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Oxidative stress and immunologic responses following a dietary exposure to PAHs in *Mya arenaria*

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Abstract

Background: The aim of this research was to investigate oxidative stress and immune responses following a dietary polycyclic aromatic hydrocarbon (PAH) exposure in a marine bioindicator organism, the soft shell clam, *Mya arenaria*. Immune parameters in hemolymph (haemocyte number, efficiency of phagocytosis and haemocyte activity) and assessment of oxidative stress using catalase (CAT) activity and levels of malondialdehyde (MDA) performed on the digestive gland were estimated as biomarkers in clams fed in mesocosm with PAH contaminated phytoplankton. MDA levels and CAT activities were also measured *in situ* in organisms sampled in a control site (Metis Beach, Québec, Canada) as well as organisms sampled in a site receiving domestic effluents (Pointe-au-Père, Québec, Canada), to assess effects of abiotic variables related to seasonal variations and mixed contamination on the selected parameters.

Results: Results on immune parameters suggest that the PAHs may interfere with the maturation and/or differentiation processes of haemocytes. MDA results showed that lipid peroxidation did not occur following the exposure. The levels of CAT activity corresponded to weak antioxidant activity (no significant differences). Recovery was noted for all the immune endpoints at the end of the experiment.

Conclusion: Results suggest that immune parameters are early biomarkers that can efficiently detect a physiological change during a short term exposure to low concentrations of PAHs. The *in situ* survey (in the natural environment) suggested that clams from the Pointe-au-Père site did not show any oxidative stress as well as the clams contaminated in mesocosm, probably due to the low concentrations of PAHs used for this study. MDA levels increased however in organisms from Metis Beach, a response probably related to domestic effluents or parasitism.

Background

Polycyclic Aromatic Hydrocarbons (PAHs) are ubiquitous in the environment. They are produced by incomplete combustion of solid and liquid fuels. High molecular weight PAHs are predominantly adsorbed on soot and particulate matter before being transported in aquatic environment. According to environmental conditions, they may accumulate in sediments [1]. Bioavailability to

marine organisms is a consequence of different mechanisms such as bioturbation, sediment resuspension and diffusion. Bivalve molluscs are suitable species to assess toxicity in the environment due to their sedentary nature, filter-feeding behavior and ability to bioaccumulate pollutants like PAHs [2-5].

Exposure to PAHs can lead to toxic effects in *Mytilus edulis* [5-10], *Mytilus galloprovincialis* [11,12], *Unio tumidus* [13], *Mya truncata* [14] and *Perna viridis* [15,16]. At cellular and molecular levels, exposure to PAHs may also trigger responses of biomarkers. For instance PAHs increase the formation of Reactive Oxygen Species (ROS) and induce detoxification systems like antioxidant defense systems present in all aerobic cells [11,17-20]. Therefore, monitoring of adverse effects as well as modifications of cellular and molecular defense systems can both be used as biomarkers [9,16,21,22].

Different antioxidant parameters, like the antioxidative enzymes including superoxide dismutase, glutathion peroxidase and catalase (CAT), are commonly used to assess exposure to xenobiotics in animals. Particularly, CAT is an enzyme promoting the conversion of hydrogen peroxide (H_2O_2) to water and molecular oxygen and can be used as a biomarker of oxidative stress in bivalves. A number of studies reported an increase of superoxide dismutase and CAT activities when an excess of ROS was observed in bivalves [16,23]. Lipid peroxidation attesting membrane damage was noticed when the antioxidant defenses were the lowest [24-27]. Malondialdehyde (MDA) is one of the final products of the membrane fatty acid degradation, mainly total thiobarbituric acid-reactive substances (TBARS), and is considered as a good biomarker of lipid peroxidation and consequently of oxidative stress [15,16,23]. The presence of pollutants in the environment increases CAT activity and MDA level in bivalves [13,21]. More specifically, exposure to PAH contaminated sediments showed the same trend in *Mytilus galloprovincialis* [28], *Mytilus edulis* [29] and *Perna viridis* [16].

The immune system is a sensitive target to the toxic action of chemicals in several organisms [19,30-32]. In bivalves, haemocytes and phagocytosis are the main components of the immune system [20,33]. An increase in the number of haemocytes has been observed after TBT contamination in *Crassostrea virginica* [34] and fluoranthene in *Mytilus edulis* [35]. However, a reduction in haemocyte count was observed with high concentrations of copper in *Sunetta scripta* and *Villorita cyprinoides* var. *cochinensis* [36]. Sediment contaminated by PAHs led to a decreased number of haemocytes and inhibited phagocytic activity in exposed *Crassostrea virginica* [37] and *Mytilus edulis* [2,3]. These responses were dependent on the species and the type of contaminant studied even if concentrations were much higher (more than 500 μg per litre per day) [2] than those found in the contaminated environment [2,32,37]. It is therefore of first importance to determine the sensitivity of immune endpoints as good markers of exposure to PAHs in bivalves. Considering antioxidant responses induced following an exposure to PAHs and the consequences on immune competence, it is also crucial to study interaction between the PAHs exposure and the physiological state of the organism.

Due to their lipophilic nature, PAHs are present in the entire marine coastal food web and we aimed in the present work to study effects of dietary exposure to PAHs on immune parameters and oxidative stress in *Mya arenaria*. It represents the first level of contamination via the trophic way, and may be of real importance in complement to other trophic level contamination studies to better understand the bioaccumulation process.

CAT activities and MDA levels were also surveyed in field animals (*in situ* experiments) to assess their natural level. Immune parameters were not measured in these organisms due to the rapid deterioration and the difficulty to preserve the haemolymph.

This work is a contribution to a crucial need to understand physiological responses of marine invertebrates facing toxicant challenges and eventually a decrease of their health conditions and survival rate.

Results and discussion

Effects of PAHs exposure on cell viability and haemocyte count

Viability of haemocytes was above 98% for both control and contaminated groups and for all samplings. For haemocyte counts (Fig. 1), a significant reduction in the number of haemocytes was observed at days 9 ($P = 0.019$) and 17 ($P = 0.004$). For the last day of the experiment, the number of haemocytes in contaminated clams was not different from the number of haemocytes in control clams with a ratio near one.

Effects of PAHs exposure on phagocytosis

Phagocytic activity representing the percentage of haemocytes having engulfed one bead or more is illustrated in Fig. 2. A significant inhibition of phagocytosis was observed for contaminated clams at days 2 ($P = < 0.001$) and 9 ($P = < 0.001$) during the contamination and at day 17 ($P = < 0.001$), 8 days after the beginning of the depuration process. Fig. 3 shows efficiency of phagocytic cells represented by the percentage of cells having engulfed three beads or more. There was a significant decrease of efficiency of phagocytic cells in contaminated organisms at days 2 ($P = < 0.001$), 9 ($P = < 0.001$) and 17 ($P = < 0.001$). At the end of the experiment, the phagocytic activ-

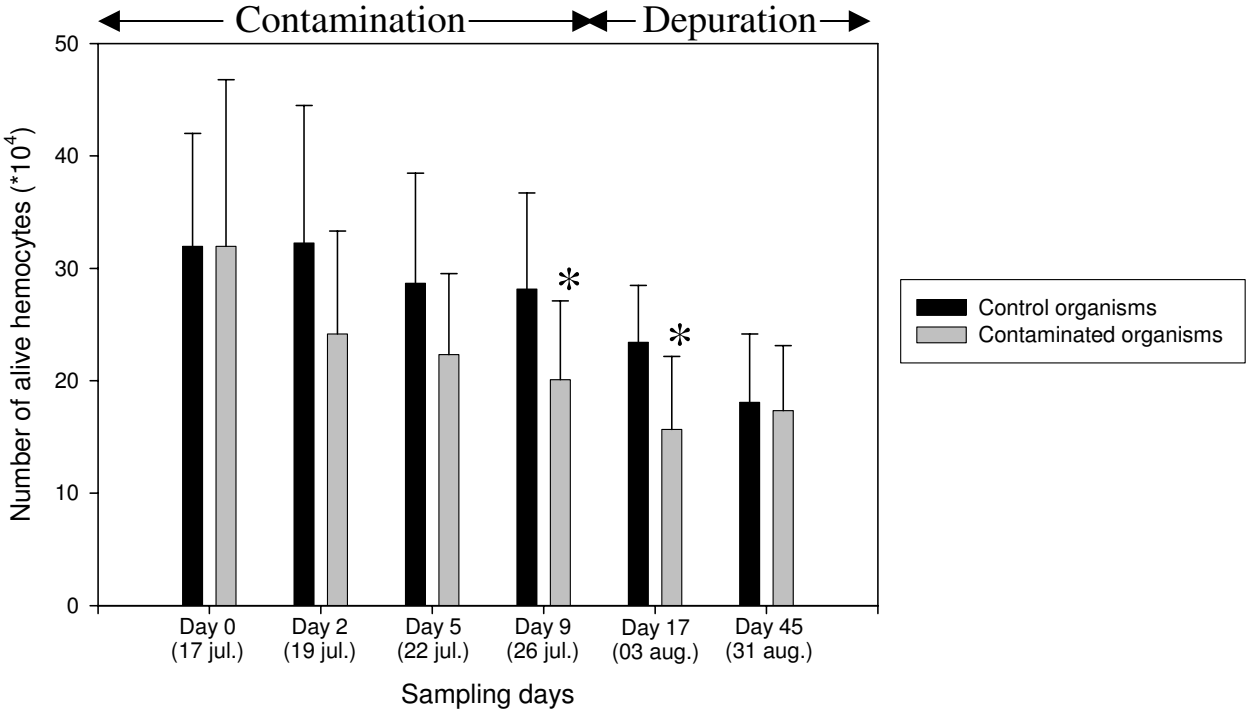

Figure 1

Effects of dietary exposure to PAHs on the number of hemocytes. Results are expressed as the mean number of live hemocytes (10^4) per 12 clams, in hemolymph collected from control and contaminated organisms. Organisms were exposed to PAHs during 9 days and the contamination was stopped for the remaining 36 days. Significant differences between control and contaminated clams are represented by * for P < 0,05. Error bars represent S.E.M.

ity of contaminated clams returned to the same level than the controls.

Biochemical markers

CAT activity following PAHs exposure showed no significant differences between control and contaminated organisms (results not shown). In both groups, significant differences were observed between days of the experiment. Differences were observed between the first day and the fifth day and between the first day and the end of the exposure period when a general increase of CAT activity in controls and contaminated organisms were observed due to uncontrolled introduction of air under high pressure during the fifth day (see "Exposure protocol").

Lipid peroxidation assessed by the MDA levels showed no significant differences (results not shown).

Samples from Pointe-au-Père and Metis beach showed no significant differences for CAT. The levels of CAT activity were similar for both sites (data not shown).

For MDA level, among 11 sampling days, 8 of them showed significant differences between sites (Fig. 4). Levels were significantly higher in the site of Metis during sampling days 2 (1st June) (Kruskal-Wallis test, H = 6,604, P = 0,01), 3 (15th June) (Kruskal-Wallis test, H = 15,384, P = < 0,001), 4 (30th June) (P = < 0,001), 6 (28th July) (P = < 0,001), 7 (10th August) (Kruskal-Wallis test, H = 14,963, P = < 0,001), 9 (12th September) (P = < 0,001) and 11 (4th October) (Kruskal-Wallis test, H = 27,539, P = < 0,001). For 26th August (sample 8), levels at Metis were significantly lower than in Pointe-au-Père (P = < 0,001).

No correlations were found using the Pearson's coefficient between PAH exposure and the biomarker responses measured for both mesocosm and *in situ* organisms, i.e. that there was no linear relationship between these two variables.

An increase in the number of circulating haemocytes is commonly observed in bivalves after an exposure to a large variety of environmental stressors or toxic sub-

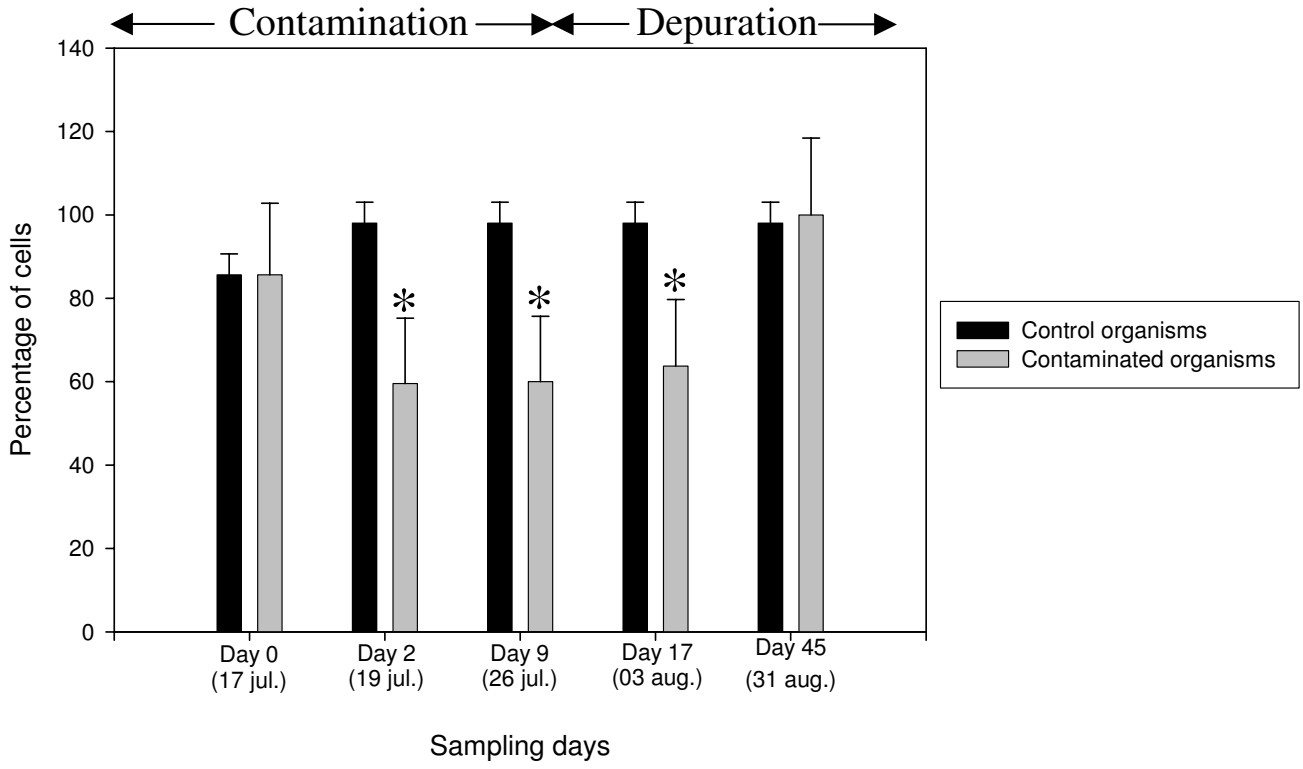

Figure 2

Effects of exposure to PAHs on phagocytic activity of haemocytes. Results are expressed as percentage of haemocytes, collected from *Mya arenaria*, having engulfed one bead or more. Organisms were exposed to PAHs during 9 days and the contamination was stopped for the remaining 36 days. Significant differences between control and contaminated clams are represented by * for P < 0,05. Error bars represent S.E.M.

stances [34,35,38,39]. However, other studies have reported a decrease in haemocyte counts, but for levels of contaminants not found in natural environment [2]. Sami et al. [37] have shown that the count of haemocytes from American oyster *Crassostrea virginica* could be modulated following *in situ* exposure to PAHs. In the present study, significant decreases were observed in days 9 and 17 between the count of haemocytes in control and contaminated organisms while no significant differences were detected between controls and contaminated at the beginning of the experiment. A slight decrease appeared during the exposure at contamination level similar to the environmental levels. The haemocyte number continued to decrease at day 17 in contaminated animals even if the exposure was stopped at the ninth day.

A significant decrease was also observed for control organisms from day 17 to the end of the experiment. For the last day of the experiment, the number of circulating haemocytes was lower comparatively to the other sampling days but similar for both groups. PAHs could possibly have affected directly the production of haemocytes in *Mya arenaria*. Organisms were sampled in June where a large majority of individuals were in spawning (spermatozoids only visible in the center of the alveoli and lower number of ripe oocytes free in the alveoli) stages (56,5% and 43,2% for males and females respectively) and in spent or past (no visible spermatogonia or oogonia) stages (39,1% and 43,2% for males and females respectively) in *Mya arenaria* from the site of Metis Beach [40]. However, mesocosm experiments were conducted from mid July to the end of August where clams were in indifferent (no visible spermatogonia) stages for males (88,5%) and in indifferent and previtellogenic (no visible oogonia and small oogonia at the periphery of the alveoli respectively) stages for females (42,4% and 36,4% respectively) [40]. These observations led us to presume that PAHs were not being sequestered/partitioned into developped ovaries and so were available to the haemocytes because clams had already spawned. However, we could suspect that the decrease of the number of circulating haemocytes in control clams was due to a few individuals that did not spawn.

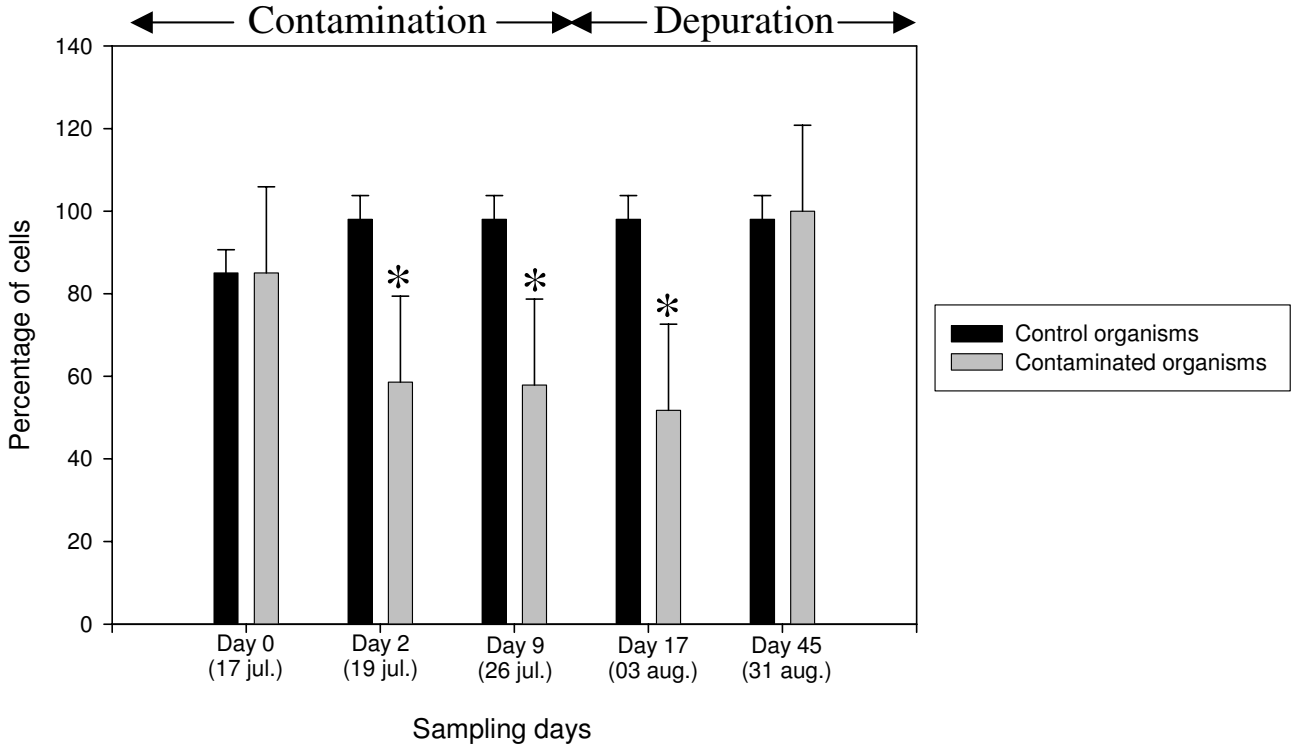

Figure 3
Effects of exposure to PAHs on the efficiency of phagocytic cells. Results are expressed as percentage of haemocytes, collected from *Mya arenaria*, having engulfed three beads or more. Organisms were exposed to PAHs during 9 days and the contamination was stopped for the remaining 36 days. Significant differences between control and contaminated clams are represented by * for P < 0,05. Error bars represent S.E.M.

Phagocytosis in bivalves exposed to PAHs tended to decrease with increasing concentrations of contaminants [2,3,5,41,42]. PAH exposure has been shown to significantly suppress phagocytic activity in *Mytilus edulis* [2,3], *Mya arenaria* and *Mactromeris polynyma* [32]. However, long-term exposure to benzo-(a)-pyrene slightly stimulated phagocytic activity in *Mercenaria mercenaria* [41]. Moreover, exposure to low concentrations of PAHs may stimulate phagocytic activity whereas increasing levels could suppress phagocytosis in *Mya arenaria* [32,43,44]. In this work, a significant difference was observed just after the beginning of the contamination showing the acute effects of PAHs on haemocyte activity (cells having engulfed one bead or more) and on the efficiency of phagocytosis (cells having engulfed three beads or more), this threshold assessing the more active population of haemocytes in phagocytosis [19,20,43-45].

There were significant differences in phagocytosis between controls and contaminated clams at days 9 and 17. Day 45 showed a return to initial conditions with a percentage of cells having engulfed one bead or more and three beads or more near to 100% attesting their great efficiency. This trend showed a strong recovery of clams. PAHs caused phagocytic inhibition at concentrations near those reported in the environment. As phagocytosis is a membrane-dependent process, PAHs affected directly the membrane stability resulting in physical disturbance of the lysosome membrane and further disrupted immune function. Other studies have already demonstrated this reduction in the lysosomal membrane stability of haemocytes of bivalves species like *Mytilus edulis* [2,3,46] and *Mya arenaria* [32,43], associated with the inhibition of phagocytic activity. PAHs may also interfere within the maturation and/or differentiation processes of haemocytes [43]. Recovery of the immune function in *Mya arenaria* during the depuration process showed that the effects of PAHs at concentrations used for this experiment were reversible.

PAHs are known to increase production of ROS leading to oxidative stress [47,48]. Oxidative stress results in increased levels of CAT activity, an antioxidant enzyme used by the organism to detoxify cells [23]. This oxidative

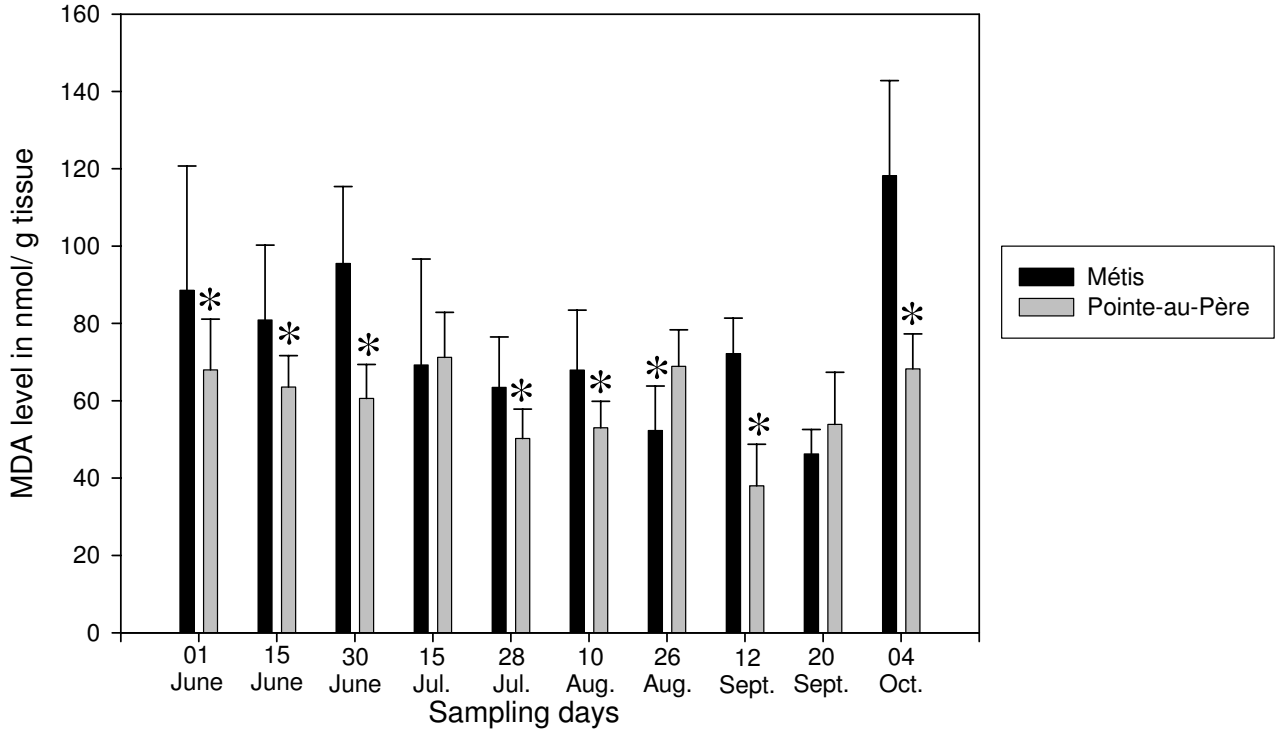

Figure 4
MDA levels in the digestive gland of *Mya arenaria* sampled in natural environment (*in situ* survey). Results are expressed as nmol per g tissue. Significant differences between control and contaminated clams are represented by * for P < 0,05. Error bars represent S.E.M.

stress can lead to cell damage caused by lipid peroxidation that can be assessed by the level of MDA [13,21]. In bivalves, several studies have associated the antioxidant activity with a contamination by PAHs [16,25,29]. For this work, the experiment showed no significant differences between control and contaminated organisms. The activity of the antioxidant system represented by CAT was constant under stress induced by the PAH exposure, probably because the PAHs concentration used for this experiment was similar to environmental conditions (the same concentration detected in seawater from the Pointe-au-Père site, representative of the St. Lawrence-Saguenay system). CAT responses were therefore indicative in this study of a no-stress exposure. Nevertheless, further studies have showed that PAHs were effectively incorporated in clams after exposure by a trophic way and that radioactive pyrene had a 14-day residence time [49]. Since no increased CAT activity was observed, another antioxidant system may be involved. An increase in the level of the superoxide dismutase enzyme was observed in bivalves as well as for CAT after an exposure to PAHs [25,29]. Moreover, the superoxide dismutase works in association with the CAT, both of them showing the same trend. This result suggests that glutathione peroxidase was used to detoxify cells in presence of this complex mixture of PAHs.

MDA concentration increases generally when stress overwhelms the antioxidant systems [50]. However, no significant differences were detected between control and contaminated organisms. The levels of CAT remaining during the exposure experiment confirmed that no oxidative stress was detected and consequently no oxidative damages to polyunsaturated fatty acids. Moreover, results of the immune parameters measured attested the hypothesis of the membrane destabilisation or of interference within the maturation and/or differentiation processes of haemocytes caused by PAH contamination. This suggests that phagocytic activity and haemocyte number, comparatively to CAT activity and MDA level, is an early biomarker that is really efficient to detect a physiological change during a short term exposure to xenobiotics like PAHs at low concentrations. There was low bioaccumulation measured in organisms (<2 nmol/g dry weight of total PAHs including phenanthrene, anthracene, fluoranthene, pyrene and benzo(a)pyrene in soft tissues) during all the experiment period reaching a maximum of 1,15 nmol/g of dry weight ± 0,42 at the 30th day during the depuration, due to the low and realistic concentrations used for the exposure. PAHs generally do not bioaccumulate in tissues or at a really low rate. However, PAHs can cause a variety of deleterious effects in exposed animals since some PAH

metabolites that are intermediates in the process of detoxication have carcinogenic, mutagenic and cytotoxic activities [51,52].

For comparison purposes between Metis and Pointe-au-Père sites, CAT activity showed no significant differences. However, when compared with data of the mesocosm experiment, the mean of CAT activity values were lower in the field study. Clams exposed to PAHs in mesocosm showed a CAT activity of 90.97 nmol/min/mg proteins while clams from Pointe-au-Père showed a CAT activity of 92.47 nmol/min/mg proteins. Interestingly, control organisms in the exposure experiment had a CAT activity of 87.87 nmol/min/mg protein while CAT activity of organisms from Metis was of 94.19 nmol/min/mg protein. The results from Pointe-au-Père clams and from clams contaminated in mesocosms showed that with a PAHs concentration near the environmental concentration detected in the St. Lawrence-Saguenay system, no oxidative stress was observed. At low concentrations, PAHs have no effects on the antioxidant enzymes such as CAT and on the lipid peroxidation assessed by MDA level of filter-feeding bivalves such as *Mya arenaria*. Moreover, clams from the two sites were exposed to changes in natural conditions such as air exposure, temperature, tide cycling and sunshine, which can cause an increase of CAT activity in bivalves [23], in contrast to the stable conditions in aquaria during the exposure experiment. These environmental changes can explain the relative differences between mesocosm and field organisms. MDA levels were significantly higher in Metis than in Pointe-au-Père for 7 samplings attesting a greater lipid peroxidation. In the site of Pointe-au-Père, no contamination effects of PAHs appeared from our results comparatively to Metis, except on 26th August where MDA levels in Metis were significantly lower probably because sampling occured when the site was partially immersed due to the tidal period. Higher levels of MDA levels in Metis can be explained by the fact that some clams can be infected by a castrator trematode *Prosorhyncus squamatus*. This parasite was observed in few clams from Metis but was not seen in clams from Pointe-au-Père [53]. Parasites are known to be major stressors for aquatic organism [54] and among them, *P. squamatus* could inhibit gonadial mitosis in *Mya arenaria* [53] *Mytilus edulis*, *Mytilus galloprovincialis*, *Pecten maximus* and *Ostrea edulis* [55,56] and therefore could trigger lipid peroxidation.

Experimental

Experimental design

In June 2004, sediment and soft shell clams (*Mya arenaria*) were collected on the south shore of the St. Lawrence Estuary, in Metis Beach (48°40'N, 68°00'W), a site exempt of direct sewage inputs or harbor activity [32]. Organisms were randomly sampled at low tide and ranged between 55 and 65 mm in shell length. Sediments were taken in the first 10 cm and sieved through a 1 mm mesh array. Clean sediments were divided in 10 aquaria of 65 L (containing 1/3 of sediments and 2/3 of water) and supplied with seawater at a constant flow rate of 500 mL per min to maintain a good renewal rate. Salinity and temperature were maintained at 28 to 30 ppm and 8 to 10°C respectively, reflecting the environmental parameters at this specific site. Organisms (n = 36 per aquarium) were transferred in each aquarium 14 days prior to the beginning of the experiment, to reduce the stress due to handling. Population density of *Mya arenaria* was of 200 clams per m^2 in each aquarium. Densities of clams are highly variable throughout the world (2–2,000 clams per m^2 and between 10 to 200 clams per m^2 in the Saguenay-St. Lawrence system) since clams can settle in various substrates, some of them are more favourable to the settlement of a high number of juvenile clams [57-59]. Clams were fed during the last week of the acclimation period with phytoplankton *Asterionnella* diluted in seawater filtered with a 0.7 μm membrane. Algae concentration was of 20 million cells per mL with a flow rate of 2 mL per min.

Clams from the reference site (Metis beach) and from a site receiving domestic waste, Pointe-au-Père (48°31'N, 68°29'W), were sampled (n = 20 per site) during five months (20 May to 4 October 2004) every two weeks, in order to measure stress responses in the natural environment and compare responses with the experimental results. Organisms were randomly sampled at low tide except on 26th August where the Metis Beach site was partially immersed due to the tidal period. Clams sampled in the salt marsh of Pointe-au-Père near the city of Rimouski (Quebec, Canada) were sporadically exposed to a domestic sewage outfall mainly in May. High level of organic carbon (5–10%) was detected in silt sediment of Pointe-au-Père site. PAHs and oil residues were also observed in sediments [60]. Total PAHs concentrations (phenanthrene, anthracene, fluoranthene, pyrene and benzo(a)pyrene) were low and less than 3 μg/g dry weight.

Exposure protocol

Phytoplankton was preincubated with a complex mixture of pure PAHs containing 0.061 g of 2-methylphenanthrene, 0.102 g of naphtalene, 0.099 g of 2-methylnaphtalene, 0.222 g of phenanthrene, 0.102 g of anthracene, 0.314 g of pyrene, 0.097 g of benzo-(a)-pyrene and 0.252 g of fluoranthrene dissolved in 200 mL of ethanol. Pure mixture of PAHs was directly added to 21 L of new phytoplankton suspension 30 minutes before its addition in the aquaria. The suspension was constantly mixed using a magnetic stirrer and bubbler. Each aquarium was supplied with new PAHs-phytoplankton mixture every 24 hours during the exposure experiment. Phytoplankton concentration was 20 million cells per mL and added at a rate of 2 mL per min giving a final concentra-

tion of PAHs of 47,6 ng/L. Flow rate of seawater in aquaria was settled at 500 mL per min. PAHs concentrations chosen are representative of values of PAHs reported in the St. Lawrence – Saguenay system in suspended particulate matter and particularly in the Pointe-au-Père site. Contaminated animals consisted of clams placed in 5 aquaria (n = 180) and fed with phytoplankton contaminated with the mixture of PAHs. Clams (n = 180) were placed randomly in five aquaria supplied with non contaminated phytoplankton and used as controls.

Organisms were exposed to PAHs during 9 days and the contamination was stopped for the remaining 36 days to study the biomarker responses during the depuration process. Control and contaminated clams (n = 12 respectively) were collected randomly in each series of aquarium and organisms were replaced by additional control organisms from Metis Beach to keep the same biomass and filtration volume throughout the experimental period. PAHs were considered as the dependant variable between aquaria since conditions in each aquarium were similar and verified daily (temperature, flow rate of phytoplankton and seawater) and sediments identical in all the aquaria. Randomization of sampling in aquaria during the experimental protocol as well as the high number of clams sampled initially in Metis Beach (n = 360) did not guarantee avoidance of pseudoreplication but certainly made it less likely [61]. Clams were sampled at day 0 (on 17th July) just before the beginning of contamination, at days 2 (on 19th July), 5 (on 22th July) and 9 (on 26th July) during the contamination period and at days 17 (on 3rd August) and 45 (on 31th August) during the depuration period. After each sampling, the organisms were placed on crushed ice and brought to the laboratory for analysis. Air was accidentally introduced under high pressure in seawater pumping and filtering system at the Pointe-au-Père station at day 5 of the experiment and caused evident stress on bivalves that specific day. Therefore, data for the sampling at day 5 were removed.

Immunologic analysis

1–2 mL of haemolymph containing haemocytes was taken from the adductor muscle of clams using a 3 mL sterile syringe with a 23 G needle [62] and kept on ice. The number and viability of haemocytes were evaluated microscopically, in replicates for each clam, on a Neubauer hemacytometer with blue Trypan solution. 40 μL of blue Trypan 10% (w/v) were incubated with 40 μL of resuspended haemolymph during 1 minute, dead hemocytes being coloured in blue.

Phagocytosis was monitored using a microplate modified cytometric method [19,32,43,63]. Cells were washed in haemolymph and the haemocyte cell concentration was adjusted, when it was necessary with seawater, to $1,1*10^6$ cells/mL. For each organism collected in the various aquaria, 100 μL of haemolymph was placed in duplicate in a 96 well microplate. A number of approximately 30 fluorescent beads, 1.716 μm diameter, (Molecular Probes Inc, Eugene, OR, USA) were added for each haemocyte. After a 230 × g centrifugation, samples were incubated in the dark for 18 h at room temperature. Following incubation, haemocytes were put on 3 mL of a 3% (w/v) bovine serum albumine (BSA) gradient, mixed and centrifuged for 10 minutes at 110 × g. Gradient medium and the free beads were removed by vacuum suction. Cells with phagocytised beads were mixed in 200 μL of a solution including 50 mL of flow cytometric solution, Hematall (Fisher, Montreal, Canada) mixed with 100 mg of sodium azide and 0.250 mL of formaldehyde. Acquisitions of phagocytic parameters were performed with a FACScan cytometer (Becton Dickinson, Immunocytochemistry Systems, Mountain View, CA, USA). Briefly, an air cooled argon laser was used to provide an excitation at 488 nm. Fluorescent emissions emitted by the beads were collected at 520 nm. Haemocyte populations were defined based on their forward (FSC) and right angle scatter (side scatter: SSC) properties. A total of 10000 events were acquired for each sample. The fluorescence (FL1) frequency distribution histogram of the haemocyte populations was obtained with the initial condition data analysed for two parameters: the complexity (SSC) and the cell size (FSC). Data collection and analysis were performed with the LYSIS-II program [19]. The results were expressed as the percentage of phagocytic cells having engulfed one bead and more, representing the haemocytes activity, and three beads and more as a marker of efficiency of phagocytic cells.

Biochemical analysis

For these analyses, both mesocosms (clams from aquaria) and *in situ* (from Pointe-au-Père and Metis Beach) organisms were used. The digestive gland of each organism was dissected and divided in two equal parts, each part was preserved and homogenised on ice in a potassium phosphate buffer 50 mM complemented with EDTA 1 mM, pH 7.4, for CAT analysis and in KCl buffer 154 mM, pH 7.4, for MDA analysis and stored at -80°C.

For CAT activity, homogenates were centrifuged for 5 minutes at 12000 × g. 1 mL of the supernatant was incubated during 30 minutes with 10.5 μL of EtOH 95% (w/v). 11.24 μL of Triton X-100 10% (w/v) was added and the samples were gently shaken for 30 seconds. All these steps were done at 4°C. 50 μL of each sample were then transferred in quartz spectrophotometer cuvettes with 2.95 mL of the potassium-phosphate-EDTA solution. The reaction was started by adding 1 mL of hydrogen peroxide solution 42 mM [23]. The CAT activity was evaluated by following during 3 minutes the disappearance of hydrogen peroxide

at a wavelengh of 240 nm with a UV spectrophotometer. One unit decomposes one micromole of H_2O_2 per minute at 25°C and pH 7.4. Results of CAT were expressed as μmol per min per mg proteins. Protein concentrations were determined using the Bradford method [64] at a wavelength of 595 nm. The colour reagent was purchased from BIORAD (Mississauga, Ontario) and Bovine Serum Albumin (BSA, fraction V) from Sigma was used as the protein standard.

Concentrations of thiobarbituric acid reactive substances (TBARS) are an index of lipid peroxidation and oxidative stress. MDA is one of these substances and the method used 1,1,3,3-tetraethoxypropane as standard [65]. Briefly, 500 μL of homogenates were transferred in 3 mL of H_3PO_4 1% (w/v) with 500 μL of the KCl buffer 154 mM. All these steps were done at 4°C. Samples were incubated in a 100°C water bath for 45 minutes. After 5 minutes, in a cold bath (10°C), 4 mL of butanol were added and samples were centrifuged at 25°C for 20 minutes at 1000 × g. The supernatant was transferred in tubes and the MDA formed was estimated at 532, 508 and 556 nm (corrected absorbance = Ab_{532} - Ab_{508} + Ab_{556}). Results were expressed as nmol per g wet tissue.

Statistical analysis

The results were analysed using SigmaStat for Windows version 3.11 (Systat software ©2004). Normality of distribution of data (Kolmogorov-Smirnov test) and homogeneity of variance (Levene Median Test) were tested. A one way analysis of variance (ANOVA) was performed to determine differences in mean values among the two groups when the normality test passed. A non-parametric (Kruskall-Wallis) one way ANOVA was performed on data depending when the normality test failed. Significance was defined at $P < 0,05$, and when significant differences were found, a Multiple Pairwise Comparison (Tukey's test) was used to isolate the different groups (significance set at $P < 0,05$). Finally, a linear regression analysis with Pearson's correlation coefficient was performed to illustrate possible relations between the PAH exposure period and the biomarkers used.

Conclusion

In summary, the exposure to a mixture of PAHs at a level normally found in coastal environment under a low contamination stress, affected processes of haemocyte production as illustrated by the immunological markers used. At the end of the experiment, recovery was observed for all the immunological parameters measured. For the measures of the oxidative stress with CAT and MDA, no significant differences were observed between control and contaminated clams, attesting a normal physiological state in spite of the bioaccumulation observed during the contamination period. However, results on the immunological markers showed that *Mya arenaria* was able to transform PAHs used for this experiment efficiently since after the exposure has been stopped, the levels of all these parameters began to return to normal conditions. The results of MDA and CAT observed in mesocosms, as well as those observed from Pointe-au-Père clams, attest that at the PAHs concentration of 47,6 ng/L, no oxidative stress occurs. The only physiological change occurred at the level of the immune system.

It is therefore of first importance to determine the sensitivity of immune endpoints as good markers of exposure to PAHs in bivalves. Considering antioxidant responses induced following an exposure to PAHs, it is also crucial to study interaction with immune competence to determine relationship (cause to effect) between exposure to PAHs, antioxidant responses and consequences on immune competence.

This work is a contribution to a crucial need to understand physiological responses of marine invertebrates facing toxicant challenges and eventually a decrease of their health conditions and survival rate. Moreover, *Mya arenaria* represents one of the first levels of the trophic way. It is a good indicator species to insure better understanding, in complement with other trophic level contamination studies, of the consequence of a low concentration PAH contamination through the trophic way. The responses of clams during the exposure period to PAHs was estimated but the more interesting responses were those of the biomarkers during the 36 day depuration period following exposure, attesting the strong recovery of immune competence.

Abbreviations

BSA: bovine serum albumin; CAT: catalase; EDTA: Ethylene glycol-bis(2-aminoethyl-ether)-N, N, N', N'-tetraacetic acid; EtOH: ethanol; MDA: malondialdehyde; PAH: polycyclic aromatic hydrocarbons; ROS: reactive oxygen species; TBARS: thiobarbituric acid reactive substances.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

NP participated in the design, collected data, performed data analysis and drafted the manuscript. SGC and PR participated in the data collection. JP, EP and MF conceived the study, participated in its design and coordination. JP and MF contributed to the draft of the manuscript. JP read the draft and suggested corrections to NP. All authors read and approved the final manuscript.

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