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ECOTOXICOLOGY OF ESTUARIES IN FRANCE AND QUÉBEC, CANADA

An integrated environmental approach to investigate biomarker fluctuations in the blue mussel *Mytilus edulis L.* in the Vilaine estuary, France

Emilie Farcy · Thierry Burgeot · Hansy Haberkorn · Michel Auffret · Laurent Lagadic · Jean-Pierre Allenou · Hélène Budzinski · Nicolas Mazzella · Romain Pete · Micheline Heydorff · Dominique Menard · Florence Mondeguer · Thierry Caquet

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Abstract Estuarine areas represent complex and highly changing environments at the interface between freshwater and marine aquatic ecosystems. Therefore, the aquatic organisms living in estuaries have to face highly variable environmental conditions. The aim of this work was to study the influence of environmental changes from either natural or anthropogenic origins on the physiological responses of *Mytilus edulis*. Mussels were collected in the Vilaine estuary during early summer because this season represents a critical period of active reproduction in mussels and of increased anthropogenic inputs from agricultural and boating activities into the estuary. The physiological status of the mussel *M. edulis* was evaluated through measurements of a suite of biomarkers related to: oxidative stress (catalase, malondialdehyde), detoxication (benzopyrene hydroxylase, carboxylesterase), neurotoxicity (acetylcholinesterase), reproductive cycle (vitelline, condition index, maturation stages), immunotoxicity (hemocyte concentration, granulocyte percentage, phagocytosis, reactive oxygen species production, oxidative burst), and general physiological stress (lysosomal stability). A selection of relevant organic contaminant (pesticides, (polycyclic aromatic hydrocarbons, polychlorobiphenyls) was measured as well as environmental parameters (water temperature, salinity, total suspended solids, turbidity, chlorophyll a, pheopigments) and mussel phycotoxin contamination. Two locations differently exposed to the plume of the Vilaine River were compared. Both temporal and inter-site variations of these biomarkers were studied. Our results show that reproduction cycle and environmental parameters such as temperature, organic ontaminants, and algal blooms could strongly influence the biomarker responses. These observations highlight the necessity to conduct integrated environmental approaches in order to better understand the causes of biomarker variations.

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E. Farcy · T. Burgeot (✉) · D. Menard
Laboratoire d'Écotoxicologie, Ifremer, Nantes, France
e-mail: thierry.burgeot@ifremer.fr

H. Haberkorn · M. Auffret
Laboratoire des Sciences de l'Environnement Marin (LEMAR), IUEM, Brest, France

L. Lagadic · M. Heydorff · T. Caquet
Équipe Écotoxicologie et Qualité des Milieux Aquatiques, Inra, Rennes, France

J.-P. Allenou
Laboratoire Côtier Morbihan Pays de Loire, Ifremer, Nantes et La Trinité-sur-Mer, France

H. Budzinski
Laboratoire de Physico-ToxicoChimie des Systèmes Naturels (LPTC), Bordeaux, France

N. Mazzella
Épuration et Qualité des Eaux, Unité de Recherche Réseaux, Cemagref, Bordeaux, France

R. Pete
UMR 5119 Ecosym (UM2, CNRS, IRD, Ifremer, UM1, Université Montpellier 2), Montpellier, France

F. Mondeguer
Laboratoire Phycotoxines, Ifremer, Nantes, France

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Introduction

Estuarine areas are complex and highly changing environments at the interface between freshwater and marine aquatic ecosystems. Despite their environmental variability, estuaries are usually characterized by high biomass due to their strong primary production, especially when compared with marine areas. These conditions enhance the development of heterotrophic populations such as filtrating molluscs. Moreover, estuaries are of special interest in ecotoxicology because they are potentially the most exposed coastal areas regarding any source of pollution (Sun et al. 2012). In order to perform ecotoxicological studies, sampling strategy has to take into account the whole complexity of the estuarine ecosystem. Many environmental variables such as temperature, salinity, or suspended matter may have a great influence on physiological processes in invertebrate species.

Worldwide chronic contamination of estuaries by inorganic toxicants (e.g., heavy metals), hydrocarbons, or persistent organic pollutants and its effect on estuarine organisms have retained much attention to date (Birch 2000; Sun et al. 2012). Information about the presence and effects of other contaminants such as pharmaceuticals and pesticides in these systems are still scarce (Power et al. 1999; Sun et al. 2012). However, there is growing evidence that point source pollution (e.g., waste water treatment plants) and runoff from agricultural areas can lead to the influx of significant amounts of various organic compounds in estuaries (Haynes et al., 2000; Shaw and Müller 2005; Lewis et al. 2009). Seasonal changes in the nature and level of contaminants found in these areas may occur, and some toxic substances may be present at higher levels at some critical periods for estuarine organisms, such as the reproductive period (Steen et al. 2001; Noppe et al. 2007). The simultaneous exposure to toxicants and to natural variation in environmental conditions may have deleterious consequences on actively reproducing organisms, leading to stress that may increase seasonal mortalities. This phenomenon is of particular importance for shellfish such as oysters or mussels that are cultivated in many estuaries and coastal zones, including on the French Atlantic coast (Renault 2011).

The blue mussel *Mytilus edulis L*. is a suitable species for linking contaminant exposure and their potential biological effects in estuaries for several reasons: (1) mussels are often farmed in estuaries, (2) due to its strong filtration ability, *M. edulis* is a good indicator of contaminants prone to bioaccumulation, and (3) the sessile nature of this species facilitates the establishment of cause–effect relationship (Ramu et al. 2007). Mussels have been used for decades as a sentinel species for the biomonitoring of metals and persistent pollutants in estuarine and coastal sites (e.g., Mussel Watch programs and current biomonitoring networks; Chase et al. 2001; Monirith et al. 2003; Andral et al. 2004; Apeti et al. 2010; Choi et al. 2010).

The catchment area of the Vilaine River covers a surface of 10,400 km^2 and is mainly characterized by extended agricultural lands mainly made up of maize crops and intensive livestock rearing (cattle, pork, and poultry). The Vilaine estuary is also characterized by mussel farming activities and boating activities, occuring mainly during summer time. In 1970, a dam was built 12 km upstream of the mouth of the river in order to help with the management of flooding and that also served as a source for drinking water in the region. It is equipped with five gates and one lock used by leisure boats coming in and out of the Arzal marina in the freshwater reservoir formed by the dam. Following heavy rains, the dam gates can be open resulting in significant changes in the physico-chemical conditions in the estuary. Changes in the hydrodynamics associated with the building of Arzal dam increased the deposition of fine particles in the estuary, which has significantly silted up since introduction of the dam. Previous analyses carried out in the Vilaine estuary between 1996 and 1999 found high levels of pesticides in water (especially atrazine, diuron, and isoproturon) as a result of intensive agriculture in the catchment area of the river (SAGE Vilaine 2001). During summer periods, contamination peaks above 2 $\mu g L^{-1}$ were previously observed (Marchand 2006).

Organic pollutants can produce a broad range of harmful effects in animals, including oxidative stress (Regoli et al. 2002). Roméo et al. (2003b) reported that metabolism of a great variety of pollutants, including polycyclic aromatic hydrocarbons (PAHs), produces reactive oxygen species. Measurement of catalase (CAT) activity was widely used in order to investigate oxidative stress in previous biomonitoring programs using bivalves molluscs and particularly using *Mytilus sp*. (Solé et al. 1995; Orbea et al. 2002; Bocquené et al. 2004; Khessiba et al. 2005; Lionetto et al. 2004; Borkovic et al. 2005; Pampanin et al. 2005; Beneditti et al. 2006; Bocchetti and Regoli, 2006; Box et al. 2007; Lima et al. 2007; Vlahogianni et al. 2007; Gorbi et al. 2008). As a consequence of oxidative stress, lipid peroxides derive from polyunsaturated fatty acids. They are unstable and decompose to form a complex series of compounds. These include reactive carbonyl compounds, of which the most abundant is malondialdehyde (MDA). Measurement of MDA has been widely used as an indicator of lipid peroxidation in *Mytilus sp*. (Viarengo et al. 1991; Pampanin et al. 2005; Martin-Diaz et al. 2009; Attig et al. 2010; Tedesco et al. 2010; Maria and Bebianno 2011). The activity of the biotransformation enzyme benzo(a)pyrene hydroxylase

(BPH) can be used as an indirect measurement of the CYP450 1A activity, which is involved in the biotransformation of xenobiotics (Snyder 2000). This biotransformation enzyme has previously been used as a biomarker of exposure to organic pollutants, especially PAHs (Porte et al. 1991; Michel et al. 1994; Solé et al. 1994; Peters et al. 1999; Akcha et al. 2000). Other xenobiotics have also been shown to increase BPH activity in molluscs, including pesticides and detergents (Baturo and Lagadic 1996). Esterases are known as relevant biomarkers of exposure to organophosphate and carbamate pesticides in marine molluscs (Ozretić and Krajnović-Ozretić 1992; Galgani and Bocquené 2000). Carboxylesterase (CbE) is a serine-dependent esterase with wide substrate specificity. This enzyme is involved in the biotransformation of xenobiotics and the activation of ester and of amide prodrugs. Acetylcholine esterase (AChE) is involved in the hydrolysis of the neurotransmitter acetylcholine. AChE is strongly inhibited by organophosphate and carbamate pesticides, and also by metals (Gill et al. 1991; Martinez-Tabche et al. 2001; Monserrat et al. 2002 ; Bainy et al. 2006), giving rise to functional disorders of the central nervous system. The measurement of AChE inhibition in marine organisms has been widely used as an indicator of environmental contamination by these compounds. A range of contaminants including heavy metals (Coles et al. 1994a; Pipe et al. 1999; Sauvé et al. 2002), PAHs (Coles et al. 1994b; Dyrynda et al. 1997; Wootton et al. 2003) and pesticides (Alvarez and Friedl 1992; Auffret and Oubella 1997; Baier-Anderson and Anderson 2000) have been investigated as potent immunomodulators in shellfish. Phagocytosis is the main cellular defense mechanism against non-self materials in invertebrates (Tiscar and Mosca 2004), and measuring this parameter has become a major biomarker of immunotoxicity in bivalves species exposed to environmental xenobiotics (Fournier et al. 2000). The measurement of lysosomal membrane stability is also considered as an indirect way to evaluate the health status of animals because this parameter integrates the effects of various stress factors, including the presence of chemical contaminants and environmental parameters (Ringwood et al. 1998; Bocchetti and Regoli 2006).

In this study, mussels were collected in spring and early summer in two sites located at the outlet of the Vilaine River. This period was chosen because it represents a critical period with elevated anthropogenic inputs in the estuary and active mussel reproduction. In bivalves, reproduction represents a major physiological event. Gametogenesis causes profound tissue and metabolic modifications, and spawning is often considered as a stressful event causing alteration of various functions, including immune function (Cho and Jeong 2005). Environmental parameters (water temperature, salinity, chlorophyll a and pheopigments concentrations, turbidity, and total suspended solids concentrations) were monitored in order to evaluate the variability of the environmental conditions in the estuary. The level of phycotoxins was also recorded in mussel digestive gland. The presence of organic pollutant was evaluated through the measurement of pesticide residue concentration in water and other organic contaminants (PAHs, polychlorobiphenyls (PCBs), polybromodiphenylethers (PBDE)) in mussel tissues. The physiological status of the mussels was evaluated through the measurement of a suite of biomarkers related to oxidative stress (CAT, MDA), biotransformation (BPH, CbE), neurotoxicity (AchE), reproductive cycle (vitelline (VIT), condition index, gonad gametogenesis stage), immunity (hemocyte concentration, granulocyte percentage, phagocytosis, reactive oxygen species (ROS) production, oxidative burst), and general stress (lysosomal stability). The challenge of this work was to study the influence of environmental changes from either natural or anthropogenic origins on the physiological responses of *M. edulis*.

Material and methods

Mussel sampling

Mussel sampling was performed twice a month between April and July 2007. Sampling dates were April 16, May 2 and 28, June 12, and July 2 and 16. An additional sampling was conducted on June 18 following heavy rains. Mussels were collected at two sites located at the outlet of the Vilaine estuary (Fig. 1): Les Granges (47°30′46.5″ N, 02°29′13.0″ W), a site exposed to the Vilaine plume, and Le Maresclé (47°27′47.3″ N, 02°29′50.4″ W), a site less influenced by the Vilaine because it is not located in the direct vicinity of its plume (Chapelle 1991; Vested et al. 2011). Mussels from the two sites were genetically comparable because they came from the same population that was transplanted in the two study sites during the winter before the study period. In order to reduce bias linked to transport stress, mantle, gills, and digestive gland were immediately dissected in the field after sampling. Dissected samples were immediately frozen in liquid nitrogen until further processing. For the analysis of fresh hemolymph (lysosomal stability and hemocyte parameters), mussels were kept in a temperature-controlled cool box during transportation. They were processed within 24 h after sampling.

Environmental parameters

Water surface temperature, salinity, and dissolved oxygen concentration were measured directly in the field using an YSI multiparameter probe (YSI Incorporated, Yellow Springs, OH, USA) on each sampling date and at each sampling site. Suspended solids, pheopigments, turbidity, and chlorophyll a concentrations were measured in water samples collected at 1 m depth in the Vilaine estuary according to routine methods implemented by Ifremer coastal laboratories (Aminot and Chaussepied 1983 and Aminot and Kerouel 2004 for suspended solids and algal pigments, respectively). For every

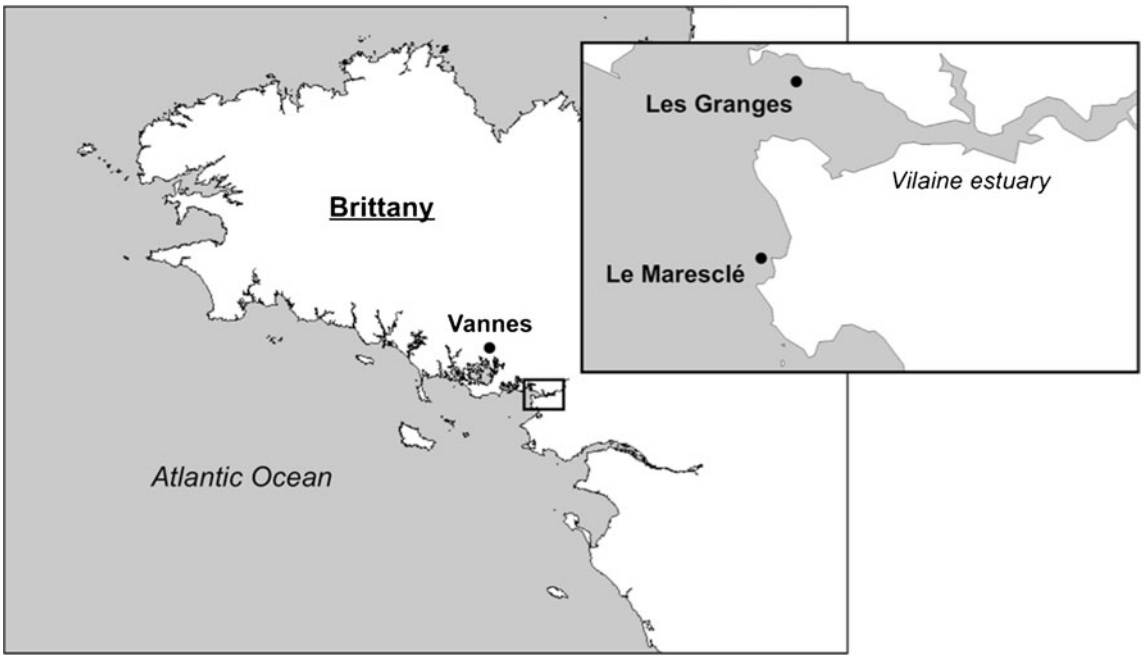

Fig. 1 Localization of the sampling sites, Les Granges and Le Maresclé, at the oulet of the Vilaine estuary, Brittany, France

parameter, a single measurement was made during each sampling event. Measurements were always conducted at low tide.

Organic contaminants

On each sampling date, one water sample per site was collected at low tide. Water samples were collected using 2-L glass bottles that were stored at 4 °C and immediately transferred to the analytical laboratory. They were processed within 24 h following sampling. Pesticide residues were analyzed using a multiresidue method based on off-line solid-phase extraction followed by reversed-phase liquid chromatographic separation and electrospray triple quadrupole mass spectrometric detection according to the protocol described in Mazzella et al. (2009). The high-performance liquid chromatography (HPLC) separation was performed on a Finnigan SpectraSYSTEM equipped with a Prontosil Spheribond ODS 2 column (150 x 4 mm, 3 μm) with a C18 (10 x 4 mm, 6 μm) guard column (Bischoff Chromatography, Germany). The HPLC system was coupled with an API 2000 (Applied Biosystems/MDS SCIEX, France) triple quadrupole mass spectrometer equipped with a turbo ion spray source. This technique allows the simultaneous determination of 30 active substances and metabolites—11 triazines (ametryn, atrazine, cyanazine, atrazine-desethyl–DEA, terbuthylazine-desethyl–DET, atrazine-desisopropyl–DIA, Irgarol 1051, prometryn, propazine, simazine, terbutylazine, terbutryn), 14 phenylureas (chlortoluron, diuron, 1-(3,4-dichlorophenyl)-3-methylurea–DCPMU, 1-(3,4-dichlorophenyl)-urea–DCPU, fenuron, isoproturon, 1-(4-isopropylphenyl)-3-methylurea–IPPMU, 1-(4-isopropylphenyl)-urea–IPPU, linuron, metobromuron, metoxuron, monolinuron, monuron, neburon), and 4 chloroacetanilides (acetochlor, alachlor, metolachlor, metazachlor). Glyphosate and its degradation product aminomethylphosphonic acid (AMPA) were assessed using high-performance liquid chromatography-fluorometric detection as described in Mazzella et al. (2009).

Analysis of PCBs and PBDEs was performed on molluscs (soft tissues) following the procedures described by Thompson and Budzinski (2000) and Tapie et al. (2008). Soft tissues were separated from the shell, freeze-dried, and homogenized. Approximately 1–2 g of dry sample, spiked gravimetrically with internal standards—CB30, CB103, CB155 and CB198 (Promochem, Molsheim, France)—were extracted within a microwave-assisted extraction system (Maxidigest MX 4350 from VWR Prolabo, Paris, France) using dichloromethane. The organic extract was filtered, concentrated to 1 mL, and further purified on a column of acidic silica gel column and eluted with a pentane–dichloromethane mixture (90:10 *v/v*). Finally, the extract was concentrated and spiked with an internal standard, octachloronaphthalene (Ultra Scientific, North Kingstown, RI, USA). PCBs analyses were performed on an HP 5890 series II gas chromatograph from Hewlett–Packard (Avondale, CA, USA) coupled to a 63Ni electron-capture detector, with a HP5-MS capillary column (60 m × 0.25 mm × 0.25 μm) (Agilent Technologies, Massy, France). The seven indicator PCBs (CB28, CB52, CB101, CB118, CB138, CB153, and CB180) and four PBDEs (BDE47, BDE99, BDE119, and BDE153) were quantified.

PAHs were analyzed by gas chromatography/mass spectrometry (GC/MS) using an Agilent 6890 gas chromatograph coupled to an Agilent 5973 mass spectrometer (Agilent, Santa Clara, CA, USA) according to the protocol

described by Baumard et al. (1999). Perdeuterated phenanthrene, fluoranthene, chrysene benzo[e]pyrene, benzo[a]pyrene, and benzo[ghi]perylene were used as internal standards gravimetrically added prior to the extraction. For PAH analysis, the extracts obtained by microwave-assisted extraction (dichloromethane) were first purified by elution on an alumina column (15 ml of dichloromethane) and then after reconcentration and transfer to iso-octane by elution on a silica column (5 ml of pentane were discarded, and the final purified extracts were collected by elution with 15 ml dicloromethane/pentane, 35/65, *v/v*). The extracts were concentrated and transferred to isooctane, and the syringe standards (perdeuterated pyrene and benzo(b)fluoranthene) were added before analysis by GC/MS. Detailed protocol was described previously (Le Goff et al. 2006).

All steps of the analytical protocol were validated in terms of reproducibility and accuracy; procedural blanks were systematically checked for every 12 samples, and two certified reference materials SRM-2977 (Mussel tissue, NIST, Gaithersburg, USA) and WM-F01 (Fish muscle, Wellington Laboratories, Guelph, Canada) were analyzed together with the real samples (Thompson and Budzinski 2000; Tapie et al. 2008).

Phycotoxins analysis

Phycotoxin content was measured in the digestive gland of mussels. Toxins were extracted from thawed tissues as described in Mondeguer et al. (2004) following a protocol validated against certified contaminated standards (AOCS-1 and MUS-2 IBM, NRC, Canada). A 4-g aliquot of the homogenate was sequentially extracted using 10, 8, and 6 ml of pure acetone. The combined acetone extracts were filled up to 25 ml with acetone and then split into two equal aliquots and evaporated to dryness. One aliquot was used for alkaline hydrolysis for detection of okadaic acid derivatives (diol or acyl esters). The dry hydrolyzed and non-hydrolyzed extracts were processed for automated solid-phase extraction on an ASPEC Xli robotized unit (Gilson Inc., WI, USA). Detection and quantification were carried out by HPLC coupled with ThermoFinnigan LCQ Classic ion trap mass spectrometer (San Jose, CA, USA) as detailed in Marcaillou-Le Baut et al. (2010).

Biomarkers

Protein concentration

The protein content was determined according to the Bradford method (Bradford 1976), using BioRad Protein Assay kit and bovine serum albumin as standard. Each sample was measured in triplicate for its absorbance at 595 nm using a Safire TECAN (Tecan Group Ltd., Switzerland) microplate reader.

Reproductive cycle

Vitelline quantification Vitelline was measured in order to investigate the vitellogenesis cycle in mussels. Since vitellogenesis is not occurring in males during normal sexual maturation, vitelline was quantified in females only. The gender of individual mussels was determined prior to analyses using a polymerase chain reaction (PCR)-based method for the detection of the *M. edulis* male-specific VCL (vitelline-coat lysin) gene and female-specific VERL (vitelline envelope receptor for lysin) gene as described in Hines et al. (2007). The forward and reverse primers used for VCL and VERL amplification are described in Table 1. This method was chosen because it allows the gender determination in both ripe and spent mussels (for additional details, see Online resource 1). Vitelline measurement was performed using Dot Blot. Microsomal fractions were prepared from mussel gonad homogenates by differential centrifugation. Dot blot was realized using microsomal proteins loaded in duplicate on a nitrocellulose transfer membrane (PROTRAN BA, Schleicher & Schuell BioScience, Germany). The membrane was probed using a polyclonal antibody directed against vitelline (1:150) and purified by Osada et al. (1992) from the ovary of the Japanese scallop *Patinopecten yessoensis*. Osada et al. (1992) have shown that ovary extracts from *M. edulis* reacted with the antibody used in our study. Then, an anti-rabbit antibody linked to alkaline phosphatase (1:500; Goat Anti-rabbit IgG AP conjugate, Pierce) was used as a secondary antibody. The BM Chemiluminescence ELISA substrate revelation kit (Roche Diagnostics) was used to reveal chemiluminescence, and final reading was done using a Fluor-STM Multimager. The intensity of the spots was calculated using Mesurim freeware (http://pedagogie.ac-amiens.fr/svt/info/logiciels/Mesurim2/Telecharge.htm). Each measurement was realized in duplicate for four females per site (for additional details, see Online resource 1).

Condition index Condition index was determined for ten individuals per site and per sampling date. It was calculated as the ratio between the dry weight of tissues and the length of the shell (Bodoy et al. 1986).

Maturation stage level A 4-mm cross-section of each sampled mussel, including digestive gland, gills, mantle, and gonad, was taken. Cross-sections were dehydrated in ascending ethanol solutions, cleared with xylene, and embedded in paraffin wax. Five-micrometer-thick sections were cut, mounted on glass slides, and stained with Harry's hematoxylin–eosin Y (Martoja et al. 1967). Slides were examined under a light microscope to determine gametogenic stages. Five stages were defined: stage 0 (resting), stage A (spawning/shedding), stage B (mature/ripe), stage

Table 1 Primer used for gender determination in *M. edulis*

Primer name	Primer sequence	PCR product length	Hybridation temperature	Reference
VCL_F	5′ CTGACGTCACTGCGCTTATGA 3′	681 pb	55 °C	Hines et al. (2007)
VCL_R	5′ CCAGTGTGTGCGTAGACTG 3′			Hines et al. (2007)
VERL_F	5′ CTGCAATGGTTTTGGTTGTG 3′	350 pb	50 °C	Hines et al. (2007)
VERL_R	5′ TTCAGTTCCATTTCCTTCGG 3′			Corrected from Hines et al. (2007)

C (recovery), and stage D (gonad resorption) (Grizel and Auffret 2003).

Oxidative stress

Catalase activity (CAT) CAT activity was evaluated through the level of hydrogen peroxide (H_2O_2) degradation, according to the Clairborne method (Clairborne 1985). Hydrogen peroxide absorbance was read every 24 s over a period of 3 min at 240 nm. Activity of catalase was expressed in micromoles of H_2O_2 degraded per min per milligram of protein. CAT activity was measured in triplicate from ten individual digestive glands per sample group (for additional details, see Online resource 1).

Malondialdehyde (MDA) The MDA assay is based on the reaction of the chromogenic reagent *N*-methyl- 2-phenylindole with MDA at 45 °C, resulting in the formation of a chromophore with absorbance at 586 nm (Esterbauer et al. 1991). A calibration curve was prepared using MDA standard as tetramethoxypropane. The results were expressed as nanomoles MDA per milligram of protein. The MDA was assayed in triplicate from ten individual gills per sample group (for additional details, see Online resource 1).

Biotransformation enzymes

Benzo(a)pyrene hydroxylase (BPH) BPH activity was assayed in the presence of NADPH using BaP as the substrate following the protocol described by Michel et al. (1994) adapted for a microplate assay. A kinetic measurement of fluorescence is realized during 5 min with an excitation wavelength of 430 nm and an emission wavelength of 510 nm. Results are expressed in picomoles of 3-OH-BaP generated per minute per milligram of microsomal protein, using an external calibration curve ranging from 0 to 100 μM of 3-OH-BaP (Promochem, Molsheim, France). BPH activity was assayed in triplicate in eight pools of three digestive glands per sample group (for additional details, see Online resource 1).

Carboxylesterase activity (CbE) CbE activities were determined by the method of Van Asperen (1962) adapted for microplates. The reaction was initiated by adding α- or β-naphthylacetate as the substrates. Absorbance was read at 530 and 490 nm for α- or β-naphthol, respectively, using a BioTek Instruments EL 311 microplate reader (Winooski, VT, USA). α- and β-CbE activities were summed and expressed as micrograms naphthol per milligram protein. CbE activity was assayed in triplicate from six individual muscles per sample group (for additional details, see Online resource 1).

Neurotoxicity

Acetylcholine esterase activity (AChE) AChE activity was measured in muscle according to Ellman et al. (1961), using acetylthiocholine iodide as the substrate. Absorbance was recorded at 412 nm for 1 min using a Uvikon 943 double-beam spectrophotometer (Kontron Instruments, Montigny-le-Bretonneux, France). The activity was corrected for the non-enzymatic hydrolysis of the substrate and calculated using least-square linear regression analysis over the first 30 s of the kinetics. AChE activity was expressed as nanomolars thiocholine per minute per milligram protein.

Lysosomal membrane stability

Hemolymph was withdrawn from the adductor muscle as described elsewhere (Auffret et al. 2006). Hemolymph was diluted (1:3) with Alsever anticoagulant (Bachère et al. 1988). Then, 0.8 % neutral red diluted in a phosphate buffer (1.5 M NaCl, 0.02 M KH_2PO_4, 0.1 M Na_2HPO_4, 0.03 M KCl, pH 7.4) was added. After 1 h at 19 °C, cells were observed under light microscopy, and destabilized cells (i.e., cells with neutral red dye located in the cytosol) were counted versus stable cells (cells with dye present in the lysosomes). A minimum of 100 cells per individual was counted, and lysosomal stability was assayed in ten individuals per sample group. The results were expressed as the percentage of destabilized cells.

Hemocyte parameters

Hemolymph was withdrawn from the adductor muscle (Auffret et al. 2006). All samples were filtered through a 80-μm mesh prior to analysis to eliminate any large debris which could potentially clog the flow cytometer. Characterization of

hemocyte sub-populations, number, and functions were performed using a FACScalibur (BD Biosciences, San Jose, CA, USA) flow cytometer equipped with a 488 nm argon laser. Two types of hemocyte variables were evaluated: descriptive variables (hemocyte viability, total, and hemocyte sub-population counts) and functional variables (phagocytosis, reactive oxygen species production, and oxidative burst).

Hemocyte viability, total, and hemocyte sub-population counts An aliquot of 100 μl of hemolymph from an individual mussel was transferred into a tube containing a mixture of 200 μl anti-aggregant solution for hemocytes (Auffret and Oubella 1997) and 100 μl filtered sterile seawater (FSSW), respectively. DNA was stained with two fluorescent DNA/RNA-specific dyes, SYBR Green I (Molecular probes, Eugene, OR, USA, 1/1000 of the DMSO commercial solution), and propidium iodide (PI, Sigma-Aldrich, final concentration of 10 $\mu g ml^{-1}$) in the dark at 18 °C for 120 min before flow cytometric analysis. PI permeates only hemocytes that lose membrane integrity and are considered to be dead cells, whereas SYBR Green I permeates both dead and live cells. SYBR Green and PI fluorescence were measured at 500–530 nm (green) and 550–600 nm (red) wavelengths, respectively, by flow cytometry. Thus, by counting the cells stained by PI and cells stained by SYBR Green, it was possible to estimate the percentage of viable cells in each sample. All SYBR Green-stained cells were visualized on a forward scatter (FSC, size) and side scatter (SSC, cell complexity) cytograms. Three sub-populations were distinguished according to size and cell complexity (granularity). Granulocytes are characterized by high FSC and high SSC, while hyalinocytes have high FSC and low SSC. Total hemocyte, granulocyte, and hyalinocyte concentrations were estimated from the flow-rate measurement of the flow cytometer as all samples were run for 30 s as described in Marie et al. (1999).

Phagocytosis An aliquot of 100 μl hemolymph, diluted with 100 μl of FSSW, was mixed with 30 μl of yellow-green 2.0 μm fluoresbrite microspheres diluted to 2 % in FSSW (Polysciences, Eppelheim, Germany). After 120 min of incubation at 18 °C, hemocytes were analyzed at 500–530 nm by flow cytometry to detect hemocytes containing fluorescent beads. The percentage of phagocytic cells was obtained from fluorescence intensity histograms and was defined as the percentage of hemocytes that had engulfed three beads or more.

Reactive oxygen species production ROS production by untreated hemocytes was measured using 2′,7′-dichlorofluorescein diacetate (DCFH-DA) (Lambert et al. 2003). A 100-μl aliquot of pooled hemolymph was diluted with 300 μl of FSSW. Four microliters of the DCFH-DA solution (final concentration of 0.01 mM) was added to each tube maintained on ice. Tubes were then incubated at 18 °C for 120 min. After the incubation period, DCF fluorescence, quantitatively related to the ROS production of untreated hemocytes, was measured at 500–530 nm by flow cytometry. Results are expressed as the geometric mean fluorescence (in arbitrary units) detected in each hemocyte sub-population.

Oxidative burst For oxidative burst measurements, ROS production was measured by flow cytometry as described above following treatment with 100 nM of the pro-oxidant phorbol myristate acetate.

Statistical analysis

Data were expressed as mean±standard deviation (SD). The homogeneity of variance was analyzed using the Bartlett's test. When the variances proved to be homogeneous, differences between the two study sites were evaluated using *t*test ($p<0.05$) for each sampling date. In the case of non-homogeneous variances, data were $\log_{10}$-transformed before performing the *t*test. Simple regression analysis was performed using the Spearman rank correlation test in order to investigate the correlation between investigated parameters. Statistical analyses were carried out using the Statgraphics Plus statistical software (Statpoint Inc, Warrenton, VA, USA). Redundancy discriminant analysis (RDA; Rao 1964) was used to further investigate the strength of relationship between measured environmental factors and biotic variables within a multivariate data set. Permutations were performed to test the significance between environmental variables and biotic composition. Analysis was performed using the Genuine Licence R software (cran.r-project.org/) and Vegan package (CRAN.R-project.org/package=vegan; Oksanen et al. 2012).

Results and discussion

Monitoring of environmental parameters

There were marked changes in water quality parameters as a function of sampling time (Fig. 2). Water temperature increased in a similar manner at both sites during the study period, and a marked increase in temperature (i.e., +6–7 °C in 15 days) was observed between May 28 and June 12, 2007 (Fig. 2a). Changes in salinity, suspended solids concentration, and turbidity were more pronounced in Les Granges than in Le Maresclé, confirming the influence of the Vilaine plume in the site Les Granges (Fig. 2b, e, and f). The decrease in salinity and the increase in turbidity and

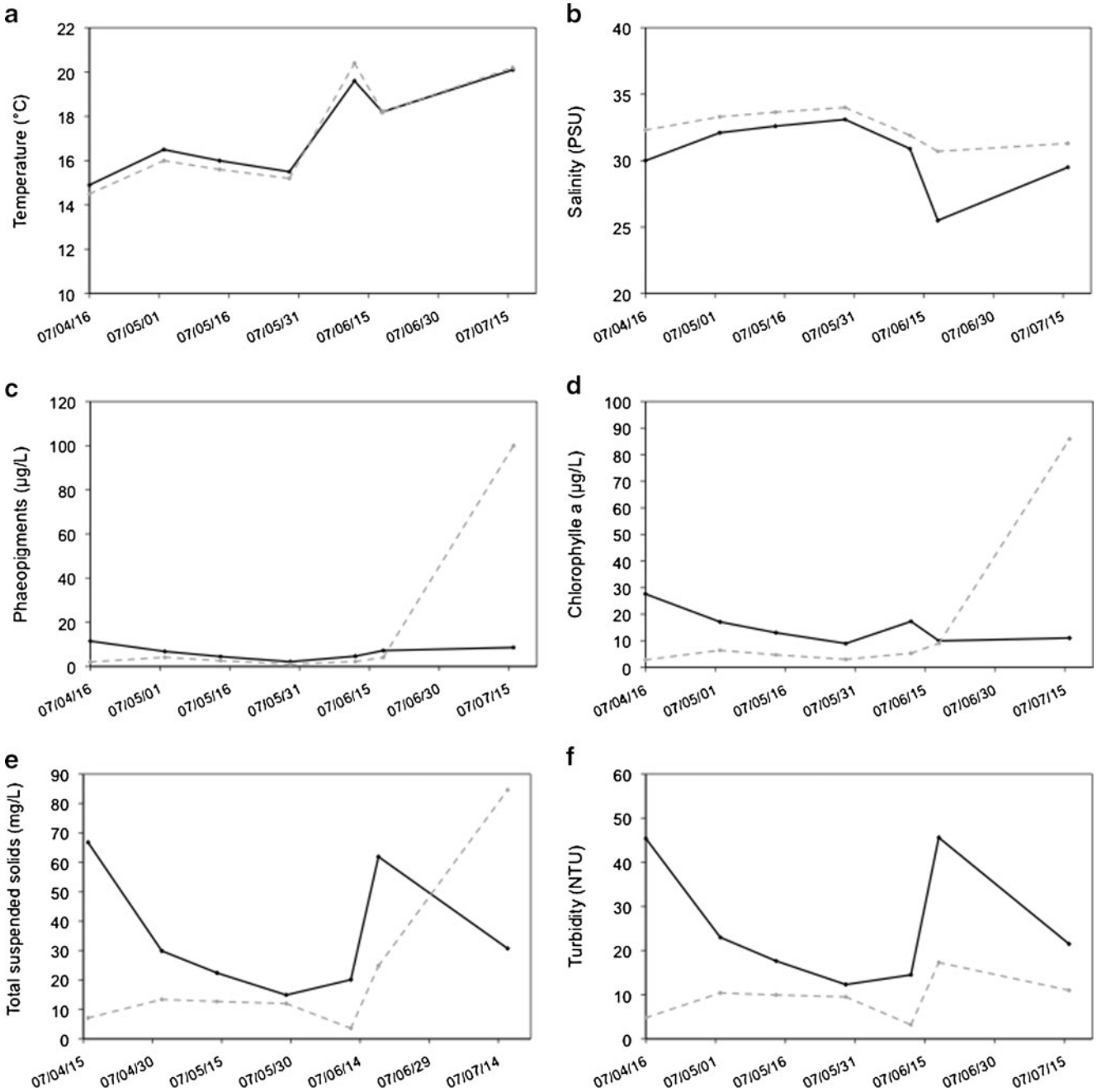

Fig. 2 Evolution of **a** water temperature, **b** salinity, **c** phaeopigment, **d** chlorophyll a concentration, **e** total suspended solids, and **f** turbidity in Les Granges (*solid black line*) and Le Maresclé (*grey dotted line*)

suspended solids concentration occuring on June 18 were the consequences of heavy rains that occurred in this area during this period (source: Météo France, data not shown). Chlorophyll a concentrations in water were higher in Les Granges than in Le Maresclé until mid-June. A strong increase of pheopigments and chlorophyll a concentrations was measured on July 16, 2007, in Le Maresclé (Fig.2c, d). This increase was due to a bloom of the dinoflagellate *Lepidodinium chlorophorum* at this period. The cellular concentration recorded in Le Maresclé on July 16, 2007, for this species reached 11 million cells/L (REPHY-Ifremer 2008). Previous monitoring studies conducted by the Ifremer have shown that *L. chlorophorum* blooms have been regularly observed in the Bay of Vilaine since the early 1980s. According to Guillaud and Lefebvre (2001), the bloom is followed by the death of the unicellular algae, and the decay of the corresponding biomasss induces a depletion of dissolved oxygen resulting in hypoxic conditions that are potentially

harmful for sessile species such as bivalve molluscs. Therefore, phytoplankton may influence the ecophysiology of bivalves in two different manners: (1) the availability of edible phytoplankton can positively influence the growth and the reproduction of bivalves (MacDonald and Thompson 1985) and (2) the occurrence of a phytoplankton bloom (harmful or not) can generate stress in bivalves leading for example, to growth abnormalities or hypoxia (Chauvaud et al. 1998; Lorrain et al. 2000; Guillaud and Lefebvre 2001). Quantitative and qualitative characteristics of phytoplankton have therefore to be measured and taken into account as potential confounding factors in the interpretation of the results of biomarker measurements.

Among diarrheic microalgal toxins that can accumulate in shellfish, okadaic acid (OA) and its derivatives (DTX1, DTX2) are occasionally subject to acylation, which results in a group of toxic acyl-esters (Yasumoto et al. 1985) collectively known as DTX3. While the processes involved in the metabolization of toxins in shellfish are still poorly understood, it is known that these compounds are never detected in the phytoplankton and that they are only formed in shellfish (Suzuki et al. 1999). High levels of total OA (i.e., OA+DTX3) were recorded in the digestive gland of *M. edulis* sampled between May 28 and July 16, 2007, at both sites (Fig. 3). The increase in okadaic acid and DTX3 concentrations was greater in the animals collected in Les Granges. The values recorded in mussels from this site exceeded the French legal toxicity threshold of 0.8 $\mu g g^{-1}$ (Yasumoto et al. 1985) at three dates: June 12, June 18, and July 2. In contrast, this threshold was exceeded only on June 18 in mussels collected at Le Maresclé. Interestingly, the patterns of free OA concentrations were comparable at both sites (increase in June, data not shown), but there was a greater metabolization of free OA to DTX3 by mussels from Les Granges (Fig. 3). In this study, the presence of phycotoxins was due to the presence of the dinoflagellate *Dinophysis sacculus* (REPHY-Ifremer 2008). Okadaic acid and dinophysistoxins are lipophilic toxins that may accumulate in shellfish, especially in digestive glands. These phycotoxins are involved in causing a gastrointestinal syndrome called diarrheic shellfish poisoning, which can occur in humans after consumption of bivalve molluscs. The exposure to okadaic acid and its derivative (DTX3) can induce histological modifications in the digestive gland and detoxification processes, as shown in *Mytilus galloprovincialis* (Auriemma and Battistella 2004).

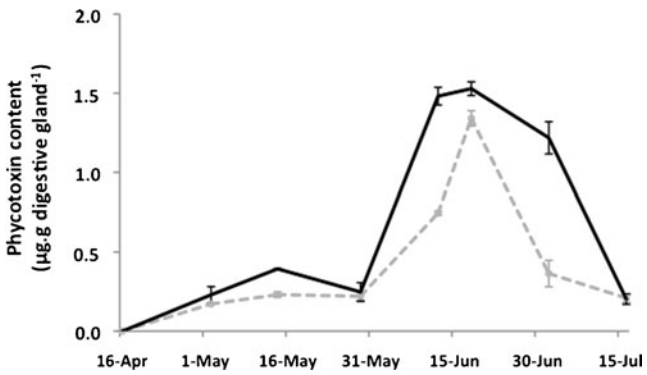

Fig. 3 Temporal variations in total okadaic acid content (free OA+DTX3) in the digestive gland of mussels collected in Les Granges (*solid black line*) and Le Maresclé (*grey dotted line*). *Error bars* represent standard deviation of three independent extracts of four digestive glands. Values are expressed as micrograms per gram per digestive gland (mean±SD)

Organic contaminants

POP contamination

There were differences in the temporal and spatial occurrence of some of the chemicals measured (Table 2). No BDEs were found above detection limits during any of the sampling events at either site (detection limits for individual compounds=0.1 ng/g dw). PAH concentrations, calculated as the sum of 21 PAHs listed as priority pollutants by the EPA, ranged from 25.9 to 91.7 $ng g^{-1}$ (dw). The highest levels of PAHs were recorded in Le Maresclé, probably due to the boating activities occuring close to this harbor. Relatively dominant PAH compounds were pyralene (7.6± 2.6 $ng g^{-1}$ in Les Granges, 10.7±13.3 $ng g^{-1}$ in Le Maresclé), phenanthrene (4.6±2.8 $ng g^{-1}$ in Les Granges, 8.9± 5.3 $ng g^{-1}$ in Le Maresclé), fluoranthene (8.1±1.7 $ng g^{-1}$ in Les Granges, 6.9±2.8 $ng g^{-1}$ in Le Maresclé), and benzo (e)pyrene (4.4±2.0 $ng g^{-1}$ in Les Granges, 6.8±5.6 $ng g^{-1}$ in Le Maresclé). However, these concentrations were relatively low compared with previous concentrations measured in mussel tissues (Roméo et al. 2003a). According to the RNO biomonitoring program (RNO 2006), fluoranthene concentrations found in this study are lower than the median calculated in mussel tissues collected on the French Atlantic Coast between 2000 and 2004 (21.4 $ng ng g^{-1}$dw). This confirms that the boating activities occurring in the studied area resulted in a small amount of PAHs. The body burden of the sum of the seven indicator PCBs ranged from 44.1 to 75.5 $ng g^{-1}$ (dw). CB153 levels measured in mussels from the two study sites are slightly higher than the median calculated for this congener in tissues of mussels collected along the French Atlantic Coast between 2000 and 2004 (19.4 $ng g^{-1}$dw, RNO, 2006). Highest ∑PCBs levels were recorded in mussels sampled in Les Granges. The two most dominant PCBs (i.e., CB153 and CB138) were also found in higher concentrations in mussels collected at Les Granges (respectively, 28.9±6.0 and 17.4±3.8 $ng g^{-1}$) as compared with Le Maresclé (respectively, 23.2±2.5 and 14.0±1.3 ng g^{-1}). PCBs are found in the environment as a result of their previous use as insulator fluids in electric equipments. The

Table 2 Concentrations (nanograms per gram dry weight) of organic contaminants: polychlorinated biphenlyls (Σ CB), polybrominated diphenyl ethers (Σ BDE), and polycyclic aromatic hydrocarbons (Σ PAH) measured in tissues of mussels (*M. edulis*) collected in Le Maresclé (MAR) and Les Granges (GRA) between April and July 2007

	∑ CB ($ng\,g^{-1}$)		∑ BDE ($ng\,g^{-1}$)		∑ PAH ($ng\,g^{-1}$)		∑ Triazines ($\mu g\,L^{-1}$)		∑ Phenylureas ($\mu g\,L^{-1}$)		∑ Chloroacetanilides ($\mu g\,L^{-1}$)	
	MAR	GRA	MAR	GRA	MAR	GRA	MAR	GRA	MAR	GRA	MAR	GRA
16 Apr07	55.4	44.5	nd	nd	43.7	33.2	nd	nd	nd	nd	–	–
02-May-07	–	–	–	–	–	–	–	nd	–	nd	nd	nd
14 May 07	44.1	62.3	nd	nd	91.7	68.4	–	nd	nd	nd	nd	nd
29 May 07	52.2	57.6	nd	nd	53.6	32.5	nd	0.05	nd	0.07	0.07	0.07
12 June 07	52.3	70.5	nd	nd	25.9	36.3	nd	0.02	nd	0.05	nd	nd
18 June 07	54.6	75.5	nd	nd	40.2	48.0	nd	0.02	nd	0.05	–	–
02 July 07	52.8	57.4	nd	nd	86.3	54.2	nd	0.02	–	nd	–	–
16 July 07	–	–	–	–	–	–	nd	nd	–	nd	–	–

Concentrations (micrograms per liter) of herbicides: triazines (Σ triazines), phenylureas (Σ phenylureas), and chloroacetanilides (Σ chloroacetanilides) measured in one single water sample collected at low tide in Le Maresclé (MAR) and Les Granges (GRA) between April and July 2007

En dash no data, *nd* not detected, ∑ *CB* CB 28, 52, 101, 118, 138, 153, 180, ∑ *BDE* BDE 47, 119, 99, 153, ∑ *PAH* naphtalene, acenaphthylene, acenaphtene, fluorene, phenanthrene, anthracene, fluoranthene, pyrene, benzo[a]anthracene, triphenylene, chrysene, benzo[b]fluoranthene, benzo[k]fluoranthene, benzo[j]fluoranthene, benzo[e]pyrene, benzo[a]pyrene, perylene, dibenz(ah)anthracene, dibenz(ac)anthracene, benzo[ghi]perylene, ∑ *triazines* ametryn, atrazine, cyanazine, DEA, DET, DIA, irgarol 1051, prometryn, propazine, simazine, terbuthylazine, terbutryn, ∑ *phenylureas* chlortoluron, diuron, DCPMU, DCPU, fenuron, isoproturon, IPPMU, IPPU, linuron, metobromuron, metoxuron, monolinuron, monuron, neburon, ∑ *chloroacetanilides* acetochlor, alachlor, metolachlor, metazachlor

production was phased out in 1987 in France, but they are still found in the aquatic environment, particularly in urbanized and industrial areas. The higher PCB levels measured in Les Granges may be attributed to the greater influence of the Vilaine plume on this site Les Granges. These results are consistent with those of Bocquené and Abarnou (2012) who showed that grey mullet and allis shad individuals collected upstream of Arzal dam in 2004 and 2005 were more contaminated by PCBs than those collected in the estuary, suggesting a contamination source located upstream the estuary. This pattern is consistent with the available data on PCBs content of sediment sampled in the Vilaine River during a national monitoring program performed in 2010 (Plan National PCB–Ministère de l'Environnement http://www.pollutions.eaufrance.fr/pcb/resultats-xls.html). The Vilaine watershed is mostly an agricultural area with no heavy industry. There is no currently active industrial site with direct or indirect PCBs release into water in this area (Registre Français des Emissions Polluantes; http://www.pollutionsindustrielles.ecologie.gouv.fr/IREP). Therefore, contamination by these substances is probably associated with various urban sources (the main source being the urban area of Rennes characterized by more than 660,000 inhabitants according to the last census of 2009, Insee Bretagne, Insee 2011) and the presence of a stock of PCBs in Vilaine sediment associated with older contamination. Finally, these results suggest a chronic non-point source pollution mainly of urban and domestic origin.

Herbicide contamination

Mussels collected from both study sites had trace concentrations of certain herbicides (Table 2). Chemicals detected included phenylureas (diuron, 0.05–0.07 $\mu g\,L^{-1}$), triazines (atrazine, diethylatrazine, and simazine, 0.02 $\mu g\,L^{-1}$), and chloroacetanilides (acetochlor, metolachlor, 0.07 $\mu g\,L^{-1}$). These substances were detected between May 28 and the June 18, 2007, at greater concentrations in mussels from Les Granges as compared with Le Maresclé, where almost no pesticides were quantified. Glyphosate and its degradation product AMPA were not detected at either site (data not shown). Previous analyses carried out in the Vilaine estuary between 1996 and 1999 found high levels of pesticides in water (especially atrazine, diuron, and isoproturon) as a result of intensive agriculture in the catchment area of the river (SAGE Vilaine 2001). During summer periods, contamination peaks above 2 $\mu g\,L^{-1}$ were previously observed (Marchand 2006). Monitoring of herbicides and booster biocide residues upstream of Arzal dam at the same study period showed that some compounds were sometimes present at levels of concern for aquatic primary producers (Caquet et al. 2012). Irgarol 1051, diuron, and, to a lesser extent, isoproturon were the substances that posed the greatest risk. The presence of Arzal dam clearly reduced downstream transfer of contaminants. It favors their retention in the reservoir where degradation may take place and may contribute to reduce downstream transfer. This retention

effect was probably reinforced by a seasonal effect leading to a reduced outflow of freshwater to the estuary during the sampling period. Additionally, there is a large degree of uncertainty associated with the measured pesticide concentrations as it is highly probable that the water sampling (grab sample) utilized in this study does not reflect the variability of pesticide contamination in the Bay of Vilaine. Estuarine hydrological systems are characterized by great dilution and dispersion phenomena due to tidal influences, and point samples only give a snapshot of the water contamination level. For future studies, more reliable concentration estimates could be obtained using polar organic chemical integrative samplers. Actually, such devices allow the determination of time-weighted average concentrations, which is a fundamental part of an ecological risk assessment process for chemical stressors (Mazzella et al. 2009).

Condition index

The condition index (CI) of mussels increased with time at both sites and was significantly greater in animals sampled in Les Granges between April and mid-June (Fig. 4a). After June 18, the CI decreased again at both sites. The CI has previously been shown to be positively influenced by seasonal factors such as food availability and sexual maturation with spawning being the most influencing parameter, leading to a marked CI decrease (Roméo et al. 2003b). Interestingly, CI values were significantly correlated to VIT level in mussel tissue (r=0.52; $p$value<0.01), confirming the relationship between sexual maturation and CI value. CI values were also significantly correlated to chlorophyll a concentration (r=0.40; $p$value<0.05) and to total suspended solids (r=0.48; $p$value<0.01). According to the results obtained for the chlorophyll a concentration, phytoplankton availability was greater at Les Granges than in Le Maresclé. This highlights the positive influence of a better availability of edible phytoplankton on the condition (i.e., increase in tissue weight) of mussels.

Gametogenesis activity and reproductive status

VIT levels were significantly higher in mussels collected in Les Granges site compared with Le Maresclé regardless of sampling time (Fig. 4b). The accumulation pattern of VIT was different between the two sites. In Les Granges, the VIT content increased from April 16 to June 12, 2007, and decreased again after June 18. This parameter was more stable in animals from Le Maresclé. These results highlight a stronger and more seasonally influenced vitellogenesis activity in mussels from Les Granges. VIT production over time was positively correlated with seasonal changes in the CI (r=0.52; $p$value<0.01). Interestingly, the decrease of CI that occurred on June 18, 2007, in mussels from Les

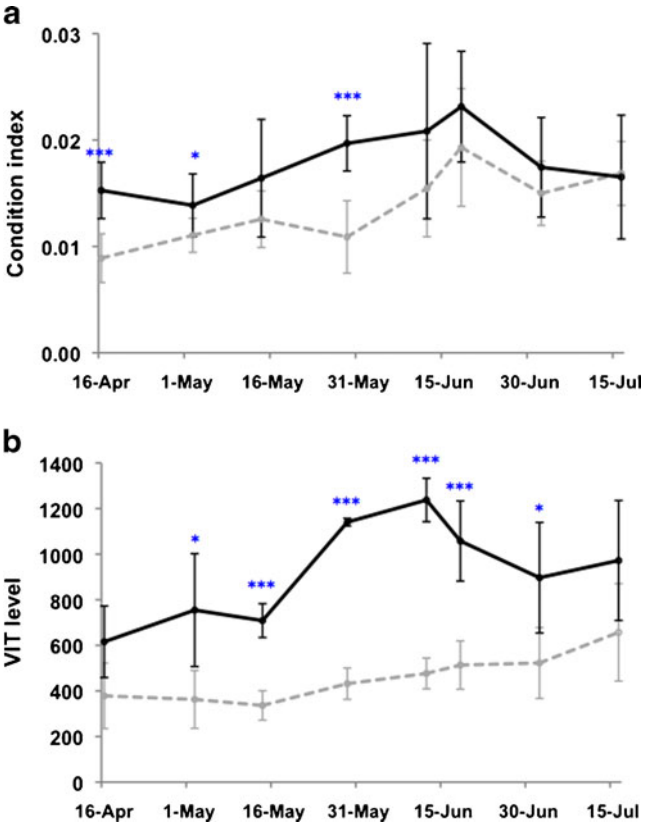

Fig. 4 Temporal variations in **a** condition index (n=10), **b** vitelline level in gonads (n=4 females) of *M. edulis* collected in Les Granges (*solid black line*) and Le Maresclé (*grey dotted line*). Significant difference between the two sites ($t$test): *p<0.05, ***p<0.001; mean ±SD

Granges was synchronized with the decrease of VIT level at the same period. These results suggest that a spawning event may have occurred in mussel from Les Granges at this period. Indeed, Li et al. (1998) showed in the oyster *Crassostrea gigas* that a decrease of VIT level occurs after spawning. The histological analysis of maturation stages of mussel gonads confirmed this hypothesis (Fig. 5).

Maturation stages A and 0, corresponding to the spawning and the resting stage, respectively, were not frequently observed during the study period in mussels from both sites. Stage A (spawning/shedding stage) was observed in early April in Le Maresclé and on May 29, 2007, in Les Granges whereas stage 0 (resting stage) was observed in July at both sites. This observation confirms that gametogenesis and spawning occurred at both sampling locations during the study period. This was further confirmed by the observation of individuals presenting maturation stages B (maximal maturation) and C (gonadal recovery) during the entire study period. Stage D (gonad resorption) was more frequent after June in both sites. Spawning started earlier and was observed in more individuals in Les Granges compared with Le Maresclé. In Le Maresclé, more mussels were

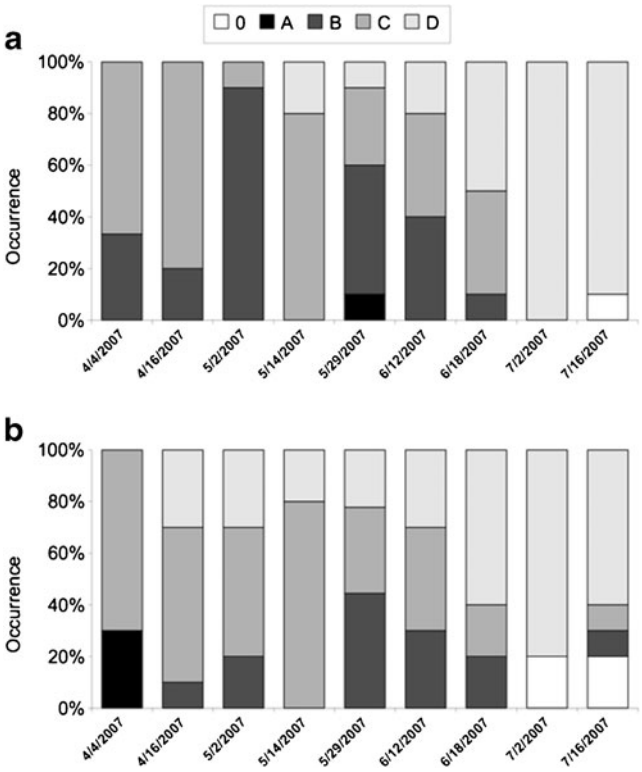

Fig. 5 Temporal variations in gametogenesis stages in mussel collected in **a** Les Granges and **b** Le Maresclé; stage *A*: spawning/shedding stage, *B*: mature/ripe stage, *C*: recovery stage, *D*: gonad resorption, *0*: resting stage (Grizel and Auffret 2003)

at maturation stages C (gonadal recovery) and D (gonad resorption).

The analysis of gametogenesis stages, condition index, and vitelline level together confirm that the study period corresponds to an active reproduction phase for *M. edulis* in the Vilaine estuary. This is in agreement with previous reports concerning *M. edulis* reproduction. Although many populations of *M. edulis* exhibit some spawning all year round, major peaks of spawning occur in spring often followed by a number of further secondary spawnings later in the summer, depending on environmental conditions (Seed and Suchanek 1992). Fecundity varies from year to year, probably reflecting adjustments to energy allocation according to variation in food supply (Thompson 1979). Although reproduction is mainly driven by environmental factors such as food and temperature, toxic substances (e.g., endocrine disruptors) can also interfere with reproduction (Ramirez 2002). Les Granges site is located in the plume of the Vilaine estuary with greater concentrations of organic particulate matter and chlorophyll a than at Le Maresclé, indicating a better food availability but also elevated exposure to contaminants from the Vilaine River. Reproductive processes of mussels may be influenced either positively by better food availability or negatively by contaminants. Our results highlight that reproductive effort was more emphasized in Les Granges, indicating that the Vilaine plume has no clear negative effects on mussel reproduction. In this study, several changes in biomarkers were observed concomitantly with the putative spawning event observed in mid-June at Les Granges site. Growing interest has been focused on the effects of spawning in bivalves, as the animals are in poor physiological condition during this period because of the energy costs of the event (Berthelin et al. 2000). Therefore, reproduction metabolism has to be considered as a major confounding factor for the interpretation of biomonitoring data.

Oxidative stress

Our results show that MDA content was slightly modified over the study period (Fig. 6a). MDA levels were significantly higher in mussels from Les Granges on April 16, and conversely, higher lipid peroxides were measured in mussels from Le Maresclé on July 16. MDA levels were positively correlated with chlorophyll a and suspended solids concentrations (r=0.37 and r=0.36, respectively, p<0.05). Moreover, the observed increase in MDA levels at Le Maresclé on July 16 was synchronized with the bloom of the dinoflagellate *L. chlorophorum* occurring at this site. Therefore, we suggest that the bloom of *L. chlorophorum* and its subsequent decay may have caused oxidative damages to lipids in mussels from Le Maresclé. CAT activity was significantly lower in mussels collected in Les Granges in early and late May and significantly higher in early June (Fig. 6b). CAT activity strongly decreased after May in mussels collected at both sites. In bivalves, CAT activity was shown to be influenced by a variety of abiotic and biotic factors, such as food availability, reproductive status, temperature, and salinity (Viarengo et al. 1991; Power and Sheehan 1996; Sheehan and Power 1999; Khessiba et al. 2005; Prevodnik et al. 2007). In our study, CAT activity was significantly correlated with various environmental factors: phycotoxins in digestive gland (r=−0.39, p<0.05), water temperature (r=−0.48, p<0.01), salinity (r=0.34, p<0.05), and PAH concentration in mussel tissues (r=0.54, p<0.01). According to previous studies in *Mytilus sp*., CAT activity may either increase or decrease following exposure to chemical stressors (Solé et al. 1995; Orbea et al. 2002; Bocquené et al. 2004; Khessiba et al. 2005; Lionetto et al. 2004; Borkovic et al. 2005; Pampanin et al. 2005; Beneditti et al. 2006; Bocchetti and Regoli, 2006; Box et al. 2007; Lima et al. 2007; Vlahogianni et al. 2007; Gorbi et al. 2008). Additionally, these studies highlighted the strong influence of seasonal parameters on CAT activity. According to Dagnino et al. (2007), CAT response profile may correspond to a bell curve. Thus, the analysis of environmental samples corresponds to a snapshot of this bell-shaped dose-effect relationship conducting to uneasy interpretation of CAT activity

changes. Consequently, we suggest that the measurement of catalase activity may be more adapted to laboratory experiments when the kinetics of exposure is known and where unexposed animals are used as controls.

Biotransformation enzymes

A marked increase of BPH activity was recorded in mussels from Les Granges on June 18, 2007 (Fig. 6c). BPH activity was significantly higher in mussels from Le Maresclé on July 16 compared with Les Granges. In this study, BPH activity was strongly and positively correlated with levels of phycotoxins in the digestive gland ($r=0.80$, $p<0.001$) and to water temperature ($r=0.76$, $p<0.001$). Significant correlations were also shown between BPH activity and PAH concentrations ($r=-0.34$, $p<0.05$) and between BPH activity and PCBs concentrations ($r=0.37$; $p<0.05$) in mussel tissues, but the correlation coefficients were lower. CbE

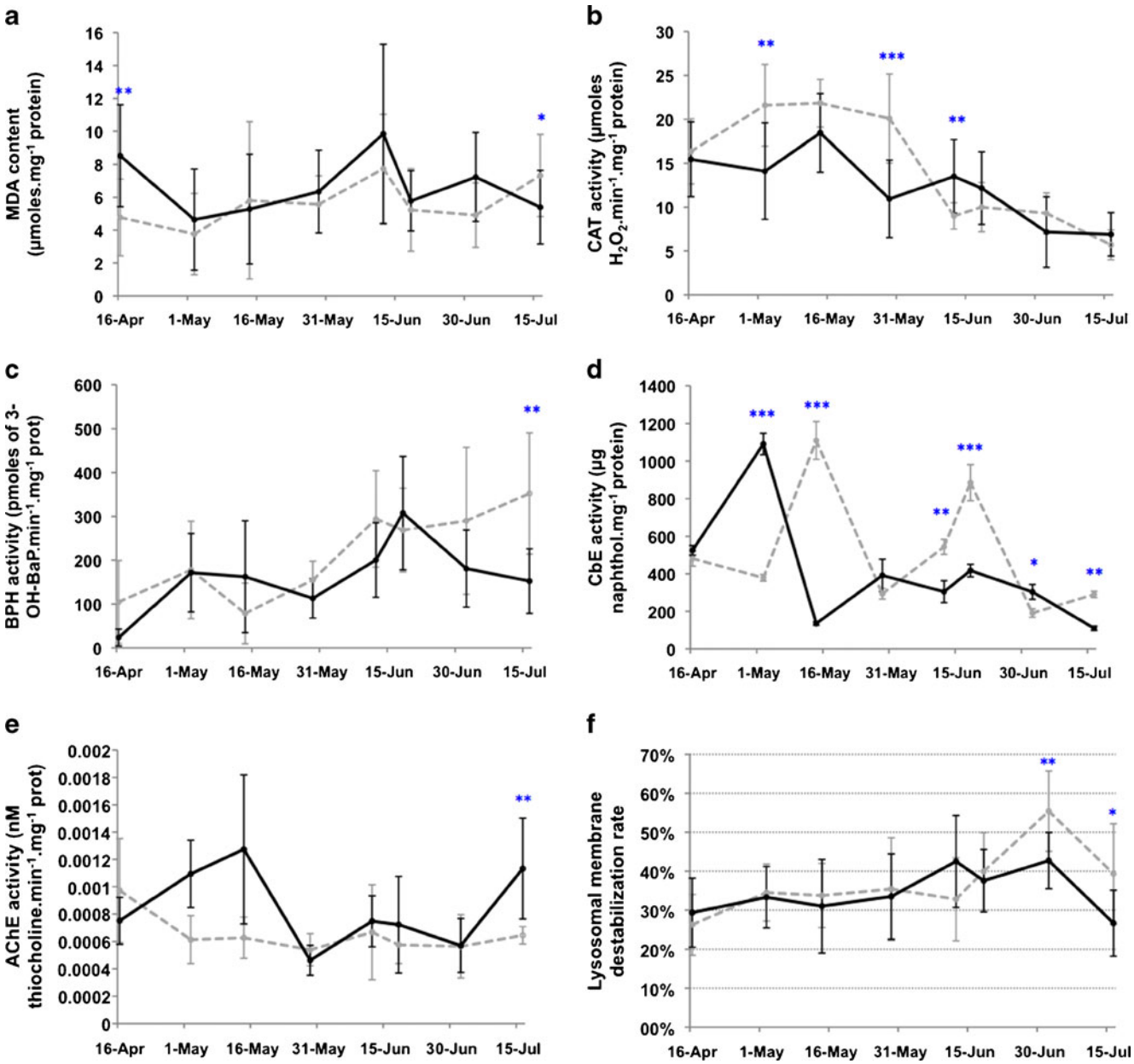

Fig. 6 Temporal variations in **a** MDA content (gill, $n=10$), **b** CAT activity (digestive gland, $n=10$), **c** BPH activity (digestive gland, $n=8$ pools of three individuals), **d** CbE activity (muscle, $n=6$), **e** AChE activity (muscle, $n=6$), **f** lysosomal membrane stability (hemocytes, $n=10$) measured in *M. edulis* from Les Granges (*solid black line*) and Le Maresclé (*grey dotted line*). Significant difference between the two sites (*t*test), $*p<0.05$, $**p<0.01$, $***p<0.001$ (mean±SD)

activity was strongly modified depending on sampling location and time, confirming that this enzyme is probably influenced by a variety of parameters (Fig. 6d). Significant correlations with phycotoxin content in digestive gland (r=0.40, p<0.05) and concentrations of chlorophyll a (r=−0.38, p<0.05), of PCBs (r=−0.60, p<0.001), and pesticides (r=−0.48, p<0.01) have been identified. Finally, changes in both studied biotransformation enzymes were slightly correlated to changes in pesticide concentrations and organic pollutants. BPH activity has previously been used as a biomarker of exposure to organic pollutants, especially PAHs (Porte et al. 1991; Michel et al. 1994; Solé et al. 1994; Peters et al. 1999 ; Akcha et al. 2000). Other xenobiotics have also been shown to increase BPH activity in molluscs, including pesticides and detergents (Baturo and Lagadic 1996). Esterases are known as relevant biomarkers of exposure to organophosphate and carbamate pesticides in marine molluscs (Ozretić and Krajnović-Ozretić 1992; Galgani and Bocquené 2000). In this study, increase in biotransformation enzymes activities was synchronized with dinoflagellate blooms (*D. sacculus* and *L. chlorophorum*) and was positively correlated to increase in phycotoxins content. This highlights that algal blooms and phycotoxins may lead to an increase in detoxification processes in mussels. This finding is consistent with the observation of Auriemma and Battistella (2004) in *M. galloprovincialis* exposed to OA and derivatives.

Neurotoxicity

AChE activity in the muscle of mussels from Le Maresclé was quite stable during the study period (Fig. 6e). Conversely, a significant increase in AChE activity was measured in mussels sampled in Les Granges on July 16 as compared with Le Maresclé. Although organophosphate and carbamate pesticides were not monitored during this study, higher amounts of pesticides were found in the site influenced by the Vilaine plume. Interestingly, a slight but significant negative correlation was found between AChE activity in muscle and pesticide levels (r=−0.27, p<0.05). This suggests that AChE activity could have been inhibited by the presence of pesticides. However, regarding AChE inhibition, no clear neurotoxic effects can be demonstrated in this study.

Lysosomal stability

The seasonal profile of lysosomal stability was comparable at both sites between April and May, with destabilization rates around 30 % (Fig. 6f). The destabilization rate increased in Les Granges up to around 40 % during the second fortnight in June. It also increased in mussels from Le Maresclé in July up to values greater than 50 %. According to Ringwood et al. (2005), destabilization rates below 30 % correspond to normal background levels of lysosomal destabilization. Rates above 30 % may indicate that animals are experiencing stressful conditions. Such values are frequently observed at polluted sites. In this study, increased destabilization rates above 40 % are observed in both sites concomitantly with several environmental changes: increase in temperature in mid-June followed by heavy rains, increase in pesticides concentrations in Les Granges, bloom of the dinoflagellate *L. chlorophorum* in Le Maresclé, higher phycotoxins levels in both study sites. These environmental changes may represent stressful events explaining the observed increase in destabilization rates. Additionally, according to Cho and Jeong (2005), spawning has also an impact on lysosomal stability in the Pacific oyster, *C. gigas*. In this study, decreases in condition index and VIT levels in late June seems to indicate that spawning has occurred concomitantly with the destabilization of lysosomal membranes. Finally, these results confirm that the measurement of lysosomal stability integrates the effects of various environmental or biological stressors (Ringwood et al. 1998; Bocchetti and Regoli 2006).

Hemocyte parameters

Most hemocyte parameters measured in mussels showed a gradual increase with time during the study period (Fig. 7). However, hemocyte viability remained stable between 82 and 92 % at both sites (data not shown), indicating that no acute environmental stress occurred to mussels during the sampling period. Total hemocyte counts increased threefold from mid-May to mid-July in both sites, and it was significantly higher in Les Granges on May 28 (Fig. 7a). Increase in total circulating hemocytes is generally considered to be an early immune response to pathogens (Ford et al. 1993; Anderson et al. 1995). However, in bivalves, densities of circulating hemocytes can also be modulated by toxicants, which may increase or decrease cellularity according to chemical characteristics and exposure concentrations (Gagnaire 2005; Auffret et al. 2006), or by exposure to the harmful algae *Alexandrium minutum* (producing paralytic shellfish toxins) which resulted in increases of circulating hemocytes (Haberkorn et al. 2010). The percentage of granulocytes showed a gradual increase during the study period at both sites (Fig. 7b). But this parameter was generally significantly higher in Les Granges as compared with Le Maresclé. Monitoring phagocytosis in mussels from the Vilaine estuary revealed significant variations with a minimum value around 20 % by mid-May toward a maximum value around 60 % 1 month later (Fig. 7c). Changes in phagocytosis activity were significantly correlated with the percentage of granulocytes (r=0.64, p<0.001). This is consistent with the fact that granulocytes are the cell type involved in phagocytosis (Cajaraville and Pal 1995). Various authors have reported that phagocytosis is a temperature-influenced process in bivalves (Oliver and Fisher 1999; Hégaret et al. 2003; Monari et al. 2007; Yang et al. 2007; Yu et al. 2009). In this study,

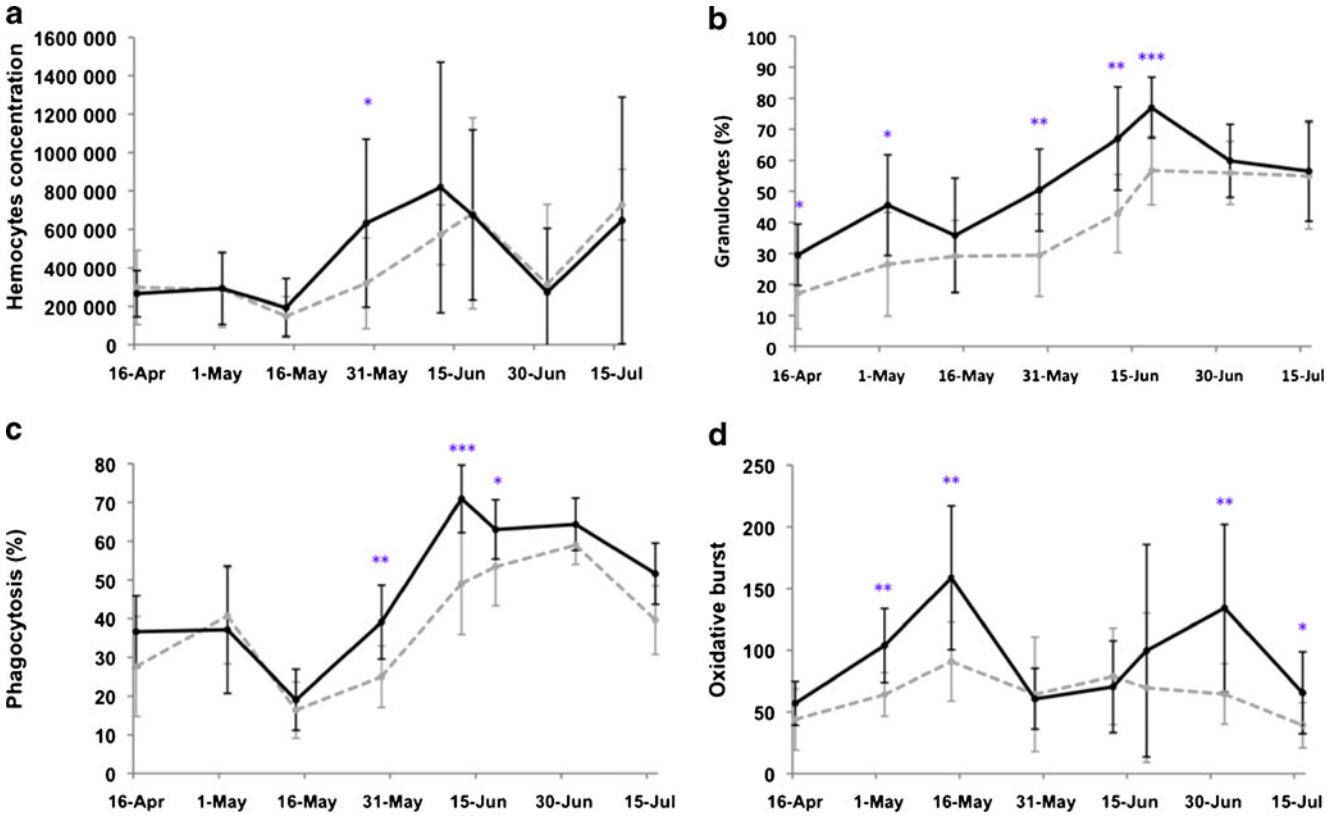

Fig. 7 Temporal variations in **a** hemocyte concentration, **b** granulocyte percentage, **c** phagocytosis, **d** oxidative burst measured in hemocytes of *M. edulis* (n=10) collected in Les Granges (*solid black line*) and Le Maresclé (*grey dotted line*). Phagocytosis is expressed as the percentage of granulocytes having engulfed one bead or more. Significant difference between the two sites ($t$test), *p<0.05, **p<0.01, ***p<0.001 (mean±SD)

phagocytosis was significantly correlated with various environmental parameters: water temperature and salinity (r=0.56 and r=−0.66, respectively, p<0.001) and phycotoxin concentration in digestive gland (r=0.43, p<0.01). Significant correlations were also found for PAH and PCB concentrations in mussel tissues (r=−0.61 and r=0.51, respectively, p<0.01). ROS levels in granulocytes were significantly higher in Les Granges on May 2, and ROS production was increased in this site at two periods: at the beginning of May and at the end of June (data not shown). Interestingly, the oxidative burst in granulocytes was significantly stronger in samples collected at the same periods in Les Granges (Fig. 7d). These results indicate that the mussels from Les Granges were exposed to biotic or abiotic factors that induced the generation of ROS. At the same time, inducibility of oxidative defense mechanisms was greater in mussel granulocytes. Increase of ROS production may have various endogenous or exogenous origins: (1) an increase of the metabolic demand due to gametogenesis and spawning (Keller et al. 2004; Delaporte et al. 2006; Meany et al. 2006); (2) the production of reactive oxidized intermediates and free radicals associated with the accumulation and metabolization of xenobiotics (Winston et al. 1996; Roméo et al. 2003b); and (3) the induction of oxidative defense systems such as inflammation in the presence of pathogens (Chu 2000; Buggé et al. 2007).

Finally, the analysis of hemocyte parameters revealed that the immune function was more stimulated in mussels collected in Les Granges than in Le Maresclé. Despite marked fluctuations of hemocyte parameters, no acute immunotoxic event occurred during the study period. Changes in immune parameters appeared to be mainly influenced by environmental parameter fluctuations, especially temperature, salinity, and phycotoxin content. Phagocytosis was also influenced by PAH and PCB concentrations, highlighting the potent immunomodulatory effects of these contaminants. This study shows that a comprehensive evaluation of environmental parameters appears necessary for a right interpretation of such hemocyte parameters in contaminated estuaries.

Influence of environmental parameters on biomarker response

A multiparametric redundancy analysis was conducted in order to investigate the strength of the relationships between measured environmental factors and biotic variables (Fig. 8). Turbidity and pheopigments were not included in the analysis because they were collinear respectively with total suspended solids (p<0.01, r=0.82) and chlorophyll a concentrations (p<0.01, r=0.88). The sum of PAHs and

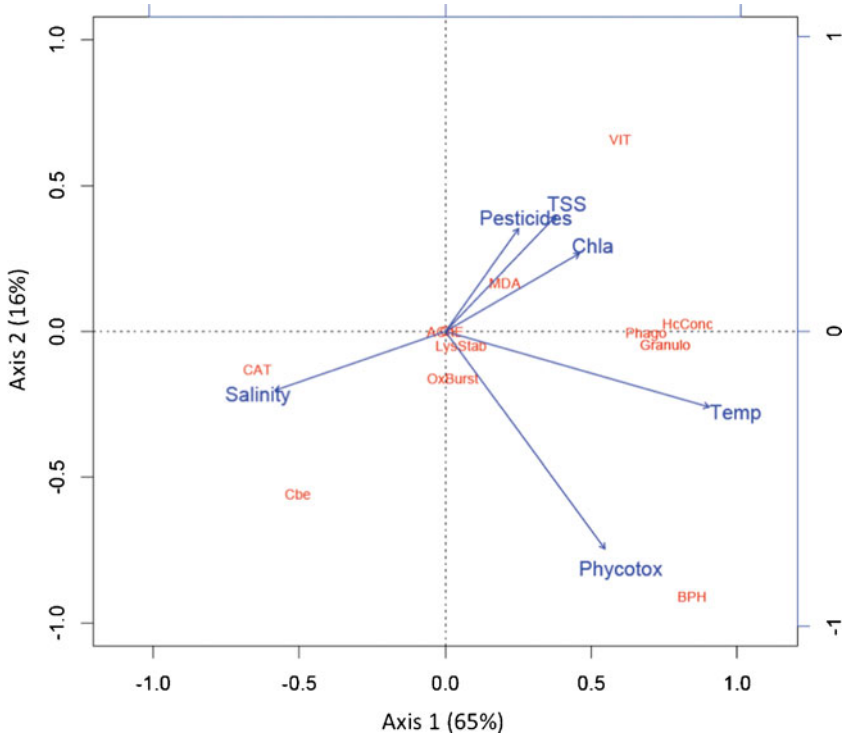

Fig. 8 Redundancy analysis (RDA) biplot of axes *1* and *2* showing environmental parameters (explanatory variables: temperature, salinity, pesticides, *phycotox*: phycotoxins, *TSS*: total suspended solids, *Chla*: chlorophylle a) and biological parameters (*CAT*: catalase, *Cbe*: carboxylesterase, *CI*: condition index, *AChE*: acetylcholine esterase, *Lys Stab*: lysosomal stability, *MDA*: malondialdehyde, *VIT*: vitelline, *HcConc*: hemocytes concentration, *Phago*: phagocytosis, *Granulo*: granulocyte percentage, *BPH*: benzopyrene hydroxylase)

sum of PCBs concentrations were also excluded because data were missing for some sampling dates and, therefore, would be a source of bias for the redundancy analysis. The two first axes of the biplot explained 81 % of the total variance (Fig. 8). The first axis, explaining 65 % of the total variance, is mainly characterized by an opposition between temperature and salinity and the second axis (16 % of explained variance) by phycotoxins. This RDA analysis revealed that the majority of biomarkers were distributed mainly according to temperature ($p$value=0.001). The sum of pesticides and phycotoxin concentrations were the second and the third parameters, respectively, explaining biomarker variations, but their influence was not statistically significant ($p$value>0.05). Total suspended solids concentration, chlorophylle a, and salinity had little influence on biomarker variations. The distributions of vitelline, BPH, and some hemocyte parameters (i.e., phagocytosis, granulocyte percentage, hemocyte concentration) were well explained by temperature. Conversely, MDA content, condition index, lysosomal stability, AChE, ROS production, and oxidative burst in granulocytes were not well explained by temperature. On the second axis, the variation of biotransformation enzymes (i.e., BPH, Cbe) was positively and significantly correlated with phycotoxin concentrations in digestive glands, and CAT variation was positively correlated to salinity. Since bivalve molluscs are poïkilothermic organisms, water temperature is one of the major factors driving metabolism, and physiological and biochemical processes (Le Gall and Raillard 1988; Chu and Greaves 1991). In blue mussels, seasonal changes in temperature and food availability govern the reproductive cycle (Seed and Suchanek 1992). Moreover, temperature can also have an influence on the biomarker responses by enhancing the production of ROS or by affecting the function of enzymes by changing their catalytic efficiency or their binding capacity (Somero, 1995). Additionally, some environmental parameters are indirectly linked to temperature. For example, an increase in temperature is susceptible to enhance the development of phytoplankton, thus modifying turbidity, total suspended solids, chlorophyll a, and pheopigment concentrations. As highlighted by the RDA analysis, temperature influences the response of biomarkers to a great extent, thus being one of the most important confounding factors in the interpretation of biomonitoring studies in bivalve molluscs. While the potentially great influence of temperature on biomarker responses was already highlighted in numerous previous studies with *Mytilus sp*. (Nesto et al. 2004; Leinio and Lehtonen 2005; Pfeifer et al. 2005, Gracey et al. 2008), its role is likely to be still underestimated in many ecotoxicological studies. One potential improvement for future studies could be a more accurate investigation of thermal changes (i.e., continuous recording using thermal probes) in order to test the possibility of predicting a change in biomarker response according to thermal time.

Biomarkers: interest and misuse

Worldwide concern over threats to natural resources has led to increased efforts to assess the effects of anthropogenic perturbations of organisms and ecosystems. Despite the

difficulty of working in complex environments such as estuaries, there is a need to understand the effect of xenobiotics in these exposed natural environments where exposure to mixture of substances, biotic and abiotic factor interactions, and seasonal fluctuations occur. This stresses the need for using selected biomarkers that are responsive to environmental stress. Although laboratory experiments are a necessary step to study regulation mechanisms and to validate biomarkers, there are a number of uncertainties associated with their use in context with ecotoxicological studies. As underlined by Forbes et al. (2006), biomarkers are unable to provide unambiguous indicators of exposure to toxicants when considered alone. As highlighted in this paper, the existence of confounding factors, i.e., natural or endogenous factors that may modify the responses of biomarkers to environmental toxicants, may lead to misinterpretation if their influence is not taken into account. It is therefore necessary to identify, evaluate, and integrate these factors in biomonitoring studies. Using suites of biomarkers in combination with the measurement of pertinent environmental parameters is more likely to provide a good assessment of the effects of environmental toxicants and of the potential factors susceptible to interfere with the response of biomarkers. This study highlighted three major confounding factors when assessing ecotoxicological impacts on mussels: (1) temperature: Due to their poikilotherm metabolism, the physiology of bivalve molluscs is greatly influenced by this parameter; (2) reproduction cycle: Gametogenesis causes profound physiologic and metabolic modifications. Additionally, spawning appears to be a stressful event in bivalve molluscs. Since reproduction represents a challenging event regarding bivalve physiology, it is very important to focus on the reproduction period for the assessment of the effects of xenobiotics; and (3) algal blooms (harmul species or not): Despite the fact that increase in primary production can be beneficial for mussel physiology due to increased food resources, our results show that the fast proliferation of algal cells and the presence of phycotoxins in the digestive gland can also be stressful and influence negatively the biomarker response in bivalve. To our knowledge, this is the first report highlighting algal blooms as potential confounding factor. Finally, we cannot definitely conclude that the biological endpoints are modified by the presence of organic contaminants because several parameters appeared to be at stake. However, based on the result of multiparametric RDA analysis, the influence of the Vilaine plume, including the pesticides drained by the river, was found to be one of the main contributors to the observed variation in biomarkers. In spite of their limitations, biomarkers are therefore useful tools for gaining insight regarding the effects of environmental contaminants in aquatic ecosystems, provided that the appropriate confounding factors (temperature, reproduction cycle, algal blooms, and phycotoxins) are controlled for.

Conclusion

We compared the biological responses of mussels exposed differently to the Vilaine River plume. Water quality parameter measurements highlighted marked differences between both study sites regarding general environmental conditions (salinity, total suspended solids, chlorophyll a) but also regarding contaminants drained by the river. Both intra- and inter-site variations of several biomarkers were detected. Our study illustrated well the fact that interpretation of environmental monitoring data in estuaries is complicated by the existence of various confounding factors. It also clearly demonstrated that changes in natural environmental conditions are responsible for a great proportion of the variation of biological endpoints chosen to monitor the effects of xenobiotics. In particular, a correlation between an increase of phycotoxin content and changes in some biomarkers was detected in blue mussels, suggesting that phycotoxins may represent a significant confounding factor that should be included into biomonitoring programs. Additionally, in spite of their relatively low concentrations in the Bay of Vilaine (especially compared with other estuaries), a possible influence of organic contaminants (PCBs, PAHs, pesticides) on the biomarker responses cannot be excluded. The use of two contrasted sample sites regarding environmental conditions helped to discriminate between the influence of anthropogenic inputs and natural variations. Implementation of monitoring programs in costal areas, and especially estuaries, still faces many difficulties. Although confirming these difficulties, our work suggests various ways to improve the accuracy of ecotoxicological studies (i.e., use of passive organic samplers, inclusion of phycotoxins content measurements, analysis of reproductive cycle, accurate investigation of the influence of temperature) and confirms the relevance of performing integrated approaches.

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