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Alterations in haemolymph parameters in the Mediterranean clam, *Ruditapes decussatus*, *in vitro* exposed to pyrene

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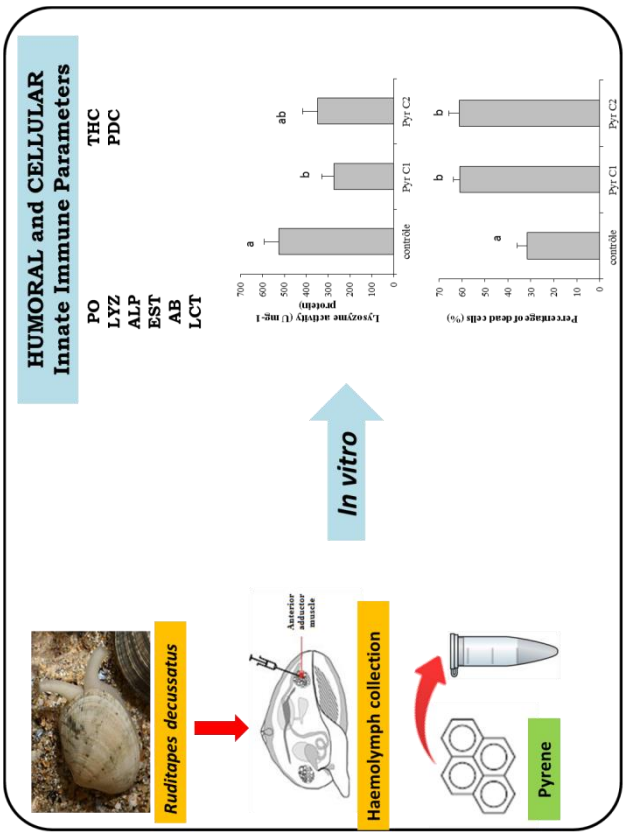
Graphical Abstract



Abstract.

The *in vitro* effects of pyrene (polycyclic aromatic hydrocarbon) on haemocyte and haemolymphatic parameters of the Mediterranean clam, *Ruditapes decussatus*, were tested using two concentrations (10^{-3} and 10^{-5} mg mL^{-1}). After 24 h contamination period, pyrene effects were monitored on individual haemolymph samples. The toxic exposure caused significant alteration in the immune parameters of clams. It increases the antibacterial activity, the total haemocyte count and the haemocyte mortality. The increase was associated with the decrease in lysozyme and esterase activities which reflects a decrease in secretion in order to reduce energy expenditure. Modulation of immune parameters in the Mediterranean clam by pyrene exposer suggested that PAH pollution may be related to enhanced susceptibility to diseases.

Keywords: *Ruditapes decussatus*; Pyrene; Immune response.

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are major contaminants that may affect biota at various trophic levels in estuaries and coastal ecosystems. These pollutants are characterized by high toxicity, high persistence in the environment and bioaccumulation capacity (Ramdine *et al.* 2012). These pollutants are well known as environmental pollutants at low concentrations and 16 of them have been classed as priorities by the United States Environmental Protection Agency (US EPA) due to their teratogenic, mutagenic and carcinogenic properties (Reynaud and Deschaux 2006, Manoli and Samara 2008). These 16 PAHs were detected in the heavy fuel oil. However, the most detected molecules are fluorene, pyrene, naphthalene and phenanthrene (Bado-Nilles *et al.* 2008).

Pyrene is one of the most predominant polycyclic aromatic hydrocarbons (PAHs) in the environment. Previous studies have investigated the toxic effects of pyrene on marine animals. Studies on the embryos of beach spawning Capelin (*Mallotus villosus*) showed that exposure to 55 µg L^{-1} of pyrene for 30 days increases embryonic mortality rates and decreases hatching success (Frantzen *et al.* 2012). Although, it has shown that pyrene inhibits the embryonic development of mussel (*Mytilus galloprovincialis*) (Bellas *et al.* 2008), reduces the offspring production in the red worm (*Limnodrilus hoffmeisteri*) (Lotufo and Fleeger 1996) and decreases the swimming performance of the common goby (*Pomatoschistus microps*) after 4 days of exposure to 0.125, 0.25, 0.5 or 1 mg L^{-1} (Oliveira *et al.* 2012).

Bivalves are commonly used as sentinel species for monitoring coastal environments (Romeo *et al.* 2003). Their worldwide distribution, their sessile nature, their filter-feeding and their ability to bioaccumulate pollutants make them ideal species for use in investigation. Host defense mechanisms in bivalve molluscs relies on haemocytes, the circulating cells present in extrapallial fluids, and soluble effectors in haemolymph. Bivalve haemocytes can perform several functions, including wound repair, nodule formation, encapsulation, phagocytosis and cytotoxicity (Cheng 1981). The secretion of soluble factors by haemocytes plays an important role in the immunity of bivalves, indirectly through the regulation of certain cellular mechanisms and directly through their antimicrobial activities. This humoral response involves various molecules such as phenoloxidase cascade, lysosomal enzymes and antimicrobial peptides. Many studies have described alterations in the cellular and biochemical parameters of the haemolymph of various bivalves (oysters, clams, mussels, etc.) exposed to pyrene (Okay *et al.* 2006, Bado-Nilles *et al.* 2008).

In vitro assays are useful to assess the biological effects of environmental samples from diverse origins, without *in vivo* physiological disturbance. This type of experimentation is usually used when little is known about a mechanism by which pollutants induce an effect *in vivo* and can sometimes understand some lesions reported. *In vitro* assays are also often used to invalidate or confirm the assumption made based on *in vivo* studies.

Taken into account these considerations, the main goal of this study was to evaluate the *in vitro* effects of pyrene on innate humoral and cellular immune characteristics in the Mediterranean clam *Ruditapes decussatus*. Therefore, the total haemocyte count, the cell viability, the phenoloxidase, lysozyme, alkaline phosphatase, lectin and antibacterial activities were measured in the haemolymph of clams after exposure to different doses of pyrene.

2. Materials and Methods

2.1. Xenobiotics

Pyrene was dissolved in cyclohexane (Sigma) at a concentration of 20 g L^{-1} (stock solution). To obtain a working solution, stock solution was diluted with cyclohexane, ensuring that the ratio cyclohexane:haemocyte suspension did not exceed 0.5% in order to avoid disturbance of the cell parameters (Bado-Nilles *et al.* 2008). Cyclohexane alone was used as solvent control in all the experiments.

2.2. Clam collection and handling

The Mediterranean clams (*R. decussatus*), 3-3.5 cm shell length, were purchased from the Bizerta shellfish farming (Bizerta, Tunisia) where they are veterinary and microbiologically controlled. Animals were maintained in aerated aquaria at 20 °C and were fed daily, 3 h before changing the water, with the alga *Nannochloripsis* ($3x10^8$ cells per day per clam). The alga was provided from the Tunisian Aquaculture Centre (Hergla, Tunisia). Clams were allowed to acclimatise for seven days before being used in the experiments.

2.3. Experimental protocol

About 1 mL of haemolymph was collected per clam from the anterior adductor muscle with a 2 mL plastic syringe and stored on ice. Two concentrations (10^{-5} and 10^{-3} mg mL^{-1}) of pyrene were tested. These two concentrations were chosen based on the available literature (Bado-Nilles *et al.* 2009). Pyrene solution was added individually at 5 µL per mL of haemolymph. Samples were incubated at 20 °C for 24 h. All the experiments including controls were carried out twice. At the end of the experiments, a volume of 25 µL of haemolymph was mixed immediately with formol then was kept at 4 °C in order to determine the total haemocyte count (THC) and the percentage of dead cells (PDC). To measure enzymatic activities, 500 µL of haemolymph were centrifuged (780 x g, 10 min, 4 °C) and the supernatant, corresponding to cell-free haemolymph, was collected and stored at -20 °C until analysis.

2.4. Cellular innate immunity

The cellular innate immunity was assessed by calculating the number of free haemocytes and the percentage of dead cells in the hemolymph using a Malassez cell counter. Briefly, a volume of 25 mL of haemolymph was mixed with the same volume of formol 6% in order to prevent cell clotting, and the formol was stained with Trypan blue to detect dead cells. Then, the total haemocyte count (THC) and dead haemocyte count (DHC) were expressed as the number of haemocytes ($x10^6$) mL^{-1} in the haemolymph. The percentage of dead cells (PDC) was expressed as follows:

PDC = (DHC/THC)*100

2.5. Humoral innate immunity

2.5.1. Phenoloxidase activity

Phenoloxidase (PO) activity assay was performed according to the method described by Asokan *et al*. (Asokan *et al.* 1997). Briefly, 50 µL of haemolymph were incubated with the same volume of SDS (sodium dodecyl sulphate, 1 mg mL^{-1}, Sigma) for 5 min at room temperature. To each well were added 50 µL of L-DOPA (3,4-dihydroxyphénylalanine, Sigma) (3 mg mL^{-1} in 0.5 M HCl containing 10 mM $CaCl_2$) as substrate. The decrease of optical density at 490 nm was followed every minute for 15 min in a plate reader. PO activity was expressed as units, where one unit represents the change in absorbance min^{-1} mg $protein^{-1}$.

2.5.2. Lysozyme activity

Lysozyme activity was measured according to the turbidimetric method adapted from Parry *et al*. (Parry *et al.* 1965). In 96-well flat-bottomed plates, 100 µL of haemolymph was mixed with the same volume of freeze-dried *Micrococcus lysodeikticus* (0.3 mg mL^{-1}, Sigma) as lysozyme substrate. Lysozyme activity was determined by recording the decrease of OD at 450 nm every minute for 15 min at 22 ºC in a plate reader. One unit of lysozyme activity was defined as a reduction in absorbance of 0.001 min^{-1}. The units of lysozyme present in haemolymph were obtained from a standard curve made with hen egg white lysozyme (HEWL, Sigma) and the enzymatic activity was expressed as µg mg^{-1} proteins.

2.5.3. Alkaline phosphatase activity

Alkaline phosphatase activity was quantified as described by Mansour *et al*. (Mansour *et al.* 2017). Aliquots of 100 µL of haemolymph were mixed with 100 µL of 4 mM p-nitrophenyl liquid phosphate (Sigma) in 100 mM ammonium bicarbonate buffer containing 1 mM $MgCl_2$ (pH 7.8, 30 ºC). ALP activity was determined by recording the increase of OD at 405 nm every minute for 1 h. ALP activity was expressed as unit mg^{-1} haemolymph proteins, where one unit represents the amount of enzyme required to release 1 µmol of p-nitrophenol phosphate product in 1 min.

2.5.4. Esterase activity

Esterase activity was measured by mixing an equal volume of haemolymph samples with 0.4 mM p-nitrophenylmyristate in 100 mM ammonium bicarbonate buffer containing 0.5% Triton X-100 (pH 7.8, 30 ºC) as described by Mansour *et al*. (Mansour *et al.* 2017). The OD was continuously measured at 1 min intervals over 3 h at 405 nm in a plate reader. Standard samples without haemolymph were used as blanks. Esterase activity was expressed as unit mg^{-1} haemolymph proteins, where one unit represents the amount of enzyme required to release 1 µmol of p-nitrophenylmyristate product in 1 min.

2.5.5. Protein determination

The total protein concentration present in the haemolymph samples was measured spectrophotometrically at 595 nm by the Bradford method (Bradford 1976) using bovine serum albumin (BSA) as a standard.

2.5.6. Antibacterial activity

Antibacterial activity was determined according to the method described by Ordás *et al*. (Ordás *et al.* 2000) modified by Mansour *et al*. (Mansour *et al.* 2017). Aliquots of 25 µL of haemolymph were

mixed with 75 µL of a suspension of *Escherichia coli* (ATCC35218) (10^8 cell mL^{-1} in Tryptone soy broth (TSB, Sigma)) in a 96 well plate. In the control, the haemolymph was substituted by TSB. After incubation for 3 h at 18 °C, 100 µL of 3-(4,5-Dimethylthiazol-2-yl) 2,5-diphenyltetrazolium bromide (MTT) (0.5 mg mL^{-1} in TSB) were added to each well. After 15 min in the dark at 18 °C, the absorbance at 600 nm was measured. All samples were run in triplicate. The antibacterial activity index (BI) was calculated as follows:

BI=sample ABS_{600}/control ABS_{600}

2.5.7. Lectin activity

Lectin (LCT) activity in haemolymph samples was determined by the reaction of agglutination (HA) following the method described by Ordás *et al.* (Ordás *et al.* 2000). This activity was measured by adding 25 µL of 3% human blood to 25 µL of serially diluted haemolymph in 96-well plates. After mixing, plates were kept at room temperature for 2 h. Agglutination was determined by the presence or the absence of a button of blood on the bottom of the well. The agglutination title (inverse of the highest haemolymph dilution factor) was recorded and expressed as its log_2. All samples were run in duplicate.

2.6. Statistical analysis

All our results were expressed as mean ± standard error (SEM). Data were statistically analysed using a one-way analysis of variance (ANOVA). Significant differences were determined at the $p<0.05$ level using Fisher test. Principal component analysis (PCA) was used to find correlations between the different biomarkers. Statistical analysis was performed using the software STATISTICA (Statsoft STATISTICA version 6.1.478.0).

3. Results and Discussion

3.1. Cellular innate immunity

3.1.1. Total haemocyte count and cell viability

Haemocytes are circulating cells involved in bivalve immune defense such as haemocytosis (increases in circulating haemocyte numbers), phagocytosis of small particles, and encapsulation of large particles and production of reactive oxygen species. In the current study, the total haemocyte count (**Fig. 1A**) was significantly affected by the presence of pyrene (Fisher test, $p<0.05$). THC was increased in clams exposed to pyrene (11.82 ± 1.73 and 12.33 ± 1.70 x 10^6 cell mL^{-1} at the concentration of 10^{-5} and 10^{-3} mg mL^{-1} respectively) compared to control samples (8.2 ± 1.17 x 10^6 cell mL^{-1}). A wide variety of studies have demonstrated similar increases in the haemocyte count for bivalves exposed to various pollutants. Indeed, *in vivo* exposure to phenanthrene resulted in an increased THC in the scallop *Pecten maximus* (Hannam *et al.* 2010). Furthermore, exposure to a diet containing a mixture of PAHs resulted in an increase in THC of the oyster *Crassostrea virginica* (Croxton *et al.* 2012). According to Auffret *et al.* 2006 (Auffret *et al.* 2006) the increase in the number of circulating haemocytes appears to be a common response to environmental stress. Hannam *et al.* 2010 (Hannam *et al.* 2010) explained the increase in the number of circulating haemocytes after exposure to phenanthrene by cell proliferation.

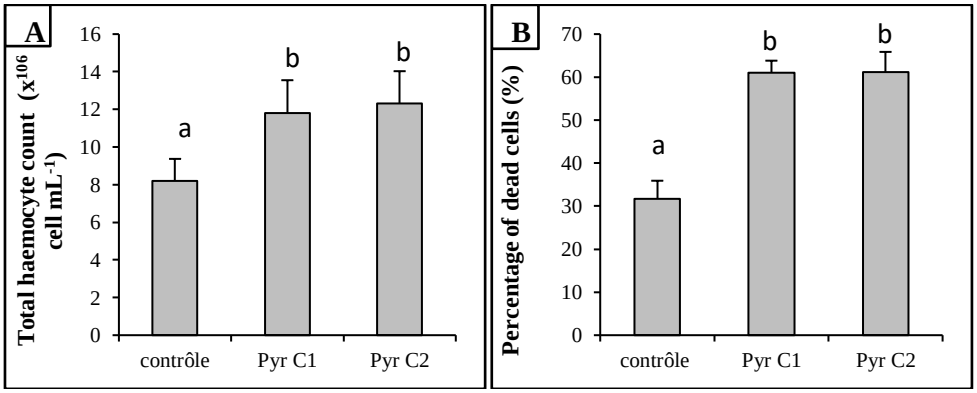


Fig. 1. Total haemocyte count (A) and percentage of dead cells (B) measured in haemolymph of clam, *Ruditapes decussatus*, after 24 h exposure to 0, 10^{-5} and 10^{-3} mg mL^{-1} of pyrene. Data represent the mean ± SEM. Different letters denote significant differences between treatment groups ($p < 0.05$).

3.1.2. Cell viability

The percentage of dead haemocytes in haemolymph is frequently used as an indicator of the physiological state of bivalves. In the current study, the percentage of dead cells (PDC) was significantly affected by the presence of pyrene (Fisher test, $p<0.05$). Compared to control samples (31.73 ± 4.18 %), pyrene (61.06 ± 2.76 and 61.18 ± 4.68 % at the concentration of 10^{-5} and 10^{-3} mg mL^{-1} respectively) significantly increased haemocyte mortality after a 24 h incubation (**Fig .1B**). A similar increase of the percentage of dead cells was recorded in the blue mussel (*Mytilus galloprovincialis*) exposed for 7 days to phenanthrene and anthracene either alone or in a mixture (Giannapas *et al.* 2012). Whereas, a decrease of the percentage of dead cells was reported in the Pacific oyster (*Crassostrea gigas*) after haemocytes *in vitro* exposure to chrysene, anthracene, indeno[1,2,3-c,d]pyrene, pyrene, phenanthrene and fluorene at 10^{-9}, 10^{-7}, 10^{-5} and 10^{-3} mg mL^{-1} at 15 °C during 24 h (Bado-Nilles *et al.* 2008).

3.2. Humoral innate immunity

3.2.1. Phenoloxidase activity

The phenoloxidase system plays a very important role in host defense mechanisms in invertebrates (Muñoz *et al.* 2006), participating in the encapsulation and melanisation of foreign bodies (Söderhäll and Cerenius 1998). Also, it exists in bivalve molluscs such as *Mytilus edulis* (Coles and Pipe 1994), *Mytilus galloprovincialis* (Carballal *et al.* 1997, Carballal *et al.* 1997), *Crassostrea gigas* (Gagnaire *et al.* 2004) and *R. decussatus* (Muñoz *et al.* 2006). In the present study, the phenoloxidase activity was sligntly influenced by pyrene exposure (Fisher test, $p>0.05$) (**Fig. 2A**). There was a tendency for this activity to increase in clams exposed to pyrene (0.308 ± 0.083 and 0.232 ± 0.060 U mg^{-1} protein, at the concentration of 10^{-5} and 10^{-3} mg mL^{-1} respectively) compared to control samples (0.147 ± 0.040 U mg^{-1} protein). In agreement with our results, in the Pacific oyster *C. gigas*, pyrene, phenanthrene and fluorene did not modulate the phenoloxidase activity after 24 h *in vitro* exposure (Bado-Nilles *et al.* 2008). However, this activity increased by benzo[b]fluoranthene (Bado-Nilles *et al.* 2008). Furthermore, Coles *et al.* (Coles *et al.* 1994) demonstrated an increase in phenoloxidase activity after *in vivo* exposure of mussels *M. edulis* to fluoranthene. Another previous study revealed that the exposure to naphthalene at 50 µg L^{-1} increases the phenoloxidase activity in the oyster *Crassostrea corteziensis* however it inhibits this activity at lower doses; 1 and 20 µg L^{-1} (Díaz-Resendiz *et al.*

2014). With regard to our results, pyrene did not modulate phenoloxidase activity, which may be explained by the absence of direct effect of this pollutant on phenoloxidase activity.

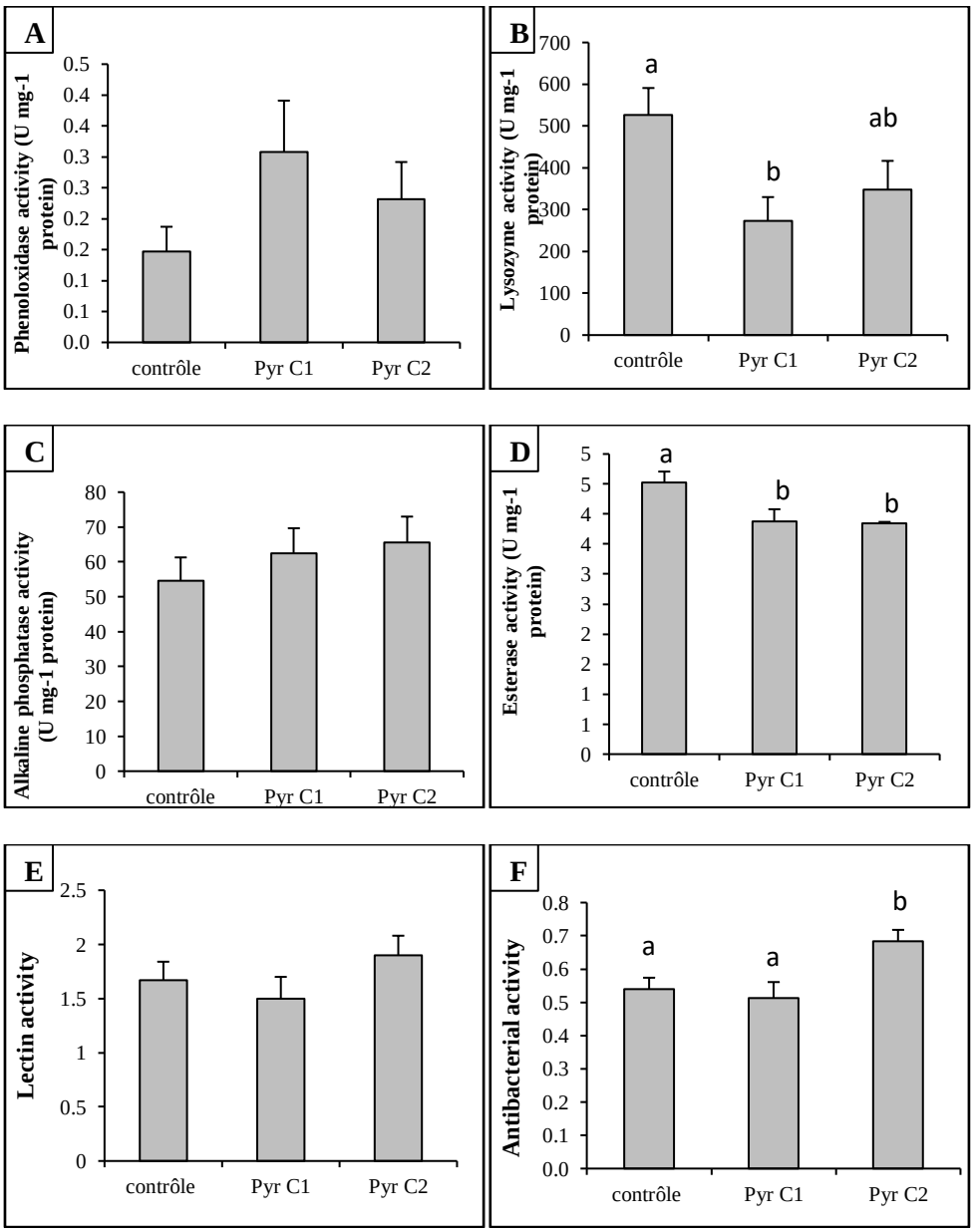


Fig. 2. Phenoloxidase (A), lysozyme (B), alkaline phosphatase (C), esterase (D), lectin (E) and antibacterial (F) activities measured in haemolymph of clam, *Ruditapes decussatus*, after 24 h exposure to 0, 10^{-5} and 10^{-3} mg mL^{-1} of pyrene. Data represent the mean ± SEM. Different letters denote significant differences between treatment groups ($p < 0.05$).

3.2.2. Lysozyme activity

The release of lysosomal hydrolytic enzymes is an important part of the extracellular killing of bacteria through the hydrolysis of components of bacterial cell walls (Canesi *et al.* 2002, Monari *et al.* 2007). Lysozyme is an bacteriolytic agents acting against several species of Gram-positive and Gram-negative bacteria; it is synthesized in bivalve haemocytes and subsequently secreted into haemolymph during phagocytosis (Cheng *et al.* 1975). In this study, lysozyme activity was measured in cell-free haemolymph to assess possible negative consequences of pyrene on enzyme release by haemocytes. In the current study, lysozyme activity was significantly affected by pyrene exposure (**Fig. 2B**). It was

significantly lower in haemolymph exposed to 10^{-5} mg mL^{-1} of pyrene (273.76 ± 56.39 U mg^{-1} protein) after a 24 h incubation compared to control samples (525.88 ± 65.26 U mg^{-1} protein). Several studies have revealed that the presence of contaminants can modulate the lysozyme activity in bivalves. For example, Bado-Nille *et al*. (Bado-Nilles *et al.* 2008) reported an increase of the lysozyme activity in the haemocytes of Pacific oyster (*Crassostrea gigas*) *in vitro* exposure to several concentrations of dibenz(a,h)anthracene. However, in the same study, pyrene, phenanthrene and fluorene had no effect on the presence of lysosome. Contrary to this, Matozzo *et al.* (Matozzo *et al.* 2009) observed a decrease in lysozyme activity in the clam *Chamelea gallina* after exposure to 0.5 mg L^{-1} of benzo[a]pyrene. Other study carried out on the haemolymph of Cortez oyster (*C. corteziensis*) reported an inhibition and increase of lysozyme activity after exposure to lower (1 µg L^{-1}) and higher (20 and 50 µg L^{-1}) doses of naphthalene, respectively (Díaz-Resendiz *et al.* 2014). Matozzo *et al.* (Matozzo *et al.* 2012) explained the decrease in lysozyme activity in animals under stress conditions by a decrease in lysozyme secretion in order to reduce energy expenditure.

3.2.3. Alkaline phosphatase activity

The alkaline phosphatase (ALP) is a lysosomal enzyme acting on the cell membrane to control trans-membrane transit dynamics (Sarkar 1992) and in the process of mineralisation of the molluscan shell (Gaume *et al.* 2011). In our *in vitro* experiments, haemocyte alkaline phosphatase activity was not significantly affected by pyrene (Fisher, $p>0.05$) after a 24 h incubation compared to control samples (**Fig. 2C**). Moreover, an increase in alkaline phosphatase activity was recorded in the digestive gland of the edible ribbed clam (*Anadara rhombea*) after exposure to tributyltin chloride (TBTCl) (Ranilalitha *et al.* 2014). By contrast, an inhibition of this activity was recorded in the freshwater bivalve (*Lamellidens marginalis*) exposed during 48 h to arsenite ($NaAsO_2$, 5 mg L^{-1}) (Chakraborty *et al.* 2013). This variation in ALP activity may be explained by a probable compromise in cellular immunity, detoxification process and mineral dynamics in animal. Taking into account, our results and those available in the literature, it is concluded that the release of alkaline phosphatase requires a higher concentration of pyrene.

3.2.4. Esterase activity

Esterase plays an important role in the immunological defense in mussels, hydrolyzing a number of choline esters, including acetylcholine (Pretti and Cognetti-Varriale 2001). In bivalves, esterase activity was inhibited by neurotoxic compounds (Ozretić and Krajnović-Ozretić 1992, Tsangaris *et al.* 2008), heavy metals (Najimi *et al.* 1997) and PAHs (Gagnaire *et al.* 2006). Therefore, esterase is considered a useful biomarker of pollution. In the present study, esterase activity was significantly influenced by pyrene exposure (Fisher test, $p<0.05$) (**Fig. 2D**). Compared to control samples (4.422 ± 0.186 U mg^{-1} protein), pyrene (3.873 ± 0.206 and 3.849 ± 0.020 U mg^{-1} protein, at the concentration of 10^{-5} and 10^{-3} mg mL^{-1} respectively) significantly decreased esterase activity after a 24 h incubation. In the Pacific oyster *C.gigas*, pyrene, phenanthrene and fluorene (at 10^{-9}, 10^{-7}, 10^{-5} and 10^{-3} mg mL^{-1}) did not modulate the percentage of non-specific esterase positive cells after 24 h *in vitro* exposure, whereas it was increased by naphthalene (Bado-Nilles *et al.* 2008). In the opposite, Gagnaire *et al.* (Gagnaire *et al.* 2006) reported a decrease in the percentage of non-specific esterase positive cells in the same species exposed to benzo[a]pyrene, phenanthrene, anthracene and fluoranthene. This difference could be due to the temperatures, PAHs and species used in each study. In agreement with our results, previous study in the clam *Mya arenaria* demonstrated that the esterase activity decreased

as the cell viability decreased (Brousseau *et al.* 1999).This decrease in esterase activity may also be explained as lysozyme activity by a decrease in secretion in order to reduce energy expenditure.

3.2.6. Lectin activity

Lectin activity plays a crucial role in eliminating potential pathogens in marine invertebrates such as bacteria and parasites (Chu 1988). In the present study, lectin activity was not significantly affected by pyrene ($p>0.05$) after a 24 h incubation compared to control samples (**Fig. 2E**). In previous study, Ahmad *et al.* (Ahmad *et al.* 2011) reported a reduction in plasma agglutination in the bivalve *Scrobicularia plana* environmentally exposed to mercury. Moreover, Chikalovets *et al.* (Chikalovets *et al.* 2010) revealed significant changes in agglutination in the mussel *Mytilus trossulus* after exposure to cadmium. Taking into account, our results and those available in the literature, it is concluded that exposure to pyrene may affect lectin activity in *R. decussatus* but at high concentrations.

3.2.5. Antibacterial activity

In the current study, antibacterial activity (**Fig. 2F**) was significantly affected by the presence of pyrene (Fisher test, $p<0.05$). It was significantly higher in haemolymph exposed to 10^{-3} mg mL^{-1} of pyrene (0.683 ± 0.035) after a 24 h incubation compared to control samples (0.540 ± 0.034). These results reflect a positive effect of pyrene on antibacterial activity in *R. decussatus*. Xue and Renault (Xue and Renault 2000) have explained the increase in antibacterial activity by an increase in lysosomal enzymes and microbicidal agent responsible for the degradation of infectious agents. However, it is not the case in our experimental conditions where we have reported an inhibition in lysozyme and esterase activities. A number of case studies have reported the antimicrobial action of several classes of organic hydrocarbons on different microorganisms (Heipieper and Martínez 2018). Therefore, this increase in antibacterial activity may be explained by Pyrene residues in haemolymph samples.

3.3. Principal component analysis

PCA analysis has been proposed as a method for obtaining an overall view of the results based on immunological parameter data obtained from all treatments. In the current study, PCA performed on biomarker data extracted two main factors which explained 60.49% of the total variance (**Fig. 3**). Factor 1 explains 39.84% of total variance. Factor 2 explains 20.65% of total variance. This axis confirmed the immunotoxic effect of the two concentrations of pyrene.

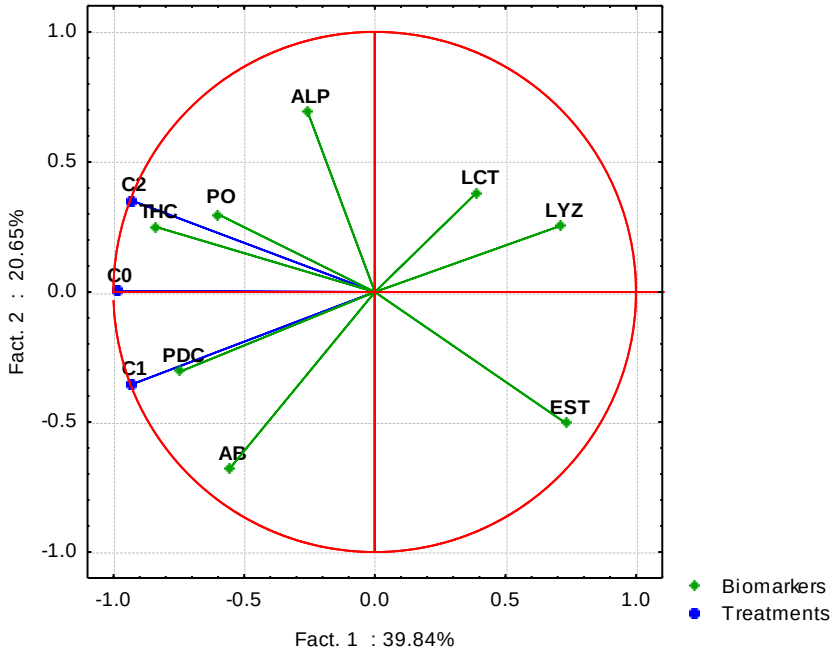


Fig. 3. Principal component analysis (PCA) of immune parameters measured in haemolymph of clam, *Ruditapes decussatus*, after 24 h exposure to 0 (C0), 10^{-5} (C1) and 10^{-3} (C2) mg mL^{-1} of pyrene. PO: phenoloxidase; LYZ: lysozyme; ALP: alkaline phosphatase; EST: esterase; LCT: lectin activity; AB: antibacterial activity; THC: total haemocyte count; and PDC: percentage of dead haemocytes.

Pearson's correlation coefficients between the biomarkers studied are shown in **Table 1**. A correlation coefficient higher than 0.5 was considered as significant at $P < 0.05$. In our experimental conditions, lysozyme activity was negatively correlated with antibacterial activity (r = -0.504) and percentage of dead haemocytes (r = -0.620). Moreover, esterase activity was negatively correlated with total haemocyte count (r = -0.764) and lectin activity was negatively correlated with antibacterial activity (r = -0.550).

Table 1 Pearson's correlation coefficients (r) of the immune-related parameters studied. Table legend: PO: phenoloxidase; LYZ: lysozyme; ALP: alkaline phosphatase; EST: esterase; LCT: lectin activity; AB: antibacterial activity; THC: total haemocyte count; and PDC: percentage of dead haemocytes.

	PO	LYZ	ALP	EST	LCT	AB	THC	PDC
PO	1							
LYZ	-0.247	1						
ALP	0.281	0.090	1					
EST	-0.446	0.415	-0.356	1				
LCT	-0.242	-0.060	-0.075	0.023	1			
AB	0.099	**-0.504**	-0.133	-0.015	**-0.550**	1		
THC	0.401	-0.490	0.339	**-0.764**	-0.237	0.334	1	
PDC	0.336	**-0.620**	-0.058	-0.384	-0.274	0.442	0.436	1

In summary, pyrene exposer showed an increase in haemocyte count while a decrease in cell viability. Moreover, our results reflect an inhibitory effect of pyrene on lysozyme and esterase activities. This decrease may be explained by a decrease in cell viability and a decrease in secretion of lysozyme and esterase in order to reduce energy expenditure whereas the increase in antibacterial activity may be explained by pyrene residues in haemolymph samples.

4. Conclusions

In conclusion, pyrene exposure caused significant alteration in the haemocyte parameters of the Mediterranean clam, *Ruditapes decussatus*. Our results showed that pyrene can increase or decrease haemocyte parameters. However, we cannot conclude whether an increase in a given haemocyte activity is related to a positive or a negative effect on immunity. These results need to be confirmed by *in vivo* experiments in order to demonstrate the effects of these potential immunomodulators in the whole animal.

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