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Hélène Collin[1], Anne-Leila Meistertzheim[2], Elise David[3], Dario Moraga[2,*] and Isabelle Boutet[4,5]

[1]Laboratoire Biologie de la Conservation, Département d'Écologie et d'Évolution, Université de Lausanne, Bâtiment de Biologie, CH-1015 Lausanne, Switzerland, [2]Laboratoire des Sciences de l'Environnement Marin (LEMAR), UMR CNRS 6539, Institut Universitaire Européen de la Mer, Université de Bretagne Occidentale, Technopole Brest-Iroise, Place Nicolas Copernic, 29280 Plouzane, France, [3]Laboratoire d'Eco-Toxicologie, UPRES-EA2069, Unité de Recherche 'Vigne et vins de Champagne: Stress et environnement' (URVVC), Université de Reims Champagne Ardenne, BP 1039, 51687 Reims Cedex 02, France, [4]Université Pierre et Marie Curie – Paris VI, Laboratoire Adaptation et Diversité en Milieu Marin, BP 74, 29680 Roscoff cedex, France and [5]CNRS UMR 7144, Station Biologique de Roscoff, Equipe Génétique de l'Adaptation en Milieux Extrêmes, BP 74, Place Georges Teissier, 29682 Roscoff cedex, France

*Author for correspondence (dario.moraga@univ-brest.fr)

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SUMMARY

Pesticide run-off into the ocean represents a potential threat to marine organisms, especially bivalves living in coastal environments. However, little is known about the effects of environmentally relevant concentrations of pesticides at the individual level. In this study, the suppression subtractive hybridisation technique was used to discover the main physiological function affected by a cocktail of three pesticides (lindane, metolachlor and carbofuran) in the Pacific oyster *Crassostrea gigas.* Two oyster populations exposed to different pollution levels in the wild were investigated. The pesticide concentrations used to induce stress were close to those found in the wild. In a time course experiment, the expression of three genes implicated in iron metabolism and oxidative stress as well as that of two ubiquitous stress proteins was examined. No clear regulation of gene or protein expression was found, potentially due to a low-dose effect. However, we detected a strong site- and organ-specific response to the pesticides. This study thus (1) provides insight into bivalve responses to pesticide pollution at the level of the transcriptome, which is the first level of response for organisms facing pollution, and (2) raises interesting questions concerning the importance of the sites and organs studied in the toxicogenomic field.

Key words: SSH library, pesticides, *C. gigas*, gene expression, oxidative stress, iron metabolism.

INTRODUCTION

Coastal ecosystems have been exposed to serious pollution for several decades because of increased human activity. Modern agriculture is a major contributor to coastal pollution, especially in Brittany (France), because it often involves the use of a wide variety of pesticides in large quantities. Run-off containing these compounds is then found in coastal systems, resulting in increased levels of pollution and potentially harming marine organisms (Banerjee et al., 1996). Some organisms, such as molluscs, bioaccumulate a large variety of pesticides (Buisson et al., 2008). This accumulation of organic compounds occurs in the gonads in particular and can cause reproductive disorders, characterised by an acceleration of sexual maturation in bivalves (Binelli et al., 2004; Buisson et al., 2008). Endocrine disruption, reflected by a disturbance of sexual differentiation and a decrease in fecundity, has also been reported in crustaceans (Kashian and Dodson, 2002). Additionally, a previous genetic study revealed differential mortality of oysters harbouring sensitive genotypes (Moraga and Tanguy, 2000). Pesticide contamination of aquatic ecosystems is thus likely to affect the fitness and repartition of aquatic species.

At individual level, the impact of pesticides has been extensively studied, especially in molluscs. Pesticides have an impact on immune responses by modulating both gene expression of proteins associated with immune function and haemocyte activity in the oyster *Crassostrea gigas* (Gagnaire et al., 2006; Gagnaire et al., 2007; Bouilly et al., 2007). Pesticides disrupt the reproductive system by increasing the level of aneuploidy in the progeny of contaminated oysters. For instance, atrazine, a widely used pesticide, has an adverse effect on mitotic spindle fibre formation, leading to aberrant chromosome segregation and chromosome loss (Bouilly et al., 2005; Bouilly et al., 2007). More generally, toxicological effects on organisms (filtration rate, atrophy of digestive gland epithelium), cytotoxic and genotoxic effects, and enzymatic activity alterations have been recorded in oysters (Buisson et al., 2008; Anguiano et al., 2010). Pesticides are also known to create pro-oxidant conditions in cells (Griffitt et al., 2006; Williams et al., 2008), leading to oxidative stress. In turn, functions such as iron metabolism can be affected (Slaninova et al., 2009). The recent development of molecular tools has allowed the identification of genes affected by pesticide exposure on a genome-wide scale (Bultelle et al., 2002; Tanguy et al., 2005), with such studies indicating that several physiological functions are involved in the response of organisms to these compounds. Previous techniques usually focused on the expression of one or a few candidate genes to describe the effect of pesticides on organisms. New techniques allow screening of a large part of the transcriptome, and have led to the identification of candidate genes affected by particular toxic compounds at the level of the transcriptome, which is the first response level of organisms exposed to pollution.

In the present study, a suppression subtractive hybridisation (SSH) method was used to answer the following two questions: (1) do pesticides modify specific gene expression or functional pathways in the oyster *C. gigas*, and (2) does the site of origin influence the response of oysters to pesticides? For this purpose, the mRNA expression of three particular genes identified in the SSH assay was analysed in two populations of oysters experimentally exposed to pesticides (lindane, metolachlor, carbofuran) at environmentally relevant concentrations. The candidate genes code for proteins implicated in iron metabolism and oxidative stress (ferritin, NifU and ribosomal protein P2-like). The expression of two stress proteins (metallothionein and heat shock protein 70) was also quantified. These candidate genes and stress proteins were expected to be up- or down-regulated during experimental contamination and thus to be differentially expressed in the two oyster populations because of their different environmental and genetic backgrounds.

MATERIALS AND METHODS

Experimental design

Adult oysters were collected in November 2005 from two sample sites differing in pollution levels, particularly pesticide content: Arcouest (low pollution) and the Vilaine estuary (high pollution) (Fig. 1). Quantification of the levels of two pesticides, lindane and DDT, in bivalve tissues over a 20 year period revealed different pesticide concentrations between these two sites (http://www.ifremer.fr/envlit/). The respective values for Arcouest and Vilaine were 2 $\mu g\,kg^{-1}$ dry mass and 6 $\mu g\,kg^{-1}$ dry mass of lindane and 4.7 $\mu g\,kg^{-1}$ dry mass and 11 $\mu g\,kg^{-1}$ dry mass of DDT. After a 10 day acclimation period in aerated, filtered (0.2 µm) sea water at constant temperature and salinity [15°C, 34 p.s.u. (practical salinity units)], one subgroup from each site was subjected to a pesticide cocktail composed of 5 $\mu g\,l^{-1}$ carbofuran, 2 $\mu g\,l^{-1}$ lindane and 10 $\mu g\,l^{-1}$ metolachlor, corresponding to the pesticide concentrations that are typically measured in the water from these two sites and to the maximal concentration authorised by European directive 98/83/CE (http://www.car-analyse.com/hydro/c9883.htm) in littoral waters. A second subgroup was maintained in aerated sea water as a control. Oysters were fed with phytoplankton (*Isochrysis galbana* and *Pavlova lutheri*) every 48 h, and no mortality was observed during the experiment. The mantle and digestive glands of 10 control and 10 exposed oysters were collected on day 0, 7, 14, 24 and 30 of the experiment and were immediately frozen (–80°C) for subsequent real-time PCR and immunoquantification analyses.

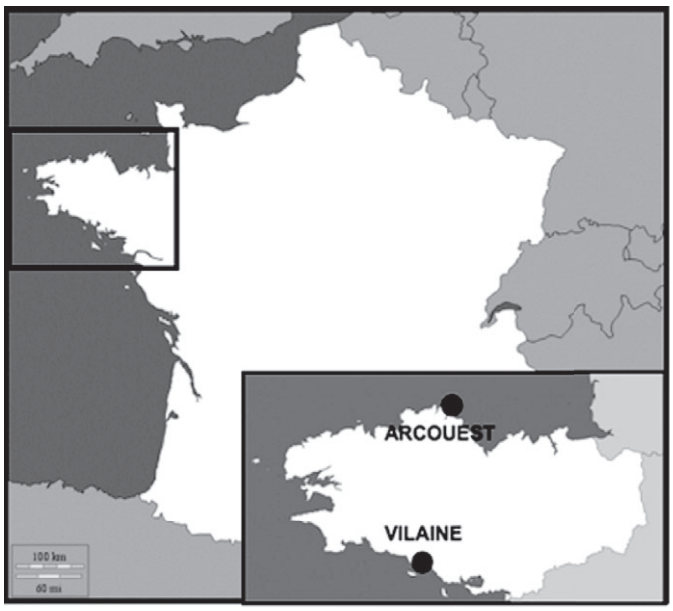

Fig. 1. Map of sampling sites in Brittany, France. Populations were sampled in 2005 at a low pollution site, Arcouest (48°49′09.00″N/3°00′28.39″W), and at a high pollution site, Vilaine (47°30′11.56″N/2°31′28.91″W).

Identification of differentially regulated genes in oysters exposed to pesticides

SSH was used to identify pesticide-regulated genes in oysters. After 24 days of pesticide exposure, total RNA was extracted from mantle pools of 10 control and 10 exposed individuals (Arcouest subset experiment) using TRIzol™ reagent (Gibco-BRL, Grand Island, NY, USA). Poly(A^+) RNA was isolated from each pool of total RNA using the PolyATract® mRNA Isolation system (Promega, Madison, WI, USA) following the manufacturer's instructions. Both forward (up-regulated genes) and reverse (down-regulated genes) subtracted libraries were made with 2 µg mRNA, using the PCR-Select™ cDNA Subtraction kit (Clontech, Mountain View, CA, USA), amplified with the Advantage® cDNA PCR kit and finally cloned into the pGEM®-T vector (Promega) following the manufacturer's instructions. The ligation mixtures were used to transform competent DH5α *E. coli* cells, and colonies were then grown in liquid ampicillin-LB medium. A total of 192 clones (96 clones for each library) were sequenced (Genomer, Roscoff, France). The resulting sequences were compared for homology to sequences in the GenBank database using BLASTX (http://www.ncbi.nlm.nih.gov/blast/) to identify the differentially expressed genes.

Expression analysis of pesticide-regulated genes

The mRNA expression of three genes (*ferritin*, *NifU*, *RpP2like*) potentially regulated by pesticides was followed by real-time PCR. Total RNA of both control and exposed oysters was extracted from the digestive gland and mantle after 0 (before treatment), 7, 14 and 30 days of the experiment (N=5 for each sampling point) using a standard guanidium isothiocyanate extraction protocol. Total RNA (5 µg) was reverse transcribed using M-MLV reverse transcriptase (Promega) and an anchor-oligo(dT) primer (5′-CGCTCTAGAACTAGTGGATCT$_{(17)}$-3′). All reactions were carried out on an ABI Prism 7300 (Applied Biosystems Inc., Foster city, CA, USA) in a final volume of 20 µl containing 150 $nmol\,l^{-1}$ primers (Table 1), 5× power SYBR® green PCR master mix (Applied Biosystems Inc.) and 5 µl diluted (1/20) reverse transcription product. Amplification conditions were as follows: 1 cycle of 10 min for enzyme activation at 95°C followed by 40 cycles comprising a denaturation step of 15 s at 95°C and an annealing–elongation step of 1 min at 60°C. Melting curves were

Table 1. Primer sequences used for real-time PCR amplification

Genes	Primers 5′–3′
NifU	Sense GGCGTGGATCAGCTATTGCTTCTAGT
	Antisense GACTGGAGGCAGACACAGTTCCTTCG
Ferritin	Sense GAGAGCGAAGCTGGCATCAACCGCCA
	Antisense TTCATCAGTTTTTCAGCATGTTCCCG
RpP2like	Sense GACCTGAAGAAAATCATCCGGGTCTGT
	Antisense CAGATGCCAGCTTTTCTTGACCTTTA
18S (endogenous control)	Sense GTCTGGTTAATTCCGATAACGAACGAGACTCTA
	Antisense TGCTCAATCTCGTGTGGCTAAACGCCACTTG

generated to verify that each primer pair amplified a unique PCR fragment, and the 18S gene was used as a reference. Gene expression was analysed using the comparative *Ct* method (Livak and Schmittgen, 2001) and presented as relative expression $RQ=2^{-\Delta\Delta Ct}$, with $\Delta Ct=Ct_{gene}-Ct_{18S}$ and $\Delta\Delta Ct=\Delta Ct_{experiment}-\Delta Ct_{control}$.

Quantification of stress proteins (heat shock proteins and metallothioneins)

Two types of stress protein, heat shock protein 70 (Hsp70) and metallothioneins (MTs), were quantified in the digestive gland and mantle of both control and exposed oysters using an immunoquantification technique based on an enzyme-linked immunosorbent assay (ELISA). ELISAs for quantification of Hsp70 and MTs were carried out according to protocols described previously (Boutet et al., 2002; Boutet et al., 2003).

Statistical analyses

Non-parametric tests (Wilcoxon–Mann–Whitney) were carried out to detect variations in mRNA expression and protein levels between populations, between organs, over time (duration of exposure) and between treatments using SPSS Statistics 17.0 (SPSS Inc., Chicago, IL, USA).

A canonical redundancy analysis (RDA) using the R-language 'rdaTest' function (http://www.bio.umontreal.ca/legendre/indexEn.html) was performed on the data obtained for the mRNA expression of three genes, protein levels (response variables), and four explanatory variables including the following experimental factors: pesticide treatment (control *vs* pesticide-exposed oysters), duration, organ and site of origin. RDA was used to estimate the fraction of the variations in gene expression and protein levels attributable to the explanatory variables. This method evaluates correlations between the response and explanatory variables and is more suitable than a general linear model as few replicates were used in this study. The explanatory variables were recorded as a set of numerical variables. Organ treatment and site of origin were considered to be quantitative variables (transformation of numerical code not required). A polynomial transformation was applied to the duration of exposure (simple=power 1, square=power and cube=power 3). In order to give the same weight to all response variables in the analysis, the gene expression levels (ΔCt) and proteins levels were standardised to a mean of 0 and a variance of 1 (*z*-score transformation) with the R-language 'decostand' function of the 'vegan' package (Vegan: Community Ecology Package version 1.8-3; http://cran.r-project.org/). Forward selection of the explanatory variables was performed using the 'packfor' R-language package (Packfor: forward selection with permutation, R package version 0.0-7; http://r-forge.r-project.org/R/?group_id=195), retaining the explanatory variables with $P\leq 0.05$ after Monte Carlo permutation tests ($n_{perm}=9999$). An overall test of significance of the canonical relationship (9999 permutations) was generated from the RDA results (Legendre and Legendre, 1998).

Table 2. Up-regulated genes identified in the SSH library after 24 days of pesticide exposure

Up-regulated genes	e-value	GenBank accession number
Cytoskeleton, structure, matrix		
Tropomyosin 1 α chain	3.00E–37	HO007348
Respiratory chain		
NADH dehydrogenase subunit 4	7.00E–20	HO007316
NADH dehydrogenase subunit 5	2.00E–19	HO007308
Cytochrome *b*	2.00E–23	HO007333–
Protein regulation and transcription		
Elongation factor 1γ	1.00E–33	HO007320
Elongation factor 1δ	7.00E–28	HO007345
Similar to Mohawk homeobox	2.00E–11	HO007356
Ribosomal proteins		
Ribosomal protein L3	1.00E–31	HO007309–HO007325–HO007341
Ribosomal protein L7	2.00E–48	HO007331
Ribosomal protein L13	2.00E–12	HO007337–HO007343–HO007362
Ribosomal protein L19	3.00E–18	HO007338–HO007344
Ribosomal protein L22	2.00E–14	HO007339
Ribosomal protein S2	7.00E–22	HO007340
Ribosomal protein S3	3.00E–47	HO007335–HO007349
Ribosomal protein S4	1.00E–41	HO007322
Ribosomal protein S6	1.00E–2	HO007350
Ribosomal protein S11	4.00E–28	HO007363
Ribosomal protein S14	3.00E–46	HO007317–HO007319–HO007365
Ribosomal protein S19	1.00E–28	HO007329
Ribosomal protein S26	7.00E–46	HO007310
Ribosomal protein P2like	5.00E–09	HO007323
Iron metabolism		
Nitrogen fixation protein NifU	8.00E–12	HO007358–HO007360–HO007367
Unknown function		
Hypothetical protein	1.00E–17	HO007332–HO007334–HO007359 HO007361–HO007364
Unknown genes		HO007306–HO007307–HO007311 to HO007315–HO007318–HO007321–HO007324–HO007326 to HO007328 HO007330–HO007336–HO007342–HO007346–HO007347–H0007351 to HO007355–HO007357–HO007366

RESULTS

Suppression subtractive hybridisation

Two SSH libraries were constructed with *C. gigas* cDNA after exposure of the oysters to a cocktail of pesticides to identify genes potentially up- or down-regulated (using a forward and a reverse SSH library, respectively). The sequences obtained were then submitted to a homology search using the BLASTX program. A total of 125 sequences were identified, including 71 corresponding to genes with an annotated function and 54 corresponding to genes with unknown functions (Tables 2 and 3). The annotated genes can be clustered into nine functional categories: (1) cytoskeleton, structure and matrix; (2) respiratory chain; (3) protein regulation and transcription; (4) ribosomal proteins; (5) energy metabolism; (6) cellular communication, membrane receptor and immune system; (7) iron metabolism; (8) oxidative stress; and (9) amino acid metabolism.

mRNA expression and protein level variations in response to pesticide exposure

Three potential candidate genes were identified. These genes encode proteins involved in responses to stress experienced by oysters during pesticide exposure: NifU, ferritin and ribosomal protein P2-like (RpP2like). These proteins are implicated in iron homeostasis and oxidative stress. A gene expression experiment was conducted on these genes using real-time quantitative PCR and immunoquantification of two stress proteins (Hsp70 and MTs) in two organs (mantle and digestive gland) of oysters originating from the two sites (Vilaine and Arcouest). The results are reported in Figs 2 and 3. A Wilcoxon–Mann–Whitney test revealed significant differences between the mantle and digestive gland of Vilaine oysters with respect to the Hsp level (U=0, Z=–3.130, P<0.05) and between the mantle and digestive gland at both sites with respect to RpP2like mRNA expression and MT levels (respectively, U=15, Z=–3.857, P<0.005 and U=8, Z=–2.108, P<0.005). Significant differences in digestive gland Hsp and mantle MT levels were found between sites (respectively, U=0, Z=–3.134, P<0.001 and U=0, Z=–3.130, P<0.005). No variation in the protein or mRNA expression was observed between treatments or over time.

Untangling the relative effects of the experiments, the organ and the site of origin on the variance of gene and protein expression across individuals

In order to clarify the effects of the experiment, the first step of the analysis consisted of selecting significant explanatory variables from

Table 3. Down-regulated genes identified in the SSH library after 24 days of pesticide exposure

Down-regulated genes	e-value	GenBank accession number
Cytoskeleton, structure, matrix		
Actin	2.00E–76	HO007400–HO007405–HO007424
α-Tubulin	5.00E–09	HO007420
β-Tubulin	9.00E–76	HO007416
Respiratory chain		
NADH dehydrogenase subunit 2	5.00E–05	HO007378
NADH dehydrogenase subunit 4	7.00E–10	HO007369
NADH dehydrogenase subunit 5	2.00E–05	HO007368
NADH dehydrogenase subunit 6	5.00E–70	HO007423
Cytochrome *b*	7.00E–20	HO007379–HO007398
Energy metabolism		
Enolase	5.00E–05	HO007380
Protein regulation		
Elongation factor 1α	2.00E–114	HO007421
Elongation factor 1δ	1.00E–28	HO007406
Ribosomal proteins		
Ribosomal protein L3	5.00E–39	HO007397
Ribosomal protein L7	3.00E–35	HO007385
Ribosomal protein L9	5.00E–25	HO007382–HO007384–HO007392
Ribosomal protein L13	3.00E–33	HO007399
Ribosomal protein L18	4.00E–31	HO007394–HO007401–HO007402
Ribosomal protein L30	4.00E–21	HO007387
Ribosomal protein L31	9.00E–57	HO007393
Ribosomal protein L34	1.00E–27	HO007386–HO007388
Ribosomal protein L35	2.00E–34	HO007403–HO007407
Ribosomal protein S3	1.00E–119	HO007425–HO007426–HO007429–HO007430
Ribosomal protein S14	5.00E–47	HO007428
Cellular communication, membrane receptor and immune system		
Tripartite motif protein TRIM2 isoform	3.00E–06	HO007422
T-cell receptor δ chain	4.00E–03	HO007419
Iron metabolism		
Ferritin	1.00E–48	HO007408
Oxidative stress		
Manganese superoxide dismutase	2.00E–04	HO007396
Amino acid metabolism		
Kinase 4	4.00E–17	HO007383
Unknown function		
Hypothetical protein	1.00E–20	HO007376–HO007381–HO007389–HO007395
Unknown genes		HO007370 to HO007375–HO007377–HO007390–HO007391–HO007404–HO007409 to HO007415–HO007417–HO007418–HO007427

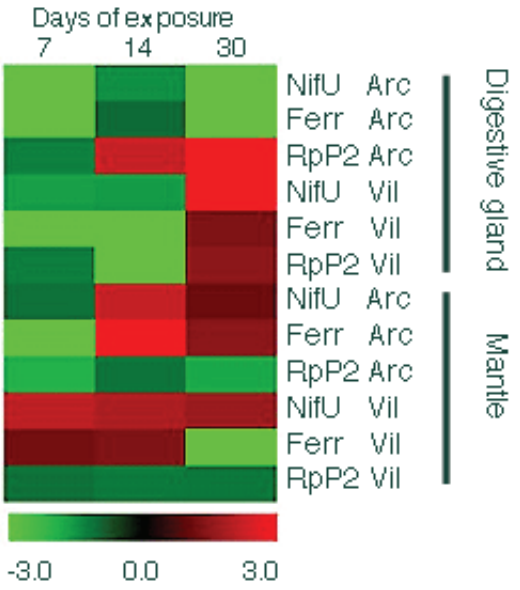

Fig. 2. Analysis of the differential expression of genes in the digestive gland and the mantle of *C. gigas* exposed to pesticides. Relative mRNA expression levels are expressed as an *x*-fold change relative to the expression levels in control oysters. The colour panel at the bottom of the figure indicates the fold change. Arc, Arcouest (low pollution); Vil, Vilaine (high pollution); NifU, iron-sulphur cluster scaffold protein; Ferr, ferritin; RpP2, ribosomal protein P2-like.

among the four that were available (treatment, duration, organ and site of origin). Using a multivariate forward selection procedure (R-language package 'packfor'), the effects of two variables remained significant (P<0.05) in explaining the variance of the five response variables across individuals: site of origin (R^2=0.282, F=48.98, P=0.001) and organ (R^2=0.215, F=52.65, P=0.001). These variables were thus used in the canonical RDA. The two axes of the RDA constrained by these two variables explained 49.7% (R^2_{adj}=49.7%, F=61.3, P=0.001) of the total variance associated with gene and protein expression.

DISCUSSION

Identification of candidate genes regulated by pesticides

In this study, Pacific oysters (*C. gigas*) originating from two populations, which differ with respect to the pollution levels they encounter in the wild and the level of pesticide exposure, were used to identify the response of oysters at both the mRNA and protein expression level.

First, suppression subtractive libraries were generated to identify differentially expressed genes in oysters exposed to pesticides. Sequencing of these libraries identified 125 regulated genes, of which half were functionally annotated.

Some genes encoded similar proteins in both the forward and reverse libraries (NADH dehydrogenase subunit 4, NADH dehydrogenase subunit 5, elongation factor 1δ, and ribosomal proteins L3, L7, L13, S3 and S14). Proteins involved in translation as well as in respiratory processes are highly expressed in cells, and some of these proteins are specific to physiological pathway activation. Therefore, the presence of similar genes encoding these types of protein was not surprising, and has also been observed in many other SSH studies (David et al., 2005; Tanguy et al., 2005; Marchand et al., 2006; Meistertzheim et al., 2007; Zapata et al., 2009), possibly due to the limitations of the assay itself.

Pesticides, especially lindane, have numerous effects on aquatic aerobic organisms. These compounds can create pro-oxidant conditions and favour oxidative stress by inducing the production of reactive oxygen species (ROS) (Ayaki et al., 2005; Valavanidis et al., 2006; Griffitt et al., 2006; Williams et al., 2008; Jin et al., 2010). ROS can alter protein function and cause lipid peroxidation and nucleic acid damage through oxidation processes (Banerjee et al., 1999). In the present work, genes coding for proteins implicated in the antioxidant response (superoxide dismutase) and in iron homeostasis regulation (NifU, ferritin and RpP2like) were

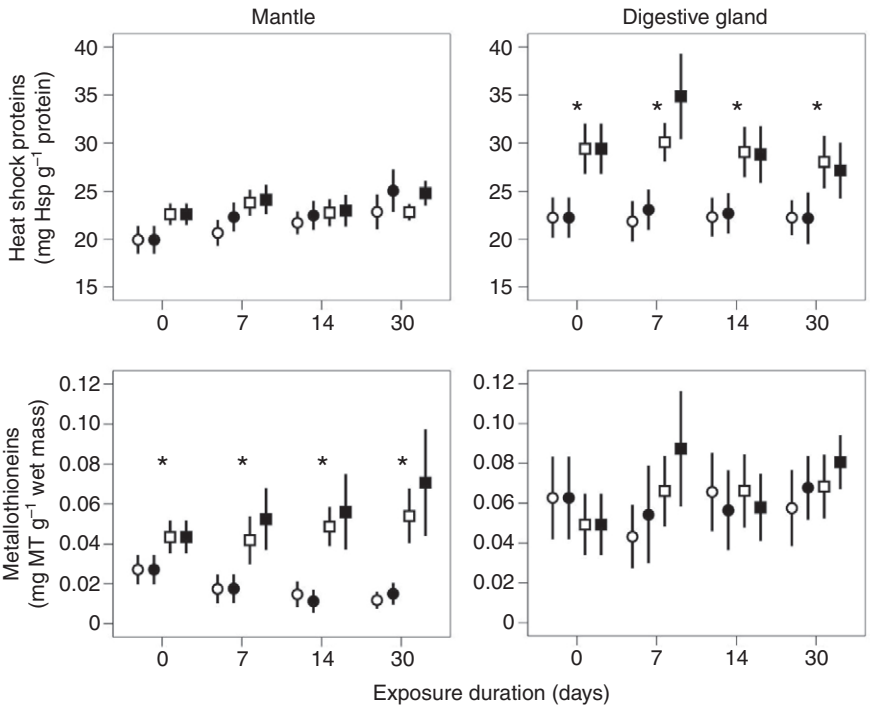

Fig. 3. Quantification of stress protein levels in the digestive gland and the mantle of *C. gigas* exposed to pesticides. The circles represent oysters originating from Arcouest (low pollution) and the squares represent oysters originating from Vilaine (high pollution). White and black symbols represent the control and exposed oysters, respectively. Hsp, heat shock protein; MT, metallothioneins. Significant differences between sites are indicated by asterisks (P<0.001).

highlighted. These results suggest that iron homeostasis is a major stress response for coping with oxidative stress caused by pesticide contamination. In our reverse library (i.e. down-regulated genes), a sequence coding for ferritin, a protein involved in iron transport in the cell, was identified. Intracellular iron is able to catalyse the Fenton reaction, which produces ROS (Fenton, 1894). Ferritin is also able to oxidise contaminants such as organic compounds. To avoid intracellular ROS production, free iron is sequestered by diverse proteins, including ferritin (Ponka et al., 1998). Ferritin can represent either a pro- or an anti-oxidant in a time-dependent manner. In fact, its early degradation contributes to the expansion of the free intracellular iron pool that, later on, activates multiple molecular mechanisms to restore ferritin content, thus limiting the pro-oxidant challenge of iron (Cairo et al., 1995; Tsuji et al., 2000).

Another way to limit iron depletion, and consequently the production of ROS, is the short-term storage of iron by an iron-binding protein or an iron-sulphur cluster scaffold protein. In the forward library, one up-regulated gene coding for the iron-binding protein RpP2like was identified. Despite its role in translation processes, this ribosomal protein is known for sequestering intracellular iron (Furukawa et al., 1992; Snyder, 1999). The forward library (i.e. up-regulated genes) also revealed a gene coding for NifU, an iron-sulphur cluster scaffold protein. This protein is broadly conserved from bacteria to mammals (Nishio and Nakai, 2000; Rouault and Tong, 2005). One potential reason for such an evolutionary pattern is that iron-sulphur clusters are included in the composition of numerous proteins implicated in iron homeostasis, such as NADPH dehydrogenase and iron regulatory protein (IRP). Thus, iron-sulphur scaffold proteins are powerful regulatory sensors supporting intracellular iron sequestration and regulation as well as protection against oxidative stress (Lill and Mühlenhoff, 2005). More specifically, an iron-sulphur cluster is present in IRP-1, a protein responsible for the expression or repression of ferritin. Under pro-oxidant conditions, when the intracellular level of dihydrogen peroxide is too high, IRP-1 is produced and activated to repress the translation of ferritin (Cairo et al., 1995; Eisenstein and Blemings, 1998). The production of a functional IRP-1 requires the assembly of iron-sulphur clusters by NifU (Eisenstein, 2000).

The results discussed above highlight the importance of the regulation of iron homeostasis under intracellular pro-oxidant conditions and when ROS production is induced by pesticide exposure. In addition to iron metabolism proteins, anti-oxidant defences involve proteins such as NADH dehydrogenase and superoxide dismutase (SOD) (Bowler et al., 1992; Lesser, 2006). During the Fenton reaction, these proteins catalyse the dismutation of superoxides and peroxide radicals to avoid the formation of more reactive free radicals (Scandalios, 1993; Storz and Imlay, 1999; Lesser, 2006; Pire et al., 2007). Thus, the presence of such genes in both libraries and the aforementioned regulation of iron homeostasis suggest that pesticide contamination might stimulate a response to oxidative stress in the oyster populations studied here.

Organ- and site-specific responses

In this study, the aim was to characterise the basic molecular and physiological responses of *C. gigas* to pesticide exposure in two organs (mantle and digestive gland) of oysters from two different sites (Arcouest and Vilaine). The experimental design allowed us to compare different candidate markers of oxidative stress following pesticide exposure. Differential expression of these markers, with site- and organ-specific responses, was observed, and 49.7% of the variance in the expression patterns was explained by these two variables.

The digestive gland is of particular importance as this organ metabolises food as well as organic xenobiotics. The mantle constitutes the main energy storage organ. Pollutants are likely to bioaccumulate in these organs, which are responsible for all detoxification processes and thus can be subjected to high levels of oxidative stress (Malanga et al., 2004). An organ-specific response was expected, as shown by David and colleagues when they tested the effect of hypoxia on *C. gigas* at the transcriptional level (David et al., 2005). Other studies on heavy metal contamination in *C. gigas* have also revealed organ-specific changes in the expression of MTs and Hsp70, with a higher level of MTs and Hsp70 in the digestive gland than in other tissues (Boutet et al., 2002; Tanguy et al., 2005; Moraga et al., 2005). Our results support these observations, as higher concentrations of MTs and Hsp70 were found in the digestive gland than in the mantle for both experimental populations.

The responses of populations from different sites to pollution are rarely studied at both the transcriptional and post-transcriptional level. Here, significant differential gene and protein expression was observed between the Arcouest and Vilaine sites, reflecting a site-specific response to pollution. Similar results were found in a study conducted on oyster larvae exposed to metal contamination (Damiens et al., 2006). The authors observed differential MT concentrations between two sites where the pollution levels differed. This result suggests that populations encountering different levels of pollution give rise to offspring that can differentially withstand the toxic effects of particular pollutants. The two sites studied in our experiment differ with respect to the pollution level, but they also differ with respect to numerous environmental components (tidal height, temperature). Additionally, as gene expression is often heritable within populations (Whitehead and Crawford, 2006; Lai et al., 2008; Pavey et al., 2010) and supposing that the Arcouest and Vilaine oyster populations have different genetic backgrounds, a combination of environmental and genetic components could explain the differential gene and protein expression observed in this study. This interesting question remains open and needs to be explored further in order to better understand oyster stress responses in general. For example, a population genetics and fitness trait approach could be integrated into a transcriptomic study like the present one to disentangle the environmental and genetic effects of pesticides on *C. gigas* populations.

Gene expression and stress protein pattern

Although three genes implicated in iron metabolism and oxidative stress defence were differentially expressed in our libraries as a result of site- and organ-specific effects, the time course experiment did not show a significant difference in the regulation of these genes in treated and control oysters over the 30 day pesticide exposure period. In a previous study, 137 genes in *C. gigas* were identified to be regulated in response to glyphosate and a mixture of three pesticides (Tanguy et al., 2005). Both SSH experiments carried out here indicated that few genes are strongly regulated. The gene expression time course analyses associated with these experiments revealed that low variations of expression occurred. Gagnaire and colleagues studied the immune response of *C. gigas* to pesticide exposure coupled with a bacterial challenge (Gagnaire et al., 2007). They found no significant difference in ROS induction in haemocytes between treated and untreated oysters. Another laboratory experiment conducted on rodent cells showed no regulation of ferritin mRNA during 24 h exposure to a herbicide known to provoke pro-oxidant conditions (Paraquat) (Ayaki et al.,

2005). These results could partly be due to a dose effect. In our study, the doses used were chosen to mimic those potentially encountered in the field. In contrast, toxicological studies usually use high pollutant concentrations in order to obtain strong effects on organisms from the molecular to the physiological level, but these high concentrations are often not ecologically or biologically relevant (Parveen et al., 2003; Jin et al., 2010).

Hsps and MTs are two ubiquitous types of stress protein, and both have been well studied and documented in the literature since the 1990s. In spite of their responses to specific types of stress – thermal stress for Hsps and heavy metal stress for Mts – expression of these proteins can be induced by a wide variety of stressors, including oxidative stress (Feder and Hofmann, 1999; Viarengo et al., 1999; Dowling et al., 2006; Micovic et al., 2009; Ivanina et al., 2009). No induction of stress protein expression (Hsp70 and MTs) was observed during the experiment in either control or treated oysters, suggesting a low-dose effect, similar to that observed in the expression experiment. The mean levels of MTs and Hsp70 observed were even lower than expected, based on previous studies (Boutet et al., 2002; Boutet et al., 2003).

CONCLUSION

The results obtained in this study are of particular importance. The SSH technique used here will provide a basis for finding new candidate genes for investigating the effects of stress on different non-model organisms in future studies. Here, it was demonstrated that pesticides induce oxidative stress, implicating the regulation of iron metabolism for detoxification and cell protection. Thus, (1) the expression of the three candidate genes linked to oxidative stress and iron homeostasis – NifU, ferritin and RpP2like mRNA level – was quantified by real-time quantitative PCR, and (2) the level of two molecular chaperone proteins, Hsp70 and MTs, was measured. No clear regulation of these candidate genes or molecular chaperones was observed during contamination, highlighting a potential low dose effect. This paper reports an organ- and site-specific effect probably linked to acclimation to pollution in different environments. These results provide new information on the response to pesticides in bivalves, especially at the level of the transcriptome, which is the first level of response for organisms exposed to pollution. Further studies using large-scale transcription methods, or similar approaches integrating environmental and genetic effects, might help to complete our understanding of the transcriptional response to environmental pollution by marine organisms.

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