior to the *Prestige* disaster. After the spill occurred (February 2003), Caión, Muxía, Punta Insua and to a lesser extent Corrubedo showed almost identical profiles with chrysene as the predominant PAH compound, together with high percentages of benzo[*b*]fluoranthene and benzo[*e*]pyrene. This indicates that the oil from the *Prestige* was the single source of this pollution, as other studies using mussels from the same area (Soriano et al. 2006, Nieto et al. 2006) or organisms from the NW coast of Portugal (Ferreira et al. 2003) have also concluded.

It is well known that the exposure of molluscs to different marine pollutants leads to an increased accumulation of reactive oxygen species (ROS) and that antioxidant defences are required to prevent oxidative stress and cellular damage (Viarengo et al. 1990; Winston and Di Giulio 1991; Manduzio et al. 2005). Antioxidant enzyme levels have been used in various studies as a biomarker for the oxidative stress produced in marine bivalves by hydrocarbons (Solé et al. 1995; Porte et al. 2001a) and other pollutants (Regoli and Principato 1995; Regoli 1998; Cheung et al. 2002; Lionetto et al. 2003; Nesto et al. 2004; Petrovic et al. 2004; Torres et al. 2002). Studies by Solé et al. (1996), Albaigés et al. (2000) and Porte et al. (2000a), carried out on mussels affected by the oil spill from the

Table 6 Mean values (±standard error,) of physiological components of the energy balance of wild mussels under standardized laboratory conditions (15°C, filtered seawater at 35 ppm, 1 mg l^{-1} of algal cells)

Sites	CR[1]		IR[2]		AR[2]		ER[2]		RR[2]		SFG[2]	
	April	November	April	November	April	November	April	November	April	November	April	November
Sta Mª Oia	5.8 ± 0.2^{a}	4.6 ± 0.1^{ab}	133.2 ± 5.0^{a}	106.5 ± 3.4^{ab}	102.6 ± 3.9^{a}	72.4 ± 2.3^{ab}	1.6 ± 0.08^{a}	0.6 ± 0.06^{a}	8.8 ± 0.4^{bc}	5.5 ± 0.2^{a}	92.2 ± 3.8^{a}	66.3 ± 2.3^{ab}
Corrubedo	4.9 ± 0.2^{b}	3.8 ± 0.2^{c}	113.8 ± 5.0^{b}	86.6 ± 3.8^{c}	87.7 ± 3.9^{b}	58.9 ± 2.6^{c}	1.2 ± 0.05^{b}	0.5 ± 0.05^{b}	6.8 ± 0.2^{d}	4.7 ± 0.2^{b}	79.6 ± 3.8^{b}	53.7 ± 2.7^{b}
Punta Insua	4.6 ± 0.1^{b}	4.9 ± 0.2^{a}	106.6 ± 2.8^{b}	112.3 ± 5.4^{a}	82.0 ± 2.1^{b}	76.3 ± 3.7^{a}	1.3 ± 0.05^{b}	0.3 ± 0.03^{b}	8.0 ± 0.2^{c}	5.7 ± 0.1^{a}	72.7 ± 2.1^{b}	70.4 ± 3.7^{a}
Muxía	5.1 ± 0.2^{ab}	4.2 ± 0.2^{abc}	117.4 ± 5.0^{b}	96.9 ± 3.7^{abc}	90.4 ± 3.8^{b}	65.9 ± 2.5^{abc}	1.7 ± 0.04^{a}	0.3 ± 0.03^{b}	9.1 ± 0.4^{b}	5.1 ± 0.2^{b}	79.6 ± 3.8^{b}	60.5 ± 2.5^{ab}
Caión	5.1 ± 0.2^{b}	4.0 ± 0.3^{bc}	116.4 ± 4.9^{b}	91.2 ± 7.2^{bc}	89.6 ± 3.8^{b}	62.0 ± 4.9^{bc}	1.2 ± 0.09^{b}	0.3 ± 0.04^{b}	10.2 ± 0.2^{a}	5.4 ± 0.3^{ab}	78.3 ± 3.9^{b}	56.3 ± 4.8^{b}

[1] 1 h^{-1}; [2] J h^{-1} for a standarized individual of 1 g of meat dry weight

Data with the same superscript indicated that they did not differ significantly at the 95% level (SNK test done after a significant ANOVA was performed at each site)

CR clearance rate, *IR* ingestion rate, *AR* absorption rate, *ER* excretion rate, *RR* respiration rate, *SFG* scope for growth

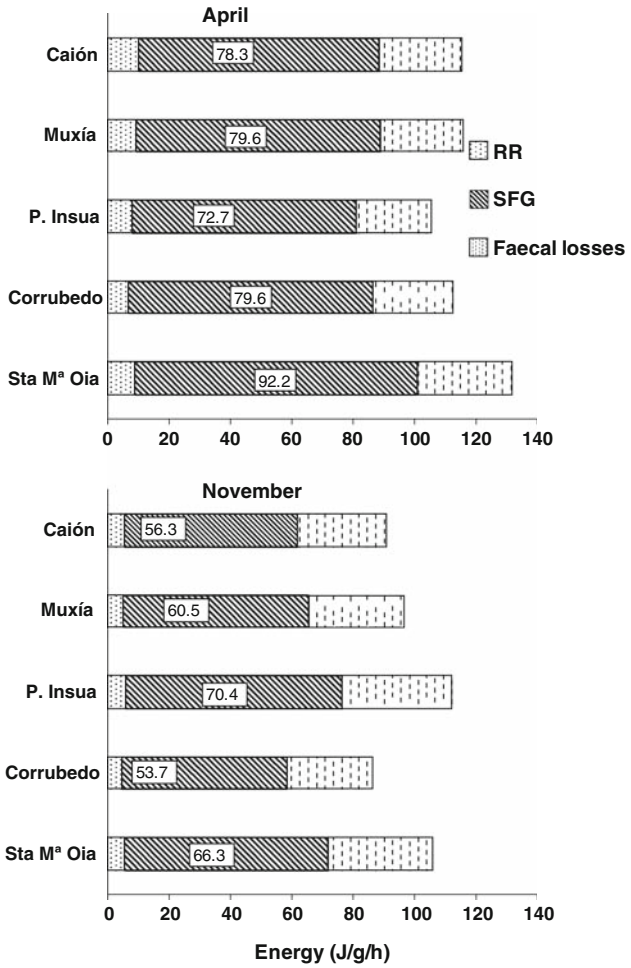

Fig. 3 Components of the energy budget of wild mussels collected from Galician coast sites affected by the *Prestige* oil spill. *Total bar* indicate ingested energy which is composed by the energy used in respiration, the energy lost in faeces production and energy available for growth SFG. SFG mean values are indicated for both seasons (April and November). Energy losses due to excretion process are not included as they suppose less than 1% of the ingested energy

Aegean Sea revealed that high body levels of PAH were related to increased responses of cytochrome P450, CYPlA-like protein, SOD or LPO. Bocquene et al. (2004) also reported high levels of DNA adducts and LPO as well as low AChE activity in *M. edulis* 6 and 12 months after the *Erika* oil spill. In the specific case of the *Prestige*, after 5 months the spill an increase in oxidative stress, as evaluated by means of enzyme determinations (EROD, GST, GR and CAT), was detected in the demersal fish species *Lepidorhombus boscii* and *Callionymus lyra*, gradually decreasing in the 3 years after the spill (Martínez-Gómez et al. 2006, 2009). Similarly, Cajaraville et al. (2006) and Orbea and Cajaraville 2006 found a high degree of disturbance in several cell and tissue biomarkers (peroxisome proliferation, lysosomal membrane stability, atrophy of digestive epithelium, acyl-CoA oxidase-AOX

activity) in mussels from the most severely affected areas in the first 12 months after the spill, although a tendency to return to previous values was noted 2 years after the disaster.

The correlation between activity levels of the different antioxidant enzymes in April and November (Table 4) reflects the existence of cooperative enzyme behaviour, indicating a greater efficiency of the antioxidant defence system, as has also been described by Manduzio et al. (2004). For this reason, this study aims to integrate all the enzymatic antioxidant levels in a single synthetic index, the IAR, which would provide a global description of this behaviour and facilitate the interpretation of the results.

In April, high antioxidant enzymatic levels in Costa da Morte sites determine that their mean IAR values were higher than at the reference site. The significantly higher IAR in Corrubedo and Muxía indicate the existence of oxidative stress at both sites, although particularly so in the case of Corrubedo. In this survey, GR activity levels were higher at the four sites along the Costa da Morte than at the reference site. Although GR is not always recognised as an antioxidant enzyme, it can be included in this category on the basis of its role in reducing oxidised glutathione (GSSG) and regenerating reduced glutathione (GSH) (Doyotte et al. 1997; Yan et al. 1997; Cheung et al. 2001), which is the most important antioxidant compound involved in ROS detoxification (Winston and Di Giulio 1991). An increase in GR activity levels as a result of exposure to organic pollutants has been demonstrated both in the laboratory (Cheung et al. 2004; Dafre et al. 2004) and in the field (Porte et al. 2001b; Box et al. 2007; Richardson et al. 2008). Thus, the high levels of GR activity at the sampling sites along the Costa da Morte appear to reflect a higher degree of conversion of GSSG to GSH, which would be a defence against ROS toxicity.

With regard to the PAH bioaccumulation, only CAT enzyme activity values in April seem to be related to PAH content of mussels sampled at the same time. CAT degrades H_2O_2 into H_2O and O_2, preventing the production of hydroxyl radicals, and is considered as an important early indicator of oxidative stress, having been signalled as one of the best and earliest antioxidant enzymes to respond to pollutant bioaccumulation (Cossu et al. 1997). CAT induction has been reported in mussels exposed to PAH and PCB (Porte et al. 1991; Richardson et al. 2008; Eertman et al., 1995); to a water-accommodated fraction of crude oil (Cajaraville et al. 1992) or after transplantation to polluted sites (Da Ros et al. 2000; Luca-Abbott et al. 2005). The relation of the CAT enzyme with PAH levels appears to indicate a specific response of this enzyme to the accumulation of hydrocarbons, one which would also seem to afford protection against LPO, as is suggested by the inverse relationship between CAT activity and LPO levels in our study (Tables 3, 5). Pampanin et al. (2005) have also detected a negative correlation between LPO levels (measured as MDA content) and CAT activity in mussels affected by petrochemical pollution.

On the contrary, IAR values and the levels of the antioxidant enzymes GRX and DTD in April are more related to PAH concentrations measured in February 2003 than to the PAH data obtained in 2004. This relationship between antioxidant response in April 2004 and PAH concentrations found 14 months before suggests the presence in the area of compounds capable of generating oxidative stress in zones of maximum exposure to hydrocarbons from the spill. After the *Aegean Sea* oil spill Albaigés et al. (2000) and Porte et al. (2000a) reported a "delayed" response of the antioxidant enzyme SOD in *M. edulis*, even though petrogenic hydrocarbon concentrations were similar to background levels found before the accident. This may be due to the existence in the environment, and particularly in the sedimentary compartment, of oxidised hydrocarbons formed through chemical and biochemical reactions that were not quantified in this study. Indeed, during the winter following the *Prestige* oil spill (November 2003–January 2004) an increase in PAH concentrations was detected in mussels from the most severely affected areas, probably as a result of storms that may have re-introduced fuel–oil residues in the water column (Viñas et al. 2009).

LPO has been included in this study as a biomarker of the possible harmful effects of ROS on membrane phospholipids, proteins or nucleic acids (Stegeman et al. 1992; Barata et al. 2005). In April, and in spite of the evidence of oxidative stress indicated by high IAR values at Corrubedo, Caión and Muxía, LPO levels were no different from those found at the reference site. The antioxidant defence system thus appears to offer an efficient protection mechanism against reactive chemical species, the induction of antioxidant enzymes being an adaptation to overcome the stress produced by exposure to pollutants (Cossu et al. 1997).

The fact that this study was performed on two clearly differentiated seasons made it possible to observe the existence of higher levels of activity of the CAT, GR and particularly SOD enzymes in spring sample (April) as opposed to the autumn sample (November). It is known that the antioxidant defence system in mussels can be influenced by seasonally fluctuating factors such as temperature, nutrient bioavailability or the reproductive cycle (Sheehan and Power 1999; Orbea et al. 2002; Petrovic et al. 2004; Bocchetti and Regoli 2006). Spring, as compared to autumm, is characterised by a higher level of metabolic activity marked by both the reproductive cycle and the improved environmental temperature and food conditions needed to complete the process of gametogenesis and enable spawning to take place. In this regard, several studies have found higher antioxidant activity and lower

levels of LPO products in mussels in spring than in winter (Viarengo et al. 1990, 1991; Sheehan and Power 1999; Romeo et al. 2003; Manduzio et al. 2004; Bocchetti and Regoli 2006), which coincides with what has been observed in this study. It is interesting to note that the antioxidant activities with greatest seasonal variability, SOD and CAT, bore a significant relation to respiration rates ($r_{CAT} = 0.784$, $P < 0.01$; $r_{SOD} = 0.588$, $P < 0.1$, data not shown). This would indicate that increased oxygen consumption, and thus an increase in the production of oxyradicals deriving from a higher rate of metabolic activity, is compensated for by an increase in antioxidant defences in April.

In November, higher enzymatic (CAT, GR, DTD and SOD) levels recorded at Caión determined that its IAR was significantly higher than that at Santa María de Oia. This was probably a response of mussels from this site to a higher exposition and bioaccumulation of hydrocarbons. Moreover, it seems to exist a relationship between the antioxidant enzyme DTD and mussel PAH accumulation. DTD belongs to the phase I mixed function oxidase (MFO) system, which is involved in the biotransformation and detoxification of organic compounds, and plays an important role in aquatic toxicology as a modulator of quinone toxicity since it allows a quinoline stable form to be produced with no passage of radicals (Cadenas 1995; Osman et al. 2004). Previous studies have shown a positive correlation between DTD and PAH concentration in *M. galloprovincialis* tissues (Livingstone et al. 1990; Porte et al. 1991, Solé et al. 1994, 1995).

On the other hand, and contrary to the April findings, LPO levels in November indicate the existence of peroxidative damage in mussels from Punta Insua and Muxía. It should also be taken into account that not only pollutants but also fluctuations in environmental factors such as temperature, salinity, oxygen rate or nutrient availability can influence the pro-oxidant/antioxidant balance (Viarengo et al. 1991; Power and Sheehan 1996; Orbea et al. 2002; Filho et al. 2001; Lau et al. 2004; Manduzio et al. 2004; Verlecar et al. 2008). In fact, in the case of mussels adapted to low or moderate pollution conditions, the above-mentioned seasonal factors can have a greater influence on antioxidant response than pollution itself, as the study by Orbea et al. (2002) in the Bay of Biscay (NE Spain) shows. Thus, a decrease in antioxidant enzyme activity levels in winter may have increased susceptibility to oxidative stress and favoured an increase in LPO in November.

Observed SFG values (70–90 J g^{-1} h^{-1} in April and 50–70 J g^{-1} h^{-1} in November) are considerably higher (maximum differences of between 20 and 30 J g^{-1} h^{-1}) than those described by other authors for mussels also kept under standardised laboratory conditions (Widdows et al. 1995, 2002; Cotou et al. 2002). The standard algal ration used in the determination of SFG in the above-mentioned studies is much lower (0.4 mg AFDW l^{-1}) than that used in the present study (1 mg AFDW l^{-1}), which would explain the higher SFG values found in the latter. On the other hand, high SFG values, similar to or even higher than those in the present study, have been described by Gardner (2000) or Honkoop et al. (2003), these also being related to a greater availability of food (1–3 mg AFDW l^{-1}).

The physiological rate with the greatest influence on the determination of SFG is the clearance rate, which determines the ingestion rate (Table 6), SFG therefore being determined by the amount of energy acquired. Numerous studies have demonstrated that the clearance rate is the most sensitive of all physiological rates to the presence of pollutants in the environment (Honkoop et al. 2003 Widdows et al. 1982; Kraak et al. 1997; Toro et al. 2003b). For this reason it has been proposed that the clearance rate alone should be used as a bioindicator of the stress produced by environmental pollution (Donkin et al. 1989; Toro et al. 2003a), given that this parameter is easier to determine than SFG, reducing the length of the experimental period, is sufficiently sensitive and the results have the same ecological significance. Nevertheless, Widdows and Staff (2006) recommend a full evaluation of the energy balance, since determining SFG reduces inter-population variability, shows a wider range of values and its significance is easier to understand than that of the clearance rate. Finally, the requirements in terms of the time and equipment needed to determine SFG are only slightly greater than those needed to determine the clearance rate, giving the SFG a clear advantage from a cost/benefit standpoint.

In the present study, no difference was found between food absorption efficiencies at the various sampling sites, since this parameter basically depends on the quantity and quality of food available (the same diet was used for all the sites studied) and is less sensitive to the presence of pollutants (Honkoop et al. 2003). On the other hand, respiration rates are often related to pollution-induced stress in animals. In general, large increases in respiration rates have been described as being associated with the presence of PAHs (Widdows et al. 1995) or TBT (Halldörsson et al. 2005), this producing a decrease in SFG, i.e. the energy left for the animal to grow. The increase in oxygen consumption rates in the presence of hydrocarbons has been related to the increase in metabolic processes induced to neutralise the presence of pollutants (Widdows et al. 1982). However, in the present study the only relationship that has been observed in the case of respiration rates is that associated with seasonality, itself related, as has been mentioned above, to the gametogenic

cycle of these animals. Finally, the respiration energy values observed in this study are similar (close to 10 J g^{-1} h^{-1}) to those published by other authors for the same species, temperature and time of year (Widdows et al. 1982, 1995).

Similarly to what was observed in the case of antioxidant response activity, the results obtained for SFG indicate that its value depends on the time of year when measurements are made, even when temperature and food availability (15°C and 1 mg AFDW l^{-1}) remain the same. SFG values for all the sampling sites fall by somewhat more than 30% in autumn when compared to the values obtained in spring. This fact is closely related to the physiological status of the mussel, which enters a period of reduced activity during the autumn months, meaning that both energy acquisition and consumption rates are lower than in spring, the period when spawning occurs and environmental food availability and temperature conditions improve (Hawkins and Bayne 1984). The slowing down of the food acquisition process (due to a decrease in food availability and quality), and hence metabolic energy consumption, during the autumn and winter months is advantageous for the animal from an energy standpoint, since when clearance and ingestion rates fall the time it takes for food to pass through the digestive system increases, and with it the efficiency of absorption of food that in addition to being scarce is also usually poor in quality (low organic matter content) (Hawkins and Bayne 1984).

Although SFG values significantly differed between Santa María de Oia and Costa da Morte sites in April, they cannot be considered as indicative of an unhealthy status since the greatest difference between sites is scarcely 10%. The classification proposed by Widdows et al. (1995) for SFG values in accordance with the level of pollution (assessment criteria) establishes that SFG values between 15 and 20 J g^{-1} h^{-1} are equivalent to a healthy status, those between 5 and 15 J g^{-1} h^{-1} imply a moderate stress on the system, and those between -5 and 5 J g^{-1} h^{-1} indicate the existence of major stress. The SFG values obtained in this study are higher than those given by Widdows et al. (1995) for the reasons cited above, and the data must therefore be relativised with regard to the maximum value, i.e. an SFG of 75–100% of the maximum value would indicate a healthy status, values in the 25–75% range would imply a moderate stress, and those under 25% would be equivalent to major stress. We can thus conclude that according to this particular biomarker both the Costa da Morte sampling sites (those affected by the oil spill) and the reference station (unaffected by the oil spill) show a healthy status. The lack of a noticeable relation between this biomarker and PAH content serves to reinforce this conclusion.

Conclusions

In spite of the extensive contamination observed along the Galician coast due to the *Prestige* oil spill, 2 years after the accident occurred PAH concentrations in mussel tissues were similar to background levels determined for this area, although their PAH profiles indicated that the oil from the *Prestige* was the main source for these hydrocarbons.

In the April sampling, antioxidant responses seem to be related to the PAH levels recorded 3 months after the accident, probably as a result of the presence in the marine environment of oxidised hydrocarbon compounds formed by the biotransformation of original hydrocarbons released in the accident. In spite of these signs of oxidative stress, no evidence of LPO was found at any of the Costa da Morte sites, probably due to the efficient antioxidant protection offered against ROS by the increase of antioxidant enzymatic activities. In November, higher levels of LPO were found, which appears to bear in relation to the seasonally-related decrease in some of the antioxidant enzymatic activities measured. However, in spite of this evidence of oxidative stress, SFG levels indicate no disturbance at a physiological level since both the mussels from the Costa da Morte sites (affected by the oil spill) and those from the reference site (unaffected by the oil spill) showed a healthy status 17 and 24 months after the accident, according to this biomarker.

Acknowledgments We are grateful to Jessica Bargiela for her technical assistance in the evaluation of PAHs, to Rocío García Muñoz for her help in the physiological determinations and to Bruno Cambeiro and Cristóbal Navarro for collaborating in the gathering of biological material. This study has been funded by the Strategic Action on R&D activities against accidental marine spills, within the framework of the Plan Nacional de I + D+I 2000-2003 (Ref. VEM2003-20068-CO5-03).

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