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Combination of a pesticide exposure and a bacterial challenge: In vivo effects on immune response of Pacific oyster, Crassostrea gigas (Thunberg)

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Abstract:

To assess the impact of pollution induced by pesticides on Pacific oyster, Crassostrea gigas, health in France, in vivo effects of combined pesticide exposure and bacterial challenge on cell activities and gene expression in hemocytes were tested using flow cytometry and real-time PCR. As a first step, an in vivo model of experimental contamination was developed. Pacific oysters were exposed to a mixture of eight pesticides (atrazine, glyphosate, alachlor, metolachlor, fosetyl-alumimium, terbuthylazine, diuron and carbaryl) at environmentally relevant concentrations over a seven day period. Hemocyte parameters (cell mortality, enzyme activities and phagocytosis) were monitored using flow cytometry and gene expression was evaluated by real-time PCR (RT-PCR). The expression of 19 genes involved in C. gigas hemocyte functions was characterized using RT-PCR. After seven days of exposure, phagocytosis was significantly reduced and the 19 selected genes were down-regulated in treated animals. As a second step, the experimental contamination method previously developed was used to study interactions between pesticide exposure and bacterial challenge by intramuscular injection of two Vibrio splendidus-related pathogenic strains. Oyster mortality and expression of 10 of the 19 selected genes were followed 4h and 24h post-injection. Oyster mortality was higher in pesticide-treated oysters compared to untreated oysters after the bacterial challenge. Gene expression was up-regulated in pesticide-treated oysters compared to untreated oysters after the bacterial challenge. We hypothesize that gene over-expression due to an interaction between pesticides and bacteria could lead to an injury of host tissues, resulting in higher mortality rates. In conclusion, this study is the first to show effects of pesticides at environmentally relevant concentrations on C. gigas hemocytes and to hypothesize that pesticides modulate the immune response to a bacterial challenge in oysters.

Keywords
Pacific oyster, Crassostrea gigas; bivalve immunity; pesticides; hemocyte; phagocytosis; pathogenesis; Vibrio splendidus; flow cytometry; gene expression.

1. Introduction

Shellfish exploitation is an historical world-wide activity and has intensified over the last century. The Pacific oyster, Crassostrea gigas, is the most frequently cultivated bivalve species and is typically reared in estuarine environments that have become increasingly threatened by exposure to pollutants. Among pollutants, pesticide contamination of shellfish has become more common in estuarine areas over the past several decades due, in part, to chemical run-off from terrestrial agriculture (Banerjee et al., 1996). Pesticides are introduced into rivers by ground "scrubbing" when rainfalls occur and then may enter marine areas, particularly estuarine and coastal zones. These pollutants may have major ecological consequences and could endanger organism growth, reproduction or survival (Banerjee et al., 1996).

Among physiological processes possibly disturbed by pollutants, the immune system is likely to be one of the more sensitive physiological systems (Fournier et al., 2000). The immune system contributes to host homeostasis by eliminating foreign particles (viruses, bacteria or parasites), killing abnormal cells and rejecting "non-self" components (Fournier et al., 2000). Pollutants or xenobiotics can interact with immune system components and interfere with protection functions and are therefore referred to as immunotoxics (Colosio et al., 2005). Xenobiotics can induce immunosuppression or stimulation, auto-immunity and decrease of disease resistance (Wong et al., 1992). In the past decades, the emergence of infectious diseases has been reported in marine species and disease outbreaks have also increased (Harvell et al., 1999). According to Snieszko (Snieszko, 1974), the development of an infectious disease results from an unbalance between the host and the pathogen due to external factors (including pollutants) and/or internal factors of both protagonists (virulence of the pathogen, susceptibility of the host). Animals presenting impaired defence mechanisms may be more susceptible to infectious diseases. Demonstration of the relationship between pollution and increase of susceptibility to infectious diseases exist in vertebrates. Harbour porpoises that died from infectious diseases presented a higher polychlorobiphenyl level than those that died from physical trauma (Jepson et al., 2005). Some studies have also attempted also to link contaminant presence and susceptibility to infectious diseases in bivalves. The bivalve defence system is mediated, in part, by hemocytes responsible for recognition, phagocytosis, and elimination by oxidation of non-self particles (Cheng, 1981). Several studies have demonstrated harmful effects of pollutants in bivalves, including PAHs (Wootton et al., 2003), PCBs (Canesi et al., 2003), pesticides (Alves et al., 2002) and heavy metals (Gagnaire et al., 2004) and in particular in the Pacific oyster, Crassostrea gigas (Gagnaire et al., 2006). Contamination of the eastern oyster, Crassostrea virginica, by polluted sediment and tributyltin increased the intensity of Perkinsus marinus infection, but no cellular or humoral parameters were modulated (Chu et al., 2002). In contrast, contamination of Mytilus edulis followed by a V. tubiashii challenge resulted in an increased hemocyte count when cadmium was used (Pipe and Coles, 1995) and in an increased phagocytosis with low concentrations of copper (Parry and Pipe, 2004). However, no clear relationship has been established between contaminants and immune response, especially in C. gigas. This phenomenon is nevertheless of interest because of its potential impact on the shellfish farming economy.

In this context, the Pacific oyster, C. gigas, was used to evaluate the impact of a pesticide mixture on some immune-related capabilities in an economically important bivalve species and to demonstrate a relationship between infectious diseases, defence capacities and pollutants. In a previous study, we have demonstrated in in vitro assays the effect of a mixture of eight pesticides on phagocytosis on C. gigas hemocytes, while none of these eight pesticides induced any effects when tested separately (Gagnaire et al., 2006). The aims of the present study were (i) to develop an in vivo model of experimental contamination using a mixture of pesticides similar to that used previously and representative of concentrations experienced in natural settings where oysters are cultured and (ii) to study effects of this mixture on the immune response to a bacterial challenge.

Oysters were exposed over a seven day period with a mixture of eight pesticides (atrazine, glyphosate, alachlor, metolachlor, fosetyl-alumimium, terbuthylazine, diuron and carbaryl). The pesticides were selected on the basis of spread amounts in the Marennes-Oleron Basin (Charente-Maritime, France), one of the most important oyster producing areas in France (Léonard, 2002; Munaron et al., 2006). As a first step, oysters were only contaminated in three distinct experiments and hemocyte biomarkers (cell mortality, esterase activity, production of Reactive Oxygen Species (ROS) and phagocytosis) were monitored using flow cytometry. The expression of 19 genes involved in hemocyte functions was also analysed using real-time PCR. In a second step, after a seven day contamination period, two pathogenic Vibrio splendidus-related strains were simultaneously injected

into the adductor muscle of oysters. Oyster mortality was daily monitored. The expression of ten genes among the 19 genes involved in hemocyte functions was followed after bacterial injection using real-time PCR.

2. Material and Methods

2.1. General Methods

2.1.1. Animals

Pacific oysters, C. gigas, with a shell height of 3-5 cm, were produced and held at the IFREMER's laboratory in La Tremblade (Charente-Maritime, France) in February 2003. Analyses were performed from February 2004 to March 2005.

2.1.2. Xenobiotics

Eight pesticides were selected: atrazine, glyphosate, alachlor, metolachlor, fosetyl-alumimium, terbuthylazine, diuron and carbaryl (Promochem®). A stock solution of each solid pesticide was separately prepared in an appropriate solvent (distilled water for glyphosate and terbuthylazine, 80 % alcohol for the six other pesticides). The eight pollutant stock solutions were then each mixed in distilled water and 10 mL aliquots of this mixture was diluted in 1,990 mL of seawater to create a working solution. This working solution was prepared just before assays and 2,000 mL were added to individual experimental (contaminated) tanks (100 L). Oysters were only exposed to the mixture of eight pesticides. The final concentrations in tanks were: 0.7 µg/L for atrazine, 0.7 µg/L for glyphosate, 0.8 µg/L for alachlor, 0.6 µg/L for metolachlor, 0.6 µg/L for fosetyl-alumimium, 0.6 µg/L for terbuthylazine, 0.5 µg/L for diuron and 0.05 µg/L for carbaryl.

2.1.3. Exposure protocol

Experiments were conducted at IFREMER's laboratory in La Tremblade (Charente-Maritime, France). Oysters were acclimated over a one week period by increasing water temperature from 11-12°C to 19-20°C using a thermostatic control (1-2°C increase per day). Seawater temperature was maintained at 19-20°C during the trial. Two tanks were used: One received 100 oysters in 100 L of seawater (untreated control) and one received 100 oysters in 100 L seawater added with pesticides (experimental treatment). Oysters were fed daily with Chaetoceros gracilis (addition of 10 L of phytoplankton solution at $5x10^6$ cells/mL in both tanks). Seawater was changed daily in both tanks. The pesticide solution was added every day after the seawater renewal in the experimental tank for seven days.

2.1.4. Bacterial challenge

Bacterial strains LGP31 and LGP32 (Vibrio splendidus-related) used in this study were isolated from oyster hemolymph during a mortality outbreak (Gay et al., 2004a). Both bacterial strains were separately grown at 20°C for 24 h in solid Zobell media that contained 2g pastone, 0.5 g yeast extract, 6g agar, 500 ml of autoclaved (121°C for 20 min) artificial seawater (ASW: 234g NaCl, 15g KCl, 12g $MgSO_4$ 7 H_2O, 2g $CaCl_2$ 2 H_2O, 1 l distilled water, use at 1/10 dilution, pH = 7.4). After 24h, bacterial strains were maintained separately in 5 mL of liquid Zobell (2g pastone, 0.5g yeast extract, 500 mL sterile ASW, pH = 7.4) and cultivated with agitation for 24h at room temperature. An equal volume of each bacterial suspension was collected and the two strains were mixed. Bacterial concentrations were evaluated using a spectrophotometer at 600 nm (UV-160, Shimadzu®). If the optical density value was 1.1, cell concentration was considered as 10^9 Colony Forming Units (CFU).ml^{-1}. Bacteria were centrifuged (30 min, 2,000 g) and re-suspended in ASW.
Before injection, oysters were anaesthetised by the addition of a 50 $g.l^{-1}$ $MgCl_2$ solution (250g $MgCl_2$, 2 l seawater, 3 l freshwater) with phytoplankton (Isochrysis sp.) to stimulate feeding over a 2-3 h period with oxygenation. Subsequently, 50 µL of the mixed bacterial strains were injected into the adductor muscle. After injection, oysters were maintained in tanks (30 oysters in 2.5 l) in aerated seawater filtered to 0.45 µm at 20°C. Animals were fed with phytoplankton daily.
These two Vibrio splendidus-related strains have a growth optimum of 19-20°C. These strains were isolated from a mortality episode which occurred at similar temperatures. We, therefore, chose to conduct our bacterial challenge experiments at these temperatures.

2.1.5. Hemocyte collection

Hemolymph (0.5-1.0 mL) was collected from the pericardial cavity with the use of a 1-mL syringe equipped with a needle (0.9x25 mm) after opening carefully the shell. Hemolymph samples were

filtered through a 60 µm mesh to eliminate aggregates and then maintained on ice to limit hemocyte aggregation for further flow cytometry analysis and RNA extraction.

2.1.6. Flow cytometry analysis

Flow cytometry protocols were previously described (Gagnaire et al., 2006). For each sample, 3,000 events were counted using an EPICS XL 4 flow cytometer (Beckman Coulter). Results were analysed on cytograms indicating cell size (FSC value) and cell complexity (SSC value) with the fluorescence channel(s) corresponding to the marker used. A gate was defined on the basis of FSC value in order to eliminate cell debris and bacteria. Excitation was performed by an argon laser at 488 nm.
Four hemocyte parameters (cell mortality, esterase activity, ROS production and phagocytosis) were monitored using flow cytometry. Analyses were conducted using 200 µL of hemocyte suspension. For cell mortality, hemocytes were incubated in the dark for 30 minutes at 4°C with 10 µL of propidium iodide (PI, 1.0 $mg.L^{-1}$, Interchim); fluorescence emission was measured at 600 nm. The EPICS XL 4 software allows differentiation between supposed populations of granulocytes and hyalinocytes based on FSC and SSC values. Esterase activity was measured using the non-specific liposoluble substrate fluoresceine diacetate (FDA, Molecular Probes). The non-fluorescent FDA diffuse in most of cells due to its liposoluble nature. Non-specific esterases hydrolyse it in a fluorescent product trapped in viable cells (Dive *et al.*, 1990). One µL of a FDA solution (400 µM) was added to 200 µL of hemocyte suspension; fluorescence emission was measured at 514 nm. Production of ROS was measured using dihydrorhodamine 123 (DHR123, Molecular Probes). DHR123 is a non-fluorescent reduced product derived from rhodamine 123. DHR 123 diffuses through cells and is oxidized in fluorescent rhodamine 123 by superoxide anion O_2^- during oxidative burst (Rothe et al., 1988). One µL of a DHR123 solution (145 µM) was added to 200 µL of hemocyte suspension; fluorescence emission was measured at 528 nm. For esterase and ROS detection, cells were incubated for 30 minutes in the dark at room temperature and the reaction was stopped on ice (5 minutes). For esterase activity and ROS, gates were defined on cytograms in order to distinguish different populations according to fluorescence intensity and statistical analysis was only conducted on the population of strongly stained cells (Gagnaire et al., 2006). For phagocytic assay, 200 µL of hemocyte suspension were incubated for 1 h in the dark at ambient temperature with 10 µL of a 1/10 dilution of Fluorospheres ® carboxylate-modified microspheres (1 µm diameter, Interchim); fluorescence emission was measured at 515 nm. Phagocytosis was measured as the proportion of cells that presented a fluorescent signal equal to or higher than the fluorescence of three beads as previously published (Gagnaire et al., 2006).

2.1.7. Gene selection

Nineteen genes involved in hemocyte functions were selected from C. gigas cDNA databases. Sixteen genes encode cell signalling proteins (ficolin, galectin, LBP/BPI and LPS/β-1,3 glucan, Vav, importin α, ECSIT, DOCK180 and C-Src kinase), cytoskeleton proteins (ankyrin, cofilin, filamin and Rho) and proteins involved in post-phagocytosis degradations and cellular protection mechanisms (Isocitrate dehydrogenase, proCL and SOD). Three other genes related to defence mechanisms in general were also monitored (Tissue Inhibitor of MetalloProteinases (TIMP), lysozyme and defensin) (Table 1).

2.1.8. Total RNA extraction

Total RNA was extracted from hemocytes using Trizol (Trizol ® reagent, Invitrogen™; 1 ml Trizol for $0,5\text{-}1.10^6$ hemocytes) and treated with 1 unit DNase I (deoxyribonuclease) (Sigma) per µg of total RNA to prevent DNA contamination. RNA concentrations were measured before and after DNase treatment at 260 nm using the conversion factor 1 OD = 40 µg RNA.

2.1.9. Reverse transcription

Reverse transcription (RT) was carried out as previously described (Huvet et al., 2003) using oligo(dT)23 anchored as a primer, 200U moloney murine leukemia virus (MMLV) reverse transcriptase (Sigma), and 2 µg of total RNA PCR samples from each condition.

2.1.10. Real-time PCR analysis

The relative levels of gene transcripts in untreated (U) and pesticide-treated (T) oysters were investigated by real-time PCR using an Icycler (Bio-Rad Laboratories Inc.). Amplification of a specific cDNA (elongation factor, coefficient of variation < 5 %) was performed to confirm the steady-state

expression of a housekeeping gene, allowing an internal control for gene expression. Primer sequences and GenBank accession numbers for all studied and housekeeping genes are reported in Table 2. Real-time PCR was performed in triplicate in a total volume of 15 µL with 5 µL of cDNA (1/10 dilution), 0.5 µL of each primer (final concentration of 0.33 µM), 1.5 µL distilled water and 7.5 µL of 2X "iQ^{TM} SYBR® Green supermix" (BioRad). The cycling conditions consisted Taq polymerase thermal activation for 3 min at 95°C, then 40 cycles of denaturation at 95°C for 30 s and annealing/elongation at 60°C for 1 min. For each individual sample, a melting curve program was carried out from 95°C to 55°C by decreasing temperature by 0.5°C each 10 s in order to confirm production of a single product. Each run included the cDNA control, negative controls (total RNA treated with DNase I) and blank controls (distilled water). PCR efficiency (E) was determined for each primer pair by performing standard curves from serial dilutions to ensure that E ranged from 99% to 100%. Cycle threshold (Ct) value corresponded to the number of cycles at which the fluorescence emission monitored in real-time exceeded the threshold limit. Ct and E were determined using the iCyclerTMiQ, Optical System Software, v. 3.0a (BioRad). The relative expression ratio of each gene was calculated using REST-384 © software (Relative Expression Software Tool, http://www.gene-quantification.de/rest-384.html) (Pfaffl et al., 2002). The relative expression ratio for a considered gene is based on the PCR efficiency (E) and the Ct of the pesticide treated (T) sample versus the untreated (U) sample expressed in comparison to the reference gene, according to Pfaffl's mathematical model (Pfaffl, 2001):

$$\text{Ratio} = \frac{(E \text{ studied gene})^{\Delta Ct \text{ studied gene (untreated-treated)}}}{(E \text{ reference gene})^{\Delta Ct \text{ reference gene (untreated-treated)}}}$$

2.2. Schedule of trials

2.2.1. Pesticide contamination

The contamination experiment was distinctly performed three times during February and March 2004. Flow cytometry analyses were performed for these three experiments to assess the four hemocyte parameters outlined in the general methods section above. At days 1, 3 and 7 after pesticide contamination, hemocytes from 20 oysters per condition (untreated (U) and treated (T)) were collected. Twenty untreated oysters were also analysed at Day 0 (before adding pesticides). For both conditions (U and T), hemolymph from four pools of five oysters were used to reduce inter-individual variation and to provide enough hemocytes. Pooled hemocytes were counted on a Malassez cell and were adjusted to 10^6 cells.mL^{-1} with ASW. After seven days of contamination for the first experiment, hemolymph from 20 oysters per condition (U and T) were collected and pooled for RNA extraction and characterization of gene expression using real-time PCR analysis of the 19 selected genes (Table 1).

2.2.2. Bacterial injection

Experiments were run in January 2005 without pesticide contamination in order to define the mortality percentages induced by different concentrations of LGP31+LGP32 Vibrio strains in control (without pesticides) oysters. Equal concentrations of each strain were inoculated into oysters (total concentrations of 2.10^8, $4.10^7$and 4.10^6 CFU/oyster). Experiments were carried out three times for each bacterial concentration and two replicates were employed in each of the three conditions (injected with 2.10^8, $4.10^7$and 4.10^6 CFU/oyster).Oyster mortality percentages were recorded for each bacterial concentration at several post-injection times (2h, 4h, 24h, 48h, 72h, 120h, 144h and 168h).

2.2.3. Pesticide contamination followed by bacterial injection

In order to observe the impact of pesticide contamination on oyster sensitivity to bacterial disease, exposure to the pesticide mixture was followed by a bacterial injection trial in March 2005. After a contamination period of seven days as outlined above, treated oysters as well as untreated oysters were injected with 4.10^7 CFU/oyster. Negative controls consisted of ten oysters (U and T) that were injected with sterile ASW. Oyster mortality was monitored for each of the four conditions (U injected with ASW, T injected with ASW, U injected with 4.10^7 CFU/oyster, T injected with 4.10^7 CFU/oyster) at several times post-injection (2h, 4h, 24h, 48h and 72h). Two replicate tanks were included for each of the four conditions. This experiment was realised one time.

Hemolymph from ten oysters for the four conditions were collected 4h and 24h post-injection for RNA extraction and gene expression analysis. In this trial, the expression of ten genes was followed by real-time PCR: ficolin, galectin and LBP/BPI, C-Src kinase, ankyrin, proCL, SOD, TIMP, lysozyme and defensin (Table 1).

Statistical analysis. For flow cytometry analysis, results were expressed as percentage of positive cells. Results were expressed as percentages for mortality measurements. Values were arcsine square root transformed to achieve normality. ANOVAs were carried out using Statgraphics ® Plus version 5.1 software. Significance was set at $p \leq 0.05$.
For real-time PCR analysis, statistical differences in gene expression between conditions were evaluated in group means by randomisation tests (Pfaffl et al., 2002) using REST-384 © software and Pair Wise Fixed Reallocation Randomisation Test©. Two thousand random allocations were performed and significant differences were considered at $p<0.05$.

3. Results

3.1. Effects of pesticide exposure on oysters

3.1.1. Hemocyte parameters

No oyster mortality was reported in any of the three experiments (data not shown). Cell mortality, esterase and ROS production showed no significant differences between U (untreated oysters) and T (treated oysters) (**Erreur ! Source du renvoi introuvable.**). Phagocytosis was significantly lower in T compared to U (i) after seven days of contamination for experiment 1, one and seven days for experiment 2, and 3 and seven days for experiment 3 ($p<0.05$, **Erreur ! Source du renvoi introuvable.**), (ii) for the whole data treated independently of time for each experiment and (iii) after seven days of contamination when pooling data of the three experiments (data not shown).

3.1.2. Gene expression

Relative expression of the 19 selected genes involved in hemocyte functions was significantly lower for all genes from treated (T) compared to those from untreated (U) animals ($p<0.001$, **Erreur ! Source du renvoi introuvable.**). Decreases in gene expression of T compared to U ranged from 60.8 % for defensin (2.5 times lower) to 96.9 % for filamin (32.6 times lower) (**Erreur ! Source du renvoi introuvable.**).

3.2. Effects of bacterial challenge on oyster mortality

The injection of 4.10^7 CFU/oyster induced 55 ± 8.8% of mortality at 168h post-injection (**Erreur ! Source du renvoi introuvable.**). Injection of 4.10^6 CFU/oyster induced significantly lower mortalities relative to other concentrations: mortality percentage was 20 ± 10.5% 168h post-injection (**Erreur ! Source du renvoi introuvable.**). Injection of 2.10^8 CFU/oyster induced the highest mortality: mortality rates were 23.3 ± 6% and 90 ± 2.6% at 2h and 168h post-injection, respectively (**Erreur ! Source du renvoi introuvable.**).

3.3. Combined effects of pesticide exposure and bacterial challenge on oysters

Preliminary experiments using only bacteria showed that 2.10^8 CFU/oyster, induced high losses 24h post-injection. In contrast, 4.10^6 CFU/oyster induced low mortality. Both bacterial concentrations were not selected for further studies because pesticide effects on mortality would have been difficult to detect under very high or low mortality conditions. The concentration of 4.10^7 CFU/oyster was selected to carry out assays including pesticide contamination and a bacterial challenge.

3.3.1. Mortality

After bacterial inoculation, mortality was significantly higher for treated oysters (T) compared to untreated oysters (U) at 48h, 72h post exposure and when cumulative mortality was examined (three way ANOVA, $F = 19.62$, $p = 0.0001$, **Erreur ! Source du renvoi introuvable.**). Seventy percent (± 5 %) of T injected with 4.10^7 CFU/oyster died by 72h post-injection, while only 25 ± 3.7 % of U injected with the same dose died (**Erreur ! Source du renvoi introuvable.**). At 24h post-injection, mortality rates were not significantly different between pesticide T and U injected with bacteria ($p>0.05$, **Erreur !**

Source du renvoi introuvable.). A few of control oysters, T or U injected with ASW, died during this trial (8 % of cumulative mortality 72h post-injection) and values were not significantly different between T and U (**Erreur ! Source du renvoi introuvable.**). Mortality was highest at 72h post-injection in all conditions (F = 50.75, p = 0, **Erreur ! Source du renvoi introuvable.**).

3.3.2. Gene expression

In our experiments, gene expression from pesticide treated animals injected with ASW or bacteria were compared to untreated animals injected with ASW or bacteria. Our experiment design allowed to follow pesticide effects, and not bacterial effects alone, on gene expression after a bacterial challenge. We monitored the expression of only ten of the 19 genes followed in pesticide contamination experiments (Table 1). We had to make a selection because of the limited quantity of RNA extracted from hemocytes for these experiments. We monitored gene expression 4h and 24h post-injection because mortality rates were not significantly different between treated injected oysters and untreated injected oysters. Samples at this time were therefore comparable.

Experimental treatments impacted gene expression. Both down and up-regulation was observed.
(i) When injected with seawater, the relative expression of five genes (galectin, C-Src kinase, ankyrin, lysozyme and defensin) was down-regulated in pesticide treated oysters (T) compared to untreated oysters (U) 4h post-injection (1.9, 2.7, 1.8, 1.7 and 2.6 times lower, respectively, $p<0.05$, **Erreur ! Source du renvoi introuvable.**). The relative expression of three genes (ficolin, SOD and TIMP) increased 24h post-injection of ASW (3.0, 4.6 and 2.4 times higher, respectively, $p<0.05$,**Erreur ! Source du renvoi introuvable.**).
(ii) When injected with bacteria, the relative expression of ficolin, galectin, C-Src kinase, ankyrin, SOD, lysozyme and defensin decreased in T compared to U 4h post-injection of 4.10^7 CFU/oyster (8.9, 6.6, 9.1, 3.3, 4.4, 23.6 and 2.2 times lower, respectively, $p<0.05$, **Erreur ! Source du renvoi introuvable.**). The expression of the ten genes (ficolin, galectin, LBP/BPI, C-Src kinase, ankyrin, proCL, SOD, TIMP, lysozyme, defensin) was up-regulated 24h post bacterial injection (3.8, 3.9, 3.4, 3.4, 2.1, 6.3, 2.5, 3.5, 2.6 and 2.9 times higher, respectively, $p<0.05$, **Erreur ! Source du renvoi introuvable.**). Among these ten genes, the expression of seven genes (galectin, LBP/BPI, C-Src kinase, ankyrin, proCL, lysozyme and defensin) was not changed 24h after ASW injection. Results are summed up on Table 3.

4. Discussion

Although often employed in successful modern agriculture, the use of pesticides leads to severe environmental pollution and can be dangerous for human safety. Dangerous amounts of pesticides and pesticide residues have been found in vegetables, milk and meat (Banerjee et al., 2001). The effects of pesticides have been evaluated most commonly by quantifying enzymatic alterations, and pathological, carcinogenic and mutagenic effects. Despite the intensive use of pesticide in coastal areas, few studies have investigated their effects on marine animals in laboratory trials (Galloway and Depledge, 2001) and have focused primarily on the effects of pesticide mixtures (Faust et al., 2001; Tanguy et al., 2005; Gagnaire et al., 2006). Although animals are exposed to complex mixtures of pollutants in the environment, most of laboratory experiments are based on the use of a unique pollutant (Banerjee et al., 2001). Studies on herbicides (Faust et al., 2001) have shown some positive interactions: toxicity of a mixture of pollutants was higher than the toxicity of individually tested chemicals. A pesticide mixture modulated C. gigas hemocyte phagocytosis in vitro whereas no modulation was reported for each molecule tested individually (Gagnaire et al., 2006). Antagonist effects have also been shown for heavy metals (Breittmayer et al., 1984).

4.1. Effects of pesticides on hemocyte parameters and defence gene expression

Although cell mortality, esterase and ROS activity were not modified by the pesticide mixture, phagocytic ability decreased after three and seven days of contact in the three experiments. Some of the selected pesticides present in the mixture have already been tested individually on phagocytosis in in vivo assays. Atrazine decreased phagocytosis in Lymnaea stagnalis (Russo and Lagadic, 2004) but had no effect on that activity in C. gigas (Gagnaire et al., 2003). Diuron increased phagocytosis of C. gigas hemocytes after 4 weeks of exposure (Bouilly et al., 2007). The combined effects of these molecules affected phagocytosis in C. gigas.

The expression of all 19 genes monitored by real-time PCR in the present study showed a decrease in mRNA levels in samples exposed to pesticides compared to untreated samples. These results support the observed decrease in phagocytosis activity reported using flow cytometry after three and seven days of contamination; some of these genes are involved in defence mechanisms and phagocytosis process. Indeed, ficolin-3 and galectin-4 are two lectins that bind β-galactoside residus (Mitta et al., 2005). LBP/BPI complex and LGBP bind LPS and activate the pro-phenoloxidase cascade (Mitta et al., 2005). LGPB is over-expressed upon bacterial infections (Tanguy et al., 2004) and was over-expressed in C. gigas after 30 days of contamination with a mixture of atrazine, diuron and isoproturon (Tanguy et al., 2005). Our results, in contrast, showed a down-regulation of these genes. Isoproturon was not used in our experiments and concentrations of other molecules were lower than in the study by Tanguy et al. (2005). Vav-3 protein, cofilin and importin-α are involved in signal cascade of lymphocytes in vertebrates (Lee et al., 2000; Andrade et al., 2003; Fujikawa et al., 2003). DOCK180 protein and c-Src kinase are involved in macrophage activation (Suzuki et al., 2000; Cote and Vuori, 2002). ECSIT is involved in the Toll/IL-1 and Rel/NF-κB signal cascade, important in innate immune response to infectious agents by the regulation of MAPK (Kopp et al., 1999), which exists in bivalves (Canesi et al., 2002). Our experiments showed a down-regulation of these six genes which, suggest a disruption of cell activation pathways, and may be related to the reduced phagocytic ability observed in the present study. Ankyrin is important in cytoskeleton organisation (Lambert and Bennett, 1993). Filamin, an actin binding protein, and Rho protein, a GTPase, are involved in the regulation of actin organisation and adhesion (Chimini and Chavrier, 2000; Van der Flier and Sonnenberg, 2001). In our study, these three genes were down-regulated by pesticides exposure, which could modify cytoskeleton activities and render hemocytes unable to phagocytose particles. Isocitrate dehydrogenase and SOD protect cells against reactive oxygen species (Kim and Park, 2003). We showed a down-regulation of these both genes, which could lead to a decrease of protection against free radicals, one of the known action mode of several pesticides (Banerjee et al., 2001). However, no modification of ROS production monitored by flow cytometry was found after the pesticide exposure (**Erreur ! Source du renvoi introuvable.**). Cathepsin L is present in lysosomes and is involved in protein degradation (Hu and Leung, 2004). Our results showed a down-regulation of this gene by pesticides which could lead to a decrease of post-phagocytosis degradations. However, cathepsin L over-expression has been observed in oysters contaminated with glyphosate after 30 days of contamination (Tanguy et al., 2005). TIMP is a protease inhibitor over-expressed after injuries and bacterial challenges in C. gigas (Montagnani et al., 2001). Lysozyme is a bactericidal enzyme largely present in bivalves (Cheng, 1981). Defensin is an anti-bacterial peptide (Gueguen et al., 2006). These

genes were down-regulated in our experiments. Pesticides may lead to decrease of bactericidal activities.
The down-regulation of these 19 genes by pesticides may therefore decrease oyster capacity to respond to an infectious agent.

4.2. Effects of pesticides and bacterial injection on oyster mortality and gene expression

Vibrio splendidus-related strains (LGP31+LGP32) used in this study were previously isolated from moribund oysters during a mortality episode. These bacteria induced a higher mortality when injected simultaneously than when inoculated separately (Gay et al., 2004a). Intramuscular injection was selected in the present study to induce high mortality rates (~50 %) (Gay et al., 2004a). This technique by-passes first lines of defence. Although bath exposure using the same bacterial strains induced mortality, losses were lower than via injection with high variations between experiments (Gay et al., 2004b).
The present study provides the first demonstration of the effects of a pesticide mixture at environmentally relevant concentrations on C. gigas exposed to a bacterial challenge. After the bacterial challenge, mortality rates in pesticide treated animals were higher than in untreated animals at 48h and 72h post-injection, indicating that pesticide exposure increased oyster susceptibility to the bacterial injection. Chou et al. (Chou et al., 1998) previously demonstrated an interaction between heavy metal exposure and viral infection in the Oriental calm, Meretrix lusoria. Higher losses were observed in M. lusoria contaminated with heavy metals and exposed to a birnavirus relative to animals that were exposed to single stressors (heavy metals or birnavirus).
An increase of the expression of the ten selected genes was reported 24h after the bacterial challenge in pesticide treated oysters. However, three of these ten genes (ficolin, TIMP and SOD) were also up-regulated 24h after ASW injection. We could hypothesize that the up-regulation of these three genes is due to the mechanical aggression due to injection. The higher expression of the seven other genes (galectin, LBP, C-Src kinase, ankyrin, proCL, lysozyme, defensin) could be first interpreted as a more efficient defence response of the oysters to the bacterial injection. Up-regulation of these genes was reported in invertebrates and vertebrates during the course of infectious diseases (Montagnani et al., 2001; Ge et al., 2004; Allam et al., 2006). However, higher mortality rates were reported in pesticide treated injected oysters compared to untreated injected oysters at 48h and 72h post-injection. Moreover, high expression of some of these seven genes observed during the course of infectious diseases has been demonstrated to correlate with host damage in vertebrates. High levels of cathepsins B, D and L were observed after a Pseudomonas aeruginosa infection in mice and resulted in corneal lesions (Dong et al., 2001). In another study, defensin concentrations were higher in human patients infected with lung diseases than in healthy human patients and have been demonstrated to contribute to disease pathogenesis (Ashitani et al., 1998). In vertebrates, when mechanisms initially developed for the host defence are not correctly controlled, these complex defence mechanisms could create pathological disorders (Haslett et al., 1989).
Pesticides modified the oyster response to a bacterial challenge by inducing an up-regulation of genes involved in hemocyte functions. Some of these genes are involved in defence mechanisms. We could therefore expect that oyster would present higher defence capacities and therefore reduced disease susceptibility. However, by analogy with vertebrates, and considering the fact that oyster mortality was higher in treated animals compared to untreated animals after the bacterial challenge, we therefore hypothesize that this up-regulation of defence genes could become harmful for the host by causing tissue damages and could be associated to pathogenesis of the disease. Pesticides could modify the regulation capacity of the response to infectious agents in oysters. To support this concept and to substantiate correlation among over-expression of genes and host damage, some tissue morpho-histological observations are needed.

5. Conclusion

This study showed that a pesticide mixture used on Pacific oyster, C. gigas, in laboratory conditions at environmentally relevant concentrations (i) decreased hemocyte phagocytosis in a reproducible way, (ii) down-regulated genes involved in hemocyte functions, particularly phagocytosis, and (iii) increased susceptibility to a bacterial challenge. This is the first study to combine two different techniques (flow cytometry and real-time PCR) to study the effects of pollutants on defence mechanisms in Pacific

oyster, C. gigas, and to demonstrate, at molecular level, a link between defence system, pesticide and susceptibility to pathogenic bacteria in oysters. However, our results were obtained using an experimental model of infection based on intramuscular injection of bacteria. This could be useful to reproduce these experiments in conditions closer of those encountered in natural infection (bath exposure). Our study showed that Galectin, LBP, C-Src kinase, ankyrin, proCL, lysozyme and defensin may be considered as markers of a bacterial infection and could be involved in disease pathogenesis. These experiments allowed establish a reproducible model of contamination and immunosuppression in C. gigas capable to weaken the organism in in vivo conditions. This model could be suitable for studying interactions between host/pathogen in other models. It would also be interesting to study the expression of the same genes after a sub-lethal injection (4.10^6 CFU/animal) of V. splendidus-related strains. Moreover, pollutants could also have an effect on pathogen virulence. Several genes potentially involved in the virulence of these bacterial strains have been identified (Saulnier et al., personal communication). The joint study of host and pathogen response to pollutants would be of interest to identify the conditions needed for a disease to be established.

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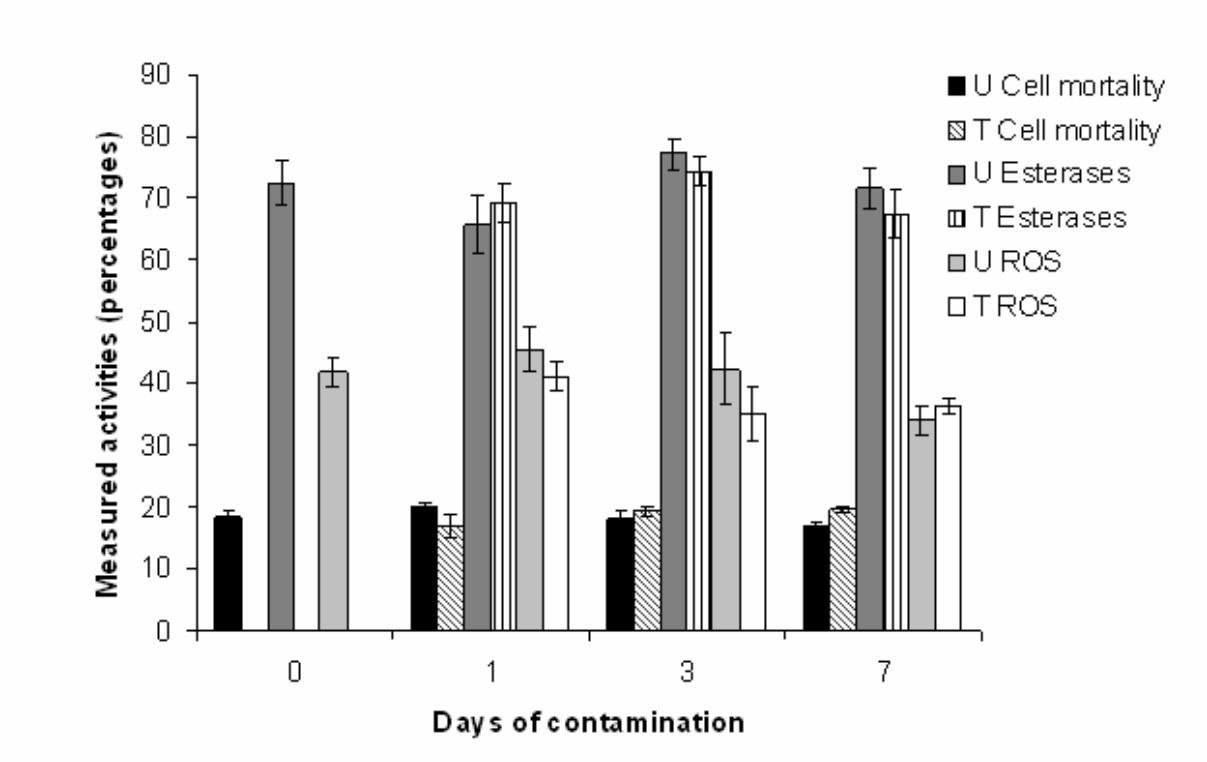

Figure 1: hemocyte parameter results obtained after pooling results from three replicates of the in vivo pesticide contamination (4 pools per replicate). U: untreated oysters, T: treated oysters. ROS = Reactive Oxygen Species. Means ± standard errors are presented. N = 12 replicates.

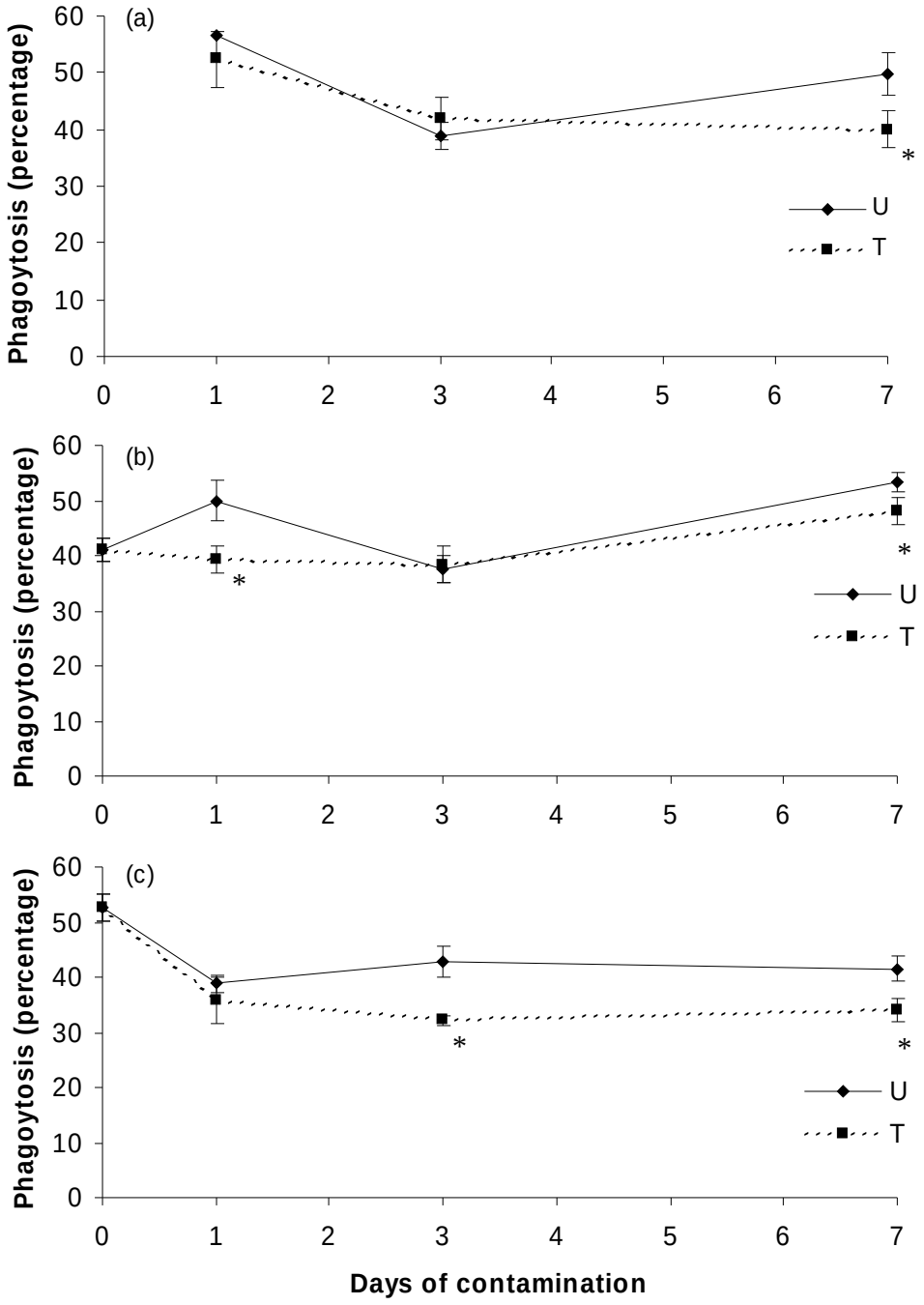

Figure 2: phagocytosis percentages obtained for each of the three in vivo pesticide contamination experiments. U: untreated oysters, T: treated oysters. (a): experiment 1; (b): experiment 2; (c): experiment 3. Means ± standard errors are presented. N = 4 replicates for each experiment. *: $p<0.05$.

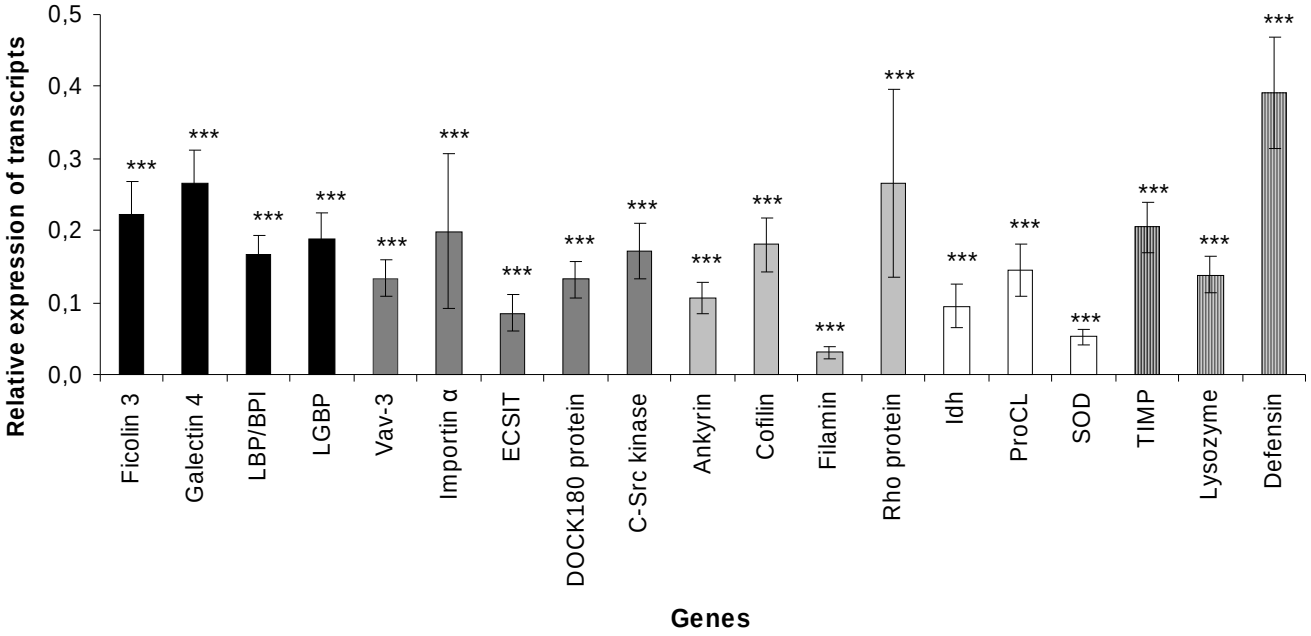

Figure 3: relative expression of the 19 gene transcripts (normalised to elongation factor I) in pesticide exposed oysters compared to controls after seven days of contamination. RNA was extracted from hemocytes of 20 oysters for each condition. N = 3 intra-experimental replicates. Standard error is presented. Value<0.5 show a significant decrease of relative expression of transcripts in treated oysters (T) compared to untreated oysters (U). All histograms presented a value <1 ($p<0.001$). See Table 1 for the category of the genes.

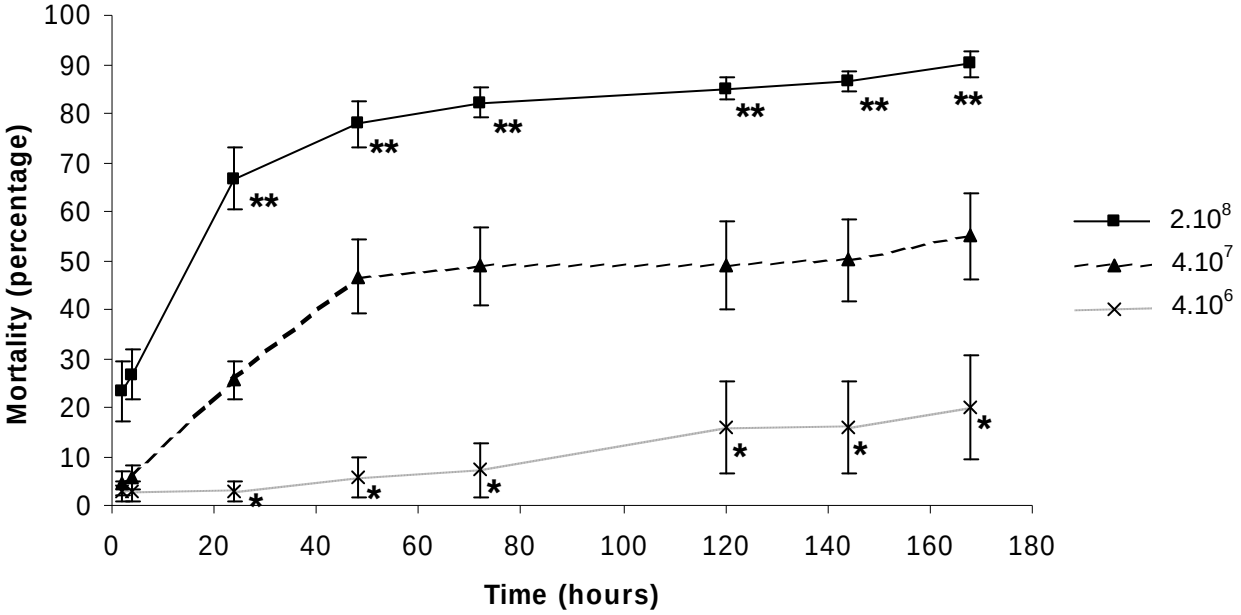

Figure 4: mortality percentages induced by several concentrations of LGP31+LGP32 strains (2.10^8, 4.10^7. and 4.10^6) at several post-injection times (2h, 4h, 24h, 48h, 72h, 120h, 144h and 168h). N = 6 replicates. Standard error is presented. *: $p<0.05$; **: $p<0.01$.

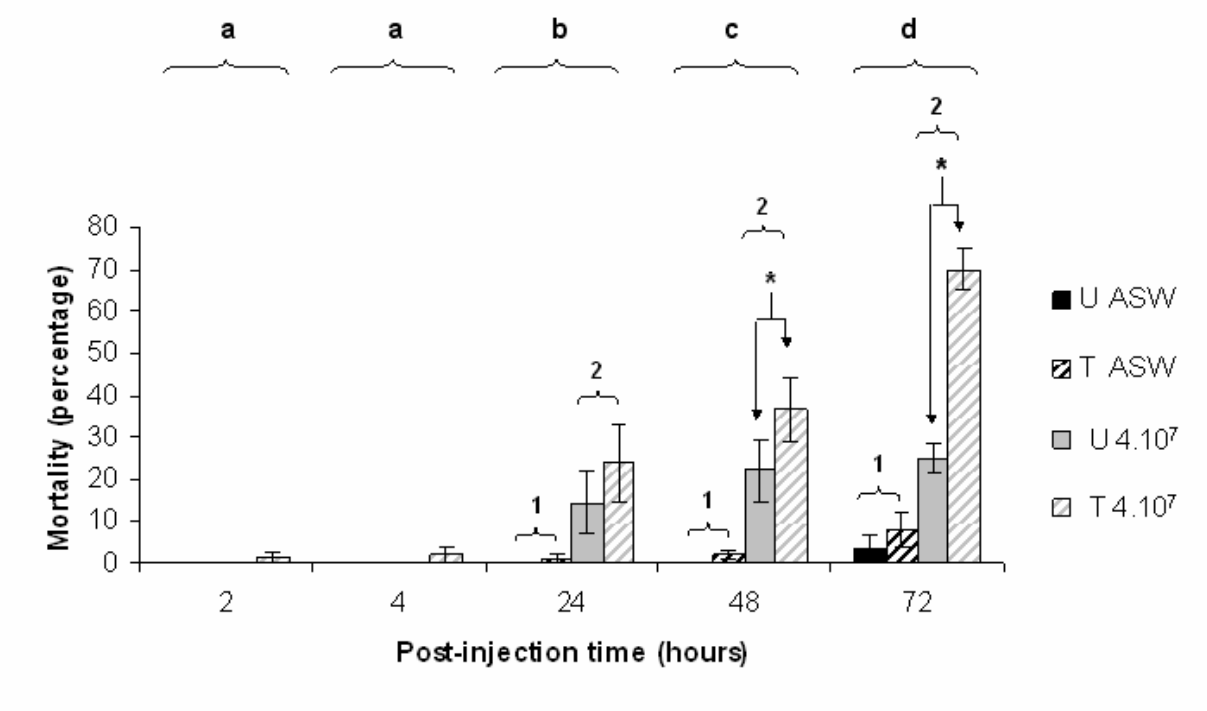

Figure 5: mortality (percent) obtained after injection of artificial seawater or 4.10^7 CFU/oyster in seven day controls and pesticide exposed oysters. N = 2 replicates. Bars represent standard error. * represents a difference between U (untreated oysters) and T (treated oysters) (U<T, $p<0.05$). Numbers 1 and 2 represent a difference between ASW and 4.10^7 (1<2, $p<0.05$). Letters a, b, c and d represent a difference between post-injection times (a<b<c<d, $p<0.05$).

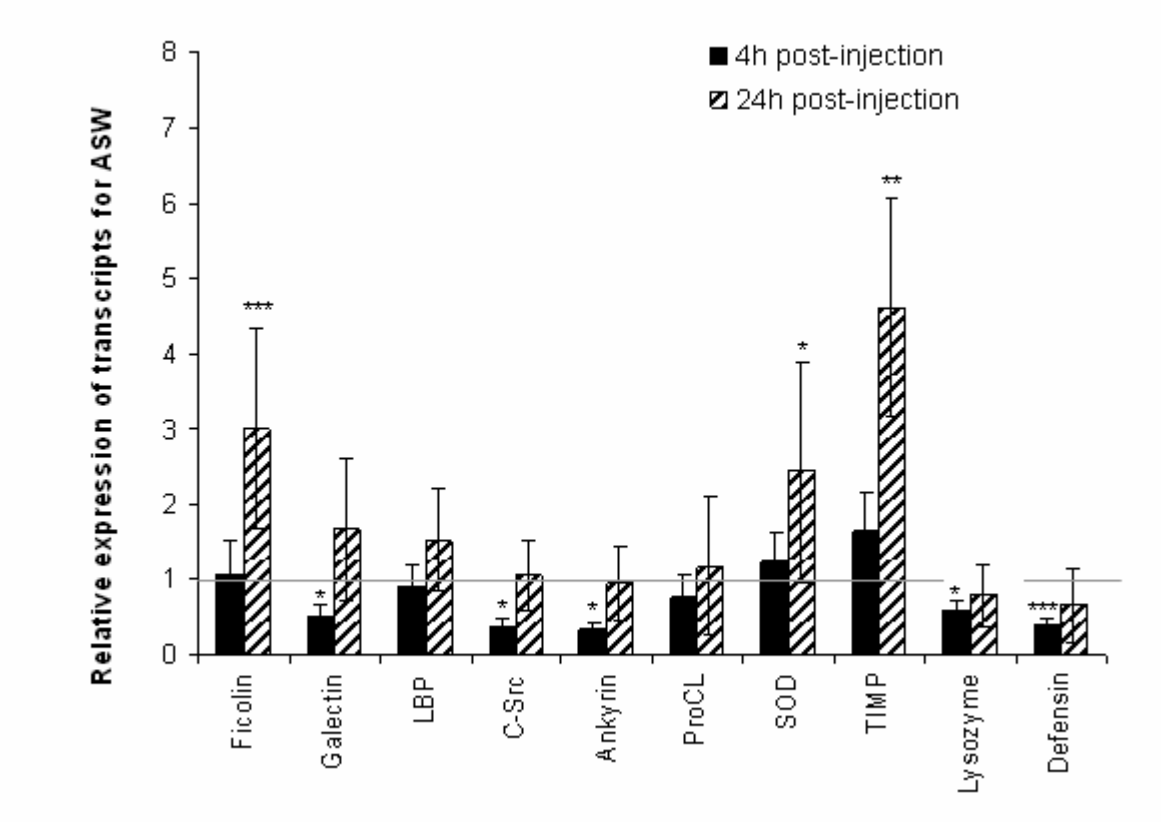

Figure 6: relative expression of the ten gene transcripts (normalised to elongation factor I) in seven days treated oysters injected with ASW compared to untreated oysters injected with ASW 4h and 24h post-injection. RNA was extracted from hemocytes of 10 oysters for each condition. N = 3 intra-experimental replicates. Bars represent standard error. The grey line (value = 1) corresponds to an identical relative expression of transcripts in U (untreated oysters) and T (treated oysters). When statistically different from 1, *: $p<0.05$; **: $p<0.01$; ***: $p<0.001$.

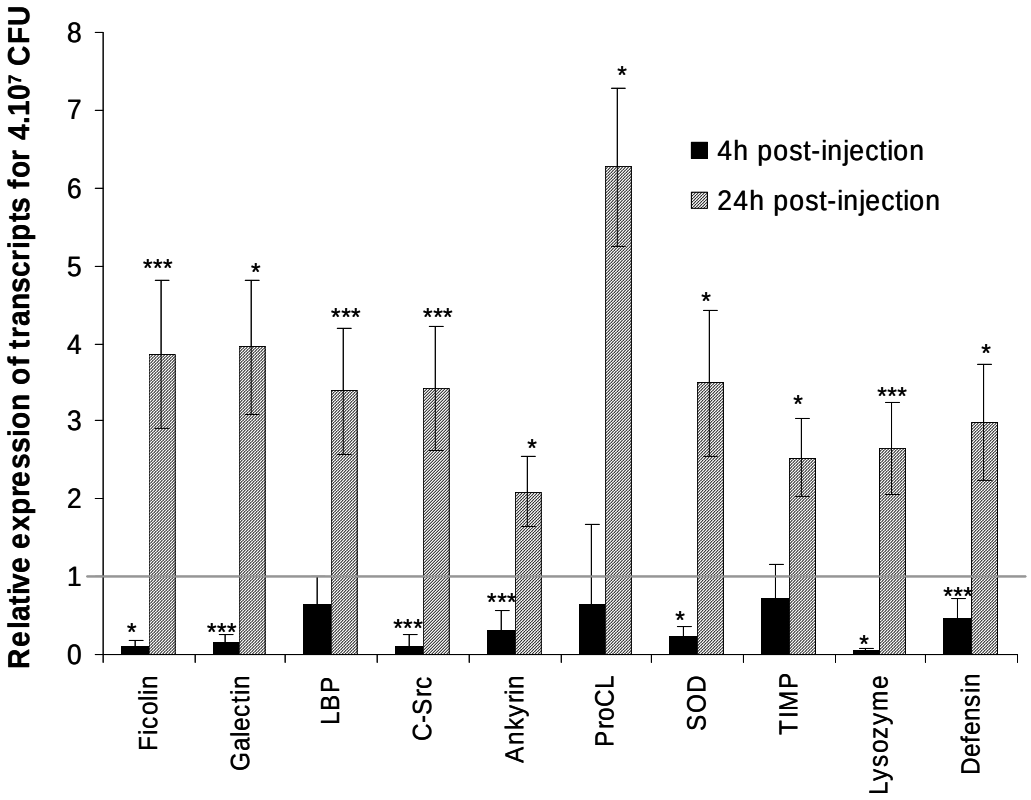

Figure 7: relative expression of the ten gene transcripts (normalised to elongation factor I) in seven days treated oysters injected with 4.10^7 CFU/oyster compared to untreated oysters injected with 4.10^7 CFU/oyster 4h and 24h post-injection. RNA was extracted from hemocytes of 10 oysters for each condition. N = 3 intra-experimental replicates. Standard error is presented. *: $p<0.05$; **: $p<0.01$; ***: $p<0.001$. The grey line (value = 1) corresponds to an identical relative expression of transcripts in U (untreated oysters) and T (treated oysters).

Table 1: the 19 C. gigas genes selected for real-time PCR analysis; these genes are involved in hemocyte functions.

Cell signaling	**Cytoskeleton proteins**	**Post-phagocytosis degradations and cellular protection mechanisms**	**Defence mechanisms**
Ficolin Galectin 4 LBP/BPI protein LPS/ß-1,3-glucan protein Vav-3 protein Importin-α ECSIT DOCK 180 protein c-Src kinase	Ankyrin Cofilin Filamin Rho protein	Isocitrate deshydrogenase pro-Cathepsin L SOD	TIMP Lysozyme Defensin

Table 2: information (primer sequences, GenBank accession numbers and references) related to the 19 genes selected for real-time PCR analysis. Elongation factor I gene was used as reference (Gueguen et al., 2003; Boutet et al., 2004; Huvet et al., 2004; Gueguen et al., 2006).

Gene		5' - 3' primer sequence	GenBank number	Reference
Ficolin 3	Forward	Gca aag gct gtg ttc tgt ga	BQ426875	Gueguen et al., 2003
	Reverse	Ctt gta atc cgt cca gtt acg g		
Galectin 4	Forward	Aga aca gac cta cca tgc cac t	BQ426390	Gueguen et al., 2003
	Reverse	Atc cgt ctt gtc cag agc ac		
LBP/BPI I	Forward	Acg gca cag aac gga tct ac	AY165040	Gueguen et al., 2003
	Reverse	Tgg ttg aca tcg ttg ctg ac		
LPS/β-1,3-glucan	Forward	Tgc ggt gaa ctc tga ctt gt	CB617438	Boutet et al., 2004
	Reverse	Aat gta gct gtg gga ggt gtg		
Vav-3 oncogene homologue	Forward	Gcg ttg act ggc tcg tta g	BQ427355	Gueguen et al., 2003
	Reverse	Gca ctc cat ctc gca aag tt		
Importin α	Forward	Acg cag cag att gaa cga c	CB617497	Boutet et al., 2004
	Reverse	Gca gga gac agt gca gaa tg		
ECSIT	Forward	Gtg tga ttc ccg atg agg ag	BQ427193	Gueguen et al., 2003
	Reverse	Act tgg gca tcc agt aca gc		
DOCK 180 protein	Forward	Caa cga ctc cgt tca aca ac	BQ426954	Gueguen et al., 2003
	Reverse	Cgc tgg aaa caa cga aca		
c-Src kinase	Forward	Aca aca gga ggc tga agg tg	BQ426966	Gueguen et al., 2003
	Reverse	Gtg tgg cgt gtt tca tcg t		
Ankyrine brank-2	Forward	Gtt cgg agc taa cgt gaa cc	BQ426701	Gueguen et al., 2003
	Reverse	Tag ctg gac gat cag gga gt		
Cofilin	Forward	Cgt ggg tga cgg ata gtc tt	BQ426293	Gueguen et al., 2003
	Reverse	Tca gtg gcg tcg tta cac tg		
Filamin	Forward	Gtg acc ttt gat ggc tgt ga	BQ426716	Gueguen et al., 2003
	Reverse	Ggt acg aag tcc gtc tcc tg		
Rho protein	Forward	Agc aaa gac cag ttc cca ga	BQ426365	Gueguen et al., 2003
	Reverse	Ccc ata atg cca act caa cc		
Isocitrate dehydrogenase	Forward	Ccg acg gaa aga ctg tcg	AY551096	Huvet et al., 2004
	Reverse	Ctg gct acc ggg ttt gtg		
Precursor of Cathepsine L	Forward	Cca gcc aga agc tgt agt cc	CF369221	Boutet et al., 2004
	Reverse	Gag cgg aag acg aag cta ga		
SOD-like	Forward	Atg tca cag gaa ccg tcc a	BQ426796	Gueguen et al., 2003
	Reverse	Tcc atg ctg tcc agg tgt ta		
TIMP	Forward	Att gcc gtg gtg aga act g	BQ427105	Gueguen et al., 2003
	Reverse	Tag cgt agc agt cgt tcg tg		
Lysozyme-like	Forward	Gca gcc gga ttc aac ct	AB179775	Nakamura et al., unpublished
	Reverse	Cgt tgt ggt ctt tgc ttc ac		
Defensin	Forward	Tgt cct tct gat ggt ttc tgc	AM050547	Gueguen et al., 2006
	Reverse	Gcc cgc tct aca act aat gg		
Elongation factor I	forward	Acc acc ctg gtg aga tca ag	BQ426516	Gueguen et al., 2003
	reverse	Acg acg atc gca ttt ctc tt		

Table 3: summary of gene expression results obtained with injection of ASW and 4.10^7 CFU/animal at 4h and 24h post-injection for the ten genes followed. NS = non significant; arrows = up- or down-regulation.

		Ficolin	Galectin	LBP	C-Src	Ankyrin	ProCL	SOD	TIMP	Lysozyme	Defensin
ASW	4h	NS	▼	NS	▼	▼	NS	NS	NS	▼	▼
	24h	▲	NS	NS	NS	NS	NS	▲	▲	NS	NS
4.10^7	4h	▼	▼	NS	▼	▼	NS	▼	NS	▼	▼
	24h	▲	▲	▲	▲	▲	▲	▲	▲	▲	▲

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