Toxicology in Vitro
September 2008, Volume 22, Issue 6, Pages 1610-1617
http://dx.doi.org/10.1016/j.tiv.2008.04.011

Effects of 16 pure hydrocarbons and two oils on haemocyte and haemolymphatic parameters in the Pacific oyster, Crassostrea gigas (Thunberg)

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Abstract:

The *in vitro* effects of polycyclic aromatic hydrocarbons (PAHs) on haemocyte and haemolymphatic parameters of the Pacific oyster, *Crassostrea gigas*, were tested using field concentrations (10^{-7} and 10^{-9} mg mL^{-1}) observed in the Marennes-Oleron Basin (Charente-Maritime, France) and high concentrations (10^{-3} and 10^{-5} mg mL^{-1}) observed during oil spills. As a first step, the effects of pollutants, after a 24 h contamination period, were monitored on individual and pooled haemolymph samples and similar results were observed for both sample types. In a second step, haemolymphs from 45 oysters were withdrawn and pooled. Eighteen pollutants were tested *in vitro* after a 4 and 24 h contamination period and 10 of them showed significant modulations of the five haemocyte parameters tested. Seven pollutants (fluorene, pyrene, anthracene, phenanthrene, chrysene, indeno[1,2,3-*c*,*d*]pyrene and heavy fuel oil (HFO)) induced a decrease in haemocyte mortality. Fluorene, pyrene and HFO significantly decreased phagocytosis activity. Percentage of non-specific esterase positive cells, phenoloxidase activity and lysosome presence were increased by naphthalene, benzo[*b*]fluoranthene and dibenz[*a*,*h*]anthracene, respectively. Modulation of immune parameters in the Pacific oyster by PAHs suggested that PAH pollution may be related to enhanced susceptibility to diseases.

Keywords: Haemocyte activity; Flow cytometry; Spectrophotometry; Pacific oyster; PAHs; Toxicity

1. Introduction

Among various chemical contaminants, the pollution caused by polycyclic aromatic hydrocarbons (PAHs) has led over recent years to numerous studies on the origin, distribution and fate of PAHs in the environment. Two main sources are identified: petrochemical (natural seepages, discharges of urban and industrial effluents, offshore oil production and oil spill) and pyrogenical (combustion processes due to human or natural fate). Concerning oil spill inputs, many authors have measured the concentrations of PAHs in the sediment and their effects on aquatic organisms before and after the event (Franco et al., 2006; Law, 1978; Morales-Caselles et al., 2006). These types of concerns were also studied in the case of chronic pollution (Budzinski et al., 1997; Halldorsson et al., 2004; Stehr et al., 2004; Van der Oost et al., 1991). Although many studies deal with PAH contamination of freshwater (Acheson et al., 1976; Tronczynski et al., 2004; WHO, 1997), only few authors have measured concentrations of the dissolved fraction of hydrocarbons in seawater (Boehm et al., 2007; Law, 1978; Maldonado et al., 1999). PAH concentrations following different oil spills ranged from 6.10^{-4} $mg.mL^{-1}$ after the *Exxon Valdez* oil spill (Boehm et al., 2007) to 17.10^{-4} $mg.mL^{-1}$ after the *Ekofisk* blowout (Law, 1978).

Anthropogenic contaminants, including PAHs, may affect the immunity of aquatic vertebrates (Reynaud et al., 2004; Yamaguchi et al., 1996) and invertebrates (Gagnaire et al., 2006a; Grundy et al., 1996a). Innate immunity in bivalve molluscs relies on haemocytes, the circulating cells present in extrapallial fluids, and haemolymph (Cheng, 1981; Johansson et al., 2000). Haemolymph contains also soluble effectors including phenoloxidase (PO) and antimicrobial peptides. Defence mechanisms, which include wound repair, coagulation, nodule formation, encapsulation, phagocytosis and cytotoxicity (Cheng, 1981), could be impaired by hydrocarbons (Coles et al., 1994; McVeigh et al., 2006; Wootton et al., 2003). Some authors described phagocytosis and other haemocyte parameters including non-specific esterase activity and lysosomal presence, as suitable biomarkers of pollution in different bivalve species (Auffret, 2005; Gagnaire et al., 2006a). PO was reported as been modified by xenobiotics in the Pacific oyster, *C. gigas* (Bouilly et al., 2006; Gagnaire et al., 2004).

Field concentrations of different PAHs were first determined in the Marennes-Oleron Basin (Charente-Maritime, France), where oyster production is an important economic activity. *In vitro* effects of selected hydrocarbons at high and field concentrations were then monitored on haemocytes of Pacific oysters, *C. gigas*. Sixteen PAHs were selected from the United States Environmental Protection Agency (US-EPA) list and two oils (heavy fuel oil (HFO) and diesel oil) were also tested. Cell mortality, phagocytosis, percentage of non-specific esterase positive cells and lysosome presence were monitored using flow cytometry. Phenoloxidase (PO) activity was studied in the acellular fraction by spectrophotometry.

2. Material and methods

2.1. Concentrations of PAHs in seawater samples

Three samples were collected in October 2005 from each site (Fig. 1). The harbour area corresponded to an oyster-farming harbour. One litre of subsurface seawater was collected at a depth of 3 m with polyethylene Nyskin's bottles and directly transferred to Duran glass bottles (Bioblock) which were heated to 500 °C before use. Samples were kept at 4 °C in the dark until analysis. Analyses of the aromatic compounds were carried out two days later.

Samples were extracted with 30 mL of dichloromethane pestipur quality (SDS). After separation of the organic and aqueous phases, water was extracted two additional times by the same volume of dichloromethane (2 X 30 mL). The organic extracts were purified and treated using gas chromatography coupled with mass spectrometry (GC-MS, Hewlett Packard HP5890 coupled with an HP5972 mass selective detector) following procedures previously described (Douglas et al., 1992).

2.2. Oysters

Two hundred Pacific oysters, *Crassostrea gigas*, 8-10 cm in shell length, were purchased from a shellfish farm (La Tremblade, Charente-Maritimes, France) in October 2005. Oysters were maintained, at Ifremer's laboratory (La Tremblade, France), for one month in tanks receiving a constant flow of external seawater (temperature 15.3-16 °C, pH 7.6, salinity 33.9-34.5 ‰, free of nitrate and nitrite).

Samples N°	Type of localisation	Latitudes Longitudes
1	Open sea	46° 05' 09.53" N 1° 16' 00.53" O
2	Open sea	46° 03' 00.10" N 1° 13' 09.80" O
3	Open sea	46° 03' 09.90" N 1° 14' 00.20" O
4	Coastal	46° 00' 09.70" N 1° 16' 00.53" O
5	Coastal	45° 00' 07.47" N 1° 09' 02.64" O
6	Coastal	45° 58' 00.00" N 1° 06' 06.50" O
7	Open sea	45° 56' 04.80" N 1° 08' 04.75" O
8	Oyster-farming	45° 52' 04.03" N 1° 10' 00.60" O
9	Oyster-farming	45° 51' 02.30" N 1° 10' 06.67" O
10	Harbour	45° 46' 09.17" N 1° 07' 02.78" O

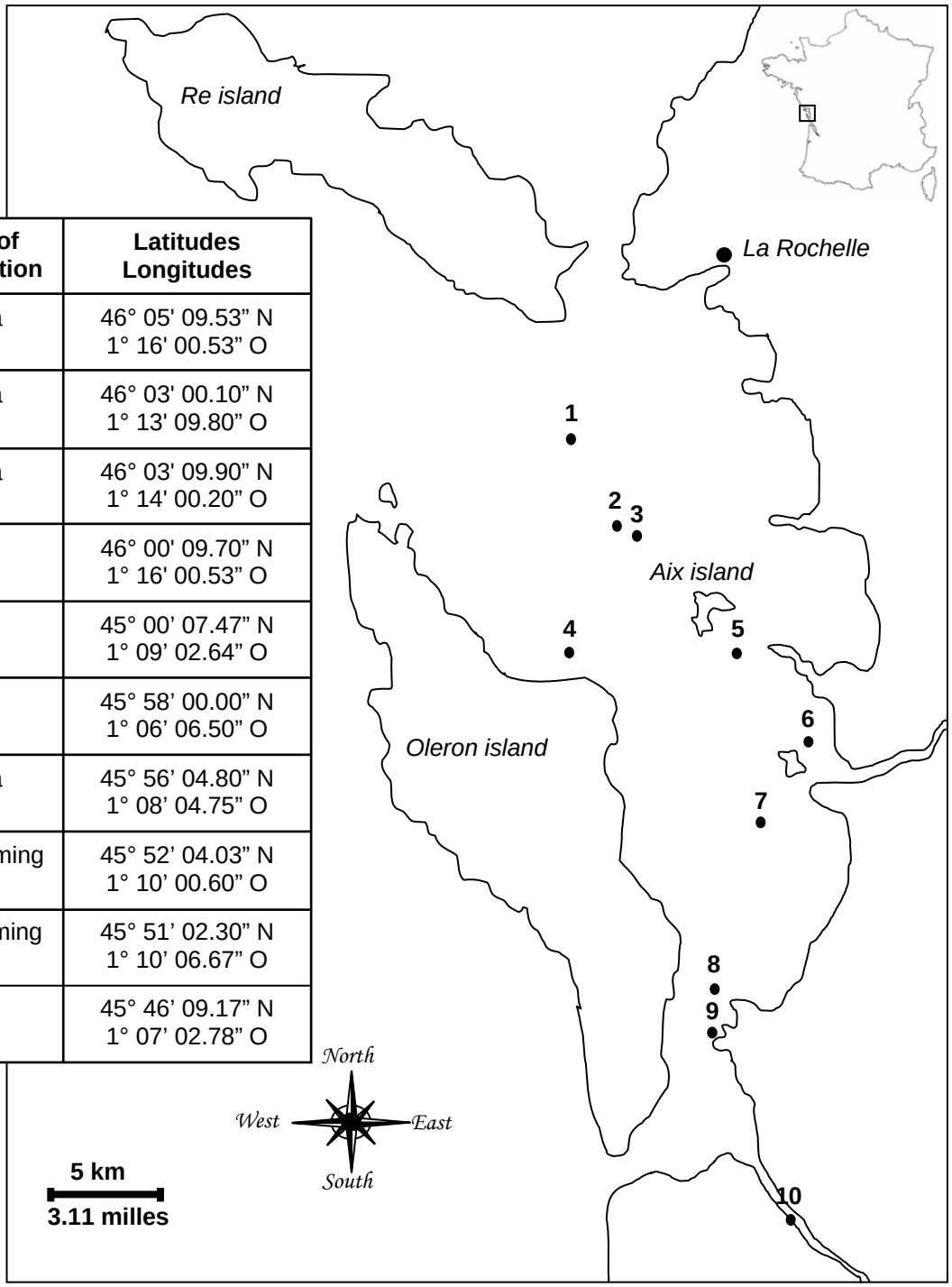

Fig. 1: Locations of the sampling sites in the Bay of Marennes-Oleron, background card issue from the National Geographic Institute.

Name of 16 PAHs US-EPA	Molecular weight ($g.mol^{-1}$)	Concentration ($\mu g.g^{-1}$ ± SE) in HFO	Concentration ($\mu g.g^{-1}$ ± SE) in diesel oil

Naphthalene	128,2	686.5 ± 51.5	1244.2 ± 49.9
Acenaphthylene	152,2	51.1 ± 1.8	72.4 ± 3.5
Acenaphthene	154,2	272.5 ± 8.4	90.7 ± 3.6
Fluorene	166,2	396.4 ± 11.5	187.2 ± 8.9
Phenanthrene	178,2	1936.9 ± 67.3	186.3 ± 15.2
Anthracene	178,2	213.6 ± 16.6	13.7 ± 0.8
Fluoranthene	202,3	125.2 ± 8.6	17.9 ± 1.1
Pyrene	202,3	516.2 ± 33.1	56.9 ± 3.5
Benz[*a*]anthracene	228,3	213.4 ± 8.0	1.4 ± 0.1
Chrysene	228,3	464.9 ± 8.2	6.5 ± 0.5
Benzo[*b+k*]fluoranthene	252,3	81.4 ± 6.8	n.d.
Benzo[*a*]pyrene	252,3	167.9 ± 4.5	n.d.
Benzo[*g,h,i*]perylene	276,3	48.1 ± 2.1	n.d.
Indeno [*1,2,3-c,d*] pyrene	276,3	16.6 ± 2.0	n.d.
Dibenz[*a,h*]anthracene	278,4	27.6 ± 3.3	n.d.

Table 1: Concentration of 16 priority PAHs of the US-EPA list in heavy fuel oil (HFO) and diesel oil. PAH detection was performed by gas chromatography coupled with mass spectroscopy (GC-MS). The results are expressed in $\mu g.g^{-1}$ (n = 3, mean ± standard error; n.d. = not detected).

2.3. Haemolymph collection

After breaching the oyster shell using pincers, approximately 2 mL of haemolymph were withdrawn from the posterior adductor muscle sinus using a 2 mL syringe equipped with a needle (0.6 x 25 mm). For each oyster, the haemolymph was filtered through a 60 µm mesh to eliminate debris and kept on ice until it was processed to reduce haemocyte aggregation (Auffret and Oubella, 1997).

For pre-experiments, which compared individual and pooled haemolymphs, 150 µL of ten haemolymph samples were pooled together. The remaining ten haemolymphs were kept for individual analysis. All samples were adjusted at 10^6 cells.mL^{-1} with artificial seawater (for 1 L: 234 g NaCl, 15 g KCl, 12 g $MgSO_4$ $4H_2O$, 1.5 g $CaCl_2$ $2H_2O$, 1.5 g $CaCl_2$ anhydrous; used at 1/10 dilution; all products come from Sigma) (Gagnaire et al., 2006a).

For the *in vitro* exposure, the haemolymph of 45 oysters was pooled, kept on ice to prevent haemocyte aggregation (Auffret and Oubella, 1997) and adjusted at 10^6 cells.mL^{-1} with artificial seawater (Gagnaire et al., 2006a).

Three pool samples were making for pre-experiments and for the *in vitro* exposure.

2.4. Experimental protocol

2.4.1. In vitro exposure protocol

For pre-experiments the difference between pool and individual contaminated samples was researched. Based on previously published results (Gagnaire et al., 2006a), two xenobiotics were selected. The effects of fluorene and pyrene were tested on cell mortality, phagocytosis percentage, percentage of non-specific esterase positive cells and lysosome presence. Both PAH were tested at 10^{-9} mg mL^{-1} at 15 °C during 24 h.

For *in vitro* exposure experiments, impact of chemicals on haemocyte parameters was monitored using 18 xenobiotics selected for their immunotoxic potential: 16 PAHs of the United States Environmental Protection Agency list (US-EPA) (WHO, 1997), and two oils (heavy fuel oil (HFO) and diesel oil). Concentrations of PAHs were determined in heavy fuel oil (HFO) and diesel oil (Table 1). High (10^{-3} and 10^{-5} mg.mL^{-1}) and field (10^{-7} and 10^{-9} mg.mL^{-1}) concentrations were tested for each PAH. The two oils were tested in their pure form and diluted to 1/1 000 and 1/10 000 in artificial seawater after a three-day contact period without mixing at ambient temperature (20°C) as previously described (Anderson et al., 1974).

Pollutants (PAHs and oils) were added individually at 5 µL per mL of haemocyte suspension. Before addition, PAHs were dissolved in cyclohexane (Sigma) and the ratio cyclohexane:haemocyte suspension did not exceed 0.5 % as recommended by manufacturers in order to avoid disturbance of cell parameters. In all experiments, the same volume of cyclohexane was used as solvent control and haemolymph alone was used as cell control. Samples were incubated at 15°C during 4 and 24 h as previously described by Gagnaire et al. (2006a). Experiments including controls were carried out three times.

2.4.2. Cell analysis by flow cytometry

Haemocyte mortality, phagocytosis percentage, percentage of non-specific esterase positive cells and lysosome presence were analysed with an EPICS XL 4 (Beckman Coulter). For each haemocyte sample, 10 000 events were counted using protocols previously described (Gagnaire et al., 2006a). Analyses were carried out on whole haemocytes without distinguishing cell subpopulation and results were expressed as percentage of positive cells.

Cell mortality was measured using FL3 (red fluorescence). Propidium iodide (PI, 1.0 g.L^{-1}, Molecular Probes) is membrane impermeant and is excluded from viable cells. Mortality was determined using 200 µL of haemocyte suspension and 10 µL of PI. Cell suspensions were incubated for 30 minutes at 4°C.

The phagocytosis percentage was determined using FL1 (green fluorescence). Fluorescent microspheres (2.7×10^{10} particles.mL^{-1}, Fluorospheres® carboxylate-modified microspheres, diameter 1 µm, Molecular Probes) were used and the fluorescence setting was established using a suspension of fluorescent beads in distilled water. Only the events showing a fluorescence of at least three beads were considered positive for phagocytic activity. Phagocytic activity of haemocyte suspensions was analysed on 200 µL of haemolymph samples and 10 µL of a 1/10 dilution of fluorescent beads. Cell suspensions were incubated for one hour at room temperature.

Percentage of non-specific esterase positive cells was analysed using the non-specific liposoluble substrate fluoresceine diacetate (FDA, Molecular Probes). One µL of a FDA solution (400 µM) was added to 200 µL of haemocyte suspension. Cells were incubated for 30 minutes in the dark at room temperature and then the reaction was stopped on ice (5 min).

Lysosome presence was analysed with a commercial kit (LysoTracker ® Green DND-26, 1mM in DMSO, Molecular Probes) which consists of a fluorophore linked to a weak base that is partially protonated at neutral pH. The LysoTracker® is freely permeant to cell membranes and typically concentrated in lysosomes. One µL of a LysoTracker marker was added to 200 µL of haemocyte suspension. Samples were incubated for two hours in the dark at room temperature and then the reaction was stopped on ice (5 min).

2.4.3. PO activity analysis

Haemolymph samples were centrifuged (260 g, 10 min, 4°C) and supernatants recovered. Detection of phenoloxidase (PO) activity in acellular fraction samples was carried out by measurement of L-3,4-dihydroxyphenylalanine (L-Dopa, Sigma) transformation in dopachromes as previously described by Gagnaire et al. (2004). Samples were distributed in 96-well microplates (Nunc, France). PO modulators were used to confirm the specificity of the detection. The purified trypsin TPCK (*N*-Tosyl-L-phenylalanine chloromethyl ketone, 1 g.L^{-1}, Sigma) was used as an activator and the β-2-mercaptoethanol (10 mM, Sigma) was used as an inhibitor. To determine the PO activity, 80 µL of cacodylate buffer (CAC buffer: sodium cacodylate (10 mM), trisodium citrate (100 mM), NaCl

(0.45 M), $CaCl_2$ (10 mM), $MgCl_2$ (26 mM), pH 7.0), 20 µL of L-Dopa (3 $mg.mL^{-1}$) and 20 µL of samples were added in each well. To measure the PO activity modulation, 60 µL of CAC buffer, 20 µL of PO modulators, 20 µL of L-Dopa and 20 µL of samples were added to each well. Control (120 µL of CAC buffer) and negative control (100 µL of CAC buffer, 20 µL of L-Dopa) wells were used to determine respectively the purity of the buffer and the autooxidation capacities of L-Dopa. Each sample was tested in nine replicates and absorbance was measured at 490 nm after a 21 h incubation period at room temperature.

2.5. Statistical analysis

Statistical tests were carried out using XLStat Pro 7.5.3. A Kruskal-Wallis test, for non normal values, was used to analyse pollutant effects and a Mann-Whitney test was applied to compare the protocol effects. In the case of the rejection of H_0, a Dunn test for non normal values was used. P values lower than 0.05 were used to identify significant differences.

3. Results

3.1. PAH concentrations in the Marennes-Oleron Basin (Charente-Maritime, France)

The concentrations of 16 PAHs in seawater samples are given in Table 2. The values ranged from 0 to 53.8 $ng.L^{-1}$ with a total concentration of 70.5 ± 10.8 $ng.L^{-1}$. Indeno[*1,2,3-c,d*]pyrene, benzo[*b*]fluoranthene and benzo[*g,h,i*]perylene were not detected at all sampling sites. Acenaphthene, chrysene, fluoranthene, fluorene, naphthalene and phenanthrene were detected at all sampling sites. Three types of sampling sites, "open sea", "coastal and harbour" and "oyster-farming", were differentiated in terms of total amounts of seawater PAH concentrations. The "open sea" sites showed the lowest concentrations with values ranging from 12.3 ± 1.4 $ng.L^{-1}$ for naphthalene to 0.1 $ng.L^{-1}$ for chrysene and with a total concentration of 39.0 ± 3.7 $ng.L^{-1}$.Twelve PAHs among the 16 analysed were detected. The "coastal and harbour" sites showed concentrations ranging from 33.8 ± 3.4 $ng.L^{-1}$ for acenaphthene to 0.8 ± 0.5 $ng.L^{-1}$ for anthracene and with a total concentration of 75.9 ± 3.3 $ng.L^{-1}$.Eight PAHs among the 16 analysed were detected. The highest total concentration (123.1 ± 18.1 $ng.L^{-1}$) was observed in the "oyster-farming" sites with values ranging from 48.7 ± 5.2 $ng.L^{-1}$ for naphthalene to 0.2 ± 0.2 $ng.L^{-1}$ for acenaphthylene. Nine PAHs among the 16 analysed were detected.

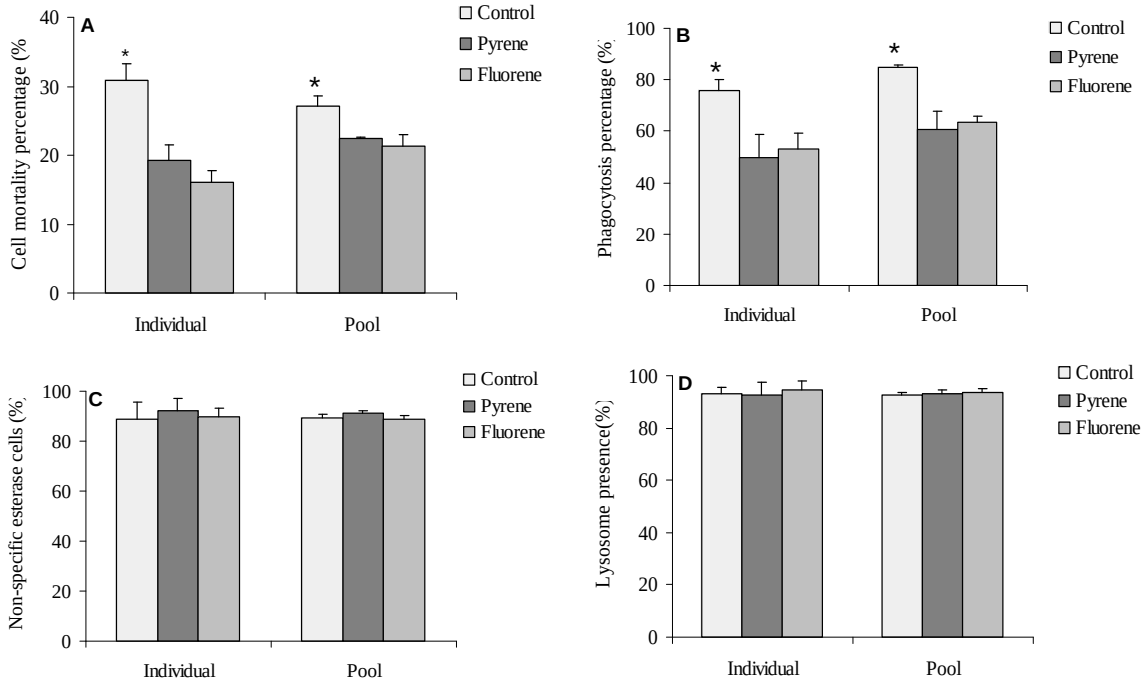

Fig. 2: Cellular mortality percentage (A), phagocytic percentage (B), percentage of non-specific positive esterase cells (C) and lysosome presence (D) of haemocytes measured by flow cytometry following an *in vitro* exposure of 24 h at 15°C to pyrene, fluorene at 10^{-9} mg.mL^{-1} for both samples (individual and pooled samples). Values are the mean of three replicates. The bars represent the standard error. * = statistical difference for $p \leq 0.05$.

Samples N° / 16 PAHs US-EPA	Open sea					Coastal and harbour					Oyster-farming			Mean (± SE)
	1	**2**	**3**	**7**	***Mean***	**4**	**5**	**6**	**10**	***Mean***	**8**	**9**	***Mean***	
Naphthalene	15.0 ± 0.6	14.4 ± 0.9	9.2 ± 0.6	10.4 ± 0.3	***12.3 ± 1.4***	25.8 ± 0.2	25.8 ± 0.4	25.7 ± 0.5	18.5 ± 0.5	***24.0 ± 1.8***	53.8 ± 0.2	43.5 ± 0.5	***48.7 ± 5.2***	**25.2 ± 4.6**
Acenaphthylene	2.6 ± 0.1	2.6 ± 0.0	2.4 ± 0.0	2.7 ± 0.1	***2.6 ± 0.1***	n.d.	n.d.	n.d.	n.d.	***n.d.***	n.d.	0.4 ± 0.0	***0.2 ± 0.2***	**0.9 ± 0.4**
Acenaphthene	8.0 ± 0.1	8.0 ± 0.0	8.2 ± 0.2	8.1 ± 0.1	***8.1 ± 0.0***	33.6 ± 0.5	29.8 ± 0.3	28.3 ± 0.2	43.4 ± 0.5	***33.8 ± 3.4***	43.5 ± 0.3	33.6 ± 0.2	***38.6 ± 5.0***	**26.3 ± 4.7**
Fluorene	3.4 ± 0.1	4.9 ± 0.1	3.3 ± 0.1	4.9 ± 0.2	***4.1 ± 0.4***	1.8 ± 0.1	2.1 ± 0.0	2.0 ± 0.0	1.5 ± 0.0	***1.9 ± 0.1***	1.2 ± 0.0	1.4 ± 0.0	***1.3 ± 0.1***	**2.6 ± 0.4**
Phenanthrene	1.4 ± 0.1	2.0 ± 0.1	0.4 ± 0.0	1.5 ± 0.1	***1.3 ± 0.3***	8.4 ± 0.1	13.1 ± 0.2	5.1 ± 0.1	6.3 ± 0.2	***8.2 ± 1.8***	6.0 ± 0.1	13.2 ± 0.2	***9.6 ± 3.6***	**6.2 ± 1.5**
Anthracene	n.d.	n.d.	n.d.	n.d.	***n.d.***	1.3 ± 0.0	n.d.	n.d.	1.8 ± 0.0	***0.8 ± 0.5***	n.d.	2.1 ± 0.0	***1.1 ± 1.1***	**0.6 ± 0.3**
Fluoranthene	3.6 ± 0.1	3.5 ± 0.0	3.3 ± 0.0	3.5 ± 0.1	***3.5 ± 0.1***	2.8 ± 0.0	0.9 ± 0.0	0.8 ± 0.1	1.0 ± 0.0	***1.4 ± 0.5***	1.2 ± 0.0	2.4 ± 0.0	***1.8 ± 0.6***	**2.2 ± 0.4**
Pyrene	3.1 ± 0.1	3.0 ± 0.1	4.0 ± 0.1	2.8 ± 0.1	***3.2 ± 0.3***	n.d.	2.7 ± 0.0	2.0 ± 0.0	3.9 ± 0.1	***2.2 ± 0.8***	2.6 ± 0.0	3.7 ± 0.0	***3.2 ± 0.5***	**2.7 ± 0.4**
Benz[*a*]anthracene	0.4 ± 0.0	0.3 ± 0.0	0.5 ± 0.0	0.4 ± 0.0	***0.4 ± 0.0***	n.d.	n.d.	n.d.	n.d.	***n.d.***	n.d.	n.d.	***n.d.***	**0.1 ± 0.1**
Chrysene	0.1 ± 0.0	0.1 ± 0.0	0.2 ± 0.0	0.1 ± 0.0	***0.1 ± 0.0***	2.0 ± 0.0	8.0 ± 0.1	3.0 ± 0.1	1.9 ± 0.0	***3.7 ± 1.4***	32.8 ± 0.3	4.7 ± 0.1	***18.8 ± 14.1***	**5.9 ± 3.2**
Benzo[*b*]fluoranthene	n.d.	n.d.	n.d.	n.d.	***n.d.***	n.d.	n.d.	n.d.	n.d.	***n.d.***	n.d.	n.d.	***n.d.***	**n.d.**
Benzo[*k*]fluoranthene	0.8 ± 0.0	0.7 ± 0.0	0.8 ± 0.0	0.8 ± 0.0	***0.8 ± 0.0***	n.d.	n.d.	n.d.	n.d.	***n.d.***	n.d.	n.d.	***n.d.***	**0.3 ± 0.1**
Benzo[*a*]pyrene	1.5 ± 0.0	1.4 ± 0.0	1.4 ± 0.0	1.4 ± 0.0	***1.4 ± 0.0***	n.d.	n.d.	n.d.	n.d.	***n.d.***	n.d.	n.d.	***n.d.***	**0.5 ± 0.2**
Benzo[*g,h,i*]perylene	n.d.	n.d.	n.d.	n.d.	***n.d.***	n.d.	n.d.	n.d.	n.d.	***n.d.***	n.d.	n.d.	***n.d.***	**n.d.**
Indeno [*1,2,3-c,d*] pyrene	n.d.	n.d.	n.d.	n.d.	***n.d.***	n.d.	n.d.	n.d.	n.d.	***n.d.***	n.d.	n.d.	***n.d.***	**n.d.**
Dibenz[*a,h*]anthracene	2.3 ± 0.0	n.d.	n.d.	2.4 ± 0.0	***1.2 ± 0.7***	n.d.	n.d.	n.d.	n.d.	***n.d.***	n.d.	n.d.	***n.d.***	**0.3 ± 0.3**
Mean (± SE)	**42.2 (± 5.2)**	**40.9 (± 2.6)**	**33.7 (± 0.9)**	**39.0 (± 0.4)**	***39.0 (± 3.7)***	**75.7 (± 2.9)**	**82.4 (± 3.4)**	**66.9 (± 2.6)**	**78.4 (± 3.2)**	***75.9 (± 3.3)***	**141.1 (± 6.1)**	**105.0 (± 5.8)**	***123.1 (± 18.1)***	**70.5 (± 10.8)**

Table 2: Concentration of 16 priority PAHs of the US-EPA list in seawater samples as function of sample localisations. PAH detection was performed by gas chromatography coupled with mass spectroscopy (GC-MS). The results are expressed in $ng.L^{-1}$ (n = 3, mean ± standard error; n.d. = not detected which corresponded to a detection limit of 0.1 $ng.L^{-1}$). With no statistical difference at $p \leq 0.05$ between each localisation type.

3.2. Pre-experiments: comparing individual and pooled haemocytes after incubation with pyrene and fluorene

Similar results were obtained between cell (haemolymph alone) and solvent controls (haemolymph plus cyclohexane) for each parameter studied (data not shown).

Cell mortality was not significantly different between individual (19.3 ± 2.2 %, 16.0 ± 1.7 % and 30.9 ± 2.3 % for pyrene, fluorene and control, respectively) and pooled haemocytes (22.5 ± 0.1 %, 21.3 ± 1.6 % and 27.1 ± 1.4 % for pyrene, fluorene and control, respectively (Fig. 2). Pyrene and fluorene at 10^{-9} mg.mL^{-1} induced a significant decrease in haemocyte mortality for both sample types.
Phagocytosis percentages were similar for individual (49.8 ± 9.1 %, 52.9 ± 6.1 % and 76.1 ± 4.0 % for pyrene, fluorene and control, respectively) and pooled haemocytes (60.7 ± 6.9 %, 63.4 ± 2.6 % and 85.0 ± 0.6 % for pyrene, fluorene and control, respectively). An equal decrease in phagocytosis percentage was shown with pyrene and fluorene at 10^{-9} mg.mL^{-1} compared to control samples.
Percentages of non-specific esterase positive cells were similar for individual (92.4 ± 4.7 %, 89.8 ± 3.6 % and 88.9 ± 6.5 % for pyrene, fluorene and control, respectively) and pooled haemocytes (91.0 ± 1.2 %, 88.9 ± 1.6 % and 89.2 ± 1.7 % for pyrene, fluorene and control, respectively). Percentages of cells presenting lysosomes were similar for individual (92.4 ± 5.3 %, 94.8 ± 3.1 % and 89.9 ± 2.3 % for pyrene, fluorene and control, respectively) and pooled haemocytes (93.2 ± 1.2 %, 93.6 ± 1.6 % and 92.4 ± 1.2 % for pyrene, fluorene and control, respectively). No significant difference in percentages of non-specific esterase positive cells and lysosome presence was observed in the presence of pyrene and fluorene compared to control samples.

3.3. *In vitro* exposure (Table 3)

Compared to control samples (28.3 ± 3.5 %), fluorene (21.0 ± 2.7 %), pyrene (20.7 ± 2.3 %), anthracene (23.6 ± 1.7 %), phenanthrene (23.0 ± 2.7 %), chrysene (23.0 ± 2.5 %), indeno[*1,2,3-c,d*]pyrene (20.1 ± 3.1 %) and HFO (21.8 ± 1.2 %) significantly decreased haemocyte mortality after a 24 h incubation. Phagocytosis activity was decreased by fluorene (28.0 ± 2.0 %), pyrene (27.6 ± 2.6 %) and pure HFO (33.5 ± 9.3 %) after a 24 h

18 pollutants used	Cell mortality (%)	Phagocytosis (%)	Non-specific esterases cells (%)	Lysosome presence (%)	PO activity ($A_{490\ nm}$)
Naphthalene			△ EC (**, 24h)		
Acenaphthylene					
Acenaphthene					
Fluorene	▼ EC (*, 24h)	▼ EC (*, 24h)			
Phenanthrene	▼ EC (*, 24h)				
Anthracene	▼ EC (*, 24h)				
Fluoranthene					
Pyrene	▼ EC (*, 24h)	▼ EC (*, 24h)			
Benz[*a*]anthracene					
Chrysene	▼ EC (*, 24h)				
Benzo[*b*]fluoranthene					△ 10^{-7}, 10^{-9} $g.L^{-1}$ (*, 24h)
Benzo[*k*]fluoranthene					
Benzo[*a*]pyrene					
Benzo[*g,h,i*]perylene					
Indeno[*1,2,3-c,d*]pyrene	▼ EC (**, 24h)				
Dibenz[*a,h*]anthracene				△ EC (**, 24h)	
HFO	▼ EC (**, 24h)	▼ pure (*, 24h)			
Diesel oil					

Table 3: Cellular mortality, phagocytosis, non-specific esterase positive cells percentages and lysosome presence of oysters measured by flow cytometry and phenoloxidase (PO) activity performed by spectrophotometry following an *in vitro* exposure of 4 and 24 h at 15°C to 18 hydrocarbons at 10^{-3}, 10^{-5}, 10^{-7} and 10^{-9} $mg.mL^{-1}$. Values are means of three replicates of 45 oysters (± standard error). * = statistical difference for $p \leq 0.05$ and ** for $p \leq 0.01$; △ = significant increase; ▼= significant decrease; EC = each concentration.

incubation compared to control samples (51.7 ± 12.0 %). On the contrary, percentage of non-specific esterase positive cells (93.2 ± 0.4 % and 89.1 ± 0.3 % for naphthalene and control, respectively) and lysosome presence (90.9 ± 2.3 % and 88.1 ± 3.0 % for dibenz[*a,h*]anthracene and control, respectively) were increased by naphthalene and dibenz[*a,h*]anthracene.
Phenoloxidase activity was increased by purified trypsin TPCK and inhibited by β-2-mercaptoethanol (data not shown). Phenoloxidase activity was significantly increased with benzo[*b*]fluoranthene at 10^{-7} (0.65 ± 0.07) and 10^{-9} mg.mL^{-1} (0.63 ± 0.08) after a 24 h incubation period compared to control samples (0.47 ± 0.10).

4. Discussion

The aim of the present work was to determine the *in vitro* effects of high and field concentrations of hydrocarbons on haemocyte parameters of Pacific oysters, *Crassostrea gigas*. The tested concentrations of hydrocarbons were determined based on the literature and experimental values (determining PAH concentrations in seawater in the Marennes-Oleron Basin). The high concentrations tested in the present study (10^{-3} and 10^{-5} mg.mL^{-1}) could be compared to those observed after an oil spill. After the *Prestige* oil spill total aromatic hydrocarbons were detected at an average concentration of 3.10^{-5} mg.mL^{-1} on the Northern Spanish coast (Gonzalez et al., 2006). The concentrations detected after the *Prestige* oil spill were in a lower range than those quoted in other accidentally polluted areas. Values up to 10^{-5} mg.mL^{-1} were reported in the coastal waters of Brittany after the *Amoco Cadiz* oil spill (Marchand, 1980). For field concentrations (10^{-7} and 10^{-9} mg.mL^{-1}), information is available for rivers, including German rivers, Dutch rivers, the Yellow River in China (WHO, 1997) and the Thames in Great Britain (Acheson et al., 1976). Field concentrations of PAHs around 10^{-9} mg.mL^{-1} were reported in the marine environment (Hellou et al., 2005; WHO, 1997). In the Marennes-Oleron Basin, no information on concentrations of hydrocarbons was available. In this study, PAH concentrations ranged from 10^{-7} and 10^{-9} mg.mL^{-1}. In concordance with results obtained in seawater on the German coast (WHO, 1997), the composition and the concentration of PAHs in the seawater of the Marennes-Oleron Basin depend of the sampling sites. Our results showed that more PAHs were detected in "open sea" sites compared to "oyster-farming" and "coastal and harbour" sites. Nevertheless, lower total PAH concentration was observed in the open sea. In all sites, no detectable concentration of indeno[*1,2,3-c,d*]pyrene, benzo[*b*]fluoranthene and benzo[*g,h,i*]perylene was found. The absence of detection in seawater may be explained by their high molecular weight which makes them less soluble and increases their affinity with particles (Meador et al., 1995). Thus, for toxicology studies, field seawater concentrations of soluble PAHs ranged from 10^{-7} and 10^{-9} mg.mL^{-1}, without emulsion or microlayers, seems to be acceptable.
Studies on Pacific oyster, *C. gigas*, haemocytes have used either individual (Auffret and Oubella, 1997; Duchemin et al., 2007; Jeong and Cho, 2005) or pooled samples (Aton et al., 2006; Gagnaire et al., 2006b; Luna-González et al., 2003). The use of individual samples allows few successive tests in similar conditions and with the same specimens but with a greater range of variation (Auffret and Oubella, 1997). On the contrary, the use of pooled samples provided a sufficient quantity of cells to carry out multiple analyses with the same group of animals and minimize effects related to inter-individual variations (Gagnaire et al., 2004). Results show that values for haemocyte parameters assessed using a pool of ten oyster haemolymphs were similar to the mean of ten individual values for both pollutants. For each immunotoxicity studies, pooled samples may be used to test a large number of pollutants and/or of cell parameters. Concerning difference between pool and individual contaminated samples for PO activity, previous results shown that no significant difference between both sample types was observed (Thomas-Guyon et al., personal communication).

Among the 16 pure hydrocarbons and the two oils tested, ten chemicals (nine pure hydrocarbons plus HFO) were able to modify at least one haemocyte parameter after a 24 h incubation period. A decrease in cell mortality was reported for four PAHs (anthracene, chrysene, indeno[*1,2,3-c,d*]pyrene and phenanthrene) and a decrease in both cell mortality and phagocytosis percentage for three hydrocarbons (fluorene, pyrene and HFO). A phagocytosis decrease could therefore not be induced by an increase in cell destruction, but rather by a decrease in haemocyte immune capacities. Gagnaire et al. (2006a) observed that benzo[*a*]pyrene, phenanthrene and anthracene tested on Pacific oyster haemocytes induced a similar decrease in cell mortality after a 24 h incubation. The impact of hydrocarbons on haemocytes was controversial. Sami et al. (1992) and Jeong and Cho (2005) reported a decrease in the number of haemocytes in the oysters, *C. virginica* and *C. gigas*, without

affecting cell mortality, when Coles et al. (1994) observed that fluoranthene increased the total cell number without modifying subpopulation percentages. Furthermore, Grundy et al. (1996b) demonstrated an *in vitro* inhibitor effect on the common mussel, *Mytilus edulis*, with anthracene, fluoranthene and phenanthrene. In marine environment, microorganisms, especially bacteria, play an important role in the aerobic biodegradation of PAHs essentially for phenanthrene, fluorene and anthracene (Kasai et al., 2002). Moreover, microorganisms were naturally present in haemolymph related to the open circulatory system in bivalves (Cheng, 1981). Then, the diminution of cell mortality in contaminated haemocytes compared to control haemocytes could be explain by two hypothesis: i) for high concentrations, bacteria are more impaired by hydrocarbons than haemocytes; ii) for field concentrations, it means lower level of hydrocarbons, PAHs are more accessible to bacteria as carbon and energy sources than haemocytes.

Naphthalene has a low molecular weight and a toxic potential due to its polycyclic nature. In our experimental conditions, each concentration of naphthalene increased percentage of non-specific esterase positive cells, after a 24 h incubation. In the opposite, decrease of percentage of non-specific esterase positive cells were shown after 4 and 24 h of oyster haemocyte exposure to benzo[*a*]pyrene, phenanthrene, anthracene and fluoranthene (Gagnaire et al., 2006a). This discrepancy could be due to the concentrations used in each study.

In this study, dibenz[*a,h*]anthracene is associated with an increase in lysosome presence after *in vitro* contact at high and field concentrations. Lysosomes are usually decreased by PAHs which induced destabilisation of lysosomal membranes (Gagnaire et al., 2006a; Grundy et al., 1996b; Grundy et al., 1996a; McVeigh et al., 2006). However, Braunbeck and Appelbaum (1999) also observed a proliferation of lysosomes on intestinal epithelium in *Cyprinus carpio* after oral contamination with an ultra-low dose of cyclodiene insecticide endosulfan. This lysosomal proliferation has to be classed as an unspecific reaction of the intestinal mucosa to stress due to xenobiotics (Braunbeck and Appelbaum, 1999). In this study, the increase in lysosomal percentage might also represent unspecific signs of stress.

The PO system is an important immune defence mechanism in invertebrates which is found in the Pacific oyster, *Crassotrea gigas* (Hellio et al., 2007). In this work, PO activity increased after 24 h of *in vitro* contact between haemolymph free of cells and benzo[*b*]fluoranthene at high concentrations. Some authors have previously described an increase in PO activity after *in vivo* pollutant exposures, e.g. fluoranthene in mussels (Coles et al., 1994), tributyltin and copper in tunicates (Tujula et al., 2001) and cadmium in Pacific oysters (Bouilly et al., 2006). On the contrary, some pollutants decreased PO activity *in vitro* such as mercury in the Pacific oysters (Gagnaire et al., 2004) and *in vivo* such as trichlorfon in prawns (Chang et al., 2006). In these *in vitro* experimentations, benzo[*b*]fluoranthene does not modulate other haemocyte parameters, thus the increase in PO activity in Pacific oysters could be due to direct action of the pollutant on this activity.

Conclusions

This work analyse the *in vitro* effects of 16 pure hydrocarbons and two oils on immune haemocyte capacities. Each parameter was modulated by at least one pollutant. In the case of fluorene and pyrene, which are very representative in HFO, they have effects on both cellular mortality and phagocytic activity. Moreover, the PAHs which had immune effects are more accurately represented in HFO than in diesel oil, which had no effect on immune parameters. It could be interesting to develop the synergic or antagonist effects of these potential immunomodulators and to research their effects in *in vivo* experimentations.

Acknowledgments

M. Girin is acknowledged for allowing the work to be conducted at *Cedre* in Brest (Brittany, France). This research was supported by the Ifremer research program ‘MORtalités ESTivales’ (France) and by the European program ‘Interreg IIIB, Erocips’. Thanks to Sally Ferguson for her reading of this document and to Dominique Vilday for his help with the seawater sampling in the Marennes-Oleron Basin.

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