Cellular and biochemical responses of the oyster Crassostrea gigas to controlled exposures to metals and Alexandrium minutum.

Hansy Haberkorn, Christophe Lambert, Nelly Le Goïc, Claudie Quéré, Audrey Bruneau, Ricardo D. Riso, Michel Auffret, Philippe Soudant

Aquatic Toxicology
February 2014, Volume 147, Pages 158–167
http://dx.doi.org/10.1016/j.aquatox.2013.12.012

Cellular and biochemical responses of the oyster *Crassostrea gigas* to controlled exposures to metals and *Alexandrium minutum*

Hansy Haberkorn[a], Christophe Lambert[a], Nelly Le Goïc[a], Claudie Quéré[b], Audrey Bruneau[a], Ricardo Riso[a], Michel Auffret[a], Philippe Soudant[a, *]

[a] Laboratoire des Sciences de l'Environnement Marin, UMR 6539, Institut Universitaire Européen de la Mer, Université de Bretagne Occidentale, Place Copernic, Technopôle Brest-Iroise, 29280 Plouzané, France
[b] IFREMER Centre de Brest, Laboratoire de Physiologie des Invertébrés, Unité Physiologie Fonctionnelle des Organismes Marins, BP 70, 29280 Plouzané, France

*: Corresponding author : Philippe Soudant, tel.: +33 2 98 49 86 23 ; fax: +33 2 98 49 86 45 ;
email address : Philippe.Soudant@univ-brest.fr

Abstract:

Effects of simultaneous exposure of Pacific oyster, *Crassostrea gigas*, to both a harmful dinoflagellate that produces Paralytic Shellfish Toxins (PST), *Alexandrium minutum*, and cadmium (Cd) and copper (Cu), were assessed. Oysters were exposed to a mix of Cd–Cu with two different diets (*i.e. A. minutum* or *Tisochrysis lutea*) and compared to control oysters fed *A. minutum* or *T. lutea*, respectively, without metal addition. Metals and PST accumulations, digestive gland lipid composition, and cellular and biochemical hemolymph variables were measured after 4 days of exposure. Oysters exposed to Cd–Cu accumulated about thirty-six times less PSTs than oysters exposed to *A. minutum* alone. Exposure to Cd–Cu induced significant changes in neutral lipids (increase in diacylglycerol – DAG – and decrease in sterols) and phospholipids (decreases in phosphatidylcholine, phosphatidylethanolamine, cardiolipin and ceramide aminoethylphosphonate) of digestive gland suggesting that lipid metabolism disruptions and/or lipid peroxidation have occurred. Simultaneously, concentrations, percentages of dead cells and phenoloxidase activity of hemocytes increased in oysters exposed to metals while reactive oxygen species production of hemocytes decreased. Feeding on the harmful dinoflagellate *A. minutum* resulted in significant decreases in monoacylglycerol (MAG) and DAG and ether glycerides (EG), as well as significant increases in hemocyte concentration and phagocytic activity as compared to oysters fed *T. lutea*. Finally, the present study revealed that short-term, simultaneous exposure to Cd–Cu and *A. minutum* may induce antagonistic (*i.e.* hemocyte concentration and phagocytosis) or synergic (*i.e.* DAG content in digestive gland) effects upon cellular and tissular functions in oysters.

Highlights

► Oysters, *C. gigas*, were exposed to both metals and PST-producer *A. minutum*. ► Oysters exposed to metals accumulated about thirty-six times less PSTs. ► Exposure to both metals and *A. minutum* induced antagonistic or synergetic effects.

Keywords: Harmful algae ; Metals ; Oysters ; Toxin accumulation ;Physiological effects

1 Introduction

In the natural environment, aquatic organisms, such as bivalves, experience numerous natural and/or anthropogenic stressors. In aquatic mollusks, interactions between different stressors, such as infectious diseases and pollution, have received increasing attention over recent years (Morley, 2010). Among pollutants, metals, often of anthropogenic origin, are known to have important effects upon fauna and flora in littoral ecosystems (Morley, 2010). Harmful microalgal blooms are another recognized biological stressor which can have important effects upon aquatic organisms and ecosystems (Landsberg, 2002).

Among HAB taxa, *Alexandrium* species are not only known to produce Paralytic Shellfish Toxins (PSTs), the most widespread shellfish-contaminating biotoxins with outbreaks occurring worldwide, but also to modify bivalve biology at different levels of organization (Huss, 2003, Hégaret et al., 2007a; Galimany et al., 2008a, b and c; Haberkorn, 2009; Haberkorn et al., 2010a and b). In France, *Alexandrium minutum* has been known to bloom in coastal waters since the 1980s (Lassus et al., 1992). PSTs produced by these blooms are neurotoxins, the mode of action of which involves a reversible and highly-specific block of sodium channel transport, disabling the action potential of excitable membranes (nerves and muscle fibers) (Narahashi, 1988). The current European Union regulatory limit for human consumption of shellfish is set at 80 µg saxitoxin equivalent 100 g^{-1} shellfish meat. *Alexandrium* species also are known to produce other toxic compounds, such as ichthyotoxins (Emura et al., 2004) and allelochemicals (Arzul et al., 1999; Tillmann et al., 2008; Lelong et al., 2011). Ford et al. (2008) assessed effects of two *A. tamarense* strains, PST and non-PST producing, upon Manila clam *Ruditapes philippinarum* and *Mya arenaria* hemocytes. This study showed that the non-PST strain had more negative effects upon hemocytes (decreased adhesion and phagocytosis) compared to the PST-producing strain of *A. tamarense* (Ford et al., 2008).

Metals also have negative effects upon bivalve physiology, including hemocyte variables and the digestive system (Zorita et al., 2006; Gagné et al., 2008; Paul-Pont et al., 2010). Metal ions can bind with organic molecules and induce reactive oxygen species formation leading to oxidative damage (Dorsey et al., 2004; Faroon et al., 2012). In France, shellfish contamination with cadmium (Cd) and copper (Cu) is reported regularly near the main estuaries (Gironde and Seine estuaries, ROCCH/RNO-IFREMER). Cadmium (Cd) is a toxic

and non-essential metal with an extremely long biological half-life, making it a cumulative toxic compound. It has been classified as a human carcinogen and is a potent multi-tissue animal carcinogen (IARC, 1993). Most bivalve mollusks are filter feeders and concentrate Cd and other metals in their soft tissues (Bouilly et al., 2006). This accumulated Cd can compromise the health of consumers of contaminated bivalves. The allowable Cd limits in seafood vary between 1 μg g^{-1} wet weight (European Union) and 3.7 μg g^{-1} WW (USA, United States Food and Drug Administration recommended guideline) (Lekhi et al., 2008). The Provisional Tolerable Daily Intake (PTDI) recommended by the World Health Organization (WHO) for human oral exposure is 7 μg kg^{-1} day^{-1} (WHO, 2006). Copper (Cu) is an essential metal for all marine organisms, but it can be toxic at elevated concentrations (Parry and Pipe, 2004). The Tolerable Daily Intake (TDI) recommended by the Dutch National Institute for Public Health and the Environment (Rijksinstituut voor volksgezondheid en milieu - RIVM) for human oral exposure is 140 μg kg^{-1} day^{-1} (Baars et al., 2001). In comparison, the TDI recommended for Cd by RIVM for human oral exposure is 5 μg kg^{-1} day^{-1} (Baars et al., 2001).

In bivalves, the digestive system appears to be a revelant target to evaluate interactions of toxic compounds such as phycotoxins and metals. Harmful microalgae were recently shown to interfere with the digestive system of bivalves. Degeneration of the digestive gland (digestive ducts and tubules) was observed in the mussel, *Mytilus edulis*, exposed to *Alexandrium fundyense* (Galimany et al., 2008a). In Pacific oysters, *Crassostrea gigas*, exposed to *A. minutum*, lipid composition of the digestive gland was altered drastically, mainly in terms of phospholipid composition (Haberkorn et al., 2010b). The digestive gland is known to accumulate most of the Cd and Cu in naturally-exposed *C. gigas* from Gironde estuary (Mouneyrac et al., 1998). Metals also appear to have profound effects upon the digestive systems of bivalves. For example, lipid peroxidation was observed in the digestive gland of the blue mussel, *M. edulis*, exposed to Cd – 200 μg l^{-1} for 21 days (Géret et al., 2002). Similarly, Ringwood et al. (1998) reported lipid peroxidation in *C. virginica* exposed to Cu – 80 μg l^{-1} for 14 days. Moreover, the digestive gland represents the major site of metal accumulation in bivalves (Pipe et al., 1999) as is also the case for PSTs (Bricelj and Shumway, 1998).

In addition to affecting the digestive system, both phycotoxins and metals are known to affect activities of circulating cells (*i.e.* hemocytes) involved in bivalve immunity and general homeostasis (Donaghy et al., 2009). Recent studies demonstrated immunomodulation in

bivalves induced by harmful microalgae (Hégaret et al., 2007a; Galimany et al., 2008b and c; Haberkorn et al., 2010a) and metals (Cherkasov et al., 2007; Dailianis, 2009; Morley, 2010). Inflammatory responses, characterized by diapedesis of hemocytes within tissues, were observed in *M. edulis* exposed to *Prorocentrum minimum* and to *Karlodinium veneficum* (Galimany et al., 2008b and c) and in *C. gigas* exposed to *A. minutum* (Haberkorn et al., 2010b). Moreover, changes in hemocyte morphology and/or functions (such as phagocytosis, reactive oxygen species production, phenoloxidase activity) were observed in *C. gigas* exposed to *A. minutum* (Haberkorn et al., 2010b), in *C. gigas* and *C. virginica* exposed to *A. catenella* and *A. fundyense* (Hégaret et al., 2007a), and in *M. edulis* exposed to *A. fundyense, P. minimum,* and *K. veneficum* (Galimany et al., 2008a, b and c). *In vitro* exposure of hemocytes of the oyster, *C. virginica,* to Cd – 50 to 1,000 µmol l^{-1} for 3 days– induced increases in apoptosis in a concentration-dependent manner (Sokolova et al., 2004). Increase in hemocyte apoptosis also was observed during *in vivo* exposure of *C. virginica* hemocytes to Cd – 50 µg l^{-1} for 45 days – (Cherkasov et al., 2007). Increase in reactive oxygen species (ROS) production was observed during *in vitro* exposure to Cd – 0.05 to 500 µmol l^{-1} for 1 h – of hemocytes of mussels, *M. galloprovincialis* (Dailianis, 2009). In flat oysters, *in vivo* exposure to a Cd and Cu mixture – 1 to 10 µmol l^{-1} and 0.75 µmol l^{-1}, respectively, for 7 days – Auffret et al. (2002) caused concentration-dependent increases in total hemocyte count and ROS production by hemocytes, suggesting a toxic stimulation of the immune system.

As in the field stressors rarely act individually, multiple-stress studies are now emerging. Few studies have investigated potential simultaneous effects of pollutants and biological stressors (such as metals/bacteria, metals/macroparasites, macroparasites/harmful microalgae or metals/macroparasites/bacteria) on defense-related activities of bivalves such as detoxification processes, endocrine system, and hemocyte responses (Pipe and Coles, 1995; Baudrimont and de Montaudouin, 2007; Hégaret et al., 2007b; Da Silva et al., 2008; Paul-Pont et al., 2010). Interactive effects of infectious diseases and pollution in aquatic mollusks were reviewed by Morley (2010). To the best of our knowledge, no study has evaluated combined effects of both metals and harmful microalgae upon bivalves.

The purpose of the present study was to explore possible combined effects of an artificial bloom of the PST-producing dinoflagellate, *A. minutum* (strain AM89BM), and a mixture of two metals, cadmium and copper (Cd-Cu) on the Pacific oyster *C. gigas*. Oysters were exposed 4 days to i) *Tisochrysis lutea* (formerly *Isochrysis* sp., clone Tahitian) as a control, ii)

143 *A. minutum* alone, iii) *T. lutea* + Cd-Cu, and iv) *A. minutum* + Cd-Cu. After exposure, toxin
144 and metal accumulations, lipid class composition, and amylase activity in digestive gland, as
145 well as concentration, morphology, viability, phagocytic activity, reactive oxygen species
146 production of hemocytes, and phenoloxidase activity (in plasma and hemocytes), were
147 measured.
148
149

2 Materials and methods

2.1 Biological material

2.1.1 Oysters

Pacific oysters, *Crassostrea gigas*, used in the experiment were obtained from an oyster producer at île de Kerner (Morbihan, France). Mean individual oyster flesh dry weight was 0.34 ± 0.03 g and mean shell length was 61.7 ± 1.9 mm (mean ± CI 5%, n = 60).

2.1.2 Algal culture

Alexandrium minutum (strain AM89BM – isolated from the Bay of Morlaix, France, in 1989) was grown in 10-liter batch culture using autoclaved seawater filtered through a 1-µm filter and supplemented with L1 nutrient enrichment (Guillard and Hargraves, 1993). Cultures were incubated at 16 ± 1°C and 100 µmol photon m^{-2} s^{-1}, with a dark:light cycle of 12:12h. *A. minutum* was harvested after 12 days, still in exponential growth phase under our conditions. At this stage, this strain produced 1.3 ± 0.1 pg saxitoxin equivalent (STX eq.) per cell (measured by the method of Oshima, 1995).

Tisochrysis lutea (formerly *Isochrysis* sp., clone Tahitian) cultures were obtained from the Argenton hatchery (Ifremer, France). Cultures were produced in 300-liter cylinders containing 1-µm filtered seawater enriched with Conway medium at 24 ± 1°C, air-CO_2 (3%) mix aerated, and with continuous light. *T. lutea* was harvested in the exponential growth phase (4-5 days) for the feeding experiments.

2.2 Experimental design of exposures

Short-term exposure period was chosen to enable comparison with previous experiments and to mimic field event of harmful microalgal bloom (Haberkorn et al, 2010a and b). The applied metals were Cd and Cu as they may have aggravating, synergistic effects. Concentrations were chosen to be in the sublethal ranges (Auffret et al., 2002). Indeed, during present experiment, no mortality was observed after four days of exposure.

To proceed, 240 oysters were placed randomly in twelve 15-liter tanks (20 oysters per tank). Oysters were acclimated for 10 days with a continuous flow of 14 ml min^{-1} of seawater (filtered through a 0.5-µm filter) with *T. lutea* at 5.10^5 cells ml^{-1} at 16 ± 1°C. After acclimation, oysters were fed continuously for 4 days at 14 ml min^{-1} with 5.10^5 cells ml^{-1} of *T. lutea* (six control tanks) and with 5.10^3 cells ml^{-1} of *A. minutum* (six *A. minutum* tanks). These

two different cell densities were used to provide the same bio-volume of microalgae to oysters as the cellular volume of *A. minutum* is about 100x higher than that of *T. lutea*.

To half of the control and *A. minutum* tanks a mixture of Cd and Cu was also added. Separate stock solutions of Cd and Cu were prepared in filtered sterile seawater (FSSW) at 250 mM and 50 mM, respectively. A volume of 1,500, 750, 375 or 187.5 µl of Cd and Cu stock solutions was added at 0, 24, 48 and 72h in each of the six tanks during 4 days (exposure). Initial concentrations of Cd and Cu at T0 were 25 µM (2.8 mg l^{-1}) and 5 µM (0.317 mg l^{-1}) and contamination pressure was expected to be maintained constant for the 4 days of experiment.

2.3 Oyster sampling

At the end of exposures (4 days), all oysters were sampled and processed as follows: from each tank, pooled digestive glands from ten oysters were used to measure toxin accumulation, Cd-Cu contents, neutral and polar lipid class composition, and amylase activity. Five oysters were used for individual plasma and hemocyte variable measurements and condition index assessments.

2.4 Digestive gland variables

Just after dissection, digestive glands were frozen immediately in liquid nitrogen, weighed, pooled (1 pool of 10 digestive glands per tank), and stored at -80°C until analysis. Later, pools were ground with a Dangoumeau apparatus in liquid nitrogen and divided for four different analyses (toxins, metals, lipids, amylase).

2.4.1 Toxin content

One gram of ground digestive gland (DG) was extracted in 2 ml of 0.1 N HCl (2 v/w) at 4°C. After centrifugation (3,000 × g, 15 min, 4°C), the pH of each extract was adjusted below 3.0. If above 3.0, pH was adjusted with 12 N HCl. After half-dilution, supernatants were ultra-filtered (20 kDa, Sartorius Centrisart) and stored at 4°C until analysis. PSTs were analyzed by ion-pairing, high-performance liquid chromatography (IPHPLC) according to the method of Oshima (1995). The molar concentration (µmol l^{-1}) was converted into µg STX eq. 100 g^{-1} of digestive gland using the conversion factors of Oshima (1995). Results were expressed as µg STX eq. 100 g^{-1} of digestive gland wet weight.

208 2.4.2 Analyses of cadmium and copper contents

209 For measuring metal concentrations, aliquots of deep frozen, ground digestive glands were
210 freeze-dried for 48 hours at -55°C (CHRIST Alpha 1-2, Bioblock scientific). One-hundred mg
211 of sample were dissolved in 2 ml nitric acid (65%, Suprapur, Merck). After dilution in 0.5 M
212 NaCl (SigmaUltra, Sigma), concentrations of copper and cadmium were assessed by stripping
213 chronopotentiometric methods. These methods are detailed in Riso et al. (1997a and b) and
214 were used previously for metal analysis in shellfish and fish tissues (Tanguy et al., 2003;
215 Evrard et al., 2010).

216 2.4.3 Lipid class contents

217 Two-hundred and fifty mg of deep-frozen ground DG were extracted in 6 ml of Folch
218 solution (chloroform:methanol 2:1). Lipid classes were analyzed by high-performance, thin-
219 layer chromatography (HPTLC) on HPTLC glass plates (1,010 mm) pre-coated with silica gel
220 60 from Merck (Darmstadt, Germany). A preliminary run was carried out to remove possible
221 impurities using hexane:diethyl ether (1:1) prior to neutral lipid analysis and using methyl-
222 acetate:iso-propanol:chloroform:methanol:KCl 0.25% (10:10:10:4:3.6) prior to polar lipid
223 analysis. Each plate was activated for 30 min at 110°C. Lipid samples (4-6 µl) were spotted
224 on the plates by the CAMAG automatic sampler (CAMAG, Switzerland).
225 The neutral lipids were separated with a solvent system containing hexane:diethyl ether:acetic
226 acid (20:5:0.5). The polar lipids were separated with a solvent system containing methyl-
227 acetate:iso-propanol:chloroform:methanol:KCl 0.25% (10:10:10:4:3.6).
228 Lipid classes appeared as black spots after dipping plates in a cupric-sulfate, phosphoric-acid
229 solution and heating for 20 min at 160°C (charring). Seven neutral-lipid classes (free fatty
230 acids, sterol esters, glycerid ethers, monoacylglycerol, diacylglycerol, triacylglycerol, sterols)
231 and seven polar lipid classes (cardiolipin = bisphosphatidylglycerol, lysophosphatidylcholine,
232 phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, phosphatidylinositol,
233 ceramide aminoethylphosphonate) were identified based upon authentic standards (Sigma–
234 Aldrich, France) and coloring techniques. The charred plates were read by scanning at 370
235 nm, and black spots were quantified using Wincats software (CAMAG, Switzerland). Results
236 were expressed as mg of each identified lipid class per g of digestive gland wet weight.

237 2.4.4 Amylase activity

238 Two hundred mg of deep frozen ground DG were homogenized in 1 ml of distilled water.
239 Two hundred µl of this solution were added to 10 µl of a 0.5 M $CaCl_2$ solution before

240 analysis. Amylase activity was then assayed by determination of starch hydrolysis according
241 to the iodine reaction (Samain et al., 1977) modified by Le Moine et al. (1997). One unit of
242 alpha-amylase was defined as the amount of enzyme that degrades 1 $mg.min^{-1}$ starch at 45°C.
243 To assess specific activity of amylase, total proteins were determined using the BCA Protein
244 Assay (Biorad). For protein extraction, 200 µl of the above solution was added to 200 µl of a
245 2N NaOH solution. Protein analysis was carried out on 10 µl of 1/10 diluted samples
246 according to the manufacturer's description. Briefly, 200 µl of dye reagent was added to 10 µl
247 of sample and incubated at 37°C for 1 hour, and the absorbance was measured at 595 nm.
248 Sample ODs were compared to a standard curve of Bovine Serum Albumin (BSA), and
249 results were expressed as mg $protein.ml^{-1}$. Results were expressed as amylase specific activity
250 (*i.e.* International Unit – IU – per mg of total protein).

251 2.5 Hemolymph variables

252 2.5.1 Hemolymph sampling
253 Hemolymph was withdrawn from individual oysters using a 1-ml plastic syringe fitted with a
254 25-gauge needle inserted through a notch made adjacent to the adductor muscle just prior to
255 bleeding. All hemolymph samples were examined microscopically for contamination (e.g.,
256 gametes, tissue debris) and then stored in micro-tubes held on ice. As recommended by the
257 flow cytometer (FCM) manufacturer, all samples were filtered through 80 µm mesh prior to
258 analysis to eliminate any large debris (>80 µm) which could potentially clog the flow
259 cytometer. Three hundred microliters (3 measures×100 µl) of each hemolymph sample were
260 used to measure hemocyte variables by flow cytometry.
261 The remaining hemolymph was separated into cellular (hemocytes) and supernatant (plasma)
262 fractions by centrifugation (800×g, 5 min, 4°C) prior to freezing (-20°C). These samples then
263 were used to measure biochemical hemocyte and plasma variables (protein content and
264 phenoloxydase activity).
265 Methods for measuring cellular (hemocyte) and humoral (plasma) variables are described
266 hereafter.
267
268 2.5.2 Measurements of hemocyte variables by flow cytometry
269 Characterization of hemocyte sub-populations, number and functions, were performed using a
270 FACScalibur (BD Biosciences, San Jose, CA, USA) flow cytometer (FCM) equipped with a
271 488 nm argon laser. Two kinds of hemocyte variables were evaluated by FCM: descriptive
272 variables (hemocyte viability and total and hemocyte sub-population counts), and functional

variables (phagocytosis and reactive oxygen species (ROS) production). Analyses were done as described below.

2.5.2.1 Descriptive variables

Hemocyte viability, total and hemocyte sub-population counts were measured individually on hemolymph samples (5 individuals per tank). An aliquot of 100 µl of hemolymph was transferred into a tube containing a mixture of Anti-Aggregant Solution for Hemocytes, AASH (Auffret and Oubella, 1995) and filtered sterile seawater (FSSW), 200 and 100 µl, respectively. Hemocyte DNA was stained with two fluorescent DNA/RNA specific dyes, SYBR Green I (Molecular probes, Eugene, Oregon, USA, 1/1,000 of the DMSO commercial solution), and propidium iodide (PI, Sigma, St Quentin Fallavier, France, final concentration of 10 µg 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 SYBRGreen I permeates both dead and live cells. SYBR Green and PI fluorescence were measured at 515–545 nm (green) and >670 nm (red) wavelengths, respectively, by flow cytometry. Thus, by counting the cells stained by PI and cells stained by SYBR Green I, it was possible to estimate the percentage of viable cells in each sample. All SYBR Green I-stained cells were visualized on a Forward Scatter (FSC, size) and Side Scatter (SSC, cell complexity) cytogram. Two main sub-populations were distinguished according to size and cell complexity (granularity). Granulocytes are characterized by high FSC and high SSC, while hyalinocytes by high FSC and low SSC. Total hemocyte, granulocyte and hyalinocyte concentrations were estimated from the flow-rate measurement of the flow cytometer (Marie et al., 1999) as all samples were run for 30 s. Results were expressed as number of cells per milliliter of hemolymph.

2.5.2.2 Functional variables

To measure phagocytosis rate, 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 515–545 nm by flow cytometry to detect hemocytes containing fluorescent beads. The phagocytosis rate was defined as the percentage of hemocytes that had engulfed three or more beads (Delaporte et al., 2003).

Reactive oxygen species (ROS) production by hemocytes was measured using 2,7-dichlorofluorescein diacetate, DCFH-DA (Lambert et al., 2003). A 100-µl aliquot of

hemolymph was diluted with 300 µl of FSSW. Four µl 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 hemocytes, was measured at 515–545 nm by flow cytometry. Results were expressed as the geometric mean fluorescence (in arbitrary units, AU) detected in each hemocyte sub-population.

2.5.3 Hemocyte and plasma phenoloxidase activities

Plasma samples were thawed on ice, and 100 µl of each was transferred to a well in a 96-well plate. For hemocytes, cells were suspended in 100 µl of FSSW and frozen and thawed on ice three times successively. Phenoloxidase activity was measured as described by Reid et al. (2003). Briefly, 50 µl of Tris–HCl buffer (0.2 M, pH = 8) and 100 µl of l-DOPA (20 mM, l-3,4-dihydrophenyl-alanine, Sigma D9628) were added to each well. The microplate was mixed rapidly for 10 s. The reaction then was measured at ambient temperature, with color change recorded every 5 min, at 492 nm, over a period of 1 h. The microplate was mixed prior to each measurement. Two controls, without sample but containing l-DOPA and Tris–SDS buffer, were measured in parallel, and these values were subtracted from test values to correct for possible auto-oxidation of the l-DOPA and buffer absorbance. To assess specific activity of phenoloxidase, total proteins in hemocyte suspension and plasma were determined using the BCA Protein Assay (Biorad). Protein analysis was carried out on 10 µl samples according to the manufacturer's description. Briefly, 200 µl of dye reagent was added to 10 µl of sample and incubated at 37°C for 1 hour, and the absorbance was measured at 595 nm. Sample ODs were compared to a standard curve of Bovine Serum Albumin (BSA), and results were expressed as mg protein.ml^{-1}. Results were expressed as specific activity of phenoloxidase (*i.e.* International Unit – IU – per mg of total protein).

2.6 Statistical analysis

Results of toxin and metal contents were analyzed statistically using one-way ANOVA. Results of each experiment were analyzed statistically using two-way ANOVA for each physiological variable and hemocyte variable as the dependent variable, and exposure conditions as independent variables.

In conjunction with two-way ANOVA, Tukey's HSD test were performed to find means that are significantly different from each other.

339 Variables expressed as percentages were transformed as arcsin(squareroot) before statistical
340 analysis, but presented as non transformed data in figures.
341 Statistical analyses were performed using Statgraphics Plus statistical software (Manugistics,
342 Inc, Rockville, MD, USA). Differences were considered significant when p-value was < 0.05.
343

3 Results

3.1 Digestive gland variables

3.1.1 Toxin content

PST content in the digestive gland was significantly higher ($p = 0.0001$, ANOVA) in oysters exposed to *A. minutum* alone than in oysters exposed to both *A. minutum* and cadmium-copper (Fig. 1). No PSTs were detected in oysters exposed to *T. lutea* with and without Cd-Cu.

3.1.2 Cadmium and copper contents

There were no significant differences in cadmium and copper contents in digestive gland of exposed oysters regardless of dietary condition (Fig. 2). Cd and Cu were not detected in oysters exposed to microalgae alone.

3.1.3 Neutral lipid contents

There was no significant difference in total neutral lipid contents between treatments (Table 1).

Monoacylglycerol (MAG) content was significantly lower in oyster fed *A. minutum* than in those fed *T. lutea*. In metal-exposed oyster, whatever the diet, the content was significantly lower than in *T. lutea* control and higher than in *A. minutum* control. MAG were significantly affected by the interaction between Cd-Cu and diet.

Diacylglycerol (DAG) content was significantly lower in oysters exposed to *A. minutum* as compared to those exposed to *T. lutea* and was significantly higher in oysters exposed to Cd-Cu as compared to non-exposed oysters (Table 1).

Content of sterols was significantly lower in oysters exposed to Cd-Cu (Table 1).

In control condition, ether glyceride content was significantly higher in oysters fed *T. lutea* than in those exposed to *A. minutum*. Interaction between diet and Cd-Cu exposure significantly affected ether glycerides with non exposed oysters fed *T. lutea* having the highest level (Table 1).

There was no significant difference in free fatty acid, triacylglycerol and sterol ester contents between treatments (Table 1).

3.1.4 Polar lipid contents

Total phospholipid content (Table 2) was significantly lower in digestive glands of oysters exposed to Cd-Cu and fed *A. minutum* as compared to non-exposed oysters.
Phosphatidylcholine (PC) and cardiolipin (CL) were significantly lower in oysters exposed to Cd-Cu and fed *A. minutum* than in non-exposed oysters (Table 2). Phosphatidylethanolamine (PE) and ceramide aminoethylphosphonate (CAEP) were significantly lower in oysters exposed to Cd-Cu as compared to non-exposed oysters (Table 2).
There was no significant difference in lysophosphatidylcholine (LPC), phosphatidylionsitol (PI) and phosphatidylserine (PS) contents between treatments (Table 2).

3.1.5 Amylase specific activity

There was no significant variation of the amylase specific activity in digestive gland between different exposure conditions.

3.2 Hemolymph variables

3.2.1 Hemocyte variables

Overall, Cd-Cu exposure had more significant effects upon hemocyte and plasma variables than *A. minutum* exposure (Table 3).
Total hemocyte counts (THC) were significantly higher in oysters exposed to Cd-Cu and fed *T. lutea* and also were affected by the interaction between Cd-Cu exposure and dietary treatment (Fig. 3A, Table 3).
Percentage of dead hemocytes was significantly higher in oysters exposed to Cd-Cu, especially in those fed *T. lutea* (Fig. 3B, Table 3).
Percentage of phagocytic hemocytes was significantly lower in oysters exposed to Cd-Cu (Fig. 3C, Table 3). Phagocytosis was also significantly higher in oysters fed *A. minutum* in relation to those fed *T. lutea*.
ROS production by granulocytes was significantly lower in oysters exposed to Cd-Cu as compared to non-exposed oysters fed *A. minutum* (Fig. 3D, Table 3). Similarly, ROS production of hyalinocytes was greatly reduced upon exposure to Cd-Cu (Table 3).

3.2.2 Hemocyte and plasma phenoloxidase (PO) specific activities

PO specific activity in hemocytes was higher in oysters exposed to Cd-Cu as compared to non-exposed oysters (Fig. 3E, Table 3). PO specific activity in plasma was higher in oysters exposed to *A. minutum* than in oysters fed *T. lutea* (Fig. 3F, Table 3). In plasma, this activity

411 was higher in oysters exposed to Cd-Cu and *A. minutum* as compared to non-exposed oysters
412 fed *T. lutea* (Fig. 3F).
413

Discussion

Effects of combined exposure to Cd-Cu and A. minutum on phycotoxin and metal accumulations.

One of the most striking results of this experiment was the difference in PST accumulation in digestive glands of Cd-Cu exposed and non-exposed oysters. Oysters exposed to Cd-Cu accumulated about thirty-six times less PSTs as compared to oysters exposed to *A. minutum* only. Such lower toxin accumulation could be explained by a decrease in feeding activity (*A. minutum* cell ingestion) and/or by alteration of digestive processes. In *Corbicula fluminea*, Tran et al. (2003a and b) observed a prolonged closure of valves in reaction to dissolved copper or cadmium, suggesting a decrease in feeding activity. Similarly, decreased filtration rate has been observed in *C. gigas* exposed to copper or cadmium (Lin et al., 1992; Lin et al., 1993). Modification of lipid composition of the digestive gland, described hereafter, may also reflect some alterations of digestive processes resulting in lower toxin accumulation by Cd-Cu exposed oysters as a lower digestive efficiency may lead to a lower toxin uptake. Another hypothesis is that alteration of phytoplanktonic cells may result in decreased ingestion by oysters. Cyst formation by *A. minutum*, however, was observed only when *A. minutum* cells were exposed to Cd-Cu at higher concentrations than those applied in this experiment (data not shown). Even though some physiological changes may have occurred in microalgae upon exposure to Cd-Cu in the oyster tank, they are unlikely to explain observed differences in toxin accumulation by oysters.

On the other hand, harmful organisms such as pathogens and toxic phytoplankton are expected to modify chemical contaminant accumulation by interfering with nutritional processes and reducing general oyster fitness. In *Cerastoderma edule*, Paul-Pont et al. (2010) observed that the presence of pathogens decreased cadmium bioaccumulation both in gills and visceral mass when cockles were concomitantly exposed to pathogens. Decrease in pollutant accumulation in parasitized individuals has been demonstrated in several host-parasite models (Evans et al., 2001; Sures, 2008). In the present study, however, exposure to a toxic dinoflagellate did not modulate accumulation of Cd-Cu.

Levels of accumulated metals in digestive glands of oysters (from 335.1 to 392.8 µg g^{-1} dry weight for Cd and 115.8 to 145.4 µg g^{-1} DW for Cu) measured in the present study were comparable to oysters reared in contaminated areas. Body burden of cadmium in field oyster

populations (*Crassostrea virginica*) ranged from 300 to 400 µg g^{-1} DW (Roesijadi, 1996). Abbe et al. (2000) observed that field Cu contamination in *C. virginica* reached 310 µg g^{-1} DW. Concentration of 850 µg g^{-1} DW for the same species was also observed by O-Connor and Lauenstein (2005). Concentration of cadmium reached 5 µg g^{-1} wet weight and concentration of copper reach at 300 µg g^{-1} digestive gland WW in *C. gigas* from a polluted estuary (Mouneyrac et al., 1998).

Effects of exposures to Cd-Cu and toxic A. minutum upon oyster physiology.

The present results demonstrated major effects of Cd-Cu upon lipid composition of the oyster digestive gland. Exposure to Cd-Cu induced decreases in sterols, PC, PE, CAEP and CL. Variations in lipid contents were probably not linked to lipid hydrolysis because no increase in FFA was observed (Chu et al., 2003). Changes in PL composition may reflect some changes in cell types or in organelles as changes in cell type composition is a general phenomenon that can take place in the digestive gland epithelia of mollusks stressed by chemical contamination, as underscored by Zaldibar et al. (2008). A recent study (Zaldibar et al., 2007) demonstrated that exposure of winkles (*Littorina littorea*) to Cd resulted in changes in the epithelia of the digestive gland (digestive ducts and tubules). These changes were characterized by a loss of digestive cells (minus 13.2%) and volume increases in both digestive and basophilic cells (plus 13.5% and 200%, respectively). Variations in PL composition could also be linked to perturbations of lipid metabolism and/or to lipid peroxidation. Ringwood et al. (1998) observed significant increase in lipid peroxidation in oysters, *C. virginica*, exposed to copper. Similarly, Géret et al. (2002) observed that exposure of *Mytilus edulis* to cadmium stimulated lipid peroxidation processes through oxidation of polyunsaturated fatty acids. Also, exposure of *Ruditapes decussatus* to cadmium led to changes in protein-expression profiles, including cell maintenance (Rab GDP dissociation inhibitor α – mediators of vesicle formation, trafficking, and fusion) and metabolism (MCAD – medium chain-CoA dehydrogenase, enzyme responsible for the metabolism of medium chain fatty acids – and ALDH – aldehyde dehydrogenase, mitochondrial precursor) suggesting potential alteration in energetic processes (Chora et al., 2009).

Effects of *A. minutum* exposure upon lipid composition of the digestive gland were mainly characterized by decreases in MAG, DAG and ether glycerides. Such effects of *A. minutum* upon oysters were reported previously by Haberkorn et al. (2010b).

Effects of Cd-Cu exposure were observed in most of the measured hemocyte variables: increases in total hemocyte count, percentage of dead hemocyte and phenoloxidase activity, as well as decreases in phagocytosis and ROS production. Oysters may possibly increase circulating hemocytes to compensate for increase in mortality. Other studies previously reported that increases in circulating hemocytes in *C. virginica* exposed to cadmium were associated with increased percentage of dead hemocytes (Cheng, 1988; Cheng, 1990). Similarly, Auffret et al. (2002) observed increases in THC in oysters, *Ostrea edulis*, exposed to Cd-Cu.

Decreased hemocyte phagocytosis upon Cd-Cu exposure also has been observed in *O. edulis* (Auffret et al., 2002). Auffret et al. (2002) similarly observed a decrease in ROS production in hemocytes in oysters, *O. edulis*, exposed *in vivo* to a high concentration of cadmium (50 µM). The present results clearly suggest an alteration of some hemocyte-based defense mechanisms by Cd-Cu exposure.

Feeding oysters on *A. minutum* resulted in less intense immunomodulation than Cd-Cu exposure. Hemocyte phagocytosis increased upon *A. minutum* exposure. Such stimulation of phagocytosis has been observed previously in hemocytes of the blue mussel, *Mytilus edulis*, exposed to *Karlodinium veneficum* (Galimany et al., 2008c). Observations suggest a stimulation of this cellular-based immune function when bivalves feed on harmful microalgae.

Combined effects and interactions of exposures to Cd-Cu and toxic A. minutum upon oyster physiology.

Oysters exposed to both metals and harmful algae accumulated large amounts of Cd-Cu but only a small quantities of PST, suggesting that effects in these exposures may be primarily from metals. Nevertheless, results of co-exposure were different than those of single "contaminant" exposure.

Effects of Cd-Cu exposure on lipids were opposite to those caused by *A. minutum* feeding: Cd-Cu exposure counteracted the decreasing effect of *A. minutum* on MAG, DAG and ether glycerides. Such interactive effects of Cd-Cu and *A. minutum* exposures on lipid metabolism within the digestive gland need further investigation.

Total hemoctye counts (THC) and percentages of dead hemocytes increased dramatically in oysters fed *T. lutea* and exposed to Cd-Cu. But when oysters were fed *A. minutum*, this effect

514 was subdued, revealing that oysters exposure to *A. minutum* interacts antagonistically with
515 Cd-Cu exposure. Similarly, Cd-Cu exposure resulted in a large decrease in hemocyte
516 phagocytosis which indeed counteracted the stimulating effect of *A. minutum* on
517 phagocytosis, revealing opposite effects.
518 The highest values of PO in plasma were found in oysters fed *A. minutum* and exposed to Cu-
519 Cd. This increase was mostly attributable to Cu-Cd exposure. This is in agreement with the
520 study of Bouilly et al. (2006) who observed an increase in PO activity in *C. gigas* exposed to
521 cadmium. Our results suggest synergistic effects of *A. minutum* and Cd-Cu upon PO activity.
522
523 As some effects upon oysters were apparently not linked to PST accumulation in Cd-Cu
524 exposed oysters (*i.e.* oysters exposed to both *A. minutum* and Cd-Cu accumulate a few PST),
525 these results suggested that responses of oysters to *A. minutum* was not only because of PST
526 but also caused by other compounds released by the microalgae or membrane bound (Arzul et
527 al., 1999; Emura et al., 2004; Ford et al., 2008;Tillmann et al., 2008; Lelong et al., 2011).
528
529 This preliminary study underscores the complexity of multiple stress interactions. Although
530 these two stressors have concentration-dependent effects (Auffret et al., 2002; Bouilly et al.,
531 2006, Hégaret et al., 2007a; Haberkorn et al., 2011), it appeared that *Alexandrium* species
532 exposures have less severe effects upon oyster physiology than Cd and Cu. This may simply
533 reflect the fact that tested concentrations of metals were quite high. Nevertheless, the present
534 results also suggest that simultaneous exposure to metal (Cd-Cu) and harmful microalgae (*A.*
535 *minutum*) can have antagonistic (for example in hemocyte phagocytosis) or synergistic (for
536 example in PO activity) effects on oysters. This is in agreement with observations previously
537 reported in multiple stressors exposure in bivalves (Hégaret et al., 2007b; Da Silva et al.,
538 2008; Paul-Pont et al., 2010). It remains to be elucidated when toxic metals and HAB are
539 interacting through oyster nutritional processes and what are the biochemical and cellular
540 involved mechanisms in these interactions.
541
542 Acknowledgment
543 The authors are grateful to Gary H. Wikfors for English corrections, as well as to anonymous
544 reviewers for their helpful comments and suggestions. This study was carried out with the
545 financial support of the National Research Agency (ANR) ‘‘MODECOPHY’’ project
546 06SEST23 (2006–2009) and of the Brittany Region, ‘‘EPHYTOX’’ project.
547

References

Abbe, G.R., Riedel, G.F., Sanders, J.G., 2000. Factors that influence the accumulation of copper and cadmium by transplanted eastern oysters (*Crassostrea virginica*) in the Patuxent River, Maryland. Mar. Environ. Res. 49, 377-396.

Arzul, G., Seguel, M., Guzman, L., Erard-Le Denn, E., 1999. Comparison of allelopathic properties in three toxic *Alexandrium* species. J. Exp. Mar. Biol. Ecol. 232, 285–295.

Auffret, M., Oubella, R., 1995. Cytological and cytometric analysis of bivalve mollusc hemocytes. Techniques in Fish Immunology. 4, 55–63.

Auffret, M., Mujdzic, N., Corporeau, C., Moraga, D., 2002. Xenobiotic-induced immunomodulation in the European flat oyster, *Ostrea edulis*. Mar. Environ. Res. 54, 585–589.

Baars A.J., Theelen R.M.C., Janssen P.J.C.M., Hesse J.M., van Apeldoorn M.E., Meijerink M.C.M., Verdam L., Zeilmaker M.J., 2001 - Re-evaluation of human-toxicological maximum permissible risk levels RIVM, Rijsinstituut voor volksgezondheid en milieu. Report 711 701 025.

Baudrimont, M., de Montaudouin, X., 2007. Evidence of altered protective effect of metallothioneins after cadmium exposure in the digenean parasite-infected cockle (*Cerastoderma edule*). Parasitology. 134, 237–245.

Bouilly, K., Gagnaire, B., Bonnard, M., Thomas-Guyon, H., Renault, T., Miramand, P., Lapègue, S., 2006. Effects of cadmium on aneuploidy and hemocyte parameters in the Pacific oyster, *Crassostrea gigas.* Aquat. Toxicol. 78, 149–156.

Bricelj, V.M., Shumway, S.E., 1998. Paralytic Shellfish Toxins in bivalve molluscs: occurrence, transfer kinetics, and biotransformation. Res. Fish. Sci. 6, 315–383.

Cheng, T.C., 1988. *In vivo* effects of heavy metals on cellular defense mechanisms of *Crassostrea virginica*: total and differential cell counts. J. Invertebr. Pathol. 51, 207–214.

Cheng, T.C., 1990. Effects of *In Vivo* Exposure of *Crassostrea virginica* to Heavy Metals on Hemocyte Viability and Activity Levels of Lysosomal Enzymes. Academic Press, San Diego, CA, USA.

Cherkasov, A.S., Grewal, S., Sokolova, I.M., 2007. Combined effects of temperature and cadmium exposure on haemocyte apoptosis and cadmium accumulation in the eastern oyster *Crassostrea virginica* (Gmelin). J. Therm. Biol. 32, 162–170.

579 Chora, S., Starita-Geribaldi, M., Guigonis, J.M.., Samson, M., Roméo, M., Bebianno, M.J.,
580 2009. Effect of cadmium in the clam *Ruditapes decussatus* assessed by proteomic
581 analysis. Aquat. Toxicol. 94, 300–308.
582 Chu, F.L.E., Soudant, P., Hale, R.C., 2003. Relationship between PCB accumulation and
583 reproductive output in conditioned oysters *Crassostrea virginica* fed a contaminated
584 algal diet. Aquat. Toxicol. 65, 293–307.
585 Da Silva, P.M., Hégaret, H., Lambert, C., Wikfors, G.H., Le Goïc, N., Shumway, S.E.,
586 Soudant, P., 2008. Immunological responses of the Manila clam (*Ruditapes*
587 *philippinarum*) with varying parasite (*Perkinsus olseni*) burden, during a long-term
588 exposure to the harmful alga, *Karenia selliformis*, and possible interactions. Mar.
589 Pollut. Bull. 50, 733–739.
590 Dailianis, S., 2009. Production of superoxides and nitric oxide generation in haemocytes of
591 mussel *Mytilus galloprovincialis* (Lmk.) after exposure to cadmium: A possible
592 involvement of Na^+/H^+ exchanger in the induction of cadmium toxic effects. Fish
593 Shellfish Immunol. 27, 446–453.
594 Delaporte, M., Soudant, P., Moal, J., Lambert, C., Quéré, C., Miner, P., Choquet, G., Paillard,
595 C., Samain, J.F., 2003. Effect of a mono-specific algal diet on immune functions in
596 two bivalve species—*Crassostrea gigas* and *Ruditapes philippinarum*. J. Exp. Biol.
597 206, 3053–3064.
598 Donaghy, L., Lambert, C., Choi, K-S, Soudant, P., 2009. Hemocytes of the carpet shell clam
599 (*Ruditapes decussatus*) and the Manila clam (*Ruditapes philippinarum*): Current
600 knowledge and future prospects. Aquaculture. 297, 10–24.
601 Dorsey, A., Ingerman, L., Swarts, S., 2004. Toxicological profile for copper. Agency for
602 Toxic Substances and Disease Registry. United States. 314 p.
603 Emura, A., Matsuyama, Y., Oda, T., 2004. Evidence for the production of a novel
604 proteinaceous hemolytic exotoxin by dinoflagellate *Alexandrium taylori*. Harmful
605 Algae. 3, 29–37.
606 Evans, D.W., Irwin, S.W.B., Fitzpatrick, S., 2001. The effect of digenean (Platyhelminthes)
607 infections on heavy metal concentrations in *Littorina littorea*. J. Mar. Biol. Ass. U.K.
608 81, 349–350.
609 Evrard, E., Devaux, A., Bony, S., Burgeot,T., Riso R., Budzinski, H., Le Du, M., Quiniou, L.,
610 Laroche, J., 2010. Responses of the European flounder *Platichthys flesus* to the
611 chemical stress in estuaries: load of contaminants, gene expression, cellular impact
612 and growth rate. Biomarkers. 15(2) 111 – 127.

Faroon, O., Ashizawa, A., Wright, S., Tucker, P., Jenkins, K., Ingerman, L., Rudisill, C., 2012. Agency for Toxic Substances and Disease Registry. United States. 487 p.

Ford, S.E., Bricelj, V.M., Lambert, C., Paillard, C., 2008. Deleterious effects of a nonPST bioactive compound(s) from *Alexandrium tamarense* on bivalve hemocytes. Mar. Biol. 154, 241–253.

Gagné, F., Auclair, J., Turcotte, P., Fournier, M., Gagnon, C., Sauvé, S., Blaise, C., 2008. Ecotoxicity of CdTe quantum dots to freshwater mussels: Impacts on immune system, oxidative stress and genotoxicity. Aquat. Toxicol. 86, 333–340.

Galimany, E., Sunila, I., Hégaret, H., Ramón, M., Wikfors, G.H., 2008a. Experimental exposure of the blue mussel (*Mytilus edulis*, L.) to the toxic dinoflagellate *Alexandrium fundyense*: histopathology, immune responses, and recovery. Harmful Algae. 7, 702–711.

Galimany, E., Sunila, I., Hégaret, H., Ramón, M.,Wikfors, G.H., 2008b. Pathology and immune response of the blue mussel (*Mytilus edulis* L.) after an exposure to the harmful dinoflagellate *Prorocentrum minimum*. Harmful Algae. 7, 630–638.

Galimany, E., Place, A.R., Ramón, M., Jutson, M., Pipe, R.K., 2008c. The effects of feeding *Karlodinium veneficum* (PLY # 103; *Gymnodinium veneficum* Ballantine) to the blue mussel *Mytilus edulis*. Harmful Algae. 7, 91–98.

Géret, F., Jouan, A., Turpin, V., Bebianno, M.J., Cosson, R.P., 2002. Influence of metal exposure on metallothionein synthesis and lipid peroxidation in two bivalve mollusks: the oyster (*Crassostrea gigas*) and the mussel (*Mytilus edulis*). Aquat. Living Resour. 15, 61–66.

Guillard, R.R.L., Hargraves, P.E., 1993. *Stichochrysis immobilis* is a diatom, not a chrysophyte. Phycologia. 32, 234–236.

Haberkorn, H., 2009. Impact du dinoflagellé toxique, *Alexandrium minutum*, sur l'huître creuse, *Crassostrea gigas* : approche intégrative. Thesis. Université de Bretagne Occidentale. 174 p.

Haberkorn, H., Lambert, C., Le Goïc, N., Guéguen, M., Moal, J., Palacios, E., Lassus, P., Soudant, P., 2010a. Effects of *Alexandrium minutum* exposure on physiological and hematological variables of diploid and triploid oysters, *Crassostrea gigas*. Aquat. Toxicol. 97, 96–108.

Haberkorn, H., Lambert, C., Le Goïc, N., Moal, J., Suquet, M., Guéguen, M., Sunila, I., Soudant, P., 2010b. Effects of *Alexandrium minutum* exposure on nutrition-related processes and reproductive output in oysters *Crassostrea gigas.* Harmful Algae. 9,

427–439.

Haberkorn, H., Tran, D., Massabuau, J-C., Ciret, V., Savar, P. & Soudant, P., 2011. Relationship between valve activity, microalgae concentration in the water and toxin accumulation in the digestive gland of the Pacific oyster, *Crassostrea gigas*, exposed to *Alexandrium minutum*. Mar. Pollut. Bull. 62, 1191–1197.

Hégaret, H., Wikfors, G., Soudant, P., Lambert, C., Shumway, S., Bérard, J., Lassus, P., 2007a. Toxic dinoflagellates (*Alexandrium fundyense* and *A. catenella*) have minimal apparent effects on oyster hemocytes. Mar. Biol. 152, 441–447.

Hégaret, H., da Silva, P.M., Wikfors, G.H., Lambert, C., De Bettignies, T., Shumway, S.E., Soudant, P., 2007b. Hemocyte responses of Manila clams, *Ruditapes philippinarum*, with varying parasite, *Perkinsus olseni*, severity to toxic-algal exposures. Aquat. Toxicol. 84, 469–479.

Huss, H.H., 2003. Aquatic biotoxins. Chapter 5.1.5. In: Huss, H.H., Ababouch, L., Gram, L. (Eds.), Assessment and Management of Seafood Safety and Quality. FAO Fisheries Technical Paper, p. 239.

International Agency for Research on Cancer (IARC). CADMIUM AND CADMIUM COMPOUNDS VOL.: 58 (1993) (p. 119)

Lambert, C., Soudant, P., Choquet, G., Paillard, C., 2003. Measurement of *Crassostrea gigas* hemocyte oxidative metabolism by flow cytometry and the inhibiting capacity of pathogenic vibrios. Fish Shellfish Immunol. 15, 225–240.

Landsberg, J.H., 2002. The effects of harmful algal blooms on aquatic organisms. Fish. Sci. 10, 113–390.

Lassus, P., Bardouil, M., Ledoux, M., Murail, I., Bohec, M., Truquet, P., Frémy, J.-M., Rohmer, V., 1992. Paralytic phycotoxin uptake by scallops (*Pecten maximus*). Aquat. Living Resour. 5, 319–324.

Lelong, A., Haberkorn, H., Le Goïc, N., Hégaret, H., & Soudant, P., 2011. A New Insight into Allelopathic Effects of Alexandrium minutum on Photosynthesis and Respiration of the Diatom Chaetoceros neogracile Revealed by Photosynthetic-performance Analysis and Flow Cytometry. Microb. Ecol. 62(4), 919–930.

Le Moine, S., Sellos, D., Moal, J., Daniel, J.Y., San Juan, S.F., Samain, J.F., Van Wormhoudt, A., 1997. Amylase on *Pecten maximus* (mollusca, bivalves): protein and cdna characterization; quantification of the expression in the digestive gland. Mol. Mar. Biol. Biotechnol. 6, 228–237.

680 Lekhi, P., Cassis, D., Pearce, C.M., Ebell, N., Maldonado, M.T., Orians, K.J., 2008. Role of
681 dissolved and particulate cadmium in the accumulation of cadmium in cultured oysters
682 (*Crassostrea gigas*). Sci. Total Environ. 393, 309–325.
683 Lin, W., Rice, M.A., Chien, P.K., 1992. The effects of copper, cadmium and zinc on particle
684 filtration and uptake of glycine in the pacific oyster *Crassostrea gigas*. Comp.
685 Biochem. Physiol. C Comp. Pharmacol. 103, 181–187.
686 Lin, W., Rice, M.A., Chien, P.K., 1993. The differential effects of three heavy metals on
687 particle filtration and amino acid uptake by the Pacific oyster, *Crassostrea gigas*. J.
688 Shellfish Res. 12, 111 p.
689 Marie, D., Partensky, F., Vaulot, D., Brussaard, C., 1999. Enumeration of phytoplankton,
690 bacteria, and viruses in marine samples. In: Curr. Protoc. Cytom. John Wiley & Sons
691 Inc., New York, pp. 11.11.11–11.11.15.
692 Morley, N.J., 2010. Interactive effects of infectious diseases and pollution in aquatic
693 mollusks. Aquat. Toxicol. 96, 27–36.
694 Mouneyrac, C., Amiard, J.C., Amiard-Triquet, C., 1998. Effects of natural factors (salinity
695 and body weight) on cadmium, copper, zinc and metallothionein-like protein levels in
696 resident populations of oysters *Crassostrea gigas* from a polluted estuary. Mar. Ecol.
697 Prog. Ser. 162, 125–135.
698 Narahashi, T., 1988. Mechanism of tetrodotoxin and saxitoxin action. In: Tiu, A.T. (Ed.),
699 Handbook of Natural Toxins. Marine Toxins and Venoms, vol. 3. Marcel Dekker Inc.,
700 New York, pp. 185–210.
701 O'Connor, T.P., Lauenstein, G.G., 2005. Status and trends of copper concentrations in
702 mussels and oysters in the USA. Mar. Chem. 97, 49–59.
703 Oshima, Y., 1995. Postcolumn derivatization liquid chromatographic method for paralytic
704 shellfish toxins. J AOAC Int. 78, 528–532.
705 Parry, H.E., Pipe, R.K., 2004. Interactive effects of temperature and copper on
706 immunocompetence and disease susceptibility in mussels (*Mytilus edulis*). Aquat.
707 Toxicol. 69, 311–325.
708 Paul-Pont, I., Gonzalez, P., Baudrimont, M., Jude, F., Raymond, N., Bourrasseau, L., Le
709 Goïc, N., Haynes, F., Legeay, A., Paillard, C., de Montaudouin, X., 2010. Interactive
710 effects of metal contamination and pathogenic organisms on the marine bivalve
711 *Cerastoderma edule*. Mar. Pollut. Bull. 60, 515–525.
712 Pipe, R.K. and Coles, J.A., 1995. Environmental contaminants influencing immune function
713 in marine bivalve molluscs. Fish Shellfish Immunol. 5, 581–595.

714 Pipe, R.K., Coles, J.A., Carissan, F.M.M., Ramanathan, K., 1999. Copper induced
715 immunomodulation in the marine mussel, *Mytilus edulis*. Aquat. Toxicol. 46, 43–54.
716 Reid, H.I., Soudant, P., Lambert, C., Paillard, C., Birkbeck, T.H., 2003. Salinity effects on
717 immune parameters of *Ruditapes philippinarum* challenged with *Vibrio tapetis*. Dis.
718 Aquat. Org. 56, 249–258.
719 Réseau d'Observation de la Contamination Chimique du milieu marin (ROCCH/RNO).
720 Institut français de recherche pour l'exploitation de la mer (IFREMER).
721 http://wwz.ifremer.fr/envlit/surveillance/contaminants_chimiques
722 Ringwood, A.H., Conners, D.E., DiNovo, A., 1998. The effects of copper exposures on
723 cellular responses in oysters. Mar Environ Res. 46, 591–595.
724 Riso, R.D., Monbet, P., Corre, P.L., 1997a. Measurement of Copper in Sea-water by Constant
725 Current Stripping Analysis (CCSA) With a Rotating Gold Disk Electrode. The
726 Analyst, 122(12), 1593–1596.
727 Riso, R.D., Le Corre, P., Chaumery, C.J., 1997b. Rapid and simultaneous analysis of trace
728 metals (Cu, Pb and Cd) in seawater by potentiometric stripping analysis. Anal. Chim.
729 Acta. 351(1-3), 83–89.
730 Roesijadi, G., 1996. Environmental factors: Response to metals. In: Kennedy,V.S., Newell,
731 R.I.E., Eble A.F. (Eds.), The Eastern Oyster *Crassostrea virginica*. A Maryland Sea
732 Grant Book. College Park Maryland, pp. 515–537.
733 Samain, J.F., Daniel, J.Y., Le Coz, J.R., 1977. Trypsine, amylase et protéines du zooplancton:
734 dosage automatique et manuel. J. Exp. Mar. Biol. Ecol. 29, 279–289.
735 Sokolova, I.M., Evans, S., Hughes, F.M., 2004. Cadmium-induced apoptosis in oyster
736 hemocytes involves disturbance of cellular energy balance but no mitochondrial
737 permeability transition. J. Exp. Biol. 207, 3369–3380.
738 Sures, B., 2008. Environmental parasitology. Interactions between parasites and pollutants in
739 the aquatic environment. Parasite. 15 (3), 434–438.
740 Tanguy, A., Boutet, I., Riso, R., Boudry, P., Auffret, M., Moraga, D., 2003. Metallothionein
741 genes in the European flat oyster *Ostrea edulis*: a potential ecological tool for
742 environmental monitoring?. Mar. Ecol. Prog. Ser. 257, 87–97.
743 Tillmann, U., Alpermann, T., John, U., Cembella, A., 2008. Allelochemical interactions and
744 short-term effects of the dinoflagellate *Alexandrium* on selected photoautotrophic and
745 heterotrophic protists. Harmful Algae. 7, 52–64.

Tran, D., Fournier, E., Durrieu, G., Massabuau, J.C., 2003a. Copper detection in the Asiatic clam *Corbicula fluminea*: optimum valve closure response. Aquat. Toxicol. 66, 333–343.

Tran, D., Ciret, P., Ciutat, A., Durrieu, G., Massabuau, J.-C., 2003b. Estimation of potential and limits of bivalve closure response to detect contaminants: application to cadmium. Environ. Toxicol. Chem. 22, 116–122.

World Health Organization. 2006. Guidelines for Drinking-water Quality: First Addendum to Third Edition, Volume 1, Recommendations. World Health Organization. http://www.who.int/water_sanitation_health/dwq/gdwq3/en/.

Zaldibar, B., Cancio, I., Marigomez, I., 2007. Reversible alterations in epithelial cell turnover in digestive gland of winkles (*Littorina littorea*) exposed to cadmium and their implications for biomarker measurements. Aquat. Toxicol. 81, 183–196.

Zaldibar, B., Cancio, I., Soto, M., Marigomez, I., 2008. Changes in cell-type composition in digestive gland of slugs and its influence in biomarkers following transplantation between a relatively unpolluted and a chronically metal-polluted site. Environ. Pollut. 156, 367–379.

Zorita, I., Ortiz-Zarragoitia, M., Soto, M., Cajaraville, M.P., 2006. Biomarkers in mussels from a copper site gradient (Visnes, Norway): An integrated biochemical, histochemical and histological study. Aquat. Toxicol. 78, 109–116.

765 **Figure captions**

766 Fig. 1. PST content (mean of 3 pools of 10 oysters, as µg STX eq. 100 g^{-1} of tissue wet
767 weight, ±CI) in digestive gland of oysters exposed to *A. minutum* and *A. minutum* + Cd-Cu.
768

769 Fig. 2. Cadmium (A) and copper (B) contents (mean of 3 pools of 10 oysters, as µg g^{-1} of
770 tissue dry weight, ±CI) in digestive gland of oysters exposed to both microalgae (*T. lutea* and
771 *A. minutum*) and metals (Cd-Cu).
772

773 Fig. 3. (A) Total hemocyte concentration (cells ml^{-1}), (B) percentage of dead hemocytes, (C)
774 phagocytosis rate, (D) ROS production in granulocytes (AU), (E) specific activity of
775 phenoloxidase (PO) in hemocytes, (F) specific activity of phenoloxidase (PO) in plasma.
776 Means of 15 individual oysters, ± CI. * Statistically significant differences according to Cd-
777 Cu exposure, *A. minutum* exposure, and interaction, respectively (two-way ANOVA ; * :
778 $p<0.05$; ** : $p<0.01$; *** : $p<0.001$). Lower-case letters (a, b and c) indicate homogeneous
779 groups (Tukey's HSD test). AU: arbitrary unit.
780

Table 1. Neutral lipid class contents (expressed as mg g^{-1} of tissue wet weight, ±CI, means of 3 pools of 10 oysters each) in oyster digestive glands according to exposure conditions. This table also indicates the results of the two-way ANOVAs testing by Cd-Cu exposure (M) and dietary effects (D). Lower-case letters (a, b and c) indicate homogeneous groups (Tukey's HSD test).

	Mean ± CI (n=3)				Two-way ANOVA		
metals (M)	control		exposed		M	D	M/D
diet (D)	*A. minutum*	*T. lutea*	*A. minutum*	*T. lutea*			
monoacylglycerols (MAG)	0.09 ± 0.03 (a)	0.27 ± 0.02 (b)	0.18 ± 0.06 (c)	0.2 ± 0.04 (c)	NS	**	**
diacylglycerols (DAG)	0.07 ± 0.04 (a)	0.15 ± 0.01 (b)	0.16 ± 0.01 (b)	0.21 ± 0.03 (c)	**	**	NS
sterols	1.69 ± 0.05 (a)	1.57 ± 0.06 (b)	1.16 ± 0.09 (c)	1.25 ± 0.11 (c)	***	NS	*
free fatty acids (FFA)	0.29 ± 0.16 (a)	0.31 ± 0.05 (a)	0.37 ± 0.29 (a)	0.25 ± 0.29 (a)	NS	NS	NS
triacylglycerols	6.07 ± 0.61 (a)	6.74 ± 1.84 (a)	7.2 ± 1.14 (a)	5.46 ± 2.8 (a)	NS	NS	NS
ether glycerides	1.04 ± 0.15 (a)	1.89 ± 0.3 (b)	1.35 ± 0.12 (a)	1.18 ± 0.44 (a)	NS	*	**
sterol esters	0.33 ± 0.06 (a)	0.3 ± 0.1 (a)	0.3 ± 0.07 (a)	0.26 ± 0.11 (a)	NS	NS	NS
Total	9.58 ± 0.62 (a)	11.22 ± 2.15 (a)	10.72 ± 0.88 (a)	8.8 ± 2.94 (a)	NS	NS	NS

* p<0.05 ; ** p<0.01 ; *** p<0.001

Table 2. Phospholipid class contents (expressed as mg g^{-1} of tissue wet weight, ±CI, means of 3 pools of 10 oysters each) in oyster digestive glands according to exposure conditions. This table also indicates the results of the two-way ANOVAs testing by Cd-Cu exposure (M) and dietary effects (D). Lower-case letters (a and b) indicate homogeneous groups (Tukey's HSD test).

	Mean ± CI (n=3)				Two-way ANOVA		
metals (M)	control		exposed		M	D	M/D
diet (D)	*A. minutum*	*T. lutea*	*A. minutum*	*T. lutea*			
lysophosphatidylcholine (LPC)	0.58 ± 0.28 (a)	0.52 ± 0.14 (a)	0.8 ± 0.56 (a)	0.64 ± 0.44 (a)	NS	NS	NS
phosphatidylcholine (PC)	6.81 ± 0.46 (a)	7.01 ± 0.18 (a)	5.8 ± 0.38 (b)	6.53 ± 0.81 (ab)	*	NS	NS
phosphatidylethanolamine (PE)	4.72 ± 0.25 (a)	4.75 ± 0.05 (a)	4.06 ± 0.12 (b)	4.35 ± 0.21 (b)	***	NS	NS
phosphatidylinositol (PI)	1.42 ± 0.12 (a)	1.38 ± 0.05 (a)	1.3 ± 0.14 (a)	1.39 ± 0.01 (a)	NS	NS	NS
ceramide aminoethylphosphonate (CAEP)	2.56 ± 0.34 (a)	2.56 ± 0.07 (a)	1.92 ± 0.12 (b)	2.08 ± 0.1 (b)	**	NS	NS
phosphatidylserine (PS)	2.12 ± 0.21 (a)	2.21 ± 0.03 (a)	1.97 ± 0.19 (a)	2.08 ± 0.06 (a)	NS	NS	NS
cardiolipin (CL)	0.81 ± 0.06 (a)	0.82 ± 0.04 (a)	0.59 ± 0.1 (b)	0.71 ± 0.12 (ab)	**	NS	NS
Total	19.03 ± 1.62 (a)	19.27 ± 0.49 (a)	16.45 ± 0.45 (b)	17.79 ± 0.87 (ab)	**	NS	NS

* p<0.05 ; ** p<0.01 ; *** p<0.001

Table 3. Effects of metal exposure and microalgal exposure on oyster hemocyte and plasma variables (n=15), tested by two-way ANOVA.

variables	metal exposure	diet	interaction
hemocytes			
total hemocyte concentration	***	*	**
% of dead hemocytes	**	NS	NS
phagocytosis rate (%)	***	*	NS
ROS production in granulocytes	**	NS	NS
ROS production in hyalinocytes	***	NS	NS
specific activity of PO in hemocytes	*	NS	NS
plasma			
specific activity of PO in plasma	NS	*	NS

* p<0.05 ; ** p<0.01 ; *** p<0.001

PO = phenoloxidase

Research highlights:

- oysters, *C. gigas*, were exposed to both metals and PST-producer *A. minutum*
- oysters exposed to metals accumulated about thirty-six times less PSTs
- exposure to both metals and *A. minutum* induced antagonistic or synergetic effects

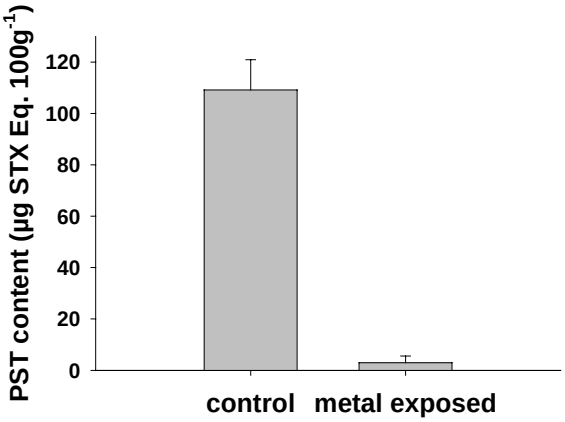

Fig. 1.

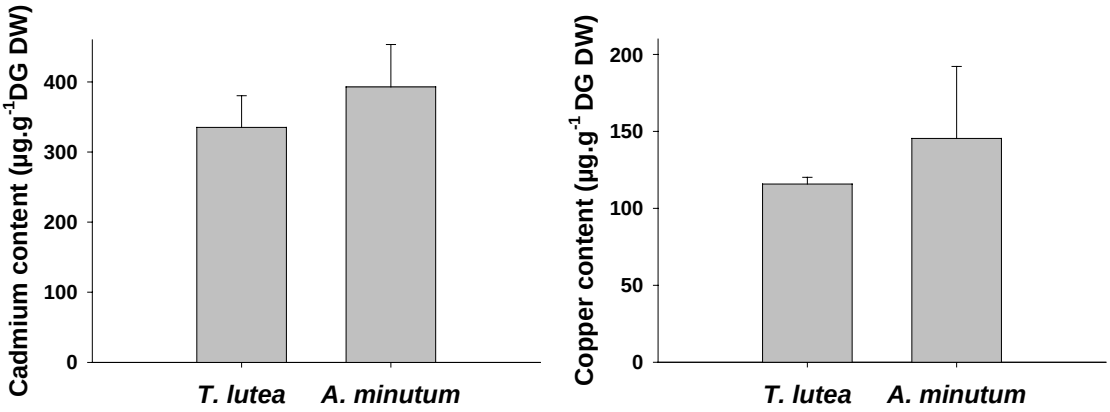

Fig. 2.

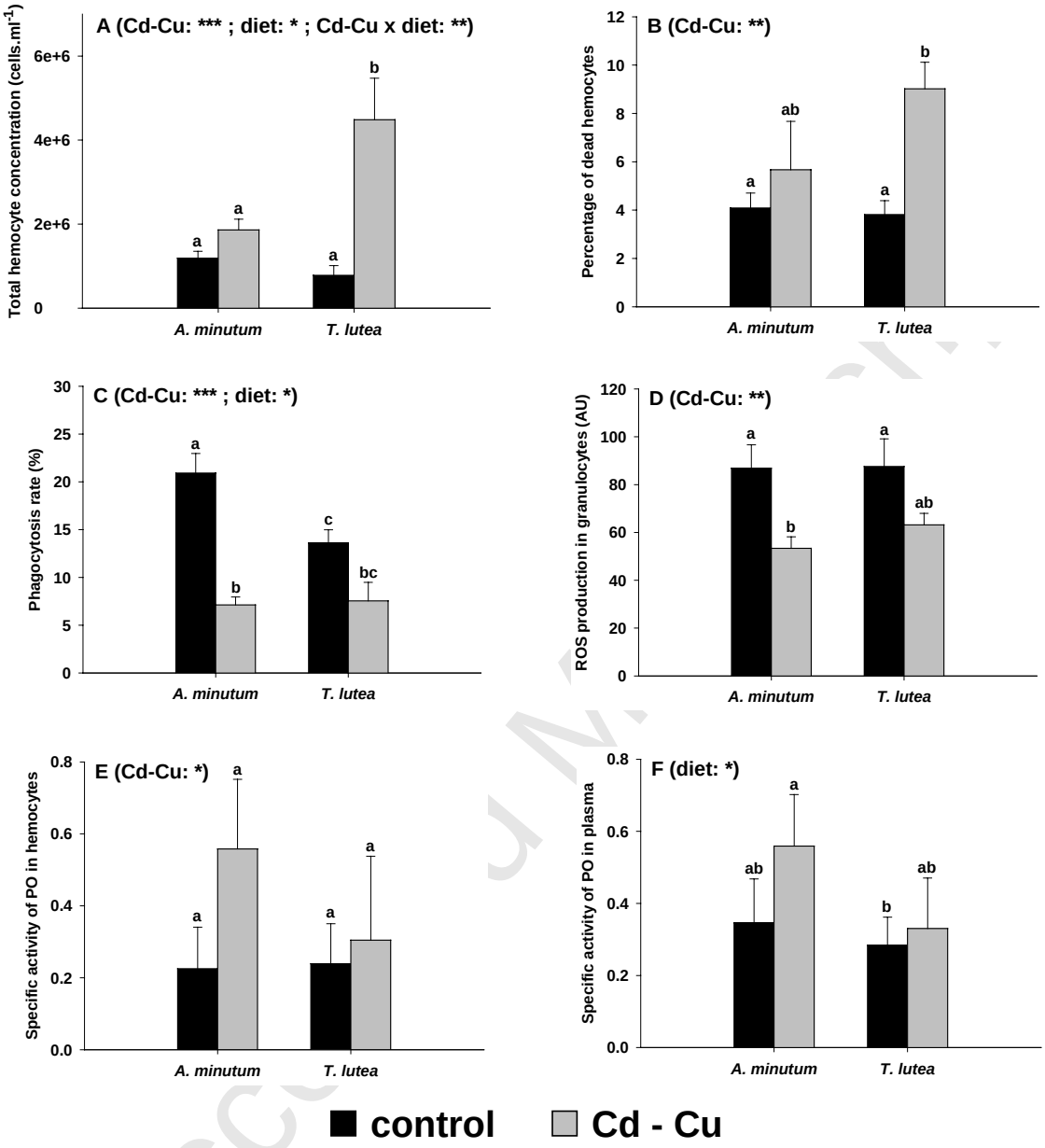

Fig. 3.