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1 **Effects of *Alexandrium minutum* exposure upon physiological and**
2 **hematological variables of diploid and triploid oysters,**
3 ***Crassostrea gigas*.**

4

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19

20 **Abstract**

21 The effects of an artificial bloom of the toxin-producing dinoflagellate, *Alexandrium*
22 *minutum*, upon physiological parameters of the Pacific oyster, *Crassostrea gigas*, were
23 assessed. Diploid and triploid oysters were exposed to cultured *A. minutum* and compared to
24 control diploid and triploid oysters fed *T.Isochrysis*. Experiments were repeated twice, in

25 April and mid-May 2007, to investigate effects of maturation stage on oyster responses to *A.*
26 *minutum* exposure. Oyster maturation stage, Paralytic Shellfish Toxin (PST) accumulation, as
27 well as several digestive-gland and hematological variables, were assessed at the ends of the
28 exposures.

29 In both experiments, triploid oysters accumulated more PSTs (approximately twice) than
30 diploid oysters. Significant differences, in terms of phenoloxidase activity (PO) and reactive
31 oxygen species (ROS) production of hemocytes, were observed between *A. minutum*-exposed
32 and non-exposed oysters. PO in hemocytes was lower in oysters exposed to *A. minutum* than
33 in control oysters in an early maturation stage (diploids and triploids in April experiment and
34 triploids in May experiment), but this contrast was reversed in ripe oysters (diploids in May
35 experiment). In the April experiment, granulocytes of oysters exposed to *A. minutum*
36 produced more ROS than those of control oysters; however, in the May experiment, ROS
37 production of granulocytes was lower in *A. minutum*-exposed oysters. Moreover, significant
38 decreases in free fatty acid, monoacylglycerol, and diacylglycerol contents in digestive
39 glands of oysters exposed to *A. minutum* were observed. Concurrently, the ratio of reserve
40 lipids (triacylglycerol, ether glycerides and sterol esters) to structural lipids (sterols)
41 decreased upon *A. minutum* exposure in both experiments. Also, several physiological
42 responses to *A. minutum* exposure appeared to be modulated by maturation stage as well as
43 ploidy of the oysters.

44

45 **Keywords:** oysters, ploidy, physiology, harmful algal bloom, *Alexandrium minutum*, PST
46 accumulation.

47

48 **1 Introduction**

49 Among harmful algae, *Alexandrium* species are known to produce Paralytic Shellfish Toxins
50 (PSTs), the most widespread shellfish-contaminating biotoxins, with outbreaks occurring
51 worldwide (Huss, 2003). In France, *Alexandrium minutum* Halim (1960) has been known to
52 bloom in coastal waters since the 1980's (Lassus et al., 1992), especially in North Brittany
53 (English Channel) during summer (Morin et al., 2000).

54
55 PSTs are comprised of approximately 20 naturally-occurring biotoxin derivatives that vary
56 widely in specific toxicity (measured by standard mouse bioassay). The basic molecular
57 structure is that of saxitoxin (STX). PSTs are neurotoxins, the mode of action of which
58 involves a reversible and highly specific block of sodium channel transport, disabling the
59 action potential of excitable membranes (nerves and muscle fibers) (Narahashi, 1988).
60 The current EU regulatory limit for human consumption of shellfish is set at 80 μg STX eq.
61 100g^{-1} shellfish meat (SM). Considering, however, possible consumption of a large portion
62 (400g) of shellfish, the European Food Safety (EFSA) recently established that the maximum
63 concentration in shellfish meat should be less than 7.5 μg STX eq. 100g^{-1} SM to avoid
64 exceeding the acute reference dose (ARfD) of 0.5 μg STX eq. kg^{-1} body weight (EFSA
65 Journal, 2009). It is also estimated that 25% of EU samples compliant with the EU limit
66 exceeded the concentration set by ARfD (EFSA Journal, 2009). The mouse bioassay (AOAC,
67 1990) protocol is the officially-prescribed method for the evaluation of STX-group toxin
68 contamination. Although MBA sensitivity (37 μg STX eq. 100g^{-1}) allows quantification of
69 STX-group toxins at the current EU regulatory limit, it is not within the range of ARfD
70 concentrations (EFSA Journal, 2009). Only the HPLC-fluorescence detection method has
71 sensitivity sufficient to quantify STX-group toxins at 1-8 μg STX eq. 100g^{-1} SM.

72

73 Several commercially-harvested bivalve species, such as oysters, are known to accumulate
74 PSTs by feeding on PST-producing phytoplankton (see review by Bricelj and Shumway,
75 1998). Bivalves show significant (up to 100-fold) inter-specific differences in accumulation
76 of PSTs, which was inversely correlated with toxin sensitivity (Bricelj and Shumway, 1998).
77 This variability in sensitivity to PSTs appeared to be related to nerve sensitivity in a dose-
78 dependant manner. Indeed, Bricelj and Shumway (1998) reported that 10^{-7} g.ml⁻¹ STX was
79 sufficient to block the action potential of nerves in eastern oysters *C. virginica* when 10^{-3}
80 g.ml⁻¹ was insufficient to block action potential in blue mussels (*Mytilus edulis*). Some
81 bivalve species possessing nerves insensitive to PST (*M. edulis*) readily feed on toxic cells
82 and thereby accumulate high toxin levels. In contrast, species such as *Crassostrea virginica*
83 are highly sensitive to PSTs, accumulating fewer toxins and exhibiting physiological and
84 behavioral mechanisms to avoid or reduce exposure to toxic cells (Bricelj and Shumway,
85 1998). The Pacific oyster *C. gigas* and the soft-shell clam *Mya arenaria* (two PST-sensitive
86 species) were reported to reduce filtration activity when feeding on PST-containing
87 microalgae (Lassus et al., 2004; Bricelj and Shumway, 1998). Differences in toxin
88 accumulation (up to five times) were also observed between different populations of the same
89 species, *M. arenaria*, and this difference was surmised to be related to nerve sensitivity
90 differences (Bricelj et al., 2005). Indeed, a natural mutation of a single amino acid residue
91 decreasing affinity (1,000-fold) of the saxitoxin-binding site in the sodium channel pore, was
92 found to be responsible for the difference in nerve sensitivity between two populations of *M.*
93 *arenaria* exposed to PST-producing *Alexandrium fundyense* (Bricelj et al., 2005).
94 Toxin composition and content in toxigenic microalgae vary greatly according to species or
95 strain and depend also environmental or culture conditions (Hégaret et al., 2009). An *A.*
96 *minutum* strain isolated in France was found to produce 1.5 pg STX eq. cell⁻¹ (Lassus et al.,
97 2004); whereas, the same species isolated in New Zealand produced 11 pg STX eq. cell⁻¹

98 (Chang et al., 1997). Chou and co-workers (2004) reported that toxin content of different
99 clones of *A. minutum* isolated in Taiwan varied from 11 pg STX eq. cell⁻¹ to 103 pg STX eq.
100 cell⁻¹. This variability in algal toxin content has consequences to feeding responses to and
101 toxin accumulation of bivalves exposed to *Alexandrium* species. Bardouil and co-workers
102 (1993) observed clearance rate in *C. gigas* decreased more drastically when oysters were
103 exposed to *A. tamarense* (7.2 pg STX eq. cell⁻¹) than to *A. minutum* (0.5 pg STX eq. cell⁻¹).
104 When exposed to less-toxic *Alexandrium* species or strains, sensitive bivalves such as *C.*
105 *gigas* can feed on and accumulate PSTs (Lassus, unpubl. obs.).

106

107 *Alexandrium* species are also known to produce other toxic compounds, such as ichthyotoxins
108 (Emura et al., 2004) and allelochemicals (Arzul et al., 1999; Tillmann et al., 2008). Ford et al.
109 (2008) tested effects of two *A. tamarense* strains, PST and non-PST producing, upon Manila
110 clam *Ruditapes philippinarum* and *Mya arenaria* hemocytes. This study showed that the non-
111 PST strain had more-negative impacts on hemocytes (decreased adhesion and phagocytosis)
112 compared to the PST-producing strain of *A. tamarense* (Ford et al., 2008). Based upon
113 biological effects of *Alexandrium* exposure clearly unrelated to PSTs, one can speculate that
114 effects of PSTs and other toxic compounds/molecules may also result in damage to organs
115 and physiological processes other than muscles. Considering this, *Alexandrium* effects upon
116 nerves and muscles of exposed bivalves can be linked to PSTs, but there appear to be
117 responses to other compounds not clearly identified.

118 Aside from toxin accumulation and associated human health issues, there is some concern
119 about the impact of *A. minutum* exposure on the physiology and health of *C. gigas*.

120 Furthermore, the physiological status of animals may also feed-back to rates of toxin
121 accumulation and effects during HAB exposure.

122 Li et al. (2002) studied the effect of *Alexandrium tamarense* (PST-containing strain) on bio-
123 energetics and growth rate of the clam *Ruditapes philippinarum* and the mussel *Perna viridis*.

124 High concentrations of toxic *A. tamarense* (resulting in high PST burdens in the tissues)
125 decreased clearance rate of the clam but not of the mussel. Absorption efficiency, however,
126 decreased for both species with diets containing PST, which resulted in a reduction in energy
127 budget. HABs occurring during specific stages of reproduction could be another major factor
128 affecting oysters, in terms of energy budget. In *C. gigas*, energy balance (evaluated through
129 scope for growth methods) has been demonstrated to decrease as gametogenesis progresses,
130 resulting in a negative scope for growth (Lambert et al., 2008). The digestive gland plays an
131 obvious, major role in nutrient digestion and assimilation, as digestive-enzyme activities in
132 bivalves can be affected by nutritional condition. Indeed, changes in enzymatic activities are
133 mechanisms used by bivalves to optimize energy gain when experiencing variation in dietary
134 input (Fernández-Reiriz et al., 2001; Labarta et al., 2002). The mussel *M. chilensis* can,
135 indeed, use toxic microalgae (*Alexandrium catenella*) as a food source by adjusting
136 carbohydrase activities (amylase, laminarinase and cellulase) and absorption mechanisms
137 (Fernández-Reiriz et al., 2008). It is, thus, pertinent to assess digestive-enzyme activities in
138 bivalves exposed to toxic microalgae. Moreover, the digestive gland is also involved in
139 energy storage, preferentially as lipids (Soudant et al., 1999). As HABs are likely to impact
140 digestive-gland structure and functions, it appears prudent to assess how HABs could
141 modulate quantities of individual lipid classes involved in energy storage in this organ.
142 Moreover, the digestive gland is the organ accumulating the most toxins compared to other
143 tissues in bivalves (Bricelj and Shumway, 1998).

144

145 In bivalves, one line of defense to noxious, harmful or pathogenic agents resides in
146 circulating cells called hemocytes that are similar to white blood cells in vertebrates (Cheng,
147 1996). Concerning hemocyte variables, Hégaret et al. (2007a, 2007b, 2008) reported that
148 harmful-algal exposure can modulate cellular immune components and functions of bivalves.

149 Moreover, Galimany et al. (2008) observed an inflammatory response in the stomach of
150 *Mytilus edulis* exposed to *A. fundyense*. These findings indicate that the bivalve immune
151 system can be activated by certain harmful algae, or conversely can be suppressed. The
152 reproductive period is also associated with changes in hemocyte variables; some are
153 depressed in *C. gigas* during gametogenesis, specifically hemocyte concentration,
154 phagocytosis, and adhesion (Lambert et al., 2008). During gametogenesis, hemocytes in
155 triploid oysters were found to have higher phagocytic, esterase and peroxidase activities than
156 those of diploids (Gagnaire et al., 2006). These differences were attributed to the reduced
157 gametogenic development of the triploids.

158 Indeed, triploid oysters are increasingly used for aquaculture because they can be marketed
159 during summer when diploid oysters are fully-ripe and not appreciated by consumers. It is
160 unknown, however, how triploidy may affect toxin accumulation.

161

162 The purpose of the present study was to determine the effects of an artificial bloom of the
163 toxin-producing dinoflagellate, *Alexandrium minutum* (strain AM89BM), upon digestive
164 parameters, and hemocyte and plasma variables of the Pacific oyster, *Crassostrea gigas*.

165 Diploid and triploid oysters were compared to assess any differences in toxin accumulation
166 and physiological responses to harmful-algal exposure. Experiments were conducted at two
167 different periods (one month apart), using the same oyster stock, to obtain a gradient of gonad
168 maturation. After 4 days of exposure to *A. minutum* or *Isochrysis* sp. (clone Tahitian T.*Iso*) as
169 a non-toxic control, toxin accumulation, reserve lipid classes and amylase activities, humoral
170 phenoloxidase variables, hemolyse/agglutination capacity, and hemocyte concentration,
171 morphology, viability, phagocytosis activity, reactive oxygen species production and
172 phenoloxydase activity, were measured.

173

174 **2 Materials and Methods**

175 **2.1 Biological material**

176 **2.1.1 Oysters**

177 Diploid and triploid Pacific oysters, *Crassostrea gigas*, used in the two experiments were
178 obtained from an oyster producer at île de Kerner (Morbihan, FRANCE) and belong to the
179 same commercial stocks (20-21 months old). For each experiment, we used 60 diploid
180 oysters and 60 triploid oysters. In April, flesh dry weight was 1.22 ± 0.12 g and 0.90 ± 0.09 g
181 in diploids and triploids, respectively. In May, flesh dry weight was 1.33 ± 0.14 g and $0.98 \pm$
182 0.09 g in diploids and triploids, respectively. At both collection times, three pools of four
183 oysters (for each ploidy) were confirmed to be free of PST contamination (no detectable
184 levels of PSTs by IP-HPLC analysis).

185 **2.1.2 Algal culture**

186 *Alexandrium minutum* (strain AM89BM) was grown in 10-liter batch culture using
187 autoclaved seawater filtered to $1\mu\text{m}$ and supplemented with L1 medium (Guillard and
188 Hargraves, 1993). Cultures were maintained at $16 \pm 1^\circ\text{C}$ and $100\ \mu\text{mol photon}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$, with a
189 dark:light cycle of 12:12h. *A. minutum* was harvested after 12 days, still in exponential
190 growth phase under our conditions. At this age, this strain produced 1.3 ± 0.1 pg STX eq. per
191 cell (measured by the method of Oshima (1995)).

192 *Isochrysis* sp., clone Tahitian (*T.Iso*), cultures were obtained from the Argenton hatchery
193 (IFREMER - FRANCE). Cultures were produced in 300-liter cylinders containing $1\text{-}\mu\text{m}$
194 filtered seawater enriched with Conway medium at $24 \pm 1^\circ\text{C}$, air-CO₂ (3%) mix aerated, and
195 with continuous light. *T.Iso* was harvested in the exponential growth phase (6-8 days) for the
196 feeding experiments.

197 **2.2 Experimental design of *A. minutum* exposures**

198 For each experiment (April and May), 120 oysters (60 diploids and 60 triploids) were placed
199 haphazardly in twelve 15-L tanks (10 oysters per tank). Oysters were acclimated for 10 days
200 with continuous flow of 14 ml.min⁻¹ of seawater (filtered to 0.5 µm) with *T.Iso*, 5.10⁵
201 cells.ml⁻¹ at 16 ± 1°C. After acclimation, diploid and triploid oysters were fed continuously
202 for 4 days 14 ml.min⁻¹ with 5.10⁵ cells.ml⁻¹ of *T.Iso* (6 control tanks with diploids and
203 triploids) and with 5.10³ cells.ml⁻¹ of *A. minutum* (equivalent to 6.5 ng STX eq. - 6 treatment
204 tanks with diploids and triploids).

205 **2.3 Oyster sampling**

206 At the end of the algal-exposure, all oysters were sampled and distributed as follow. For each
207 tank (10 oysters), gonads of two oysters were used for histological analysis of maturation
208 stage. Pooled digestive glands of four oysters were used to measure toxins, reserve lipid
209 contents, and amylase activity. Four oysters were used for individual plasma and hemocyte
210 variable measurements and condition index.

211 **2.4 Qualitative analysis of maturation stages**

212 Gonads were dissected and transferred into Bouin fixative (for 48 h). Fixed gonads were
213 dehydrated in ascending ethanol solutions, cleared with xylene and embedded in paraffin
214 wax. Five-micrometer thick sections were cut, mounted on glass slides, and stained with
215 Harry's hematoxylin-Eosin Y (Martoja et al., 1967). Slides were examined under a light
216 microscope to determine gametogenic stage according to the reproductive scale reported by
217 Mann (1979). In this scale, four stages are defined: stage 0 (inactive), stage 1 (early
218 gametogenesis), stage 2 (late gametogenesis) and stage 3 (ripe).

219 **2.5 Condition index**

220 To assess oyster-flesh dry weight, soft tissues were removed from shells and placed in a pre-
221 weighed aluminum cup. Shell and flesh were dried for 48h at 70°C and then weighed.

222 Condition index of individual oysters was then calculated as described previously (Lucas and
223 Beninger, 1985), following the formula: (g dry flesh weight / g dry shell weight) x 100.

224 **2.6 Digestive gland variables**

225 Just after dissection, digestive glands were immediately frozen in liquid nitrogen, weighed,
226 pooled (1 pool of 4 digestive glands per tank), and stored at -80°C until analysis. Later on,
227 pools were ground with a “Dangoumau” homogenizer into liquid nitrogen and divided for
228 three different analyses.

229 **2.6.1 Toxin content**

230 One gram of ground digestive gland was extracted in 2 ml of 0.1 N HCl (2 v/w) at 4°C. After
231 centrifugation (3,000 × g, 15 min, 4°C), the pH of extracts was adjusted. If above 3.0, pH was
232 adjusted to 3.0 with 12 N HCl. After half-dilution, supernatants were ultra-filtered (20 kDa,
233 Sartorius Centrisart) and stored at 4°C until analysis. PSTs were analyzed by ion-pairing,
234 high-performance liquid chromatography (IPHPLC) according to the method of Oshima
235 (1995). The molar concentration ($\mu\text{mol.l}^{-1}$) was converted into $\mu\text{g STX eq. } 100 \text{ g}^{-1}$ of
236 digestive gland by using the conversion factors of Oshima (1995). Results were expressed in
237 $\mu\text{g STX eq. } 100 \text{ g}^{-1}$ of digestive gland wet weight.

238 **2.6.2 Reserve lipid content**

239 Ground digestive gland (250 mg) were extracted in 6 ml of Folch solution
240 (chloroform:methanol 2:1). Lipid classes were analyzed by high-performance, thin-layer
241 chromatography (HPTLC) on HPTLC glass plates (1,010 mm) pre-coated with silica gel 60

242 from Merck (Darmstadt, Germany). A preliminary run was carried out to remove possible
243 impurities using hexane:diethyl ether (1:1), and the plate was activated for 30 min at 110°C.
244 Lipid samples (4 µl) were spotted on the plates by the CAMAG automatic sampler. The
245 neutral lipids were separated using a double development with hexane:diethyl ether:acetic
246 acid (20:5:0.5) as first solvent system followed with hexane:diethyl ether (93:3) as a second
247 solvent system. Lipid classes appeared as black bands after dipping plates in a cupric-sulfate,
248 phosphoric-acid solution and heating for 20 min at 160°C (charring). Seven neutral lipid
249 classes (categorized as storage lipids: free fatty acids, sterol esters, glycerid ethers,
250 monoacylglycerol, diacylglycerol and triacylglycerol; considered as structural lipids: sterols)
251 were identified based upon standard (Sigma–Aldrich, France) and coloring techniques. The
252 charred plates were read by scanning at 370 nm, and black bands were quantified by Wincats
253 software. Results were expressed as mg of each identified neutral lipid class per g of
254 digestive gland wet weight.

255 **2.6.3 Amylase activity**

256 Ground digestive gland (200 mg) was homogenized in 1 ml of distilled water and 200 µl of
257 this solution were added to 10 µl of 0.5 M CaCl₂ solution before analysis to assess amylase
258 activity. Amylase activity was then assayed by determination of starch hydrolysis according
259 to the iodine reaction (Samain et al., 1977) modified by Le Moine et al. (1997). One unit of
260 alpha-amylase was defined as the amount of enzyme that degrades 1 mg.min⁻¹ starch at 45°C.
261 To assess specific activities, total proteins were determined using the BCA Protein Assay
262 (Biorad). For protein extraction, 200 µl of the above solution were added to 200 µl of 2 N
263 NaOH solution. Protein analysis was carried out on 10 µl of 1/10 diluted samples according
264 to the manufacturer's description. Briefly, 200 µl of dye reagent was added to 10 µl of
265 sample, incubated at 37°C for 1 hour and the absorbance was measured at 595 nm. Sample
266 ODs were compared to a standard curve of Bovine Serum Albumin (BSA), and results were

267 expressed as mg of protein.ml⁻¹. Amylase activity was expressed as UI of amylase activity
268 per mg of total protein (specific activity).

269 **2.7 Hemolymph variables**

270 **2.7.1 Hemolymph sampling**

271 Hemolymph was withdrawn from individual oysters using a 1 ml plastic syringe fitted with a
272 25-gauge needle inserted through a notch made adjacent to the adductor muscle just prior to
273 bleeding. All hemolymph samples were examined microscopically for contamination (e.g.,
274 gametes, tissue debris) and then stored in micro-tubes held on ice. As recommended by flow
275 cytometer (FCM) manufacturer, all samples were filtered through 80 µm mesh prior to
276 analysis to eliminate any large debris (> 80 µm) which could potentially clog the flow
277 cytometer. Three hundred microliters (3 measures x 100 µl) of each hemolymph sample were
278 used to measure hemocyte variables by flow cytometry. The remaining hemolymph was
279 separated into cellular (hemocytes) and supernatant (plasma) fractions by centrifugation
280 (800×g, 5 min, 4°C) prior to freezing (-20°C). These samples then were used to measure
281 biochemical hemocyte and plasma variables (protein content, phenol-oxydase activity and
282 hemolysis/agglutination titers). Methods for measuring cellular (hemocyte) and humoral
283 (plasma) variables are described hereafter.

284 **2.7.2 Measurements of hemocyte variables by flow cytometry**

285 Characterization of hemocyte sub-populations, number and functions were performed using a
286 FACScalibur (BD Biosciences, San Jose, CA USA) flow cytometer (FCM) equipped with a
287 488 nm argon laser. Two kinds of hemocyte variables were evaluated by FCM: descriptive
288 variables (hemocyte viability and total and hemocyte sub-population counts), and functional
289 variables (phagocytosis and reactive oxygen species (ROS) production). Analyses were done
290 as described below.

291

292 *Descriptive variables: Hemocyte viability, total and hemocyte sub-population counts*

293 These variables were measured individually on hemolymph samples (4 individuals per tank).

294 An aliquot of 100 μ l of hemolymph from an individual oyster was transferred into a tube

295 containing a mixture of Anti-Aggregant Solution for Hemocytes, AASH (Auffret and

296 Oubella, 1995) and filtered sterile seawater (FSSW), 200 μ l and 100 μ l respectively.

297 Hemocyte DNA was stained with two fluorescent DNA/RNA specific dyes, SYBR Green I

298 (Molecular probes, Eugene, Oregon, USA, 1/1000 of the DMSO commercial solution), and

299 propidium iodide (PI, Sigma, St Quentin Fallavier, France, final concentration of 10 μ g.ml⁻¹)

300 in the dark at 18°C for 120 minutes before flow-cytometric analysis. PI permeates only

301 hemocytes that lose membrane integrity and are considered to be dead cells; whereas, SYBR

302 Green I permeates both dead and live cells. SYBR Green and PI fluorescences were

303 measured at 500-530 nm (green) and at 550-600 nm (red), respectively, by flow-cytometry.

304 Thus, by counting the cells stained by PI and cells stained by SYBR Green, it was possible to

305 estimate the percentage of viable cells in each sample. All SYBR Green-stained cells were

306 visualized on a Forward Scatter (FSC, size) and Side Scatter (SSC, cell complexity)

307 cytogram. Three sub-populations were distinguished according to size and cell complexity

308 (granularity). Granulocytes are characterized by high FSC and high SSC, hyalinocytes by

309 high FSC and low SSC, while agranulocytes have low FSC and SSC. Total hemocyte,

310 granulocyte, hyalinocyte, and agranulocyte concentrations estimated from the flow-rate

311 measurement of the flow-cytometer (Marie et al., 1999) as all samples were run for 30 sec.

312 Results were expressed as number of cells per milliliter of hemolymph.

313

314 *Functional variables*

315 These variables were measured individually on hemolymph samples, for each condition.

316

317 Phagocytosis

318 An aliquot of 100 µl hemolymph, diluted with 100 µl of FSSW, was mixed with 30 µl of YG,
319 2.0-µm fluoresbrite micro-spheres, diluted to 2% in FSSW (Polysciences, Eppelheim,
320 Germany). After 120 minutes of incubation at 18°C, hemocytes were analyzed at 500-530 nm
321 by flow cytometry to detect hemocytes containing fluorescent beads. The percentage of
322 phagocytic cells was defined as the percentage of hemocytes that had engulfed three or more
323 beads (Delaporte et al., 2003).

324

325 Reactive oxygen species production

326 Reactive oxygen species (ROS) production by untreated hemocytes was measured using 2',7'-
327 dichlorofluorescein diacetate, DCFH-DA (Lambert et al., 2003). A 100-µl aliquot of pooled
328 hemolymph was diluted with 300 µl of FSSW. Four µl of the DCFH-DA solution (final
329 concentration of 0.01 mM) was added to each tube maintained on ice. Tubes were then
330 incubated at 18°C for 120 minutes. After the incubation period, DCF fluorescence,
331 quantitatively related to the ROS production of untreated hemocytes, was measured at 500-
332 530 nm by flow-cytometry. Results are expressed as the geometric-mean fluorescence (in
333 arbitrary units, AU) detected in each hemocyte sub-population.

334 **2.7.3 Biochemical hemocyte and plasma variables**

335

336 *Hemocyte and plasma phenoloxidase activities*

337 Plasma samples were thawed on ice, and 100 µl of each was transferred in ninety-six-well
338 plates. For hemocytes, cells were suspended in 100 µl of FSSW and frozen and thawed on ice
339 three times successively. Phenoloxidase activity was measured as described by Reid (2003).
340 Briefly, 50 µl of Tris-HCl buffer (0.2M, pH = 8) and 100 µl of l-DOPA (20 mM, L-3,4-

341 dihydrophenyl-alanine, Sigma D9628) were added to each well. The micro-plate was rapidly
342 mixed for 10 s. The reaction was then measured at ambient temperature, with color change
343 recorded every 5 min, at 492 nm, over a period of 1 h. The micro-plate was mixed prior to
344 each measurement. Two controls, without sample but containing l-DOPA and Tris–SDS
345 buffer, were measured in parallel, and these values were subtracted from test values to correct
346 for possible auto-oxidation of the l-DOPA.

347 To access phenoxidase specific activity, protein analysis was carried out as described for
348 digestive glands (see paragraph 2.6.3), except that proteins were not extracted with NaOH
349 and samples were not diluted. Results were expressed as phenoxidase-specific activity
350

351 *Agglutination and hemolysis titers in plasma*

352 Agglutination titer (indicative of the presence of lectins) and hemolysis titer (indicative of red
353 blood cell lysis factors) were measured on a sub-sample of plasma (supernatant) fraction.
354 Quantification of agglutination titer was performed according to the protocol from Barracco
355 et al. (1999), using horse red blood cells. Briefly, 50- μ l plasma samples were added to U-
356 shaped wells of 96-well-microtiter plates, and a two-fold, serial dilution (pure solution to 1/2
357 dilution) was prepared using Tris-buffered saline (containing 0.15 M NaCl). The same
358 volume of a 2% suspension of horse red blood cells in TBS was added to each well and
359 incubated for 3 h at room temperature. In controls, oyster plasma was replaced with TBS.
360 Agglutination titer and hemolysis titer were expressed as the log (base 2) of the reciprocal of
361 the highest dilution showing a positive pattern of agglutination or hemolysis of red cells,
362 respectively.

363

364 **2.8 Statistical analysis**

365 Differences between experiments (April and May) were assessed using Student's T-test.
366 Results of each experiment were analyzed statistically using Multifactor-ANOVA
367 (MANOVA) for each physiological parameter and hemocyte variable as the dependent
368 variable, and feeding treatment and ploidy as independent variables. Whenever a clear trend
369 appeared on the graphs, a Student's T-test was also used within ploidy groups to assess
370 differences linked to dietary treatment for a dependent variable. We used Statgraphics Plus
371 statistical software (Manugistics, Inc, Rockville, MD, USA). Results were considered
372 significant when the P-value was < 0.05 .

373

374 **3 Results**

375 **3.1 Gonad maturation stages**

376 Oysters fed *T.Iso* and *A. minutum* were combined to assess oyster gonad maturation (Fig. 1).
377 Maturation of both diploid and triploid oysters was more advanced during the May
378 experiment than during April. In both experiments, triploids were less mature than diploids.
379 Oyster groups in both experiments can be classified according to gonad maturation stage
380 (from less mature to more mature) first were triploids in April, triploids in May, then diploids
381 in April, and at last diploids in May.
382 Triploids in the April experiment were mostly at the undifferentiated stage, and one third
383 were in early and late gametogenesis; triploids in the May experiment were in early and late
384 gametogenesis, diploids in the April experiment were dominated by late gametogenesis with
385 20% mature oysters; and finally, diploids in the May experiment were mainly (60%)
386 observed to be sexually mature while 40% were in late gametogenesis.

387 **3.2 Wet weight of digestive gland and condition index**

388 There was no significant difference, in term of condition index (CI), attributable to diet or
389 ploidy in both experiments (Table 1). Whole-oyster dry weight (DW) was significantly
390 higher in diploid oysters than in triploid oysters for both experiments (in April $p=0.0045$ and
391 in May $p=0.0021$, MANOVA). DW of both diploid and triploid oysters did not change
392 significantly between the two experiments. Wet weights (WW) of digestive glands were
393 similar in the April experiment regardless of diet or ploidy. In the May experiment, mean
394 WW of digestive gland was significantly higher in diploids than in triploids. Exposure to *A.*
395 *minutum* in this experiment resulted in a significant decrease in digestive gland WW
396 compared to *T.Iso* feeding. A significant interaction between ploidy and diet was also noted;
397 lower digestive gland WW was found in triploid oysters exposed to *A. minutum*.

398 **3.3 Toxin content**

399 PST content in digestive gland was significantly higher in May than in April ($p=0.003$, T-
400 test). In both experiments, triploid oysters accumulated more toxin -- about twice -- than
401 diploids (Fig. 2); April experiment $p=0.032$ and May experiment $p=0.047$, T-test).
402 Concomitantly, *A. minutum* cells were observed in digestive gland and bio-depots.

403 **3.4 Digestive gland parameters**

404 **3.4.1 Reserve lipid content**

405 Neutral lipid classes detected in digestive glands were free fatty acids, sterol esters, ether
406 glycerides, sterols, monoacylglycerols, diacylglycerols and triacylglycerols (Table 2). A
407 reserve/structure ratio was determined as the ratio between reserve lipids (sterol ester +
408 glycerid ether + triacylglycerol content) and a structural lipid (sterol content).

409

410 In both experiments, monoacylglycerol, diacylglycerol and free fatty-acid contents (Fig. 3)
411 were significantly lower in both diploid and triploid oysters fed *A. minutum* as compared to
412 those fed *T.Iso* (Table 2). In May, contents of sterols, triacylglycerols, and sterol esters, as
413 well as the reserve/structure ratio, were higher in diploids than in triploids.

414

415 In April, the reserve/structure ratio was significantly lower (Fig. 4) in triploid oysters exposed
416 to *A. minutum* as compared to triploids fed *T.Iso* ($p=0.0427$, T-test). Exposure to *A. minutum*
417 similarly resulted in a significant decrease of this ratio in diploids in May ($p=0.0067$, T-test).

418 **3.4.2 Amylase-specific activity**

419 Amylase-specific activity (ASA) was significantly higher in May than in April ($p=0.0222$, T-
420 test) (Fig. 5). In both experiments ASA was higher in triploids than in diploids (April
421 experiment $p=0.0263$; May experiment $p=0.0134$, MANOVA). ASA was higher in *A.*
422 *minutum*-exposed than in control, diploid oysters in April ($p=0.0467$, T-test), but was similar
423 in exposed and non-exposed triploid oysters. In May, only triploid oysters showed a
424 significant increase of ASA upon *A. minutum* exposure ($p= 0.0337$, T-test).

425 **3.5 Hemocyte and plasma variables**

426 Overall, ploidy had more significant impacts on hemocyte and plasma variables than algal
427 exposure (Table 3).

428 **3.5.1 Hemocyte characteristics and functions analyzed by flow cytometry**

429 Total hemocyte concentration (THC) was significantly higher in May than in April ($p=0$, T-
430 test). THC increased significantly upon *A. minutum* exposure in diploid oysters in the April
431 experiment ($p=0.013$, T-test) and triploid oysters in the May experiment ($p=0.042$ with
432 $\alpha=0.05$, T-test) (Fig. 6). This increase was mainly attributable to variation in granulocyte

433 counts, especially in April when granulocyte counts drastically increased upon *A. minutum*
434 exposure.

435 Sizes (FSC) of granulocytes and hyalinocytes (Fig. 6) of oysters in the April experiment were
436 significantly higher in triploids than in diploids, but hemocyte size was not affected by *A.*
437 *minutum* exposure. In May, size and complexity (SSC) of both granulocytes and hyalinocytes
438 were higher in triploids than in diploids. In the same experiment, *A. minutum* resulted in a
439 significant increase in granulocyte and hyalinocyte size and in hyalinocyte complexity.

440 ROS production in granulocytes and hyalinocytes (Fig. 6) was significantly higher in May
441 than in April ($p=0.0002$ for granulocytes and $p=0.0011$ for hyalinocytes, T-test). In both
442 experiments, granulocytes of triploids produced more ROS than granulocytes of diploids
443 ($p=0.0034$ in April and $p=0.0012$ in May, MANOVA). The same difference was observed for
444 hyalinocytes but was only significant in April ($p=0.0215$, MANOVA).

445 *A. minutum* exposure resulted in opposite effects in the two experiments. In the April
446 experiment, granulocytes of oysters fed *A. minutum* produced more ROS than those of
447 control oysters ($p=0.0119$, MANOVA). In the May experiment, granulocytes and
448 hyalinocytes of oysters fed *A. minutum* produced less ROS than those of control oysters
449 ($p=0.0067$ and $p=0.0358$ respectively, MANOVA).

450 Neither phagocytosis nor percentage of dead cells was affected by algal exposure or ploidy.

451 **3.5.2 Hemocyte and plasma phenoloxidase (PO) activities**

452 PO in plasma was higher in April than in May ($p=0.001$, T-test). In April, PO in plasma
453 decreased in *A. minutum*-exposed oysters, significantly only for diploids ($p=0.008$, T-test).

454 There were no significant variations in PO in plasma in May. In hemocytes, PO (Fig. 7) was
455 significantly higher in May than in April ($p=0.0152$, T-test). PO in hemocytes was higher in
456 triploids than in diploids in both April and May experiments (respectively $p=0.0108$ and
457 $p=0.046$, MANOVA). In April, PO in hemocytes was lower in oysters fed *A. minutum* than in

458 control oysters ($p=0.0312$, MANOVA). In May, PO in hemocytes was higher in diploids fed
459 *A. minutum* as compared to control diploids ($p=0.0189$, T-test) and lower in triploids
460 ($p=0.0458$, T-test).

461 **3.5.3 Agglutination and hemolysis**

462 There were no significant differences in agglutination or hemolysis titer according to algal
463 exposure or ploidy.

464 **4 Discussion**

465 The effects of *Alexandrium minutum* exposure for 4 days, specifically on toxin accumulation
466 and several physiological parameters, were evaluated in diploid and triploid Pacific oysters,
467 *Crassostrea gigas*. Although triploid oysters are increasingly used for aquaculture, it was
468 unknown how triploidy may affect toxin accumulation and physiological responses to
469 harmful algal blooms. Two experiments were conducted during two consecutive months to
470 assess the possible impact of reproductive stage on toxin accumulation and physiological
471 responses to *A. minutum* exposure. As *A. minutum* toxins are released in oyster digestive
472 glands, this organ could be expected to be impacted. The second physiological compartment
473 expected to be impacted by *A. minutum* exposure was the circulatory system, containing
474 hemocytes.

475 The most striking result in these two experiments was the difference in PST accumulation in
476 digestive glands between diploid and triploid oysters. In both experiments, triploid oysters
477 accumulated twice the toxin of diploids. One could be quick to attribute higher toxin content
478 in triploids to lower gametogenesis, but this hypothesis can be rejected as oysters in the May
479 experiment had both more-advanced gonad development and higher toxin contents than
480 oysters in the April experiment. Gametogenetic stage is not, however, the only trait that
481 distinguishes triploid from diploid oysters. The augmentation in genetic material and gene

482 copies in triploids also has physiological implications by changing heterozygosity. This
483 increased heterozygosity in triploids can lead to additive and non-additive effects upon gene
484 expression (Riddle et al., 2006; Johnson et al., 2007). Indeed, in parallel to toxin
485 accumulation, amylase activity, ROS production, and phenoloxidase activities were found to
486 be higher in triploid oysters than in diploids. Esterase and and peroxidase activities were also
487 found to be higher in triploid oysters than in diploid oysters (Gagnaire et al., 2007). This
488 tends to support the hypothesis that triploids accumulate more toxin than diploids because
489 they are metabolically more active. As triploid heterozygosity is higher, it is thought to have
490 positive influences on feeding rate, absorption efficiency, and growth efficiency (Magoulas et
491 al., 2000). Thus, one can speculate that the difference in toxin accumulation may reflect
492 differences in metabolic and/or feeding activities between diploid and triploid oysters.

493
494 The increase in toxin accumulation between April and May experiments is also possibly a
495 result of an increase in feeding and digestive activities. Considering together *A. minutum*-
496 exposed and non-exposed oysters, amylase activity, ROS production, and PO activity were
497 found to be higher in May than in April. Even though oysters in both experiments originated
498 from the same stock and were acclimated for 7 days prior to exposure to *A. minutum*, an
499 additional month in field rearing conditions appeared to impart subsequent physiological
500 status, including reproductive processes in the conditioned oysters. Indeed, during this
501 additional month, temperature increased from 11 to 15°C and photo-period increased from
502 13h06 per day on 5 April to 14h38 per day on 2 May. These temperature and photo-period
503 changes allowed diploid oysters to develop from early gametogenesis to a large proportion of
504 mature gonads within one month. This is in good agreement with the study of Fabioux et al.
505 (2005). The percentage of gonad occupation in *C.gigas*, in field conditions, increased from
506 15% to 50% between April and May (Fabioux et al., 2005). Gonad development and energy

507 allocation in the scallop *Pecten maximus* were shown to be modulated by temperature and
508 photoperiod (Saout et al, 1999). Similarly Fabioux et al. (2005) experimentally demonstrated
509 that the gametogenic cycle of *C. gigas* can be controlled by coupled modifications of
510 temperature and photoperiod. In marine invertebrates, it is postulated that the nervous system
511 under environmental influences has an effect on the endocrine regulation of reproduction
512 (Olive, 1995, Lafont, 2000). In bivalves, fluctuations of neurotransmitters (monoamines)
513 appeared to be related to seasonality and reproductive cycle (Lopez-Sanchez et al., 2009).
514 Although specific functions of monoamines are not clearly identified yet, they are thought to
515 be involved in meiosis re-initiation in bivalve oocytes (Guerrier et al., 1993), control of
516 ciliary movement (Carroll and Catapane, 2007), or spawning induction (Velez et al., 1990,
517 Fong et al. 1996, Velasco et al., 2007).

518 In the present study, the general increase in measured physiological parameters is certainly
519 determined by environmental conditions (temperature, salinity and food in the field) and we
520 speculate that metabolism is further maintained during the experiment possibly through
521 endocrine control. From all the above, we infer that increasing toxin accumulation paralleled
522 increasing metabolic and physiological activities determined by ploidy and preceding field
523 conditions.

524

525 Major changes in free fatty acids (FFA), monoacylglycerols (MAG) and diacylglycerols
526 (DAG) contents in the oyster digestive gland were clearly attributable to algal exposure. In
527 both experiments, concentrations of FFA, MAG and DAG were reduced upon *A. minutum*
528 exposure in both diploids and triploids. These compounds are generally absent in gonad and
529 muscle, and only transiently observed in digestive glands of oysters (Soudant et al., 1999).
530 The biological significance of FFA, DAG and MAG contents is still unclear as little has been

531 published on the subject. These lipid classes are thought to be intermediate products in the
532 synthesis or catabolism of both structural and reserve lipids.

533 Upon exposure to *A. minutum*, the ratio of reserve lipids (TAG, EGLY and SE) to structural
534 lipids (sterols) decreased in both experiments. This depletion of reserve lipids upon *A.*
535 *minutum* exposure, however, appeared to partially depend upon maturation stage. The most-
536 drastic decrease upon *A. minutum* exposure occurred when oyster gametogenesis was almost
537 absent (April experiment triploids) or predominantly terminated (May experiment diploids).
538 In these physiological conditions, more reserve lipids resided in the digestive gland, where
539 these energy reserves are potentially available to respond to stressful conditions. In contrast,
540 when oysters were in late gametogenesis (April experiment diploids and May experiment
541 triploids), reserve lipids were likely intensively transferred to the gonad and may thus be only
542 slightly affected by *A. minutum* exposure.

543 As FFA, MAG and DAG concomitantly decreased with the reserve/structure ratio, we
544 speculate that *A. minutum* negatively affects digestion of dietary lipids and/or synthesis of
545 storage lipids. Also, it has to be noted that contents of TAG and EGLY in *A. minutum*-
546 exposed oysters decreased as toxin accumulation increased according to oyster groups.

547 Nevertheless, at this stage, it is difficult to establish clear relationships between reserve lipid
548 changes and toxin accumulation. To further progress on these aspects, it would be interesting
549 to combine this biochemical information with histological analyses.

550 Amylase-specific activity (ASA) was also modulated upon *A. minutum* exposure. Overall,
551 ASA was higher in oysters exposed to *A. minutum* than in *T.Iso*-fed oysters, but also higher
552 in triploids than in diploids. As mentioned earlier, ASA, along with other metabolic activities,
553 could partially explain the differences in toxin accumulation between diploids and triploids
554 and between April and May experiments. Digestive-enzyme activities are important in
555 maximizing absorption and food conversion efficiencies (Huvet et al., 2003). In *C. gigas*, a

556 positive correlation has been established between high specific amylase activity and high
557 food assimilation (Prudence et al., 2006). In the present study, digestive-enzyme activities
558 were likely controlled by physiological status, which varied with ploidy and times of
559 experiments. Thus, we speculate that higher amylase activity would result in higher *A.*
560 *minutum* digestion and toxin release and assimilation.

561
562 These results highlight the complexity of relationships between oyster physiology and toxin
563 accumulation. Toxin accumulation certainly depends upon the physiological status and
564 metabolic activities of the oysters; concomitantly, toxin accumulation can interfere with the
565 same physiological processes.

566
567 Regarding hemocyte variables, changes upon *A. minutum* exposure were mainly observed in
568 hemocyte cell density, phenoloxidase, and ROS production. Except in mature oysters
569 (diploids in experiment 2), *A. minutum* exposure resulted in increases in numbers of
570 circulating hemocytes. Increase in total circulating hemocyte counts is generally considered
571 to be an immune response to pathogens (Chu et al., 1993; Chu and La Peyre, 1993; Ford et
572 al., 1993; Anderson et al., 1995). Many toxic chemicals can modulate densities of circulating
573 hemocytes, which may increase or decrease according to chemical characteristics and
574 concentrations (Auffret et al., 2002; Gagnaire, 2005; Auffret et al., 2006). Further,
575 modulation of hemocyte counts upon *A. minutum* exposure appeared to vary according to
576 maturation stage of the oysters. Indeed, mature diploid oysters responded in an opposite
577 manner compared to other oyster groups by decreasing hemocyte count upon *A. minutum*
578 exposure. Ripe oysters are known to be especially sensitive to stress and to summer mortality
579 (Samain et al., 2007). *A. minutum* exposure, serving as an additional stress to reproductive
580 effort, may have re-enforced the decrease in hemocyte counts occasionally observed in fully-

581 mature oysters (Delaporte et al., 2006). This makes changes of hemocyte concentration
582 difficult to interpret. The strongest increase in hemocyte concentration occurred in the oyster
583 groups showing the strongest increase in amylase activity upon *A. minutum* exposure.
584 Hemocytes have been thought for a long time to be involved in digestion processes and
585 digestive activities (Cheng, 1996). The observed parallel between hemocyte counts and
586 amylase activities may simply reflect involvement of hemocytes in microalgal digestion and
587 nutrient assimilation.

588 Reactive oxygen species (ROS) production and phenoloxidase (PO) specific activity in
589 circulating cells were both affected by *A. minutum* exposure. ROS production is associated
590 with internal chemical destruction of engulfed pathogens or foreign particles within
591 hemocytes (Cheng, 1996; Cheng, 2000; Chu, 2000). Hemocyte ROS production may also be
592 activated by high reproductive effort, leading some to consider reproductive activity as a
593 physiological stress (Delaporte et al., 2006; Delaporte et al., 2007). The elevated energy
594 demand for gamete production leads to a marked increase of whole-animal oxygen
595 consumption during gametogenesis, corresponding to an elevated basal metabolism
596 (Shumway et al., 1988). ROS production was thus hypothesized to reflect an oxidative stress
597 during periods of high energy expenditure, such as active gametogenesis. ROS production
598 was previously observed to increase in ripening oysters, especially in oysters known to be
599 genetically more sensitive to summer mortalities (Delaporte et al., 2007). In the present
600 study, ROS production was two times higher in oysters in the May experiment than in the
601 April experiment. This suggests that ROS production may reflect increases in metabolic
602 activities and energy expenditure formerly “programmed” by temperature and photo-period
603 conditions of their rearing site. Temperature and photo-period may accelerate metabolic
604 activities, even for triploid oysters which produced only few mature gametes and remained on
605 average at intermediate stages of gametogenesis.

606 Upon *A. minutum* exposure, hemocyte ROS production significantly increased in the April
607 experiment and significantly decreased in the May experiment, similarly in diploid and
608 triploid oysters. In the April experiment, the increase in ROS production may reflect an
609 increase in metabolic activities responding to *A. minutum* exposure. On the contrary, in the
610 May experiment, we speculate that higher toxin accumulation exceeded the “tolerance” of
611 oysters, affecting directly and more profoundly physiological and metabolic activities leading
612 to reduced ROS production. At this point, we can only confidently conclude that changes in
613 ROS production upon *A. minutum* exposure do not depend directly upon reproductive
614 activities, as near fully-matured diploid oysters responded similarly to early-maturing triploid
615 oysters. Also, as oysters in the May experiment accumulated twice the toxin of those in the
616 April experiment, we cannot disprove the possibility that released toxins may act as a
617 stimulant when present at low levels, while resulting in inhibitory effects when accumulated
618 at higher concentrations. Similar observations were made in ecotoxicological studies
619 assessing impacts of toxic chemicals upon immune functions in bivalves (Auffret and
620 Oubella, 1997; Fournier et al., 2001; Auffret et al., 2002; Gagnaire et al., 2006). Several
621 heavy metals were demonstrated to have a stimulatory effect upon hemocyte counts,
622 chemotaxy, and mobility in field study. In contrast, in experimental studies, high
623 concentrations of the same heavy metals were inhibitory to the same or related immune
624 parameters (Auffret et al., 2002).

625 Phenoloxidase is activated by microbial substances and is thought to have a role in host
626 defense in *C. gigas* (Hellio et al., 2007). PO specific activity was drastically reduced upon *A.*
627 *minutum* exposure in both diploid and triploid oysters in the April experiment, but PO
628 increased in diploids and decreased in triploids in the May experiment. Thus, mature diploid
629 oysters respond in an opposite manner, compared to other oyster groups, to *A. minutum*
630 exposure. *A. minutum* challenge seems to decrease the activity of PO in hemocytes from

631 oysters at early and intermediate maturation stages, and to increase activity in oysters with
632 advanced maturation. Notably, PO activity decreased upon *A. minutum* exposure when
633 concomitantly hemocyte counts increased. This suggests that hemocytes newly mobilized in
634 the circulatory system may not be fully functional and are less able to produce PO activity.
635 Triploid oyster hemocytes were significantly larger (higher FSC) than those of diploid
636 hemocytes, possibly because of their higher nuclear DNA content. Additionally, triploid
637 oyster hemocytes were also more complex (higher SSC) than diploid hemocytes. As cell
638 complexity is related to granule content, higher granule content in triploid hemocytes may
639 parallel the higher hemocyte activities (ROS production as well as PO activity).
640 Triploid oysters were previously reported to have statistically-higher hemocyte phagocytosis,
641 esterase, and peroxidase activities than diploid oysters (Gagnaire et al., 2007). If higher
642 hemocyte chemical activities can confer a better tolerance to toxin accumulation, then triploid
643 oysters may have a better ability to respond to *A. minutum* exposure than diploid oysters. As
644 low toxin sensitivity results in high toxin accumulation (Bricelj and Shumway, 1998),
645 triploids would be able to accumulate more toxin than diploids. Triploid hemocyte size and
646 complexity were reduced upon *A. minutum* exposure in the May experiment but not in the
647 April experiment. We therefore speculate that toxin accumulation was high enough to affect
648 cell physiology, possibly through cell degranulation, upon toxin exposure.

649

650 To summarize, hemocyte responses to *in vivo* *A. minutum* exposure depended upon
651 reproductive status, toxin accumulation, but also upon oyster physiological status in the field
652 prior to collection for experiments. When considering only *A. minutum*-exposed oysters,
653 positive linear correlations between PST accumulation and both hemocyte ROS production
654 (granulocytes: $R^2= 0.55$, $p<0.01$; hyalinocytes: $R^2= 0.6$, $p< 0.005$) and PO activity (total
655 hemocytes: $R^2= 0.74$, $p< 0.005$) were found (data not shown). These relationships indicate

656 that these activities can be considered as good “markers” of metabolic activity useful in
657 interpretation of physiologically-dependent differences in toxin accumulation. As stated
658 above, however, these oxidative activities also may be modulated by other sources of stress,
659 such harmful algae. Thus, it is difficult to unravel the respective influences of physiological
660 status *vs* stress encountered by oysters.

661
662 Bivalve physiological responses to *Alexandrium* spp. exposure often are thought to be related
663 to toxins (saxitoxin and derivatives) affecting human health. It is not known, however, if these
664 compounds are the ones affecting bivalves, or if other compounds produced by harmful algae
665 can affect shellfish at a greater extent. Lush et al. (1997) reported that juvenile greenback
666 flounder (*Rhombosolea taparina*) exposed to an *A. minutum* whole cell suspension showed
667 gill damage characterized by severe epithelial swelling that was not related to PSTs. In
668 addition to this ichthyotoxic effect of *A. minutum*, it has been reported that *A. minutum*
669 showed potent toxic effects upon brine shrimp (*Artemia salina*) (Lush et al., 1996) and a
670 harpacticoid copepod (*Euterpina acutifrons*) (Bagoien et al., 1996), independently of
671 paralytic-toxin effects. Moreover, Ford et al. (2008) found no measurable effect of a PST-
672 producing strain of *Alexandrium tamarense* on hemocytes of two bivalve species. Instead,
673 extract from a non-PST-producing strain had a strong and consistent negative effect on
674 hemocytes from two clam species, resulting in significantly-lower adherence and
675 phagocytosis compared to a PST-producing strain and filtered seawater controls. These
676 studies allow us to suggest that PSTs are not the only compounds responsible of *A. minutum*
677 effects upon oyster physiology, but other active compounds are likely bio-active as well.

678

679 **5 Conclusion**

680 The most striking result of this study was the difference in PST accumulation between diploid
681 and triploid oysters: triploids accumulate about twice the toxin as diploids. This difference
682 may be attributable to differences in physiology linked to ploidy, especially during
683 reproduction. This finding can have important implications, in terms of oyster production and
684 risk management.

685 Despite the finding that *A. minutum* exposure was not lethal to oysters, exposure to a toxin-
686 producing microalga can significantly impact oyster physiology, as compared to non-toxic
687 algae (*T.Iso*). *A. minutum* exposure affected several digestive and hematological parameters,
688 and these responses were modulated by ploidy and maturation stage. Indeed, for some
689 physiological parameters such as phenoloxydase activity and hemocyte concentration, ripe
690 oysters responded in an inverted manner as compared to maturing oysters. We highlight,
691 however, that observed effects of *A. minutum* were not only related to PSTs, but likely also to
692 other bioactive compounds produced by *A. minutum*.

693 Results of the present study showed that analyses of digestive-gland activities and
694 composition (neutral lipids for example) can provide information on effects of *A. minutum*
695 exposure upon oysters. Also, it could be productive to investigate other lipid classes, such as
696 cell membrane constituents (polar lipids). Finally, biochemical approaches developed here
697 can be complementary to histo-pathological observations as such methods have been
698 successfully applied to assess impacts of toxic microalgae upon mollusks (Galimany et. al,
699 2008).

700

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708

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- 958

959 **Figure captions**

960 **Table 1:** Oyster condition index, body dry weight and digestive gland wet weight according
 961 to ploidy and microalgal exposure, in April and May experiments. This table also includes the
 962 results of the MANOVAs testing ploidy and microalgal exposure effects separately in both
 963 experiments.

964
 965 **Table 2:** Neutral lipid class contents (expressed as mg.g^{-1} of tissue wet weight) in oyster
 966 digestive glands according to ploidy and microalgal exposure, in April and May experiments.
 967 This table also includes the results of the MANOVAs testing ploidy and microalgal exposure
 968 effects separately in both experiments.

969
 970 **Table 3:** Effects of ploidy and microalgal exposure on oyster hemocyte and plasma variables,
 971 tested by MANOVA in April and May experiments.

972
 973 **Fig. 1:** Oyster maturation stages (expressed as %, $n = 12$ oysters) according to ploidy in April
 974 (A) and May (B) experiments, regardless of dietary conditioning (*T.Iso* or *A. minutum*).
 975 Stages 0, 1, 2 and 3 correspond to reproductively inactive, early gametogenesis, late
 976 gametogenesis and ripe, respectively.

977
 978 **Fig. 2:** PST content (mean of 3 pools of 4 oysters each, as $\mu\text{g STX equiv. } 100 \text{ g}^{-1}$ of tissue
 979 wet weight, \pm CI) in digestive gland of diploid and triploid oysters exposed to *A. minutum* in
 980 April (A) and May (B) experiments.

981
 982 **Fig. 3:** Sum of monoacylglycerol (MG), diacylglycerol (DG) and free fatty acid (FFA)
 983 contents (mean of 3 pools of 4 oysters each, as mg.g^{-1} of tissue wet weight, \pm CI) in oyster
 984 digestive gland according to ploidy and microalgal exposure, in April (A) and May (B)
 985 experiments. α indicates statistically significant difference according to microalgal exposures
 986 (MANOVA).

987
 988 **Fig. 4:** Ratio between reserve (sterol ester + glycerid ether + triacylglycerol content) lipids
 989 and structural (sterol content) lipids (\pm CI) in oyster digestive gland according to ploidy and
 990 microalgal exposure, in April (A) and May (B) experiments. # and α indicate statistically
 991 significant differences according to ploidy or microalgal exposure, respectively (MANOVA);
 992 * indicates statistically significant difference according to microalgal exposure (T-test).

993
 994 **Fig. 5:** Amylase-specific activity expressed as amylase activity as IU per mg of total protein
 995 (mean of 3 pools of 4 oysters each, \pm CI) in oyster digestive gland according ploidy and
 996 microalgal exposure, in April (A) and May (B) experiments. # indicates statistically

997 significant difference according to ploidy (MANOVA); * indicates statistically significant
998 difference according to microalgal exposure (T-test).

999

1000 **Fig. 6:** Total hemocyte count (cells.ml⁻¹), ROS production in granulocytes (AU), and size of
1001 hyalinocytes (AU) according to ploidy and microalgal exposure in April (A) and May (B)
1002 experiments (mean of 12 individual oysters, ± CI). # and α indicate statistically significant
1003 differences according to ploidy and microalgal exposure, respectively (MANOVA); *
1004 indicates statistically significant difference according to microalgal exposure (T-test). AU:
1005 Arbitrary unit.

1006

1007

1008 **Fig. 7:** Specific activity (SA) of phenoloxidase (PO) expressed as PO activity (IU) per mg of
1009 total protein in hemocytes (mean of 12 individual oysters ± CI) according to ploidy and
1010 microalgal exposure, in April (A) and May (B) experiments. # and α indicate statistically
1011 significant differences according to ploidy and microalgal exposure, respectively
1012 (MANOVA); * indicates statistically significant difference according to microalgal exposure
1013 (T-test).

1014

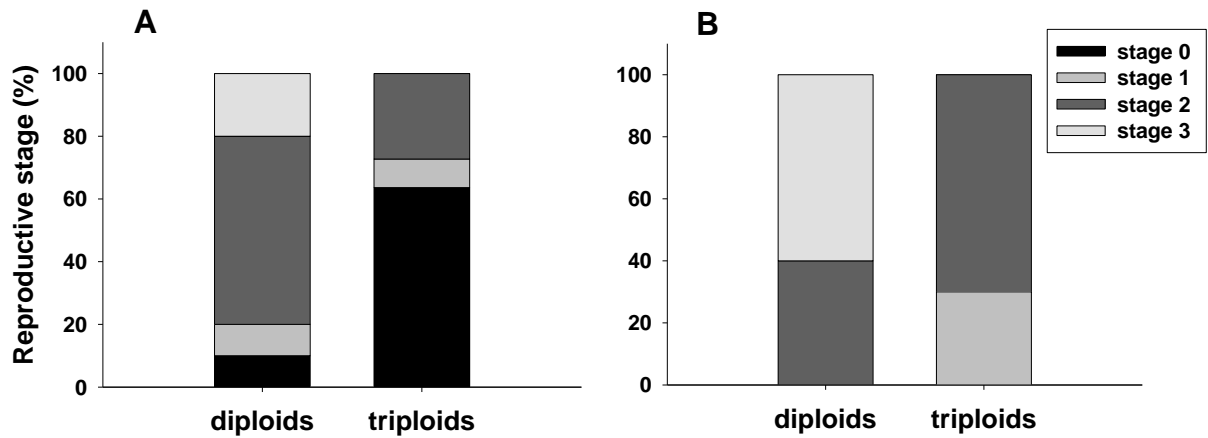


Fig. 1

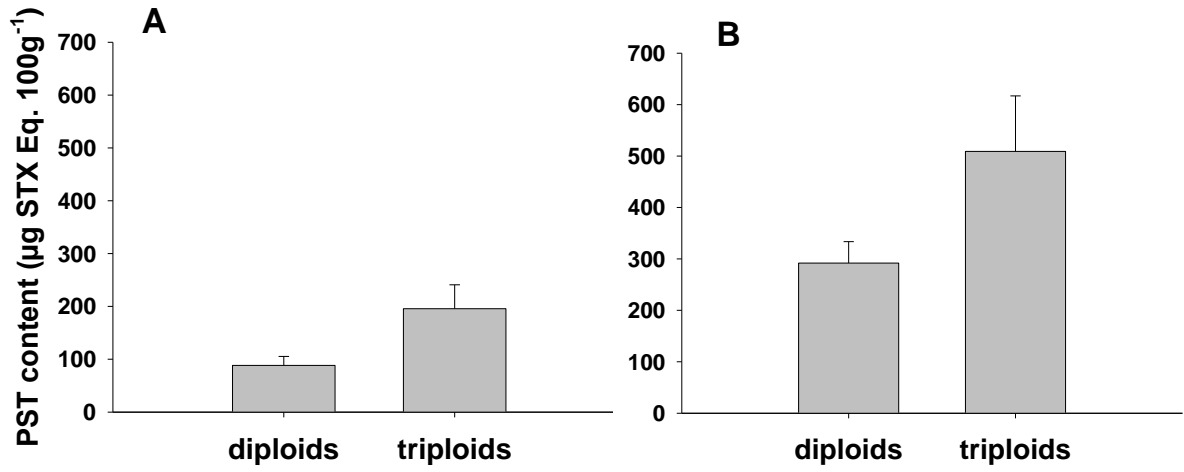


Fig. 2

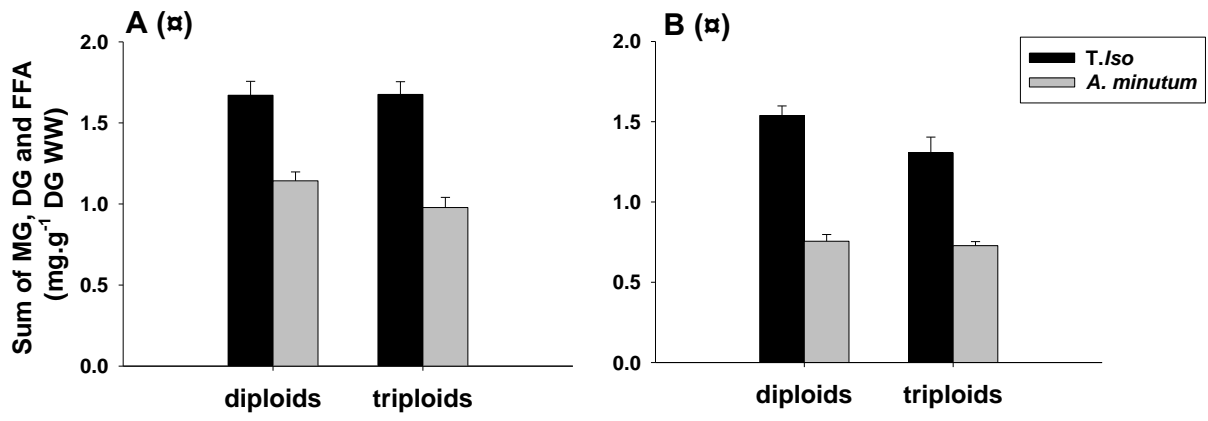


Fig. 3

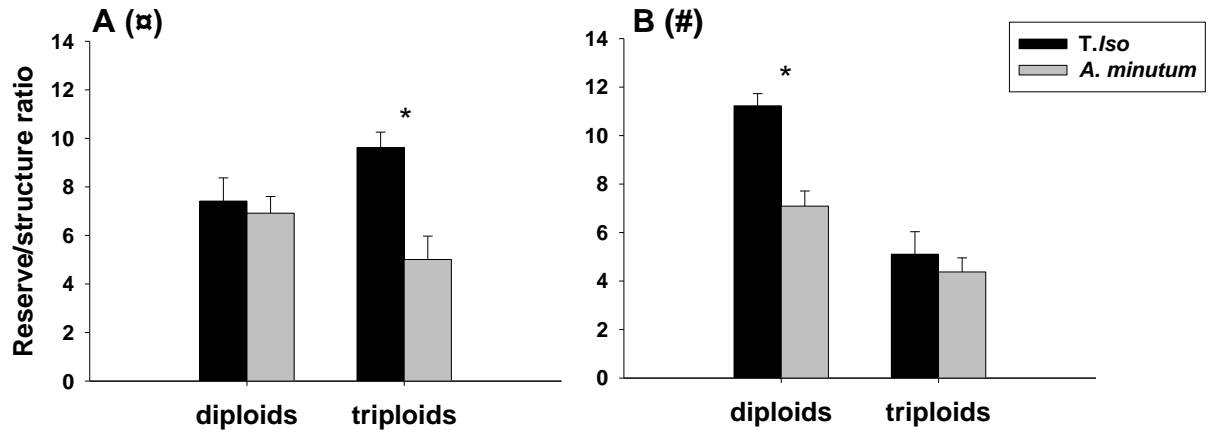


Fig. 4

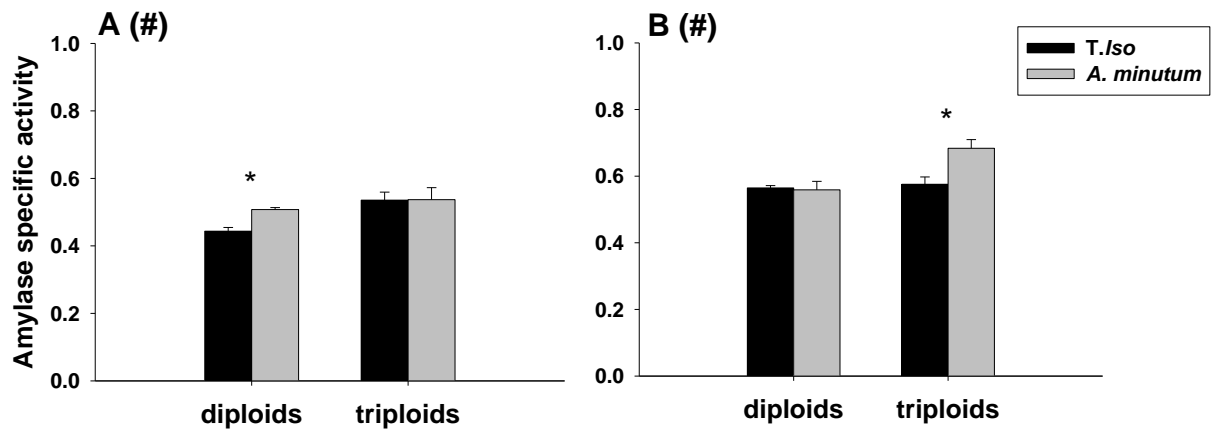


Fig. 5

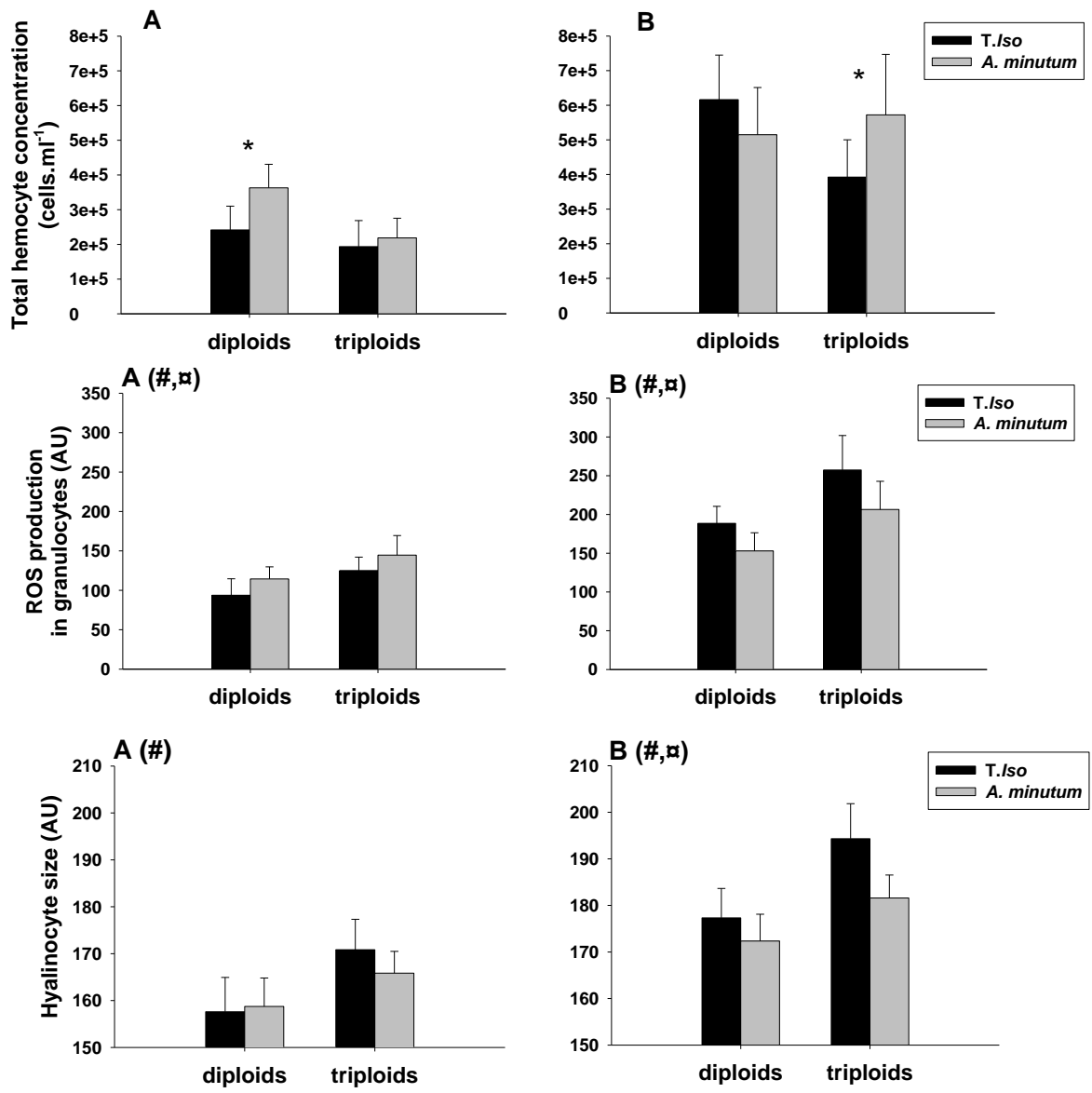


Fig. 6

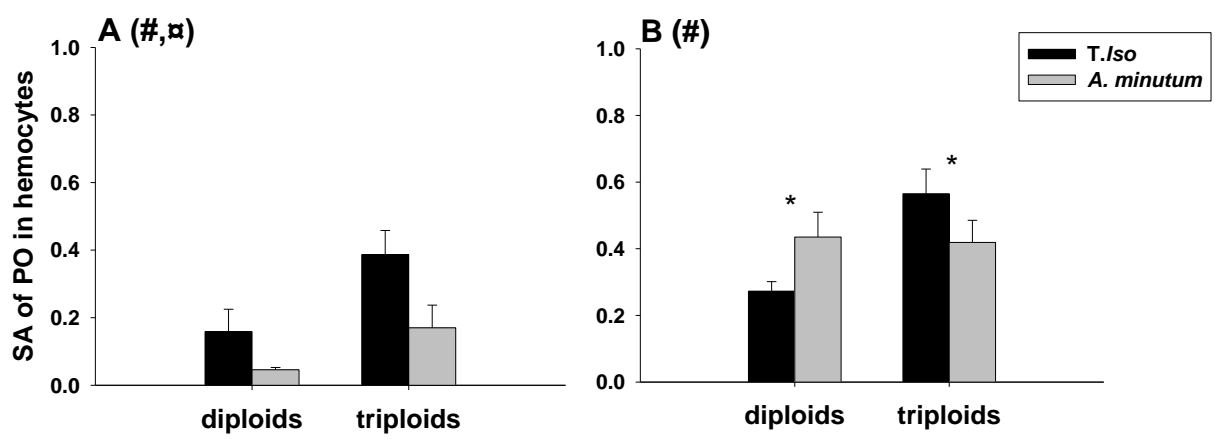


Fig. 7

Table 1:

	April experiment							May experiment						
	mean \pm CI				MANOVA			mean \pm CI				MANOVA		
	diploids		triploids		P	D	P/D	diploids		triploids		P	D	P/D
	<i>A. minutum</i>	<i>T. Iso</i>	<i>A. minutum</i>	<i>T. Iso</i>				<i>A. minutum</i>	<i>T. Iso</i>	<i>A. minutum</i>	<i>T. Iso</i>			
condition index	4.3 \pm 0.3	4.6 \pm 0.3	3.9 \pm 0.1	3.9 \pm 0.3	NS	NS	NS	4.3 \pm 0.3	4.2 \pm 0.2	3.6 \pm 0.2	4.2 \pm 0.2	NS	NS	NS
body dry weight (in g)	1.4 \pm 0.1	1.3 \pm 0.09	1 \pm 0.1	1 \pm 0.07	**	NS	NS	1.2 \pm 0.07	1.2 \pm 0.07	1 \pm 0.08	0.8 \pm 0.06	**	NS	NS
digestive gland wet weight (in g)	0.7 \pm 0.04	0.7 \pm 0.06	0.6 \pm 0.04	0.6 \pm 0.04	NS	NS	NS	0.5 \pm 0.04	0.5 \pm 0.03	0.4 \pm 0.02	0.5 \pm 0.02	***	*	*

P = ploidy ; D = diet; P/D; interaction ploidy and diet; Significant differences are indicated by * when $p < 0.05$, ** when $p < 0.01$, *** when $p < 0.001$; NS non-significant

Table 2:

	April experiment							May experiment						
	mean \pm CI				MANOVA			mean \pm CI				MANOVA		
	diploids		triploids		P	D	P/D	diploids		triploids		P	D	P/D
	<i>A. minutum</i>	<i>T.Iso</i>	<i>A. minutum</i>	<i>T.Iso</i>				<i>A. minutum</i>	<i>T.Iso</i>	<i>A. minutum</i>	<i>T.Iso</i>			
monoacylglycerols	0.3 \pm 0.02	0.6 \pm 0.04	0.2 \pm 0.00	0.6 \pm 0.03	NS	***	NS	0.1 \pm 0.02	0.6 \pm 0.05	0.1 \pm 0.00	0.4 \pm 0.07	NS	***	NS
diacylglycerols	0.3 \pm 0.04	0.4 \pm 0.06	0.2 \pm 0.03	0.4 \pm 0.01	NS	*	NS	0.1 \pm 0.02	0.2 \pm 0.01	0.1 \pm 0.00	0.2 \pm 0.05	NS	*	NS
sterols	2.1 \pm 0.06	1.9 \pm 0.07	2.06 \pm 0.1	1.8 \pm 0.2	NS	NS	NS	1.6 \pm 0.1	1.5 \pm 0.2	0.9 \pm 0.1	0.9 \pm 0.08	*	NS	NS
free fatty acids	0.5 \pm 0.03	0.7 \pm 0.04	0.6 \pm 0.06	0.7 \pm 0.08	NS	*	NS	0.5 \pm 0.03	0.8 \pm 0.03	0.5 \pm 0.03	0.7 \pm 0.04	NS	*	NS
triacylglycerols	13.1 \pm 1.5	12.5 \pm 1.5	9.8 \pm 0.9	14.6 \pm 0.2	NS	NS	NS	9.9 \pm 1.2	14.6 \pm 1	3.5 \pm 0.08	4.2 \pm 0.8	**	NS	NS
ether glycerides	1.4 \pm 0.2	1.3 \pm 0.2	1.04 \pm 0.1	2.07 \pm 0.3	NS	NS	NS	0.8 \pm 0.1	1.6 \pm 0.2	0.2 \pm 0.02	0.4 \pm 0.1	NS	NS	NS
sterol esters	0.3 \pm 0.01	0.3 \pm 0.01	0.2 \pm 0.05	0.2 \pm 0.01	NS	NS	NS	0.4 \pm 0.00	0.5 \pm 0.05	0.1 \pm 0.01	0.2 \pm 0.02	**	NS	NS
ratio reserve/structural	6.9 \pm 0.7	7.4 \pm 0.9	5.01 \pm 0.9	9.6 \pm 0.6	NS	*	NS	7.09 \pm 0.6	11.2 \pm 0.5	4.4 \pm 0.6	5.1 \pm 0.9	*	NS	NS

P = ploidy ; D = diet; P/D; interaction ploidy and diet; Significant differences are indicated by * when $p < 0.05$, ** when $p < 0.01$, *** when $p < 0.001$; NS non-significant

Table 3:

variables	April experiment			May experiment		
	ploidy	diet	interaction	ploidy	diet	interaction
concentration of granulocytes	*	**	NS	NS	NS	NS
concentration of agranulocytes	**	NS	NS	*	NS	NS
concentration of agregats	*	NS	NS	**	NS	NS
size of granulocytes	*	NS	NS	***	**	NS
size of hyalinocytes	**	NS	NS	***	**	NS
complexity of granulocytes	NS	NS	NS	***	NS	NS
complexity of hyalinocytes	NS	NS	NS	***	*	NS
arcin (% phagocytic hemocytes)	NS	NS	NS	***	NS	NS
ROS production of granulocytes	**	*	NS	**	**	NS
ROS production of hyalinocytes	*	NS	NS	NS	*	NS
hemocyte phenoloxidase SA	*	*	NS	*	NS	NS

Significant differences are indicated by * when $p < 0.05$, ** when $p < 0.01$, *** when $p < 0.001$; NS non-significant.

The following variables were not presented in this table as these were not significantly affected by ploidy or microalgal exposure or the interaction (MANOVA): concentration of total hemocytes, concentration of hyalinocytes, % of dead hemocytes, and plasma variables (phenoloxydase specific activity, agglutination titer and hemolysis titer)