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Acute toxicity of phenanthrene on the clam *Ruditapes decussatus*

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Graphical Abstrac

Abstract.

Phenanthrene, a major component of crude oil, is one of the most predominant polycyclic aromatic hydrocarbons (PAHs) in aquatic ecosystems.The present study aimed to assess the*in vitro*effects of phenanthreneon the clam, *Ruditapes decussatus*.For this purpose, haemolymph samples were exposed for 24 h to two concentrations (10^{-3} and 10^{-5} mg mL^{-1}) of this pollutant. Immune function was assessed using a combination of humoral and cellular responses.The toxic exposure caused alteration in the immune parameters of clamswith a significant increase(ANOVA, $p<0.05$)in total

	haemocyte count and haemocyte mortality and a significant decrease(ANOVA, $p<0.05$)inlysozyme and esterase activities. This study investigates the direct effects of phenanthrene on humoral and cellularimmune functions in clam *Ruditapes decussatus*.Further research is necessary to clarify the immunomodulatoryeffect of this contaminanton the whole animal. **Keywords:** Clams,*Ruditapes decussatus*;Phenanthrene; Immunetoxicity.

1. Introduction

Marine bivalve molluscs, such as clams, mussels and oysters are often usedas 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.

Polycyclic aromatic hydrocarbons (PAHs) are a ubiquitous class of organic contaminants found throughout the marine environment that may affect biota at various trophic levels in estuaries and coastal ecosystems. PAHs are characterized by high toxicity, high persistence in the environment and bioaccumulation capacity (Ramdine *et al.* 2012). PAHs 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).

Phenanthrene is one of the most predominant PAHs in the environment. This low molecular weight PAH is an important component of crude oil, with experiments indicating that phenanthrene dominates weathered oil after the volatile naphthalenes have been lost(Kennedy and Farrell 2005).In marine organisms, the toxic effects of phenanthrenehave been well studied. For example, a study carried out on the Marine medaka fertilized eggs (*Oryziasmelastigma*) showed that 25 d of chronic exposure to phenanthrene (0-800 μgL^{-1})prolongsthe embryonic incubation period,increasesthe deformityrates, and can cause up to 100% mortality(Mu *et al.* 2014).An additional study revealed a decrease ingrowthand alteration inhematological propertiesin the olive flounder, *Paralichthysolivaceus*exposed to waterbornephenanthrene(Jee *et al.* 2004). Moreover, many authors have pointed outalterations in the cellular and biochemical parameters of the haemolymph of various bivalves (oysters, clams, mussels, etc.) exposed to phenanthrene(Gagnaire *et al.* 2006, Bado-Nilles *et al.* 2008, Hannam *et al.* 2010).

Therefore, the general aim of the present study was to evaluate the *in vitro* effects of phenanthreneon innate humoral and cellular immune characteristics in the clam *Ruditapes decussatus*. Therefore, the total haemocyte count, cell viability, phenoloxidase, lysozyme, alkaline phosphatase, lectin and antibacterial activities were measured in the haemolymph of clams after exposure to different doses of phenanthrene.

2. Material and methods

2.1. Clams

Clams*Ruditapes decussatus*, 3-3.5 cm shell length, were purchased from the Bizerta shellfish farming (Bizerta, Tunisia) where they are veterinary and microbiologically controlled. Clams were maintained in aerated aquaria at 20 °C and were fed daily with the alga *Nannochloripsis* ($3x10^8$ cells

per day per clam) 3 h before changing the water. Clams were allowed to acclimatise for seven days before being used in the experiments.

2.2. Pollutant

Phenanthrene 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.

2.3. Experimental design

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 phenanthrenewere tested. These two PAH concentrations were chosen based on the available literature (Bado-Nilles *et al.* 2008, Bado-Nilles *et al.* 2009). Phenanthrenewere 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 haemolymphwas mixed immediately with formol then was kept at 4 °Cin order to determine the total haemocyte count (THC). To measure enzymatic activities, 500 µLof 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 evaluated by calculating the number of free haemocytes and the percentage of dead haemocytesin the hemolymph using a Malassez cell counter. Briefly, a volume of 25 mL of haemolymph was mixed with the same volume of formol6% 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 was measured spectrophotometrically by recording the formation of dopachrome from L-DOPA (Sigma), according to Asokan et al. (Asokan *et al.* 1997). Samples of 50 µL of haemolymphwere incubated for 5 min at room temperature with the same volume of SDS (sodium dodecyl sulphate, 1 mg mL^{-1}, Sigma). Then, 50 µL of L-DOPA (3 mg mL^{-1} in 0.5 M HCl containing 10 mM $CaCl_2$) were added as substrate.After a 5 min incubation period at room temperature in the dark, the decrease of optical density at 490 nm was followed every minute for 15 min in a plate

reader. Enzymeactivity 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 described by Parry *et al.*(Parry *et al.* 1965)with some modifications. 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.The reduction in absorbance at 450 nm was measured 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 U mg^{-1}haemolymph proteins.

2.5.3. Alkaline phosphatase activity

Alkaline phosphatase (ALP) activity was measured as described by Mansour *et al*. (Mansour *et al.* 2017). Aliquots of 100 µL of haemolymph were mixed with with the same volume of 4 mM p-nitrophenyl liquid phosphate (Sigma) in 100 mM ammonium bicarbonate buffer containing 1 mM $MgCl_2$ (pH 7.8, 30 ºC). The increase in absorbance at405 nmwas measured everyminute for 1 hin a plate reader, using standard samples without haemolymph as blanks. 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 as described by Mansour *et al*. (Mansour *et al.* 2017)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). 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. 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).A 25 µL volume of haemolymph and 75 µL of a suspension of *Escherichia coli* (ATCC35218) (10^8 cell mL^{-1} in TSB) were mixed in a 96 well plate. In the control, the haemolymph was substituted by Tryptone soy broth (TSB, Sigma). Then, the plate was incubated for 3 h at 18 °C. After that, 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 anti-bacterial activity index (BI) was calculated as follows:

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

2.5.6. 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). Briefly,25 µL of 3% human blood were mixed with the same volume 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. Protein determination

The total protein concentration present in the haemolymph samples was measured spectrophotometricallyat 595 nm by the Bradford method (Bradford 1976) with a reactive agent (BioRad). Bovine serum albumin was used as standard.

2.7. 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

In the current study, the total haemocyte count (**Table 1**) was significantly affected by the presence of phenanthrene(Fisher test, $p<0.05$). THC was increased in clams exposed to phenanthrene(13.74 ± 1.92 and 13.87 ± 2.41 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 number of case studies have reportedsimilar increases in the haemocyte count for bivalves exposed to various pollutants. For example, an increase in the total haemocyte count was recorded in the scallop *Pectenmaximus*after *in vivo* exposure to phenanthrene(Hannam *et al.* 2010). Moreover, an increase in THC in the oyster *Crassostreavirginica* was recordedafter exposure to a diet containing a mixture of PAHs (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.

3.1.2. Cell viability

The percentage of dead haemocytes (PDC) 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 phenanthrene(Fisher test, $p<0.05$). Compared to control samples (31.73 ± 4.18 %), phenanthrene(52.80 ± 4.43 %at the concentration of 10^{-5} mg mL^{-1}) significantly increased ($p=0.012$) haemocyte mortality after a 24 h incubation (**Table 1**).A similar effect on PDC was also been observed in the blue mussel (*Mytilusgalloprovincialis*) exposed for 7 days to phenanthrene and anthracene either alone or in a mixture (Giannapas *et al.* 2012). Whereas, study carried out on the Pacific oyster (*Crassostreagigas*) revealed a decrease of the percentage of dead cells 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} during 24 h (Bado-Nilles *et al.* 2008). In these *in vitro* experimentations, the high levels of cell death observed in clams exposed to phenanthrene revealed the induction of severe cellular damages.

Table 1 Total haemocyte count (THC), percentage of dead cells, phenoloxidase, lysozyme, alkaline phosphatase, esterase, antibacterial and agglutinating activities measured in haemolymph of European clam after 24 h exposure to 0, 10^{-5} and 10^{-3} mg mL^{-1} of phenanthrene. Data represent the mean ± S.E. Different letters denote significant differences between treatment groups ($p < 0.05$).

	Control	Phenanthrene (10^{-5} mg mL^{-1})	Phenanthrene (10^{-3} mg mL^{-1})
THC (x10^{6} cell mL^{-1})	8.2 ± 1.17a	13.74 ± 1.92b	13.87 ± 2.41b
Percentage of dead cells (%)	31.73 ± 4.18ab	52.80 ± 4.43b	30.57 ± 3.26a
Phenoloxidase activity (U mg^{-1} protein)	0.147 ± 0.040a	0.086 ± 0.069a	0.245 ± 0.027a
Lysozyme activity (U mg^{-1} protein)	525.88 ± 65.26a	366.03 ± 96.54ab	291.30 ± 139.74b
Alkaline phosphatase activity (U mg^{-1} protein)	54.537 ± 6.748a	57.576 ± 5.482a	68.715 ± 6.643a
Esterase activity (U mg^{-1} protein)	4.422 ± 0.186a	3.984 ± 0.236b	4.095 ± 0.183ab
Antibacterial activity	0.540 ± 0.034a	0.483 ± 0.011a	0.485 ± 0.034a
Agglutinating activity	1.67 ± 0.17a	2.20 ± 0.15a	1.50 ± 0.24a

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). Our results showed that the phenoloxidase activity was sligntly influenced by phenanthreneexposure (Fisher test, $p>0.05$) (**Table 1**). There was a tendency for this activity to decrease in clams exposed to phenanthrene(0.086 ± 0.069 U mg^{-1} protein,at the concentration of 10^{-5} and mg mL^{-1}) compared to control samples (0.147 ± 0.040 U mg^{-1} protein). Similar findings have also been reported in the Pacific oyster *C. gigas*, wherepyrene, phenanthrene and fluorene did not modulate the phenoloxidase activity after 24 h *in vitro* exposure (Bado-Nilles *et al.* 2008). Whereas, in the same study, exposure to benzo[b]fluoranthene increased this activity(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 *Crassostreacorteziensis*

however it inhibits this activity at lower doses; 1 and 20 µg L^{-1}(Díaz-Resendiz *et al.* 2014). With regard to our results, phenanthrenedid not modulate phenoloxidase activity, which may be explained by the tested dose.

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 phenanthreneon enzyme release by haemocytes. In our experimental conditions, lysozyme activity was significantly affected by phenanthreneexposure (**Table 1**). It was significantly lower in haemolymph exposed to 10^{-3} mg mL^{-1} of phenanthrene(291.30± 139.74 U mg^{-1} protein) after a 24 h incubation compared to control samples (525.88 ± 65.26 U mg^{-1} protein). A wide variety of studies have demonstratedthat the presence of contaminants can modulate the lysozyme activity in bivalves. Indeed, Bado-Nille*et al*. (Bado-Nilles *et al.* 2008) reported an increase of the lysozyme activity in the haemocytes of Pacific oyster (*Crassostreagigas*) *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 *Chameleagallina* 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 the current study, haemocyte alkaline phosphatase activity was not significantly affected by phenanthrene(ANOVA, $p>0.05$) after a 24 h incubation compared to control samples (**Table 1**). Contrarily to what observed in the present study,Chakrabarty*et al.*(Chakraborty *et al.* 2013)reported an inhibition in the alkaline phosphatase activity in the freshwater bivalve (*Lamellidensmarginalis*) exposed during 48 h to arsenite($NaAsO_2$, 5 mg L^{-1}).In the opposite,an increase in this activity was recorded in the digestive gland of the edible ribbed clam (*Anadararhombea*) after exposure to tributyltin chloride (TBTCl) (Ranilalitha *et al.* 2014). This variation in ALP activity may be explained by a probable compromise in cellular immunity, detoxification process and mineral dynamics in animal. With regard to this, it is concluded that the release of alkaline phosphatase requires a higher concentration of phenanthrene.

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 phenanthreneexposure (Fisher test, p=0.048) (**Table 1**). Compared to control samples (4.422 ± 0.186 U mg^{-1} protein), phenanthrene(3.984 ± 0.236 U mg^{-1} protein,at the concentration of 10^{-5} mg mL^{-1}) significantly decreased (ANOVA, p=0.0483) 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). Whereas, a decrease in the same biomarker was recorded in the same species after 24 h *in vitro* exposure to benzo[a]pyrene, phenanthrene, anthracene and fluoranthène (Gagnaire *et al.* 2006). This difference could be due to the temperatures, PAHs dose and species used in each study.On the other hand,our results are in accordance with previous study carried out in clams *Myaarenaria*which also demonstrated that the esterase activity decreased as the cell viability decreased (Brousseau *et al.* 1999).This decrease in esterase activity may also be explained by a decrease in secretion in order to reduce energy expenditure.

3.2.5. Lectin activity

Lectin activity plays a crucial role in eliminating potential pathogens in marine invertebrates such as bacteria and parasites (Chu 1988). In the current study, lectin activity was not significantly affected by phenanthrene(ANOVA, p>0.05) after a 24 h incubation compared to control samples (**Table 1**).Study carried out on the bivalve *Scrobiculariaplana*reported a reduction in plasma agglutination in animals environmentally exposed to mercury (Ahmad *et al.* 2011). Moreover, Chikalovets*et al.*(Chikalovets *et al.* 2010) revealed significant changes in agglutination in the mussel *Mytilustrossulus* after exposure to cadmium. Taking into account, our results and those available in the literature, it is concluded that exposure to phenanthrenemay affect lectin activity in *R. decussatus* but at high concentrations.

3.2.6. Antibacterial activity

A wide variety of studies have demonstrated the antimicrobial action of several classes of organic hydrocarbons on different microorganisms (Heipieper and Martínez 2018). However, in our experimental conditions, antibacterial activity (**Table 1**) was not significantly affected by the presence of phenanthrene(ANOVA, p>0.05).

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 52.57% of the total variance (**Fig. 1**). Factor 1 explains 29.14% of total variance. Factor 2 explains 23.43% of total variance. This axis

confirmedthe immunotoxic effect of phenanthrene and showed that toxic effects occur in a dose-dependent manner.

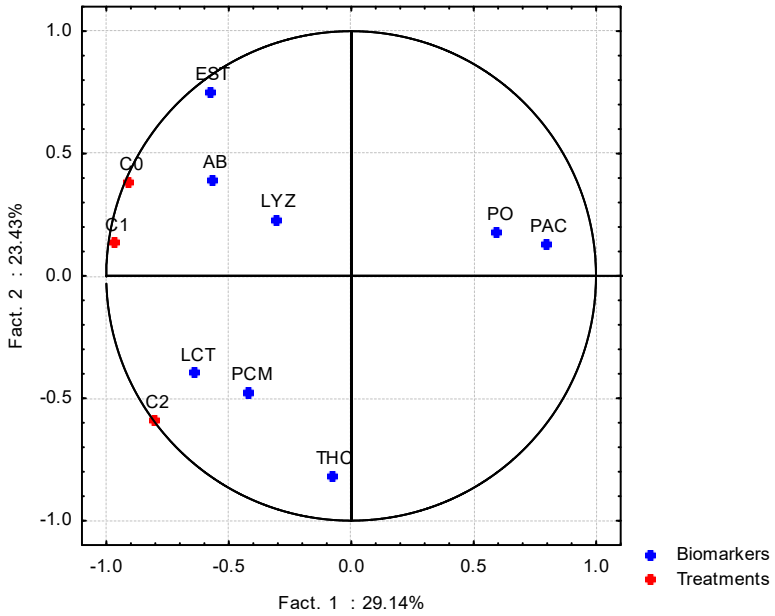

Fig. 1. Principal component analysis (PCA) of immune parameters measured in haemolymph of clam, *Ruditapes decussatus*, after 24 h exposure to 0 (C0), 10^{-5} (C1) and 10^{-3} (C2) mg mL^{-1} of phenanthrene. PO: phenoloxidase; LYZ: lysozyme; ALP: alkaline phosphatase; EST: esterase; LCT: lectin activity; 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 2**. A correlation coefficient higher than 0.5 was considered as significant at $P < 0.05$. In our experimental conditions, alkaline phosphatase activity was negatively correlated with lectine activity (r = -0.529) and slightly correlated with antibacterial activity (r = -0.440). Moreover, esterase activity was positively correlated with antibacterial activity (r = 0.591) and slightly correlated with total haemocyte count (r = -0.484).

Table 2 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**	**THC**	**PDC**	**AB**	**LCT**
PO	1							
LYZ	-0.155	1						
ALP	0.237	-0.092	1					
EST	-0.267	0.360	-0.262	1				
THC	-0.312	-0.235	-0.109	-0.484	1			
PDC	-0.118	0.092	-0.336	-0.123	0.238	1		
AB	-0.213	-0.228	-0.440	0.591	-0.085	0.091	1	
LCT	-0.326	0.227	-0.529	0.075	0.209	0.304	-0.040	1

In summary, phenanthrene exposer showed an increase in haemocyte count while a decrease in cell viability.Moreover, our results reflect an inhibitory effect of phenanthreneon 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.

4. Conclusions

Overall, phenanthreneexposure caused significant alteration in the haemocyte parameters of the clam, *Ruditapes decussatus*. The results from this study demonstrate revealed that phenanthrene 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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