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Effects of the Erika oil spill on the common starfish *Asterias rubens*, evaluated by field and laboratory studies.

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## **Abstract**

Impacts of the 'Erika' oil spill on the common starfish *Asterias rubens* were investigated in the field, and using laboratory experiments based on contamination via food at different stages of the starfish reproductive cycle. Two months after the shipwreck, levels of hydrocarbons characteristic of 'Erika' fuel were significantly higher in pyloric caeca and body wall of *A. rubens* from a contaminated site, compared with control animals from an unpolluted reference area. Concomitant immunological responses and detoxification enzyme activity (CYP1A) were enhanced in the impacted starfish, suggesting rapid biotransformation processes. This was confirmed by laboratory experiments which showed a fast PAH uptake during the 10 first days of contamination, and the start of biotransformation processes from the third day. Our study confirms benzo(a)pyrene hydroxylase activity (BPH) in *A. rubens* and demonstrates the influence of CYP1A in the conversion of insoluble PAHs into soluble derivatives in this species for the first time. The rapidity of decontamination could explain

why starfish growth, level of motile activity, reproductive investment, energy storage and larval development were not significantly affected by these contaminants.

Keywords: *Asterias rubens*; Erika oil spill; Polycyclic aromatic hydrocarbons; biomarkers; growth; reproduction.

## INTRODUCTION

In December 1999, the oil tanker “Erika” sank off the coast of southern Brittany (France), breaking in two and releasing approximately 19 000 metric tons of heavy fuel oil (N° 2 or 6 according to the French or British classifications, respectively) along 400 km of the French Atlantic coast (Laubier et al., 2004, Fig. 1). The substance released was not a true crude oil, but a residue of petroleum transformation characterized by high molecular weight PAHs, a specific gravity similar to seawater and a high viscosity (20,000 cSt at 10°C). It was composed of about 50% PAHs with a high predominance of chrysene, phenanthrene, pyrene and benzo(a)anthracene (INERIS, National Institute for Environment and Industrial Risk, Diderich, 2000). The main oil discharges from the wrecked tanker occurred between 24 and 27 Dec and fouled the upper parts of the shorelines of Loire Atlantique and Vendée counties. In order to study the ecological and ecotoxicologic consequences of this oil spill, the French Ministry of Ecology and Sustainable Development launched a monitoring program, managed by IFREMER (French Research Institute for the Exploitation of the Sea) and INERIS. Two to three months after the oil spill, PAHs were found in every compartment of the environment, the main types detected being alkyl-substituted phenanthrenes, pyrenes, chrysenes and heterocyclic sulfur compounds as dibenzothiophenes and its alkyl derivatives (Tronczynski et al., 2004). Moreover, evidence of contamination associated with Erika oil was still detectable

in water and suspended particulate matter in May 2002. However, the shallow subtidal sediments exhibited little sign of oil contamination at any time.

Mass mortalities of the starfish species *Asterias rubens* and *Marthasterias glacialis* were observed on the highly polluted shore of the Croisic headland (Loire Atlantique) a few days after the spill (Bruet, personal communication). *A. rubens* is a common marine predator of the Atlantic coast and has been frequently used as a bio-indicator of contamination of the benthic environment by heavy metals and polychlorinated biphenyls (Coteur et al., 2003b,c; Danis et al., 2004b; den Besten et al., 2001; Guns et al., 1999; Stronkhorst et al., 2003; Temara et al., 1998). The expression of *A. rubens* cytochrome P450 immunopositive protein (Danis et al., 2004a; den Besten et al., 1993) and immune response (Coteur et al., 2005) are among the biomarkers used for such toxicity studies. Moreover, contaminated sediments from the North Sea were proven to have deleterious effects on embryo development of this species (Coteur et al., 2003b). The finding of sublethal effects on *A. rubens* skeletogenesis and growth following contamination by heavy metals (Temara et al., 1997, 1998) suggests that pollution could also have an impact on the demographic parameters of the species.

The literature contains few reports about the effects of oil spillage on starfish populations, apart from some monitoring carried out after the Exxon Valdez oil spill (Dean et al., 1996; Hooten and Highsmith, 1996; Jewett et al., 1996). However, as these field studies dealt with density measurements, it is difficult to dissociate effects of the oil from those of other factors like post-spill shoreline treatments, the use of dispersants, or even natural cycles. Previous experimental studies have mostly been focused on the impact of the water accommodated fraction (WAF) or water soluble fraction (WSF) of crude oil on the survival, behavior or feeding of predatory starfish including *Asterias rubens* (Georgiades et al., 2003; O'Clair and Rice, 1985; Ordzie and Garofalo, 1981; Temara et al., 1999; reviewed by O'Clair and Rice, 1985). Locomotive function, prey localization ability, feeding rates and growth have all been

found to be reduced at low oil concentrations, even though sensitivity was shown to vary with the type of oil and/or starfish species. Ability to avoid oiled sediment was also demonstrated in one bottom-dwelling starfish (Ryder et al., 2004).

The present study, therefore, aimed not only to quantify the effects of the Erika oil spill on biomarker expression in *Asterias rubens*, i.e. cytochrome P450 (CYP1A) and immune responses, but also to determine whether such pollution would have an impact on the growth or reproductive cycle. Effects on growth and reproduction were investigated through controlled laboratory experiments, using oil-polluted mussels as a food supply for starfish. These experiments also aimed to investigate PAH uptake and loss over time in the digestive structures of *A. rubens*, in order to determine possible relationships between the presence of PAHs in starfish and the biological effects observed.

## **MATERIALS AND METHODS**

### **Field survey**

The field survey of the effects of the Erika oil spill on *Asterias rubens* populations was conducted by sampling starfish from two intertidal populations: Piriac (Croisic headland), contaminated by the Erika oil spill on December 25 1999 (Laubier et al., 2004), and Aber (Bay of Douarnenez, Fig. 1) which remained free of pollution.

Both field sites were visited at low tide to collect starfish on days 40 (Feb 00), 58 (Feb 00), 221 (Aug 00), and 2 years after the oil spill (Jan 02). Individuals with a 50 to 70-mm-long ray were sampled (the ray corresponds to the size of the longest arm measured from mouth to tip with a Vernier caliper). On day 40 no starfish were found at Aber, and the 5 starfish collected at Piriac the same day were used for immune response measurements. On days 58, 221 and 2 years after the oil spill, 4 starfish from each site were dissected in the field for cytochrome P450 immunopositive protein induction measurement (CYP1A): 2 ml of pyloric caeca were

blotted, immediately frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$  until analysis. On days 58 and 221, another ten starfish were brought to the laboratory where they were kept for 24 hours in aquaria supplied with clean running sea water prior to immune response measurements. On day 58 and 2 years after the oil spill, 10 starfish from each site were dissected for PAH analysis; 2 pools of pyloric caeca and 2 pools of body wall were made (10 starfish per pool) and stored at  $-20^{\circ}\text{C}$ .

#### *Cytochrome P450 immunopositive protein induction measurement (CYP)*

The P450 immunopositive protein (CYP1A-IPP) contained in the post-mitochondrial fraction of the pyloric caeca was quantified using a competitive ELISA method as described by Danis et al. (2004a). The final results were expressed as inhibition factors, i.e. the ratio of CYP1A-IPP levels between experimental and control groups.

#### *Immune response measurement*

The two essential parameters of the starfish immune system considered in this study were coelomic amoebocyte concentration (CAC) and reactive oxygen species (ROS) production by resting (unstimulated) or stimulated amoebocytes. Amoebocyte ROS production was measured by the peroxidase luminol-enhanced chemiluminescence (PLCL), method developed by Coteur et al. (2002, 2004, 2005). Measurements were normalized using the actual amoebocyte concentration in each sample, and expressed as the sum of all 10-min interval measurements (in Relative Light Units RLU, an internal scale of the instrument) for  $10^6$  cells  $\text{ml}^{-1}$  (total chemiluminescence) of resting or stimulated amoebocytes.

#### *PAH measurements*

For quantification of the PAHs listed in Table 1, tissue samples were freeze-dried and ground prior to analysis. Among the alkylated PAHs, only certain compounds of high molecular weight, known to be representative of the crude oil from the Erika, and highly abundant, were chosen for measurement. PAHs were extracted from 1 to 3 g of dry tissues using the

microwave-assisted extraction method described by Budzinski et al. (1999). Filtration of the extracts, reduction of their volume and purification were performed as described by Baumard and Budzinski (1997). Experimental blanks were also passed to assess the risk of potential contamination. Extracts were analyzed by HP GC/MSD according to the procedure used by Geffard et al. (2004) in the Erika monitoring program, except for the oven temperature which was programmed to increase by 2°C/min from 70 °C to 300 °C in the present study. Different perdeuterated PAHs were used as internal standards for each class of aromaticity (Baumard and Budzinski, 1997). The response factor of a given PAH relative to the perdeuterated one was determined for each GC/MS sequence by injecting 1 µL of a standard solution containing a mixture of PAHs (SRM 2260, National Institute of Science and Technology, Gaithersburg, MD, USA) and the solution of perdeuterated PAHs used for sample spiking. The precision of this type of quantification has been evaluated to be between 88% and 98%, depending on the compound, with reproducibility between 2% to 5%. The entire analytical procedure was performed several times with certified mussel tissue, SRM 2974 (NIST, Gaithersburg, MD, USA). The recoveries on this SRM were between 70% and 110% for five replicates with reproducibility ranging from 7 to 17% depending on the compound.

### **Laboratory studies**

Our laboratory investigations complemented the field study by aiming to assess to what extent starfish somatic growth, reproduction, and development of gametes and larvae were affected by oil exposure via food. Experiments were performed on starfish at different stages of development and reproductive cycle. Uptake and loss of PAHs over time were also analyzed.

#### *Experimental procedure*

Mussels were exposed for 3 days to 'Erika oil' samples, supplied by the CEDRE (Centre of documentation, research and experimentation on accidental water pollution, Brest, France). A preliminary study had first been carried out to establish a protocol to produce a PAH

concentration in mussels that would be close to the maximum PAH content observed in the mussels in the Bay of Vilaine after the oil spill (sum PAH =2536  $\mu\text{g kg}^{-1}$  dw in February 2000, Tronczynski et al., 2004). A PAH concentration of 3420  $\mu\text{g kg}^{-1}$  (268.5  $\mu\text{g g}^{-1}$  lipids)  $\pm$  900  $\mu\text{g kg}^{-1}$  (mean PAH concentration in 3 replicates of 30 g WW of mussel soft tissue) was obtained after 3 days of contamination with the following method: 1500g of mussels were placed in an aquarium containing 2 l seawater and 50 g Erika oil (25  $\text{g.l}^{-1}$  oil). The aquarium was covered and the soluble phase homogenized by high air bubbling. After contamination, the mussels were carefully brushed and cleaned to eliminate any traces of oil. The PAH concentration in the control mussels was 14  $\mu\text{g g}^{-1}$  lipids. Contaminated and uncontaminated mussels of the same origin were frozen following treatment and prior to use as food. One should note that after 24 hours in running seawater, the frozen mussels showed no significant decrease in PAH content ( $222 \pm 134.3 \mu\text{g g}^{-1}$ ). Starfish were fed at the rate of 1 mussel per individual per day.

Two treatments were used in the laboratory experiments: ‘contaminated treatment’ and ‘control treatment’ depending on whether the mussels supplied as food to starfish were contaminated or not (details of the separate experiments are given below under *Experiment descriptions*). For each experiment, the aquaria were filled with clean running seawater from the nearby Bay of Brest; salinity ranged from 33 to 35 PSU. Once a week, the photoperiod was adjusted to match the natural one. The mussels fed to each starfish were chosen according to the size of the starfish under test, according to the relation established by Sommer et al. (1999) (mussels of 2 to 50-mm shell length for starfish of 5 to 65 mm ray length).

#### *Parameters measured*

The parameters studied were: size structure, gonad and pyloric caeca indices, PAH concentrations, righting activity coefficient (RAC), ovocyte diameter, fertilization success,

embryo development and adambulacral spine regeneration. The parameters assessed depended on the particular experiments.

Before the experiments were started, starfish size distribution was carefully checked for likeness between the groups used for the different treatments. Pyloric and gonad indices were calculated as follows:  $organ\ index = organ\ dry\ weight / body\ wall\ dry\ weight$ . Gonads and pyloric caeca were dissected, and the body wall was carefully cleaned. Body compartments were dried separately at 60°C for 48 hours and weighed.

RAC is indicative of the level of motion activity (Watts and Lawrence, 1990). Here, the righting time corresponded to the time required by a starfish to turn back over once it had been placed on its aboral face. RACs were calculated as follows:  $RAC = 1000 / righting\ time\ (s)$ . Among individuals, those showing no sign of righting response initiation within 10 minutes were assigned an RAC value of  $1000 / (2 * 600)$ .

Histological observations were made to detect any effect that oil pollution had on ovocyte diameter. Ovary samples were embedded in paraffin, sectioned into 5- $\mu$ m-thick slices and colored using Trichrome staining. For each female, digital pictures of 100 ovocytes exhibiting a nucleolus and a nucleus within a same section were taken with a CCD camera (Iris Sony) and measured with Visilog 4.0 software; diameters were then obtained by conversion.

Fertilization assays were made to assess the capacity of starfish ovocytes to be fertilized, as well as the quality of subsequent larval development. Starfish were sexed by puncture of gonadic material through the integument. Spawning and fertilization were performed as described by Coteur et al. (2003b). Individual female clutches were fertilized by a pool of sperm (3 males). Fertilization success was assessed after 1h by counting the number of eggs with a fertilization membrane in 3 subsamples of 100 eggs each and the embryo development quality was determined after 72 h. For each fertilization, the different types of larvae (viable larvae, abnormal larvae and dead eggs) were counted in each well of a six-well plate .

Adambulacral spine regeneration was induced and studied according to the methods developed by Dubois and Jangoux (1990).

### *Experiment descriptions*

#### Impact of PAH uptake on adults at the gametogenic stage: a 3-month experiment

Two laboratory experiments (named 1A and 2A, respectively) were carried out over three months in two successive years. These were conducted during the period of gonadal growth from December to the end of February. In each treatment (contaminated and control), 60 starfish of 35-45 mm ray length were used (10 per aquarium).

#### Monitoring PAH uptake and its potential physiological effects: a short 15-day experiment

This experiment (named PAH uptake) was conducted in June during the agametogenic stage on starfish of 50-70 mm ray length. PAH accumulation in pyloric caeca (pool of 10 starfish per aquarium) and the RAC values were measured on days 0, 1, 2, 5, 7, 10 and 15 of exposure to oil. The regenerating adambulacral spines were sampled on five starfish in each treatment, 15 days after fracture, and the regeneration size (RAS) measured with a scanning electron microscope as previously described by Dubois and Jangoux (1990).

For the sum of all measured PAHs, the sum of parental PAHs and PAHs of particular interest in this study, PAH uptake *vs.* time was tentatively modeled with the following quadratic equation:

$$C_{PAH} = aT^2 + bT + c ,$$

where  $C_{PAH}$  is the concentration of the PAH concerned,  $T$  is the time-length expressed in days of exposure to contamination, and  $a$ ,  $b$ ,  $c$  are constants. The inflection point of the curve,  $I$ , corresponded to  $I = -b/2a$ . The transfer factor (TF) of a given PAH in this study corresponded to the ratio of its concentration in the pyloric caeca (i.e. the mean of data at days 10 and 15) to that in the food ( $\mu\text{g g}^{-1}$  lipids).

#### Decrease of PAHs in the pyloric caeca: a 24-day experiment

Juvenile starfish of about 20 mm ray length were contaminated over 4 months (from May to September) in the conditions previously described. In September they had grown to 40-65 mm ray length. Contaminated and control starfish were then both fed with pollution-free mussels for 23 days. Temperature was constant and verified daily during the experiment ( $17.5 \pm 0.15$  °C). Pyloric index (fresh weight) and RAC value were determined on 4 starfish per treatment on days 0, 1, 2, 4, 7, 10, 14, 18 and 23 of the PAH loss experiment. For the sum of all measured PAHs, the sum of parental PAHs and PAHs of particular interest in this study, PAH loss by starfish was tentatively modeled using the following equation:

$$C_{PAH} = d \times e^{-kT} + g$$

where  $C_{PAH}$  is the concentration of the concerned PAHs,  $T$  is the number of days under PAH loss conditions, and  $d$ ,  $k$  and  $g$  are constants. The biological half lives of the different compounds were estimated as  $T_b = \ln 2/k$ .

### **Statistical analysis**

The measured immune responses (ROS production and CAC) were compared between treatments using nonparametric tests since the data that did not fit a normal distribution and CYP1A expression were tested with two-way ANOVA. In the laboratory contamination trials, gonad and pyloric indexes, PAH levels and physiological parameters were inter-compared and statistically tested using t-tests or ANOVAs, depending on the number of samples. In experiments requiring the use of several aquaria for each treatment, differences in the variables (measured on all individuals) were tested using a nested ANOVA with “aquarium” as a nested factor within “treatment”. The nested factor allows one to distinguish any discrepancy between pseudo-replicates within each experimental treatment (Zar, 1996).

PAH uptake and loss were modeled by quadratic curve fitting with Systat 9 software. Residuals were plotted, and checked to ensure there was no serial correlation.

Whenever several statistical comparisons were performed either on the same set of data or on subsets, we used the Bonferroni criterion and divided by the number of comparisons.

## RESULTS

### Field survey

Fifty-eight days after the oil spill, the PAH levels in the pyloric caeca and body wall of starfish from Piriac were higher than those in starfish from Aber (Fig. 2). The amounts were particularly high in pyloric caeca compared with the body wall. The most abundant PAHs in the fuel transported by the Erika were analyzed in starfish samples; these were alkyl substituted homologues of phenanthrene (Phe), dibenzothiophene (DBT) and chrysene (Chrys) (Tronczynski et al., 2004), and their unsubstituted PAHs. Chrysene and its alkylated homologues were far more abundant in starfish from Piriac than in controls for both organs studied (Table 2). Moreover, the pyloric caeca of Piriac starfish contained more Phe-alkylated homologues and less DBT and DBT-alkylated homologues than did the body walls.

Figure 3 illustrates the changes occurring over time in the responses of the biomarkers according to sampling site. At day 58, ROS production by both resting and stimulated amoebocytes (Mann-Whitney  $p \leq 0.005$ ,  $n = 19$ ), CAC and CYP1A levels were significantly higher in starfish from Piriac than in those from Aber. However, at day 221, only CAC (Mann-Whitney  $p \leq 0.005$ ,  $n = 37$ ) and CYP1A levels were more elevated (two-way ANOVA, factor: site  $p = 1 \cdot 10^{-8}$ ,  $n = 16$ ).

The immune response parameters showed contrasting results (Fig. 3): at Piriac, ROS production by both resting and stimulated amoebocytes was significantly higher at day 40 than at day 58 (Kruskall-Wallis  $p = 0.007$ ,  $n = 23$ ), CAC response however showed no significant difference between the two dates. ROS production by both resting and stimulated amoebocytes and CAC levels did not significantly change between days 58 and 221 at Piriac.

At Aber in contrast, amoebocyte ROS production (of both types) was higher on day 221 than on day 58 (Mann-Whitney  $p \leq 0.01$ ,  $n = 19$ ). CYP1A levels were higher on day 58 than on day 221 in both populations (two-way ANOVA, factor: time,  $p = 23 \cdot 10^{-8}$ ,  $n = 16$ ).

In January 2002 (after 2 years), starfish from both sites had a low PAH content with respect to lipids (Fig. 2); moreover, no significant difference was found between mean CYP1A expression at Aber ( $2.12 \pm 0.87$ ) and Piriac ( $1.90 \pm 0.71$ ) ( $t$ -test  $p = 0.70$ ).

## Laboratory studies

### Impact of PAH uptake on adults at the gametogenic stage: a 3-month experiment

The three-month experimental contamination (experiment 1A) showed an accumulation of PAHs in the pyloric caeca of contaminated starfish ( $49.3 \pm 18.8 \mu\text{g g}^{-1}$  lipids vs.  $1.7 \pm 0.7 \mu\text{g g}^{-1}$  lipids in the control) and in their gonads ( $32.2 \pm 6.7 \mu\text{g g}^{-1}$  lipids vs.  $0.4 \pm 0.2 \mu\text{g g}^{-1}$  lipids in the control). Differences were highly significant between treatments (two-way ANOVA,  $p = 1 \cdot 10^{-4}$ ) but not significantly different between body compartments ( $p = 0.170$ ) or treatment x body compartment interaction ( $p = 0.199$ ). PAH uptake was the same in pyloric caeca and gonads. Apart from RAC values, which were higher in experiment 1A in contaminated starfish than in controls ( $t$ -test,  $p = 0.02$ ,  $n = 36$ ), no other significant differences were shown between control and contaminated treatments in the parameter measured: i) mean size ( $t$ -test,  $p = 0.81$ ,  $0.13$  with  $n = 63$ ,  $50$  for experiments 1A and 2A respectively), ii) gonad index ( $t$ -test,  $p = 0.43$  with  $n = 63$  in experiment 1A), iii) pyloric index ( $t$ -test,  $p = 0.26$ ,  $0.26$  with  $n = 63$ ,  $50$  for experiments 1A and 2A respectively) and iv) ovocyte diameters ( $t$ -test,  $p = 0.81$  with  $n = 10$  in experiment 1A).

Spawning and fertilization were induced at the end of these experiments. At least 90% of eggs presented a fertilization membrane demonstrating their fertilization quality. No significant difference was found in the percentage of dead eggs and viable larvae between larval batches

produced from contaminated or uncontaminated parents (ANOVA,  $p = 0.76$ ;  $t$ -test,  $p = 0.95$ ,  $n = 12$ ).

#### Monitoring PAH uptake and its potential physiological effects: a short 15-day experiment

Starfish in the contamination treatment consumed the same number of mussels as control starfish. During this experiment the changes in daily sea water temperature were low ( $15.4 \pm 0.4^\circ\text{C}$ ). The patterns of PAH accumulation in the pyloric caeca appeared to be the same for unsubstituted and alkylated PAHs (Fig. 4; Table 3): the marked uptake observed in the first days in the two pools analyzed from contaminated starfish was followed by a decrease between days 9 and 10. Transfer factors were always less than 0.2. The evolution over time in the ratios of benzo(e)pyrene (BeP) to benzo(a)pyrene (BaP), and the ratios of some parent PAHs: chrysene, phenanthrene and dibenzothiophene to their more hydrophobic methyl-substituted compounds showed a preferential accumulation of the more soluble PAHs over the first 3 days of the experiment, except for the chrysene family (Fig. 5). Such an evolution is indicative of a biotransformation process. A slight rise in chrysene was noted throughout the experiment. An accumulation of DBT and Phe during the first days was followed by a large increase in methyl-substituted compounds from the 5<sup>th</sup> day. From the 2<sup>nd</sup> day BeP increased faster than BaP indicating preferential metabolism of BaP compared with BeP. After the 7<sup>th</sup> day, none of these ratios changed very much. Overall contamination of starfish under controlled conditions showed no evolution over time though, with the sum of analyzed PAHs remaining within the range  $0.3$  to  $0.6 \mu\text{g g}^{-1}$  lipids.

No differences were observed in RAC, pyloric indexes or size of regenerating adambulacral spines between the two groups of contaminated starfish ( $t$ -tests,  $p > 0.05$ ), and mean RAC showed no temporal evolution whatever the treatment (Pearson correlation coefficients  $r = -0.492$ ,  $-0.573$  and  $-0.502$  respectively for control starfish, contaminated starfish in aquaria 1 and 2,  $p \geq 0.2$ ,  $n = 7$  for each). As the starfish consumed food during the experiment and

*accumulated reserves throughout the agametogenic stage, their pyloric indices under both contaminated and control conditions were higher compared to those measured at the beginning of the experiment (Day 0) (ANOVA,  $p = 4.10^{-4}$ ,  $n = 40$ ), but no difference was detected between treatments ( $t$ -test,  $p > 0.1$ ). Similarly, neither RAC values, nor the sizes of regenerating adambulacral spines were found to differ between contaminated and control starfish ( $t$ -test,  $p = 0.68$  and  $0.344$ ,  $n = 30$  and  $14$  respectively).*

#### Decrease of PAHs in the pyloric caeca: a 24-day experiment

Mean daily sea water temperature in the tanks ( $17.5 \pm 0.1^\circ\text{C}$ ) did not change during this experiment. PAH levels decreased rapidly in the pyloric caeca (Fig. 6), and calculated half-lives were within 2 and 4 days (Table 4). After 23 days of PAH loss, most of the individual PAHs of particular interest were close to zero apart from a few PAHs including Pyrene, Chrysene and phenanthrene that had concentrations much higher than in the control. No significant differences in RAC were observed between treatments or days (two-way ANOVA  $p = 0.23$ ,  $0.39$  and  $0.75$  for the factors “treatment”, “days” and their interaction, respectively,  $n = 74$ ). No significant difference was detected in pyloric indices between decontaminated starfish ( $\text{PI} = 0.496 \pm 0.099$ ) and the control ( $\text{PI} = 0.440 \pm 0.064$ ;  $t$ -test,  $p = 0.12$ ,  $n = 74$ ).

## **DISCUSSION**

### **Biomarker expression and PAH dynamics in *Asterias rubens* pyloric caeca**

This study has provided evidence for contamination of the pyloric caeca and body wall of *Asterias rubens* in the field by the oil from the Erika, spilt along the shoreline of South Brittany over the first weeks following the shipwreck. Less than two months after the event, high PAH levels characteristic of fuel from the Erika were detected in pyloric caeca of starfish at Piriac. Reactive oxygen species (ROS) production by resting or stimulated amoebocytes and coelomic amoebocyte concentration (CAC) were simultaneously enhanced in the

impacted starfish. A high induction of CYP1A expression was also observed. CYP1A converts particularly insoluble organic compounds such as PAHs and PCBs (Bucheli and Fent, 1995) into soluble derivatives. The induction of CYP1A expression by exposure to PCBs had already been reported in the pyloric caeca of *A. rubens* (Danis et al., 2004a,b) but the present study is the first time that a high reactive signal was clearly observed in presence of PAHs. These biomarker expression patterns suggest a rapid initiation of the biotransformation process.

Laboratory experiments confirm this hypothesis and allow a better understanding of the dynamics of some specific aromatic hydrocarbons. While PAH uptake within the pyloric caeca of adult starfish fed with oil-polluted mussels was fast (the peak concentration was reached in 8 to 10 days after the start of contamination), elimination and biotransformation were also rapid; even in conditions with a constant supply of contaminated food. The most soluble compounds, apart from the chrysene family, were preferentially accumulated over the first few days. Indeed, among the PAH parent compounds under study, chrysene was the only one that showed a continuous increase in relation to its alkylated homologues during the 15 days of the PAH uptake experiment. This indicated that it was not biotransformed, in contrast to benzo(a)pyrene, whose biotransformation was induced in the first days of contamination. Benzo(a)pyrene is more reactive and oxidizable than benzo(e)pyrene (Baumard et al. 1999) and the observation of benzo(a)pyrene biotransformation is in agreement with the study of den Besten et al. (1993) where benzo(a)pyrene hydroxylase activity (BPH) was enhanced in the *A. rubens* pyloric caeca microsomes in the days immediately following injection of benzo(a)pyrene into the animals. Moreover, the low transfer factors of PAHs (<1) in the pyloric caeca indicate that there was no biomagnification through the mussel-starfish trophic link.

Following the end of the experimental pollutant exposure, half of the total PAHs were eliminated in the next 2 to 4 days. However, 20 days later, a certain amount of PAHs remained stored in the pyloric caeca. Experimental observations therefore suggest that despite clear evidence for rapid biotransformation processes, the longer the exposure to pollution continues, the greater the level of PAHs accumulated in the pyloric caeca. Table 5 shows that a constant supply of oil-polluted food lowered the biotransformation capability of starfish. Measured PAH concentrations were much lower in the field than under experimental conditions (24 times lower in the field 2 months after the oil spill than in the laboratory after 15 days of controlled contamination). It is also worth noting that the high oil concentrations found in mussels following the oil spill did not remain as constant as the contamination in controlled laboratory conditions.

Several biomarkers were used in this study to monitor the physiological response of starfish to PAHs in the field. All showed a significant enhancement in the impacted starfish compared with controls a few weeks after the oil spill. But although the CYP1A signal is clear, ROS production by starfish from the contaminated field site were within the range of the values found by Coteur et al. (2004) in natural uncontaminated populations, and CAC values were below those previously measured in heavily polluted locations (Coteur et al., 2003a,b). The kinetics of PAH loss previously described suggest that rapid decontamination could possibly be occurring and thus that there may be a reduction in ROS production during the 24h that elapsed between starfish collection and response measurement. Such an explanation cannot account for the CAC result though, since immune cell turnover in marine invertebrates exceeds 24 hours (McIntosh and Robinson, 1999). CAC is known to be quite a robust biomarker for stress (Coteur et al., 2004), and the differences observed between the two populations would indeed have resulted from the PAH level in the field. However, as it is well known that environmental factors such as temperature can influence immune response in

starfish (Coteur et al., 2004), the immune biomarker levels could have been modulated by changes in water temperature, notably in the impacted field site between days 40 and 58.

### **Effect of PAHs on *Asterias rubens* growth, reproduction and embryo development**

As observed in the present study, irrespective of which experiment is considered, neither growth nor reproductive capability of starfish was affected by PAH exposure, despite an increase of PAH content in the pyloric caeca. PAH accumulation in the gonads had no effect on ovocyte morphometry, and fertilization rates were only very slightly affected. To our knowledge, no previous study has dealt with the impact of oil contamination through food. Oil has previously been shown to induce damage to starfish locomotive function (O'Clair and Rice, 1985 and references therein, Ordzie and Garofalo, 1981), feeding rate and adult growth (Crapp, 1971; Georgiades et al., 2003; O'Clair and Rice, 1985; Temara et al., 1999), and embryo development (Davis et al., 1981). However, in these studies, the starfish were exposed either to the water accommodated fraction (WAF) of different types of oil or to a complex mixture of hydrocarbons and dispersants. Volatile and soluble oil fractions may have greater direct effects on echinoderms than compounds obtained via food. In the sea urchin, *Paracentrotus lividus*, the righting response was enhanced by the most volatile compounds of crude oil, but not by weathered crude oil (Axiak and Saliba, 1981). The effects of WAF are more harmful on embryo development in sea urchins (references in Kobayashi, 1995): adult exposure to polluted effluents can lead to egg abnormalities at the first cleavage stage (Vashchenko, 1980) or affect egg fertilization and sperm motility (Krause, 1994). It is not easy to determine whether the reported differences resulted from exposure conditions, pollutant sensitivity of the organism under study or levels of contamination, because none of these authors qualified or quantified the level of parental contamination. Once again, without additional data about the relative toxicity of PAHs, it does not seem relevant to speculate on

whether volatile and soluble oil fractions are more or less toxic or bioaccumulated than PAHs taken in with food. Only Barron and et al. (1999) have suggested that PAHs are not necessarily the major determinant of WAF toxicity.

In conclusion, this study has demonstrated that uptake of PAHs via food is an important route for contamination of the common starfish *A. rubens*. Short-term impacts of oil contamination were easily quantified using detoxification biomarkers, immunological responses and by monitoring PAH uptake and loss in the storage organs. Moreover, *A. rubens* and suitable biomarkers (e.g. CAC or CYP1A) can be judged to be suitable for use as early warning signals for exposure to PAHs. However, behavior, reproduction and growth did not appear to be affected by PAH exposure through food. Finally, the present data showed no significant long-term effects of the Erika oil spill on *A. rubens* dynamics. The predation power of *A. rubens* thus appears to be unaffected in the mussel beds of the intertidal rocky temperate communities structured by this starfish.

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Table 1. List of PAH compounds studied with the internal standards used for GC/MS analysis

Compound	Abreviation	Internal standard
naphtalene		Nd8
acenaphtylene		Nd8
acenaphtene		Nd8
fluorene		Pd10
phenanthrene	Phe	Pd10
anthracene		Pd10
fluoranthene		Fluod10
benzo(a)anthracene		Chrys d12
pyrene	Pyr	Pyrd10
triphenene + chrysene	Chrys	Chrys d12
benzo(b + j + k) fluoranthene		BePd12
benzo(e)pyrene	BeP	BePd12
benzo(a)pyrene	BaP	BaPd12
perylene		BaPd12
indeno(1,2,3-cd)pyrene		BPd12
dibenz(a)anthracene + DaC		BPd12
benzo(ghi)peryleneP		BPd12
<i>Sum of unsubstituted PAHs</i>	<i>uns-PAHs</i>	
Sum methylnaphtalene		Nd8
Sum methylphenanthrenes and dimethylphenanthrene	MPhe	Pd10
methylchrysene	MChrys	Chrysd12
<i>Sum of methylated PAHs</i>	<i>M-PAHs</i>	
dibenzothiophene	DBT	DBTd8
methyl dibenzothiophene	MDBT	DBTd8
2,1 benzonaphthothiophene		Chrysd12
<b>Sum of all analysed PAHs</b>	<b>Sum PAHs</b>	

Table 2 Characterisation of PAH levels in organs of starfish

	Body wall		Pyloric caeca	
	Aber	Piriac	Aber	Piriac
uns-PAHs	38 %	32 %	41 %	30 %
M-PAHs	62 %	68 %	59 %	70 %
Phe	712 10 <sup>-4</sup>	506 10 <sup>-4</sup>	346 10 <sup>-4</sup>	262 10 <sup>-4</sup>
Alkyl-Phe-	319 10 <sup>-3</sup>	349 10 <sup>-3</sup>	180 10 <sup>-3</sup>	428 10 <sup>-3</sup>
DBT	31 10 <sup>-4</sup>	78 10 <sup>-4</sup>	39 10 <sup>-4</sup>	24 10 <sup>-4</sup>
MDBT	<10 <sup>-4</sup>	122 10 <sup>-4</sup>	156 10 <sup>-4</sup>	176 10 <sup>-4</sup>
Chrys	56 10 <sup>-4</sup>	478 10 <sup>-4</sup>	146 10 <sup>-4</sup>	734 10 <sup>-4</sup>
MChrys	<10 <sup>-4</sup>	1787 10 <sup>-4</sup>	39 10 <sup>-4</sup>	1984 10 <sup>-4</sup>

Piriac : oil-exposed population; Aber : control. All data are means, n = 2 pools (10 starfish by pool).

uns- (unsubstituted) and M-PAH (methylated PAH) are expressed as percent of the sum of all analyzed PAHs.

Phe (phenanthrene), DBT (dibenzothiophene), Chrys (chrysene) and Alkyl-Phe (Alkyl-phenanthrene), -DBT and -Chrys are expressed in  $\mu\text{g g}^{-1}$  lipids. Lipid contents are those measured at day 58 (Fig.2).

Table 3. Kinetics of PAH uptake and results of the quadratic curve.

	$p$	Curve Fitting		TF
		$R^2$	I (days)	
Sum PAHs	$<1 \cdot 10^{-3}$	0.823	9.48	0.110
uns-PAHs	$<1 \cdot 10^{-3}$	0.881	9.11	0.125
Pyr	$<1 \cdot 10^{-3}$	0.909	9.19	0.121
Phe	$<1 \cdot 10^{-3}$	0.874	8.82	0.159
DBT	$<1 \cdot 10^{-3}$	0.756	9.37	0.118
Chrys	$<1 \cdot 10^{-3}$	0.849	10.12	0.086

(n = 2 pools of 10 starfish) fed contaminated mussels during the agametogenic stage (1 mussel per individual starfish per day. PAH:  $268.5 \pm 78.5 \mu\text{g/g}$  lipids).

(I): inflection point of the fitted curve, (TF): transfer factor,  $P$ : curve fitting probability,  $R^2$ : correlation coefficient, Sum PAHs: sum of all analyzed PAHs, uns-PAHs: sum of analyzed unsubstituted PAHs, Pyr: pyrene, Phe: phenanthrene, DBT: dibenzothiophene, Chrys: chrysene.

Table 4. PAH loss in the pyloric caeca of starfish and result of the curve fitting for certain PAHs.

	$p$	$R^2$	D	g	h
Sum PAHs	$<1 \cdot 10^{-3}$	0.958	92.84	3.50	3.03
uns-PAHs	$<1 \cdot 10^{-3}$	0.983	21.323	1.45	2.77
Pyr	$<1 \cdot 10^{-3}$	0.980	2.97	-0.0318	3.50
Phe	$<1 \cdot 10^{-3}$	0.976	7.05	0.284	2.02
DBT	$2 \cdot 10^{-3} < p < 5 \cdot 10^{-3}$	0.856	2.27	-0.0065	2.12
Chrys	$<1 \cdot 10^{-3}$	0.926	4.744	0.0117	3.42

n = 1 pool of 4 starfish fed with 2 uncontaminated mussels per individual per week over 23 days.

$P$ : curve fitting probability,  $R^2$ : correlation coefficient, D,g: parameters of the fitted equation (in  $\mu\text{g g}^{-1}$  of lipids), h: half-life of the compounds (in days). Sum PAHs: sum of all analyzed PAHs, uns-PAHs: sum of analyzed unsubstituted PAHs, Pyr: pyrene, Phe: phenanthrene, DBT: dibenzothiophene, Chrys: chrysene.

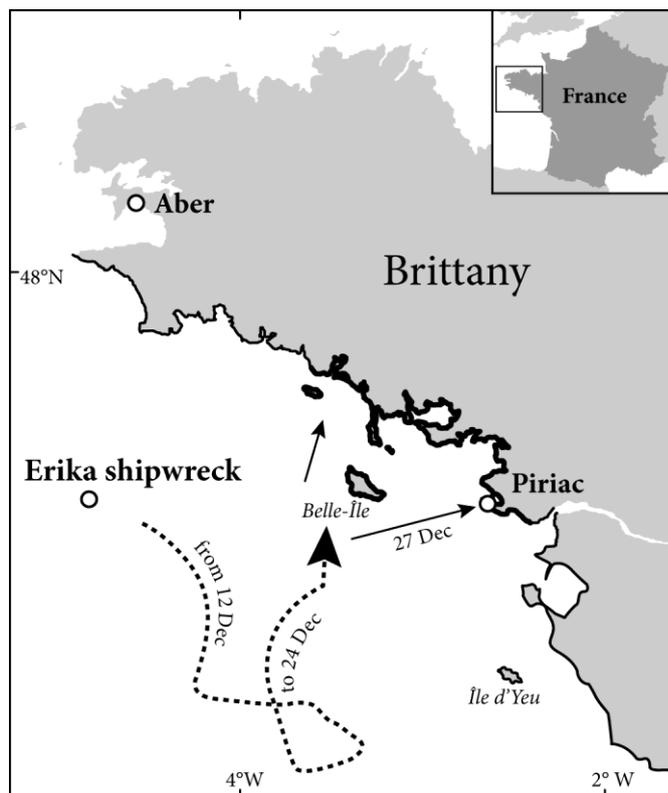
Table 5. Comparison of PAH load in starfish pyloric caeca in the field and under experimental conditions.

	Field		In-lab experiments		
	Day 58 Piriac	Short term contamination <sup>1</sup>	Long term contamination		End of the PAH loss experiment <sup>4</sup>
			Adults <sup>2</sup>	Juveniles <sup>3</sup>	
Sum PAHs	0.97	23.16 (24)	49.27 (51)	86.15 (89)	3.50 (4)
uns-PAHs	0.29	6.53 (23)	11.37 (40)	21.43 (75)	1.45 (5)
Pyr	0.062	0.79 (13)	1.19 (19)	2.73 (44)	-0.032 (-1)
Phe	0.026	2.29 (87)	3.12 (119)	7.12 (272)	0.28 (11)
DBT	0.0024	0.46 (193)	0.54 (228)	1.92 (803)	-0.0065 (-3)
Chrys	0.073	1.00(14)	1.72 (23)	3.97 (54)	0.012 (0.2)

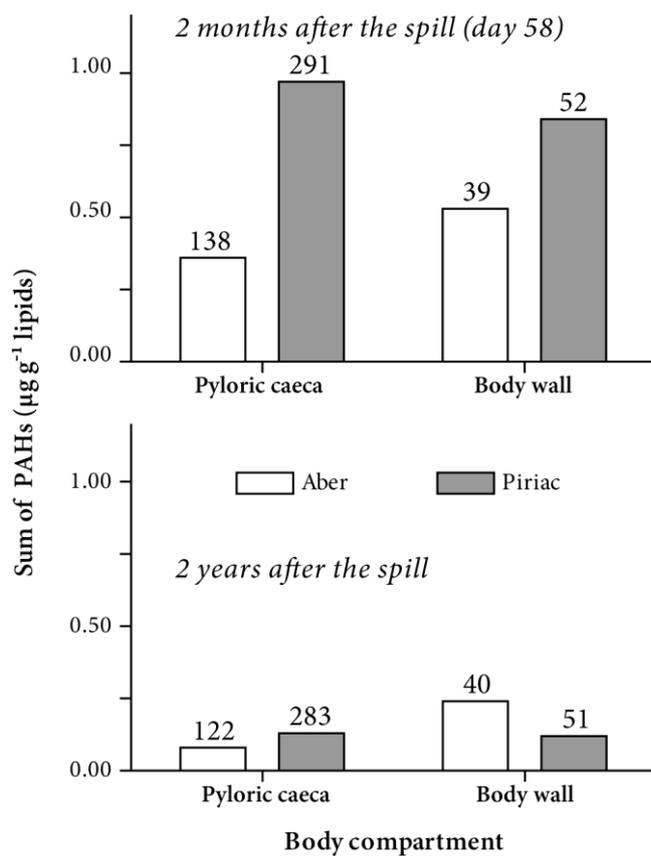
Laboratory experiments: <sup>1</sup>day 15 of PAH uptake experiment, <sup>2</sup>end of the first experiment on adults (1A), <sup>3</sup>day 0 of the PAH loss experiment, <sup>4</sup>day 23 of the PAH loss experiment (fitted values in g).

Sum PAH: sum of all analyzed PAHs, uns-PAHs: sum of analyzed unsubstituted PAHs, Pyr (pyrene), Phe (phenanthrene), DBT (dibenzothiophene), Chrys (chrysene) are expressed in  $\mu\text{g g}^{-1}$  of lipids. Numbers between brackets are the PAH concentrations expressed in multiples of the field-measured concentrations

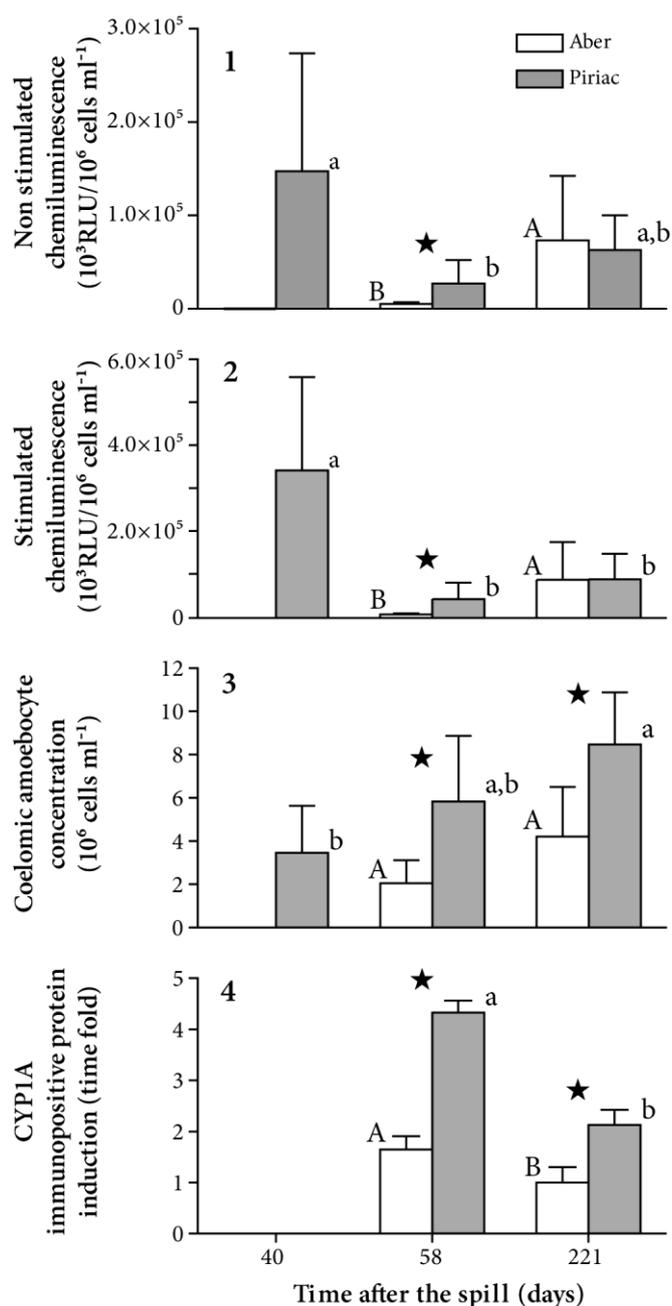
### Figure Captions



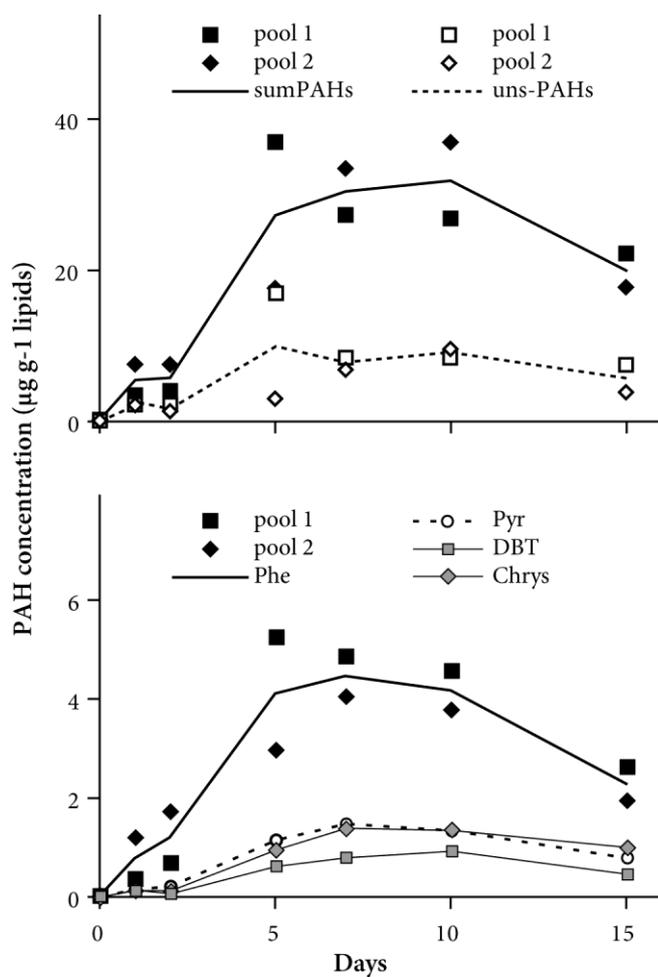
**Figure 1:** Map showing the location of field sites and of the Erika shipwreck. Piriac was contaminated by oil from Erika, whereas Aber remained free of this contamination. —: shoreline affected by the oil spill; dotted line: the main oil slick drift from December 12 to December 24, 1999 (from Laubier et al., 2004).



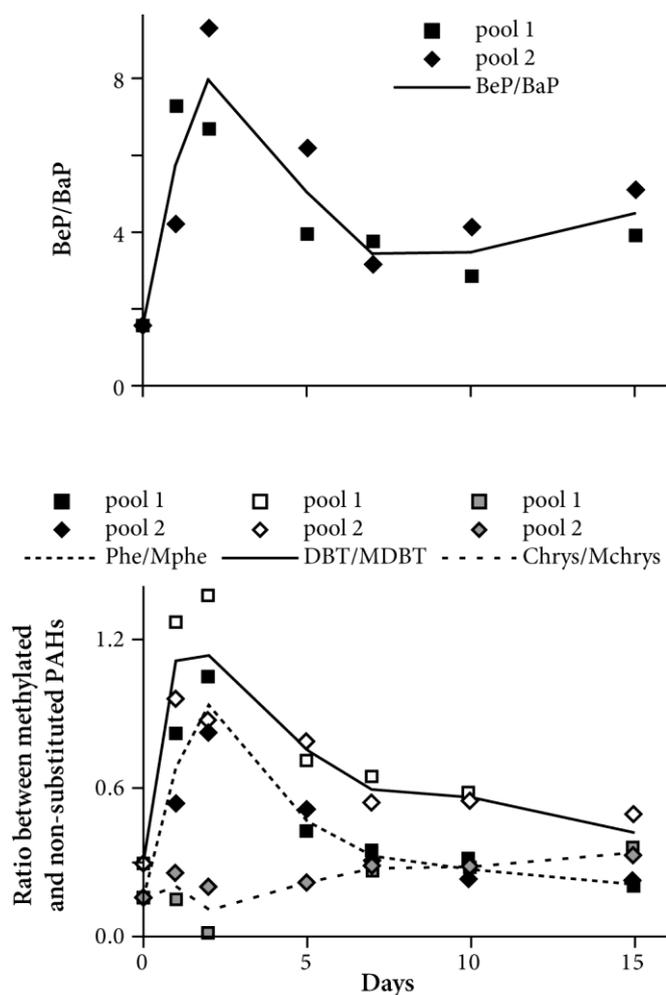
**Figure 2:** Total PAH levels in field-collected starfish 58 days and two years after the Erika oil spill. Piriac: oil-exposed site; Aber: reference site. All data are means,  $n = 2$  pools (10 starfish per pool). The numbers written above the histogram bars indicate the lipid contents ( $\text{mg g}^{-1}$ ) of the organs.



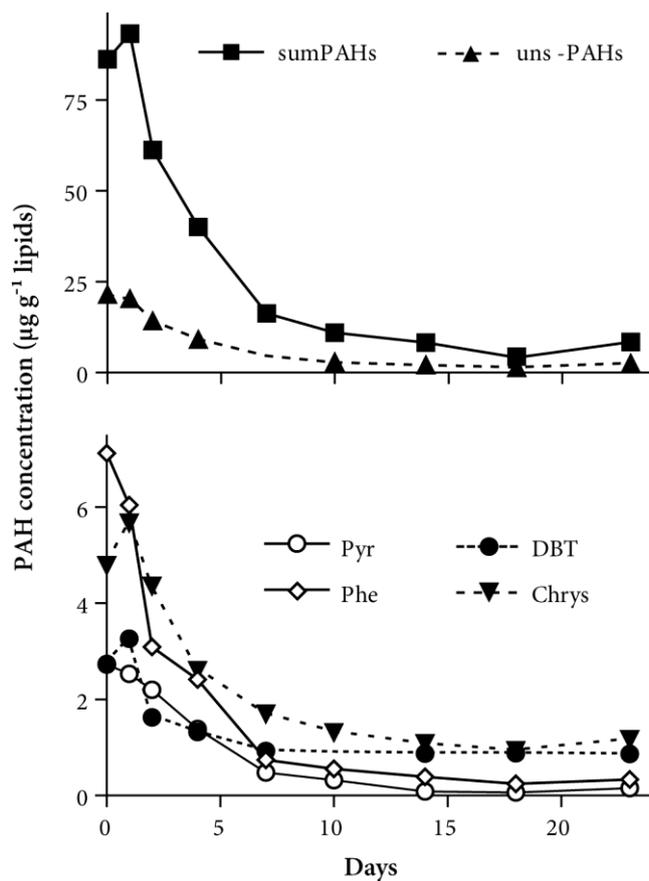
**Figure 3:** Temporal changes in biomarker responses after the oil spill in field-collected starfish from Piriac: oil-exposed site and Aber: reference site. All data are means  $\pm$  standard deviations. 1) ROS production by resting amoebocytes ( $n = 5$  or  $10$ ), 2) ROS production by stimulated amoebocytes ( $n = 5$  or  $10$ ), 3) Coelomic amoebocyte concentration ( $n = 5$  or  $10$ ) and 4) CYP1A induction ( $n = 4$ ). Histogram bars with the same letters are not significantly different. Lower case letters are for comparison of dates at Piriac, whereas capital letters are for comparison of dates at Aber, and ★ indicates a statistical difference between the sites at a given date. (ROS: reactive oxygen species; CYP1A: Cytochrome P450 immunopositive protein content).



**Figure 4:** Kinetics of PAH uptake in the pyloric caeca of starfish ( $n = 2$  pools of 10 starfish) fed with contaminated mussels over the agametogenic stage. Sum PAHs: sum of PAHs analyzed in this study, uns-PAHs: sum of unsubstituted PAHs, Pyr: pyrene, Phe: phenanthrene, DBT: dibenzothiophene, Chrys: chrysene.



**Figure 5:** Temporal changes in biotransformation ratios in the pyloric caeca of starfish (n = 2 pools of 10 starfish) fed with contaminated mussels. BeP: Benzo(e)pyrene, BaP: Benzo(a)pyrene, Phe: phenanthrene, MPhe: methyl-phenanthrene, DBT: dibenzothiophene, MDBT: methyl-dibenzothiophene, Chrys: chrysene, MChrys: methyl-chrysene.



**Figure 6:** Loss kinetics of PAH in pyloric caeca of starfish ( $n = 1$  pool of 4 starfish) fed with uncontaminated mussels over 23 days. sumPAHs: sum of all analysed PAHs, uns-PAHs: sum of non-substituted PAHs, Pyr: pyrene, Phe: phenanthrene, DBT: dibenzothiophene, Chrys: chrysene.