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

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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

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41 **1 Introduction**

42

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

50

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

68

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

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75 and non-essential metal with an extremely long biological half-life, making it a cumulative
76 toxic compound. It has been classified as a human carcinogen and is a potent multi-tissue
77 animal carcinogen (IARC, 1993). Most bivalve mollusks are filter feeders and concentrate Cd
78 and other metals in their soft tissues (Bouilly et al., 2006). This accumulated Cd can
79 compromise the health of consumers of contaminated bivalves. The allowable Cd limits in
80 seafood vary between 1 $\mu g\ g^{-1}$ wet weight (European Union) and 3.7 $\mu g\ g^{-1}$ WW (USA,
81 United States Food and Drug Administration recommended guideline) (Lekhi et al., 2008).
82 The Provisional Tolerable Daily Intake (PTDI) recommended by the World Health
83 Organization (WHO) for human oral exposure is 7 $\mu g\ kg^{-1}\ day^{-1}$ (WHO, 2006). Copper (Cu)
84 is an essential metal for all marine organisms, but it can be toxic at elevated concentrations
85 (Parry and Pipe, 2004). The Tolerable Daily Intake (TDI) recommended by the Dutch
86 National Institute for Public Health and the Environment (Rijksinstituut voor
87 volksgezondheid en milieu - RIVM) for human oral exposure is 140 $\mu g\ kg^{-1}\ day^{-1}$ (Baars et
88 al., 2001). In comparison, the TDI recommended for Cd by RIVM for human oral exposure is
89 5 $\mu g\ kg^{-1}\ day^{-1}$ (Baars et al., 2001).
90
91 In bivalves, the digestive system appears to be a revelant target to evaluate interactions of
92 toxic compounds such as phycotoxins and metals. Harmful microalgae were recently shown
93 to interfere with the digestive system of bivalves. Degeneration of the digestive gland
94 (digestive ducts and tubules) was observed in the mussel, *Mytilus edulis*, exposed to
95 *Alexandrium fundyense* (Galimany et al., 2008a). In Pacific oysters, *Crassostrea gigas*,
96 exposed to *A. minutum*, lipid composition of the digestive gland was altered drastically,
97 mainly in terms of phospholipid composition (Haberkorn et al., 2010b). The digestive gland is
98 known to accumulate most of the Cd and Cu in naturally-exposed *C. gigas* from Gironde
99 estuary (Mouneyrac et al., 1998). Metals also appear to have profound effects upon the
100 digestive systems of bivalves. For example, lipid peroxidation was observed in the digestive
101 gland of the blue mussel, *M. edulis*, exposed to Cd – 200 $\mu g\ l^{-1}$ for 21 days (Géret et al.,
102 2002). Similarly, Ringwood et al. (1998) reported lipid peroxidation in *C. virginica* exposed
103 to Cu – 80 $\mu g\ l^{-1}$ for 14 days. Moreover, the digestive gland represents the major site of metal
104 accumulation in bivalves (Pipe et al., 1999) as is also the case for PSTs (Bricelj and
105 Shumway, 1998).
106 In addition to affecting the digestive system, both phycotoxins and metals are known to affect
107 activities of circulating cells (*i.e.* hemocytes) involved in bivalve immunity and general
108 homeostasis (Donaghy et al., 2009). Recent studies demonstrated immunomodulation in

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109 bivalves induced by harmful microalgae (Hégaret et al., 2007a; Galimany et al., 2008b and c;
110 Haberkorn et al., 2010a) and metals (Cherkasov et al., 2007; Dailianis, 2009; Morley, 2010).
111 Inflammatory responses, characterized by diapedesis of hemocytes within tissues, were
112 observed in *M. edulis* exposed to *Prorocentrum minimum* and to *Karlodinium veneficum*
113 (Galimany et al., 2008b and c) and in *C. gigas* exposed to *A. minutum* (Haberkorn et al.,
114 2010b). Moreover, changes in hemocyte morphology and/or functions (such as phagocytosis,
115 reactive oxygen species production, phenoloxidase activity) were observed in *C. gigas*
116 exposed to *A. minutum* (Haberkorn et al., 2010b), in *C. gigas* and *C. virginica* exposed to *A.*
117 *catenella* and *A. fundyense* (Hégaret et al., 2007a), and in *M. edulis* exposed to *A. fundyense*,
118 *P. minimum*, and *K. veneficum* (Galimany et al., 2008a, b and c). *In vitro* exposure of
119 hemocytes of the oyster, *C. virginica*, to Cd – 50 to 1,000 µmol l^{-1} for 3 days– induced
120 increases in apoptosis in a concentration-dependent manner (Sokolova et al., 2004). Increase
121 in hemocyte apoptosis also was observed during *in vivo* exposure of *C. virginica* hemocytes
122 to Cd – 50 µg l^{-1} for 45 days – (Cherkasov et al., 2007). Increase in reactive oxygen species
123 (ROS) production was observed during *in vitro* exposure to Cd – 0.05 to 500 µmol l^{-1} for 1 h
124 – of hemocytes of mussels, *M. galloprovincialis* (Dailianis, 2009). In flat oysters, *in vivo*
125 exposure to a Cd and Cu mixture – 1 to 10 µmol l^{-1} and 0.75 µmol l^{-1}, respectively, for 7 days
126 – Auffret et al. (2002) caused concentration-dependent increases in total hemocyte count and
127 ROS production by hemocytes, suggesting a toxic stimulation of the immune system.
128
129 As in the field stressors rarely act individually, multiple-stress studies are now emerging. Few
130 studies have investigated potential simultaneous effects of pollutants and biological stressors
131 (such as metals/bacteria, metals/macroparasites, macroparasites/harmful microalgae or
132 metals/macroparasites/bacteria) on defense-related activities of bivalves such as detoxification
133 processes, endocrine system, and hemocyte responses (Pipe and Coles, 1995; Baudrimont and
134 de Montaudouin, 2007; Hégaret et al., 2007b; Da Silva et al., 2008; Paul-Pont et al., 2010).
135 Interactive effects of infectious diseases and pollution in aquatic mollusks were reviewed by
136 Morley (2010). To the best of our knowledge, no study has evaluated combined effects of
137 both metals and harmful microalgae upon bivalves.
138
139 The purpose of the present study was to explore possible combined effects of an artificial
140 bloom of the PST-producing dinoflagellate, *A. minutum* (strain AM89BM), and a mixture of
141 two metals, cadmium and copper (Cd-Cu) on the Pacific oyster *C. gigas*. Oysters were
142 exposed 4 days to i) *Tisochrysis lutea* (formerly *Isochrysis* sp., clone Tahitian) as a control, ii)

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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.
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149

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149 **2 Materials and methods**

150 2.1 Biological material

151 2.1.1 Oysters

152 Pacific oysters, *Crassostrea gigas*, used in the experiment were obtained from an oyster
153 producer at île de Kerner (Morbihan, France). Mean individual oyster flesh dry weight was
154 0.34 ± 0.03 g and mean shell length was 61.7 ± 1.9 mm (mean ± CI 5%, n = 60).

155 2.1.2 Algal culture

156 *Alexandrium minutum* (strain AM89BM – isolated from the Bay of Morlaix, France, in 1989)
157 was grown in 10-liter batch culture using autoclaved seawater filtered through a 1-µm filter
158 and supplemented with L1 nutrient enrichment (Guillard and Hargraves, 1993). Cultures were
159 incubated at 16 ± 1°C and 100 µmol photon m^{-2} s^{-1}, with a dark:light cycle of 12:12h. *A.*
160 *minutum* was harvested after 12 days, still in exponential growth phase under our conditions.
161 At this stage, this strain produced 1.3 ± 0.1 pg saxitoxin equivalent (STX eq.) per cell
162 (measured by the method of Oshima, 1995).
163 *Tisochrysis lutea* (formerly *Isochrysis* sp., clone Tahitian) cultures were obtained from the
164 Argenton hatchery (Ifremer, France). Cultures were produced in 300-liter cylinders containing
165 1-µm filtered seawater enriched with Conway medium at 24 ± 1°C, air-CO_2 (3%) mix aerated,
166 and with continuous light. *T. lutea* was harvested in the exponential growth phase (4-5 days)
167 for the feeding experiments.

168 2.2 Experimental design of exposures

169 Short-term exposure period was chosen to enable comparison with previous experiments and
170 to mimic field event of harmful microalgal bloom (Haberkorn et al, 2010a and b). The applied
171 metals were Cd and Cu as they may have aggravating, synergistic effects. Concentrations
172 were chosen to be in the sublethal ranges (Auffret et al., 2002). Indeed, during present
173 experiment, no mortality was observed after four days of exposure.
174 To proceed, 240 oysters were placed randomly in twelve 15-liter tanks (20 oysters per tank).
175 Oysters were acclimated for 10 days with a continuous flow of 14 ml min^{-1} of seawater
176 (filtered through a 0.5-µm filter) with *T. lutea* at 5.10^5 cells ml^{-1} at 16 ± 1°C. After
177 acclimation, oysters were fed continuously for 4 days at 14 ml min^{-1} with 5.10^5 cells ml^{-1} of *T.*
178 *lutea* (six control tanks) and with 5.10^3 cells ml^{-1} of *A. minutum* (six *A. minutum* tanks). These

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179 two different cell densities were used to provide the same bio-volume of microalgae to oysters
180 as the cellular volume of *A. minutum* is about 100x higher than that of *T. lutea*.
181 To half of the control and *A. minutum* tanks a mixture of Cd and Cu was also added. Separate
182 stock solutions of Cd and Cu were prepared in filtered sterile seawater (FSSW) at 250 mM
183 and 50 mM, respectively. A volume of 1,500, 750, 375 or 187.5 µl of Cd and Cu stock
184 solutions was added at 0, 24, 48 and 72h in each of the six tanks during 4 days (exposure).
185 Initial concentrations of Cd and Cu at T0 were 25 µM (2.8 mg l^{-1}) and 5 µM (0.317 mg l^{-1})
186 and contamination pressure was expected to be maintained constant for the 4 days of
187 experiment.

188 2.3 Oyster sampling

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

194 2.4 Digestive gland variables

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

199 2.4.1 Toxin content

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

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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

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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

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273 variables (phagocytosis and reactive oxygen species (ROS) production). Analyses were done
274 as described below.
275
276 2.5.2.1 Descriptive variables
277 Hemocyte viability, total and hemocyte sub-population counts were measured individually on
278 hemolymph samples (5 individuals per tank). An aliquot of 100 µl of hemolymph was
279 transferred into a tube containing a mixture of Anti-Aggregant Solution for Hemocytes,
280 AASH (Auffret and Oubella, 1995) and filtered sterile seawater (FSSW), 200 and 100 µl,
281 respectively. Hemocyte DNA was stained with two fluorescent DNA/RNA specific dyes,
282 SYBR Green I (Molecular probes, Eugene, Oregon, USA, 1/1,000 of the DMSO commercial
283 solution), and propidium iodide (PI, Sigma, St Quentin Fallavier, France, final concentration
284 of 10 µg ml^{-1}) in the dark at 18°C for 120 min before flow cytometric analysis. PI permeates
285 only hemocytes that lose membrane integrity and are considered to be dead cells, whereas
286 SYBRGreen I permeates both dead and live cells. SYBR Green and PI fluorescence were
287 measured at 515–545 nm (green) and >670 nm (red) wavelengths, respectively, by flow
288 cytometry. Thus, by counting the cells stained by PI and cells stained by SYBR Green I, it
289 was possible to estimate the percentage of viable cells in each sample. All SYBR Green I-
290 stained cells were visualized on a Forward Scatter (FSC, size) and Side Scatter (SSC, cell
291 complexity) cytogram. Two main sub-populations were distinguished according to size and
292 cell complexity (granularity). Granulocytes are characterized by high FSC and high SSC,
293 while hyalinocytes by high FSC and low SSC. Total hemocyte, granulocyte and hyalinocyte
294 concentrations were estimated from the flow-rate measurement of the flow cytometer (Marie
295 et al., 1999) as all samples were run for 30 s. Results were expressed as number of cells per
296 milliliter of hemolymph.
297
298 2.5.2.2 Functional variables
299 To measure phagocytosis rate, an aliquot of 100 µl hemolymph, diluted with 100 µl of FSSW,
300 was mixed with 30 µl of Yellow-Green, 2.0-µm fluoresbrite microspheres, diluted to 2% in
301 FSSW (Polysciences, Eppelheim, Germany). After 120 min of incubation at 18°C, hemocytes
302 were analyzed at 515–545 nm by flow cytometry to detect hemocytes containing fluorescent
303 beads. The phagocytosis rate was defined as the percentage of hemocytes that had engulfed
304 three or more beads (Delaporte et al., 2003).
305 Reactive oxygen species (ROS) production by hemocytes was measured using 2,7-
306 dichlorofluorescein diacetate, DCFH-DA (Lambert et al., 2003). A 100-µl aliquot of

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307 hemolymph was diluted with 300 µl of FSSW. Four µl of the DCFH-DA solution (final
308 concentration of 0.01 mM) was added to each tube maintained on ice. Tubes were then
309 incubated at 18°C for 120 min. After the incubation period, DCF fluorescence, quantitatively
310 related to the ROS production of hemocytes, was measured at 515–545 nm by flow
311 cytometry. Results were expressed as the geometric mean fluorescence (in arbitrary units,
312 AU) detected in each hemocyte sub-population.

313

314 2.5.3 Hemocyte and plasma phenoloxidase activities

315 Plasma samples were thawed on ice, and 100 µl of each was transferred to a well in a 96-well
316 plate. For hemocytes, cells were suspended in 100 µl of FSSW and frozen and thawed on ice
317 three times successively. Phenoloxidase activity was measured as described by Reid et al.
318 (2003). Briefly, 50 µl of Tris–HCl buffer (0.2 M, pH = 8) and 100 µl of l-DOPA (20 mM, l-
319 3,4-dihydrophenyl-alanine, Sigma D9628) were added to each well. The microplate was
320 mixed rapidly for 10 s. The reaction then was measured at ambient temperature, with color
321 change recorded every 5 min, at 492 nm, over a period of 1 h. The microplate was mixed
322 prior to each measurement. Two controls, without sample but containing l-DOPA and Tris–
323 SDS buffer, were measured in parallel, and these values were subtracted from test values to
324 correct for possible auto-oxidation of the l-DOPA and buffer absorbance. To assess specific
325 activity of phenoloxidase, total proteins in hemocyte suspension and plasma were determined
326 using the BCA Protein Assay (Biorad). Protein analysis was carried out on 10 µl samples
327 according to the manufacturer's description. Briefly, 200 µl of dye reagent was added to 10 µl
328 of sample and incubated at 37°C for 1 hour, and the absorbance was measured at 595 nm.
329 Sample ODs were compared to a standard curve of Bovine Serum Albumin (BSA), and
330 results were expressed as mg protein.ml^{-1}. Results were expressed as specific activity of
331 phenoloxidase (*i.e.* International Unit – IU – per mg of total protein).

332 2.6 Statistical analysis

333 Results of toxin and metal contents were analyzed statistically using one-way ANOVA.
334 Results of each experiment were analyzed statistically using two-way ANOVA for each
335 physiological variable and hemocyte variable as the dependent variable, and exposure
336 conditions as independent variables.
337 In conjunction with two-way ANOVA, Tukey's HSD test were performed to find means that
338 are significantly different from each other.

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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.
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343 **3 Results**

344

345 3.1 Digestive gland variables

346

347 3.1.1 Toxin content

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

352

353 3.1.2 Cadmium and copper contents

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

357

358 3.1.3 Neutral lipid contents

359 There was no significant difference in total neutral lipid contents between treatments (Table
360 1).

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

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

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

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

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

375

376 3.1.4 Polar lipid contents

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377 Total phospholipid content (Table 2) was significantly lower in digestive glands of oysters
378 exposed to Cd-Cu and fed *A. minutum* as compared to non-exposed oysters.
379 Phosphatidylcholine (PC) and cardiolipin (CL) were significantly lower in oysters exposed to
380 Cd-Cu and fed *A. minutum* than in non-exposed oysters (Table 2). Phosphatidylethanolamine
381 (PE) and ceramide aminoethylphosphonate (CAEP) were significantly lower in oysters
382 exposed to Cd-Cu as compared to non-exposed oysters (Table 2).
383 There was no significant difference in lysophosphatidylcholine (LPC), phosphatidylionsitol
384 (PI) and phosphatidylserine (PS) contents between treatments (Table 2).
385
386 3.1.5 Amylase specific activity
387 There was no significant variation of the amylase specific activity in digestive gland between
388 different exposure conditions.
389
390 3.2 Hemolymph variables
391
392 3.2.1 Hemocyte variables
393 Overall, Cd-Cu exposure had more significant effects upon hemocyte and plasma variables
394 than *A. minutum* exposure (Table 3).
395 Total hemocyte counts (THC) were significantly higher in oysters exposed to Cd-Cu and fed
396 *T. lutea* and also were affected by the interaction between Cd-Cu exposure and dietary
397 treatment (Fig. 3A, Table 3).
398 Percentage of dead hemocytes was significantly higher in oysters exposed to Cd-Cu,
399 especially in those fed *T. lutea* (Fig. 3B, Table 3).
400 Percentage of phagocytic hemocytes was significantly lower in oysters exposed to Cd-Cu
401 (Fig. 3C, Table 3). Phagocytosis was also significantly higher in oysters fed *A. minutum* in
402 relation to those fed *T. lutea*.
403 ROS production by granulocytes was significantly lower in oysters exposed to Cd-Cu as
404 compared to non-exposed oysters fed *A. minutum* (Fig. 3D, Table 3). Similarly, ROS
405 production of hyalinocytes was greatly reduced upon exposure to Cd-Cu (Table 3).
406
407 3.2.2 Hemocyte and plasma phenoloxidase (PO) specific activities
408 PO specific activity in hemocytes was higher in oysters exposed to Cd-Cu as compared to
409 non-exposed oysters (Fig. 3E, Table 3). PO specific activity in plasma was higher in oysters
410 exposed to *A. minutum* than in oysters fed *T. lutea* (Fig. 3F, Table 3). In plasma, this activity

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

414

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

417 One of the most striking results of this experiment was the difference in PST accumulation in
418 digestive glands of Cd-Cu exposed and non-exposed oysters. Oysters exposed to Cd-Cu
419 accumulated about thirty-six times less PSTs as compared to oysters exposed to *A. minutum*
420 only. Such lower toxin accumulation could be explained by a decrease in feeding activity (*A.*
421 *minutum* cell ingestion) and/or by alteration of digestive processes. In *Corbicula fluminea*,
422 Tran et al. (2003a and b) observed a prolonged closure of valves in reaction to dissolved
423 copper or cadmium, suggesting a decrease in feeding activity. Similarly, decreased filtration
424 rate has been observed in *C. gigas* exposed to copper or cadmium (Lin et al., 1992; Lin et al.,
425 1993). Modification of lipid composition of the digestive gland, described hereafter, may also
426 reflect some alterations of digestive processes resulting in lower toxin accumulation by Cd-Cu
427 exposed oysters as a lower digestive efficiency may lead to a lower toxin uptake. Another
428 hypothesis is that alteration of phytoplanktonic cells may result in decreased ingestion by
429 oysters. Cyst formation by *A. minutum,* however, was observed only when *A. minutum* cells
430 were exposed to Cd-Cu at higher concentrations than those applied in this experiment (data
431 not shown). Even though some physiological changes may have occurred in microalgae upon
432 exposure to Cd-Cu in the oyster tank, they are unlikely to explain observed differences in
433 toxin accumulation by oysters.

434

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

443

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

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447 populations (*Crassostrea virginica*) ranged from 300 to 400 µg g^{-1} DW (Roesijadi, 1996).
448 Abbe et al. (2000) observed that field Cu contamination in *C. virginica* reached 310 µg g^{-1}
449 DW. Concentration of 850 µg g^{-1} DW for the same species was also observed by O-Connor
450 and Lauenstein (2005). Concentration of cadmium reached 5 µg g^{-1} wet weight and
451 concentration of copper reach at 300 µg g^{-1} digestive gland WW in *C. gigas* from a polluted
452 estuary (Mouneyrac et al., 1998).
453
454 ***Effects of exposures to Cd-Cu and toxic A. minutum upon oyster physiology.***
455 The present results demonstrated major effects of Cd-Cu upon lipid composition of the oyster
456 digestive gland. Exposure to Cd-Cu induced decreases in sterols, PC, PE, CAEP and CL.
457 Variations in lipid contents were probably not linked to lipid hydrolysis because no increase
458 in FFA was observed (Chu et al., 2003). Changes in PL composition may reflect some
459 changes in cell types or in organelles as changes in cell type composition is a general
460 phenomenon that can take place in the digestive gland epithelia of mollusks stressed by
461 chemical contamination, as underscored by Zaldibar et al. (2008). A recent study (Zaldibar et
462 al., 2007) demonstrated that exposure of winkles (*Littorina littorea*) to Cd resulted in changes
463 in the epithelia of the digestive gland (digestive ducts and tubules). These changes were
464 characterized by a loss of digestive cells (minus 13.2%) and volume increases in both
465 digestive and basophilic cells (plus 13.5% and 200%, respectively). Variations in PL
466 composition could also be linked to perturbations of lipid metabolism and/or to lipid
467 peroxidation. Ringwood et al. (1998) observed significant increase in lipid peroxidation in
468 oysters, *C. virginica*, exposed to copper. Similarly, Géret et al. (2002) observed that exposure
469 of *Mytilus edulis* to cadmium stimulated lipid peroxidation processes through oxidation of
470 polyunsaturated fatty acids. Also, exposure of *Ruditapes decussatus* to cadmium led to
471 changes in protein-expression profiles, including cell maintenance (Rab GDP dissociation
472 inhibitor α – mediators of vesicle formation, trafficking, and fusion) and metabolism (MCAD
473 – medium chain-CoA dehydrogenase, enzyme responsible for the metabolism of medium
474 chain fatty acids – and ALDH – aldehyde dehydrogenase, mitochondrial precursor)
475 suggesting potential alteration in energetic processes (Chora et al., 2009).
476 Effects of *A. minutum* exposure upon lipid composition of the digestive gland were mainly
477 characterized by decreases in MAG, DAG and ether glycerides. Such effects of *A. minutum*
478 upon oysters were reported previously by Haberkorn et al. (2010b).
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480 Effects of Cd-Cu exposure were observed in most of the measured hemocyte variables:
481 increases in total hemocyte count, percentage of dead hemocyte and phenoloxidase activity,
482 as well as decreases in phagocytosis and ROS production. Oysters may possibly increase
483 circulating hemocytes to compensate for increase in mortality. Other studies previously
484 reported that increases in circulating hemocytes in *C. virginica* exposed to cadmium were
485 associated with increased percentage of dead hemocytes (Cheng, 1988; Cheng, 1990).
486 Similarly, Auffret et al. (2002) observed increases in THC in oysters, *Ostrea edulis*, exposed
487 to Cd-Cu.
488 Decreased hemocyte phagocytosis upon Cd-Cu exposure also has been observed in *O. edulis*
489 (Auffret et al., 2002). Auffret et al. (2002) similarly observed a decrease in ROS production in
490 hemocytes in oysters, *O. edulis*, exposed *in vivo* to a high concentration of cadmium (50 µM).
491 The present results clearly suggest an alteration of some hemocyte-based defense mechanisms
492 by Cd-Cu exposure.
493 Feeding oysters on *A. minutum* resulted in less intense immunomodulation than Cd-Cu
494 exposure. Hemocyte phagocytosis increased upon *A. minutum* exposure. Such stimulation of
495 phagocytosis has been observed previously in hemocytes of the blue mussel, *Mytilus edulis*,
496 exposed to *Karlodinium veneficum* (Galimany et al., 2008c). Observations suggest a
497 stimulation of this cellular-based immune function when bivalves feed on harmful
498 microalgae.
499
500 ***Combined effects and interactions of exposures to Cd-Cu and toxic A. minutum upon***
501 ***oyster physiology.***
502 Oysters exposed to both metals and harmful algae accumulated large amounts of Cd-Cu but
503 only a small quantities of PST, suggesting that effects in these exposures may be primarily
504 from metals. Nevertheless, results of co-exposure were different than those of single
505 “contaminant” exposure.
506
507 Effects of Cd-Cu exposure on lipids were opposite to those caused by *A. minutum* feeding:
508 Cd-Cu exposure counteracted the decreasing effect of *A. minutum* on MAG, DAG and ether
509 glycerides. Such interactive effects of Cd-Cu and *A. minutum* exposures on lipid metabolism
510 within the digestive gland need further investigation.
511
512 Total hemoctye counts (THC) and percentages of dead hemocytes increased dramatically in
513 oysters fed *T. lutea* and exposed to Cd-Cu. But when oysters were fed *A. minutum,* this effect

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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
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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.
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780 Table 1. Neutral lipid class contents (expressed as mg g^{-1} of tissue wet weight, ±CI, means of
781 3 pools of 10 oysters each) in oyster digestive glands according to exposure conditions. This
782 table also indicates the results of the two-way ANOVAs testing by Cd-Cu exposure (M) and
783 dietary effects (D). Lower-case letters (a, b and c) indicate homogeneous groups (Tukey's
784 HSD test).
785

	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

786 * p<0.05 ; ** p<0.01 ; *** p<0.001
787

788 Table 2. Phospholipid class contents (expressed as mg g^{-1} of tissue wet weight, ±CI, means of
789 3 pools of 10 oysters each) in oyster digestive glands according to exposure conditions. This
790 table also indicates the results of the two-way ANOVAs testing by Cd-Cu exposure (M) and
791 dietary effects (D). Lower-case letters (a and b) indicate homogeneous groups (Tukey's HSD
792 test).
793

	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

794 * p<0.05 ; ** p<0.01 ; *** p<0.001
795

796 Table 3. Effects of metal exposure and microalgal exposure on oyster hemocyte and plasma
797 variables (n=15), tested by two-way ANOVA.
798

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

799 PO = phenoloxidase
800

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800 **Research highlights:**

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

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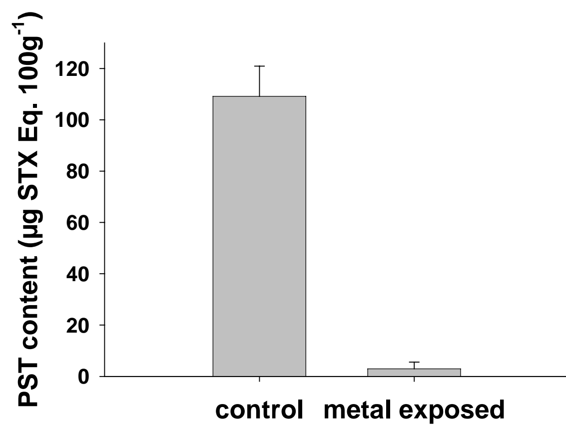

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807 Fig. 1.
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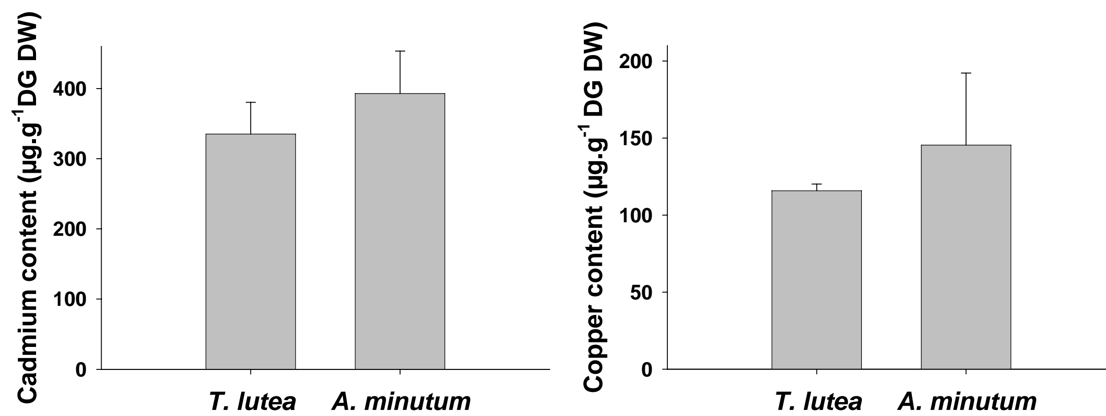

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809 Fig. 2.
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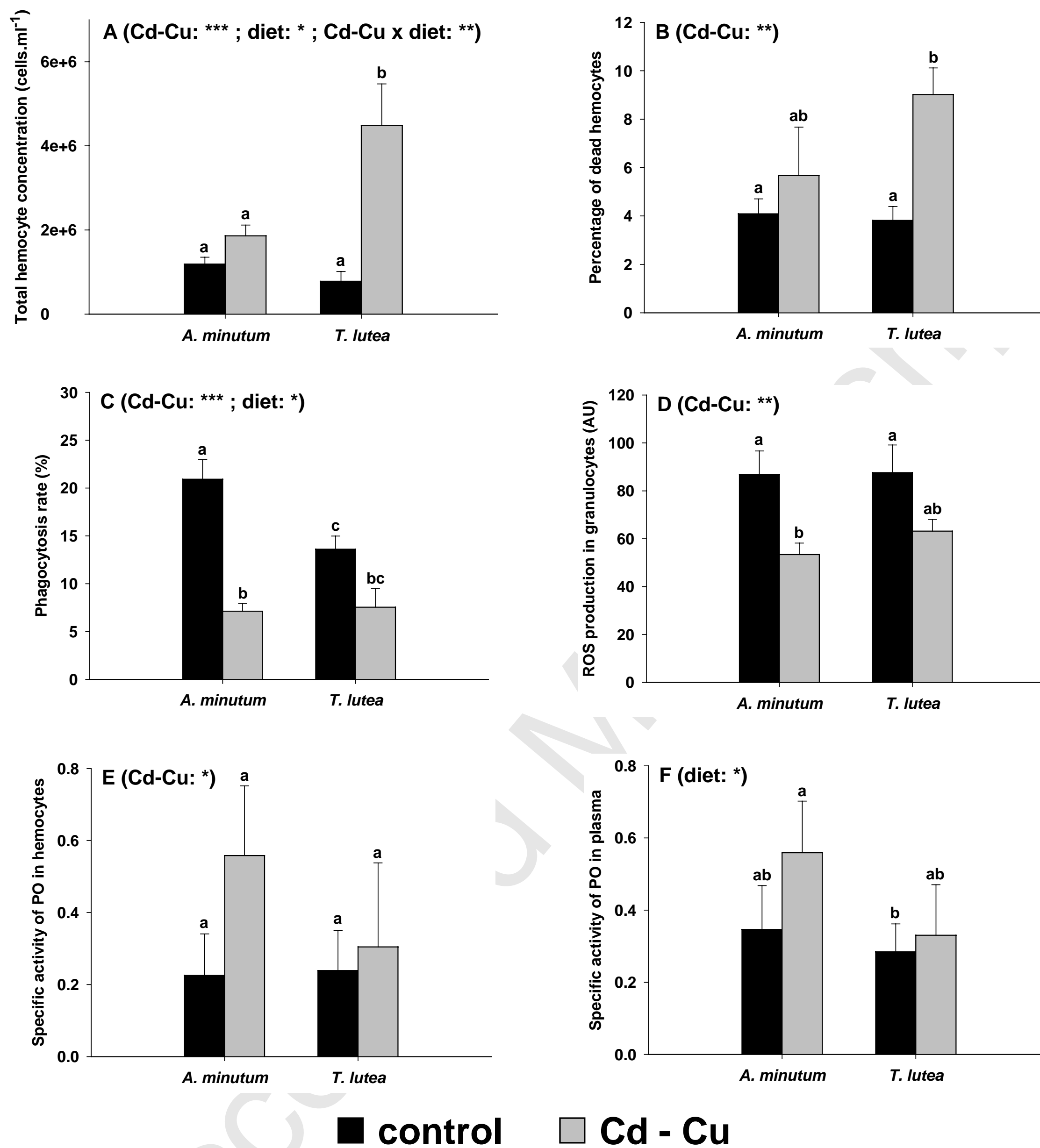

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811
812 Fig. 3.
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