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1 Relationship between valve activity, microalgae concentration in the water  
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3 of the Pacific oyster *Crassostrea gigas* exposed to *Alexandrium minutum*.

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20

## Abstract

The complexity of the relationships between *Alexandrium minutum* (*A.m.*) concentration in the water ( $[A.m.]_w$ ), Paralytic Shellfish Poisoning contamination in the digestive gland ( $[PSP]_{dg}$ ) and valve behavior was explored in oysters *Crassostrea gigas*. Two experiments were conducted, during which oysters' valve behaviour were analyzed. Oysters, first acclimated for 10-days with the non harmful microalgae *Heterocapsa triquetra* (*H.t.*) were exposed to 4 microalgae mixtures at constant total concentrations of  $10 \cdot 10^3$  cells.ml<sup>-1</sup> (experiment-1) and  $5 \cdot 10^3$  cells.ml<sup>-1</sup> (experiment-2): 100% *A.m.*; 50% *A.m.*-50% *H.t.*; 25% *A.m.*-75% *H.t.*; 100% *H.t.* At the end of experiment-2,  $[PSP]_{dg}$  were measured.

At  $10 \cdot 10^3$  cells.ml<sup>-1</sup>, the microalgal ingestion decreased ( $p < 0.05$ ) with increasing  $[A.m.]_w$  but not at  $5 \cdot 10^3$  cells.ml<sup>-1</sup> ( $p > 0.05$ ). The frequency of microclosures specifically increased with  $[A.m.]_w$  ( $p < 0.05$ ) and the opening duration with  $[PSP]_{dg}$  ( $p < 0.0001$ ). Oysters exhibiting the maximum increase in opening duration also exhibited the highest  $[PSP]_{dg}$ . The results are discussed in terms of oyster physiology and origin of the behavioral response.

## 35 Introduction

36 Recent studies have tested modification of shell valve activity in bivalves following  
37 harmful microalgal exposure (Nagai et al., 2006; Basti et al., 2009; Tran et al., 2010). The  
38 scallop *Pinctada fucata* exposed to *Heterocapsa circularisquama* showed an increased  
39 frequency of valve adductions or microclosures (Nagai et al., 2006). Similarly, *Ruditapes*  
40 *philippinarum* increased the frequency of valve adductions and decreased amplitude of valve  
41 openings upon exposure to *H. circularisquama* (Basti et al., 2009). In oysters, *Crassostrea*  
42 *gigas*, exposed to *Alexandrium minutum*, Tran et al. (2010) also described an increased  
43 frequency of microclosures as well as an increase in valve-opening duration. This increased  
44 interest in behavioral responses has two major aims: to improve our understanding of the  
45 physiological impact of harmful microalgae on bivalve physiology and ecology; to test the  
46 putative interest of behavioral change to detect the presence of harmful microalgae or to  
47 monitor depuration processes in the field and oyster farms. However, much remains to  
48 explore, both in the lab and in the field. The aim of the present study was to gain more  
49 insights into the relationship between *C. gigas* behavior, characterized by shell valve activity,  
50 concentration of the harmful microalgae *A. minutum* in the ambient water and accumulation  
51 of PSPs in the digestive gland.

52 Among harmful microalgae, *Alexandrium* species are known to produce Paralytic  
53 Shellfish Toxins (PSPs). Several commercial bivalve species, such as oysters and mussels,  
54 accumulate PSPs by feeding on phytoplanktonic PSP producers (Oshima et al., 1990; Bricelj  
55 and Shumway, 1998). PSP accumulation and detoxification kinetics, as well as  
56 biotransformation of the toxins, were reviewed in Bricelj and Shumway (1998) and have been  
57 well documented for the oyster, *Crassostrea gigas*, exposed to *Alexandrium minutum* or *A.*  
58 *catenella* (Lassus et al., 2005, 2007; Guéguen et al., 2008).

59 Beyond behavioral studies on valve activity, authors have mainly focused on bivalve  
60 feeding and/or digestive responses (Bardouil et al., 1993; Wildish et al., 1998; Li et al., 2001;  
61 Bougrier et al., 2003; Lassus et al., 2004; Navarro et al., 2008; Fernández-Reiriz et al., 2008).  
62 In the oyster, *C. gigas* (Bardouil et al., 1993; Wildish et al., 1998; Lassus et al., 2004) and  
63 mussel, *Mytilus chilensis*, (Navarro et al., 2008) an inhibition of feeding activity was reported  
64 when animals were exposed to harmful *Alexandrium* species. In *M. chilensis* exposed to *A.*  
65 *catenella*, the inhibition appeared to be reversible after a few days, even though exposure  
66 continued, suggesting that mussels can acclimate to feeding on toxic microalgae (Navarro et  
67 al., 2008).

68 Relationships between the accumulation potential of different bivalve species for PSPs and  
69 the ingestion and absorption rates were described by Bricelj et al. (1990) and Bricelj and  
70 Shumway (1998). These studies demonstrated an inverse relationship between sensitivity to  
71 toxin and potential toxin accumulation. For example, the oyster *C. virginica*, with high nerve  
72 sensitivity to PSPs, potentially accumulates fewer toxins by decreasing ingestion and  
73 absorption rates (Bricelj and Shumway, 1998). On the contrary, the mussel *Mytilus edulis*,  
74 which has a low nerve sensitivity to PSPs, accumulates a higher amount of toxins. Another  
75 level of complexity has been discussed by Lassus et al. (2000) at the intra-population level.  
76 In *C. gigas* exposed to *A. minutum*, they suggested that the variability of contamination status  
77 could be related to inter-individual differences in valve and/or clearance activities. This inter-  
78 individual variability was used by Bougrier et al. (2003) to investigate the relationship  
79 between PSP content and clearance rates of *C. gigas* exposed to *A. minutum*.

80 The objective of this work was to go further to the previous study performed by Tran  
81 et al., (2010) by i) exposing oysters to different concentrations of *A. minutum* and ii)  
82 measuring PSP concentrations in oyster digestive glands. This allows to evaluate if behavioral  
83 responses of the Pacific oyster *C. gigas* are related to the presence and concentration of  
84 harmful microalgae in water and/or to toxin concentration in their digestive gland. This is part  
85 of a more general work in which the impact of toxic micro-algae on bivalves physiology and  
86 the underlying mechanisms explaining behavior variability in the field are studied (Schmitt et  
87 al. 2011; Tran et al., 2011).

88

## 88 **Materials and methods**

89

### 90 **Oyster characteristics**

91 Two experiments were carried out at two different periods, in November-December  
92 (Experiment 1) and January-February (Experiment 2), with Pacific oysters, *Crassostrea gigas*  
93 at the University of Brest. Oysters were obtained from a shellfish farmer in the bay of Brest  
94 (France). Two homogenous groups of oysters (n = 32 per experiment) were chosen *a priori*  
95 according to the shell length (75 mm shell length,  $40 \pm 1$  g total weight). No death was  
96 observed during the experiments.

97

### 98 **Microalgal cultures**

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

105 The dinoflagellate *Heterocapsa triquetra* (strain HT99PZ - Ehrenberg, 1840) was  
106 grown in 10 L batch cultures in autoclaved,  $1\mu\text{m}$ -filtered seawater enriched with L1 nutrients.  
107 Cultures were maintained for 5 days at  $16 \pm 1^\circ\text{C}$  and  $100 \mu\text{mol photon m}^{-2} \text{ s}^{-1}$ , with a  
108 dark:light cycle of 12:12h.

109

### 110 **Experimental procedure for oyster exposure**

111 The non-toxic dinoflagellate *H. triquetra* was chosen as a control because of its  
112 similarity to *A. minutum*, in terms of size and shape characteristics: *H. triquetra* cell size (19-  
113  $28 \mu\text{m}$ ) is similar to *A. minutum* cell size (23-29  $\mu\text{m}$ ). Importantly, *A. minutum* and *H.*  
114 *triquetra* have different green auto-fluorescence characteristics which allow their individual  
115 quantification in mixtures.

116 The experiments were conducted in an isolated room with minimal human activity to  
117 limit inadvertent stimulation of oysters. Experimental tanks were installed on antivibrating  
118 benches to minimize any external disturbance that could interfere with the behavior of the  
119 oysters. Experiments were carried out with a photoperiod of 12h light / 12h dark and seawater  
120 was maintained at a temperature of  $16 \pm 1^\circ\text{C}$ . Prior to the experiments, oysters were

121 distributed randomly into eight 10-liter tanks (four tests and four controls), with four oysters  
122 per tank. Oysters were maintained in the same tanks during the entire experiment to avoid  
123 behavioral disturbance from handling. Each tank was individually supplied with cultured  
124 microalgal suspensions using a multichannel peristaltic pump. Central air-lifts were used to  
125 homogenize microalgal concentration and water in the tanks. Present experiments were  
126 performed at two different total microalgae concentrations ( $10 \cdot 10^3$  cells $\cdot$ ml $^{-1}$  and  $5 \cdot 10^3$   
127 cells $\cdot$ ml $^{-1}$ ), which correspond with bloom concentrations observed on French coast (Belin and  
128 Raffin, 1998).

129

130 Experiment 1. As a first step to characterize a relationship between concentration of *A.*  
131 *minutum* in water and behavior of oysters, oysters were exposed to four different microalgal  
132 mixtures, at an identical total concentration of microalgae of  $10 \cdot 10^3$  cells $\cdot$ ml $^{-1}$ . Three different  
133 phases were considered: acclimation period (Acc.;  $t_0 - t_{10}$ ), exposure period (Exp.;  $t_{11} - t_{12}$ )  
134 and recovery period (Rec.;  $t_{13} - t_{18}$ ). During the 10-day acclimation period, each tank received  
135 a continuous flow of  $12.5 \text{ L}\cdot\text{day}^{-1}$  of seawater (filtered to  $0.5 \mu\text{m}$ ) with *H. triquetra* at  $1 \cdot 10^4$   
136 cells $\cdot$ ml $^{-1}$ . Following the acclimation period, four supply tanks were used to distribute  
137 microalgal suspensions into the respective, replicated experimental tanks (two tanks per  
138 condition). Supply tanks were prepared with each of four microalgal mixtures: i) 100% *A.*  
139 *minutum* ( $1 \cdot 10^4$  cells $\cdot$ ml $^{-1}$ ), ii) 50% *A. minutum*- 50% *H. triquetra* ( $5 \cdot 10^3$  cells $\cdot$ ml $^{-1}$  of *A.*  
140 *minutum* and  $5 \cdot 10^3$  cells $\cdot$ ml $^{-1}$  of *H. triquetra*), iii) 25% *A. minutum*- 75% *H. triquetra* ( $2.5 \cdot 10^3$   
141 cells $\cdot$ ml $^{-1}$  of *A. minutum* and  $7.5 \cdot 10^3$  cells $\cdot$ ml $^{-1}$  of *H. triquetra*) and iv) 100% *H. triquetra*  
142 ( $1 \cdot 10^4$  cells $\cdot$ ml $^{-1}$ ). Each tank received a continuous flow of  $12.5 \text{ L}\cdot\text{day}^{-1}$  of each microalgal  
143 treatment. The exposure period continued for two days, followed by five days of recovery  
144 ( $12.5 \text{ L}\cdot\text{day}^{-1}$  of seawater with *H. triquetra* at  $1 \cdot 10^4$  cells $\cdot$ ml $^{-1}$ ).

145 Experiment 2. To test the possible relationship between behavior and toxin  
146 accumulation, toxin contents in the digestive glands of the oysters were determined at the end  
147 of the exposure period. Two different phases were considered: acclimation period (Acc.,  $t_0 -$   
148  $t_{10}$ ) and a 5-days exposure period (Exp.,  $t_{11} - t_{15}$ ). During the 10-day acclimation period, each  
149 tank received a continuous flow of  $12.5 \text{ L}\cdot\text{day}^{-1}$  ( $8.7 \text{ ml}\cdot\text{min}^{-1}$ ) of seawater (filtered to  $0.5 \mu\text{m}$ )  
150 with *H. triquetra* at  $5 \cdot 10^3$  cells $\cdot$ ml $^{-1}$ . Following the acclimation period, four microalgal supply  
151 tanks were used to distribute microalgal suspensions into the respective, replicated  
152 experimental tanks (two tanks per condition). Supply tanks were prepared with each of four  
153 microalgal mixtures: i) 100% *A. minutum* ( $5 \cdot 10^3$  cells $\cdot$ ml $^{-1}$ ), ii) 50% *A. minutum*- 50% *H.*  
154 *triquetra* ( $2.5 \cdot 10^3$  cells $\cdot$ ml $^{-1}$  of *A. minutum* and  $2.5 \cdot 10^3$  cells $\cdot$ ml $^{-1}$  of *H. triquetra*), iii) 25% *A.*

155 *minutum*- 75% *H. triquetra* ( $1.25 \cdot 10^3$  cells.ml<sup>-1</sup> of *A. minutum* and  $3.75 \cdot 10^3$  cells.ml<sup>-1</sup> of *H.*  
156 *triquetra*) and iv) 100% *H. triquetra* ( $5 \cdot 10^3$  cells.ml<sup>-1</sup>). Each tank received a continuous flow  
157 of 12.5 L.day<sup>-1</sup> of each microalgal treatment. At the end of the exposure period, the digestive  
158 glands of individual oysters were dissected, weighed, frozen immediately in liquid nitrogen,  
159 and stored at -80°C until analysis. Later, digestive glands were ground with a “Danguomeau”  
160 homogenizer into liquid nitrogen and this sample was used to measure toxin content.

161

### 162 ***C. gigas* valve-activity measurement**

163 To evaluate the effect of *A. minutum* on the valve behavior of *C. gigas* throughout the  
164 experiment, we recorded the valve activity continuously with a laboratory made valvometer.  
165 The oysters were equipped (at  $t_0$ ) with light weight ( $\approx 1$  g) electromagnetic electrodes glued  
166 onto both shells. The electrodes allow the oysters to move their valves without constraint.  
167 More details are described in Tran et al. (2003) and Chambon et al. (2007), as well as data  
168 about the required adaptation periods before experimental set-up (Tran et al., 2003). The  
169 record of valve activity started at  $t_0$ .

170 The free ends of the electrodes were connected to an electronic apparatus composed  
171 mainly of a multiplexer that switched the current every 300 msec from one pair of electrodes  
172 to another, and a computer driving the apparatus via a data acquisition card (LAB PC 1200;  
173 National Instruments, Austin, TX, USA), using LabView 8.0 software (National Instruments).

174

### 175 **Phytoplankton sampling**

176 During exposure periods, 1-ml water samples were collected from supply tanks, both  
177 at the input and within the oyster tanks, in order to determine microalgal cell densities.  
178 Samples were collected and fixed in 3% formaldehyde (final concentration) at 1, 3, 5, 6, 7, 23,  
179 24 and 27h30 after the beginning of the exposure for experiment 1 and at 1, 15, 17, 20, 24, 26,  
180 40, 48 and 64 h for experiment 2. Samples were analyzed by flow cytometry within one day  
181 of sampling.

182

### 183 **Measurements of dinoflagellate cell densities by flow cytometry**

184 Measurements of dinoflagellate cell concentrations were performed using a  
185 FACScalibur (BD Biosciences, San Jose, CA USA) flow cytometer (FCM) equipped with a  
186 488 nm argon laser. Threshold was set to FL3 (red fluorescence, 550-600 nm) to detect only  
187 chlorophyll-containing cells. Settings were adjusted to visualize dinoflagellate cells on two  
188 cytograms with i) Forward Scatter (FSC, related to cell size) vs Side Scatter (SSC, related to



189 cell internal complexity), and ii) red auto-fluorescence (FL3) vs green auto-fluorescence  
190 (FL1) as parameters. Cells of *A. minutum* and *H. triquetra* possess similar FSC and SSC  
191 characteristics, but *A. minutum* has higher FL1 in comparison to *H. triquetra*, making the two  
192 cells distinguishable in a mixed suspension. Cell densities were estimated from flow-rate  
193 measurement of the flow-cytometer (Marie et al., 1999), as all samples were run for 1 min.  
194 Results were expressed as number of cells per ml.

195

### 196 **Toxin content**

197 Digestive gland (ground, 0.5 g) was extracted in 1 ml of 0.1 N HCl (2 v/w) at 4°C.  
198 After centrifugation (3,000 × g, 15 min, 4°C), pH of extracts was adjusted to 3.0 with 12 N  
199 HCl. After half-dilution, supernatants were ultra-filtered (20 kDa, Sartorius Centrisart) and  
200 stored at 4°C until analysis. PSPs were analyzed by ion-pairing, high-performance liquid  
201 chromatography (IP-HPLC) according to the method of Oshima (1995). The molar  
202 concentration ( $\mu\text{mol.l}^{-1}$ ) was converted into  $\mu\text{g STX equiv. } 100 \text{ g}^{-1}$  of digestive gland by using  
203 the conversion factors of Oshima (1995). Results were expressed in  $\mu\text{g STX equiv. } 100 \text{ g}^{-1}$  of  
204 digestive gland wet weight.

205

### 206 **Statistical analysis**

207 Results are expressed as mean  $\pm$  confidence interval (CI). Differences between variables  
208 before and during *A. minutum* exposure were determined using the T-test, after checking  
209 assumptions (normality and homoscedasticity of the error term).

210 Homogenous groups were identified using multiple range comparison tests (ANOVA) to  
211 determine which means were significantly different from which others and Fisher's least  
212 significant difference (LSD) procedure discriminated among the means. On graphics, the  
213 same letter was used for means which were not statistically different.

214 A statistical regression was used to correlate toxin content and behavioral variables. The fit  
215 between predicted and measured values was tested statistically using ANOVA. For all  
216 statistical results, a probability of  $p < 0.05$  was considered significant. Statistical analyses  
217 were performed using Statgraphics Plus (Manugistics, Inc, Rockville, MD, USA).

218

## 218 Results

219

220 Under acclimation conditions, in tanks fed with  $10 \cdot 10^3$  cells.ml<sup>-1</sup> without *A. minutum*,  
221 oysters consumed  $92.5 \pm 4.6$  % of the microalgae supplied in the tanks (Fig. 1A). This  
222 percentage decreased with increasing ratio of *A. minutum* added ( $79.5 \pm 9.9$  % at 25% *A.m./75*  
223 % *H.t.*;  $81.7 \pm 6.8$  % at 50% *A.m./50* % *H.t.* and  $48.8 \pm 12.6$  % at 100% *A.m./0* % *H.t.*). On  
224 the contrary, when animals were fed with half the concentration of microalgae,  $5 \cdot 10^3$  cells.ml<sup>-1</sup>,  
225 all microalgae were consumed, independent of the *Alexandrium* concentration in the input  
226 water (Fig. 1B). In all conditions, no microalgal sedimentation was observed and no  
227 noticeable amount of feces and/or pseudofeces were found in the tanks. Similar decreases of  
228 both microalgae in the mixture (*A. minutum* and *H. triquetra*) were measured showing that *C.*  
229 *gigas* fed equally on both species.

230 Figure 2 illustrates the similarity and replicability of the present experimental  
231 conditions: no statistical difference was observed between any of the 8 tanks that were  
232 running in parallel, during both acclimation conditions ( $p > 0.05$ ) or during the second day of  
233 recovery ( $p > 0.05$ ). Similarly, paired comparison between the two series of replicate did not  
234 reveal statistical difference. This allowed evidencing dose-response relationship between  
235 behavioral responses and water concentration of *A. minutum* in *C. gigas*. During the 2-day  
236 exposure period, the oysters exhibited significantly more valve micro-closures in the presence  
237 of *A. minutum* (see inserts in Fig. 2). The number of valve micro-closures was 3.5 times  
238 higher in the presence of 100 % *A. minutum*, 3.1 times higher at 50 % and 1.9 times higher at  
239 25 %, in relation to the acclimation period of the oysters in the same tanks. It is only  
240 following the exposure to 25% *A. minutum* that valve micro-closure was not significantly  
241 different from the acclimation period and the 2 days of recovery. Figure 3 presents the mean  
242 values in the above series of replicates (Experiment 1) and demonstrates how the number of  
243 micro-closures statistically increased at higher *A. minutum* concentrations. During recovery, it  
244 significantly decreased towards acclimation values (see inserts in Fig. 2).

245

246 Figure 4A and C shows the absence of a relationship between PSP concentration in the  
247 digestive gland and concentration of *A. minutum* in the water supplying the tanks ( $p > 0.05$ )  
248 while figure 4B and D (data analysis either performed per oyster, 4B, or per tank, 4D) shows  
249 a positive correlation between toxin accumulation and daily opening duration. Figure 5A and  
250 C demonstrates that the contamination status was not related to the opening-duration behavior

251 prior exposure (mean value during the 5 last day acclimation period,  $p > 0.05$ ). This is  
252 coherent with the absence of significant difference within the whole set of reference and  
253 recovery conditions (low acclimation variability). This low variability completely vanished  
254 under exposure conditions. The more “responsive” animals (Fig 5B) or tanks (Fig. 5D),  
255 identified as those showing the most dramatic differences in daily opening duration between  
256 acclimation and exposure phases, were those exhibiting the highest accumulation of PSP in  
257 their digestive gland (Fig. 5B;  $p < 0.0001$ ,  $R^2 = 0.67$ ). A comparison between the opening  
258 duration between the acclimation period (Fig. 5A, per individual, or 5C, per tank) and the  
259 exposure period (Fig. 4B, per individual, or 4D, per tank) shows a greatly increased  
260 variability in the presence of *A. minutum*. Under acclimation conditions the min-max values  
261 were 18-40 % of opening duration while they were 19-85 % with *A. minutum*.

262

## 262 Discussion

263 The objective of this work was to evaluate if behavioral responses of the Pacific oyster  
264 *C. gigas* are related to the presence and concentration of harmful microalgae in water and/or  
265 to toxin concentration in their digestive gland. A dose-response relationship was observed  
266 between the frequency of micro-closures and the concentration of *A. minutum* in the ambient  
267 water but no dose-response relationship between micro-closures and the PSP concentration in  
268 the digestive gland. On the contrary, opening-duration showed a dose-response relationship to  
269 the PSP concentration in the digestive gland but not to the concentration of *A. minutum* in the  
270 water. In addition, data showed that the inter-individual variability of opening-duration  
271 increased during exposure in comparison to the acclimation period. Oysters exhibiting the  
272 largest increase in opening duration were those with the largest toxin content in the digestive  
273 gland. There was no relationship between acclimation and intensity of response during  
274 exposure as regard to the opening duration.

275

276 Behavioral changes versus total microalgae concentrations

277 Present experiments were performed at two different total microalgal concentrations  
278 ( $10 \cdot 10^3$  cells·ml<sup>-1</sup> and  $5 \cdot 10^3$  cells·ml<sup>-1</sup>), with the aim to experimentally manipulate oyster  
279 feeding activity. Analysis of experiment 1 ( $10 \cdot 10^3$  cells·ml<sup>-1</sup>) suggested that feeding activity  
280 slightly decrease with increasing proportion of *A. minutum* in the mixture. Such decreases of  
281 filtration rate were previously reported in *C. gigas* exposed to the toxic dinoflagellate  
282 *Alexandrium tamarense* (Bardouil et al., 1993) and *A. minutum* (Lassus et al., 1999). Lassus et  
283 al. (2004) also reported an inhibition effect at  $10 \cdot 10^3$  cells·ml<sup>-1</sup> in *C. gigas* fed with *A.*  
284 *minutum*. Present results showed that feeding was partly inhibited at  $10 \cdot 10^3$  of *A. minutum* but  
285 not at  $5 \cdot 10^3$  cells·ml<sup>-1</sup>. This could be the result of a balance between feeding requirements and  
286 a protective response.

287

288 Impact of the *A. minutum* concentration in the water

289 Among the variables measured to describe oyster behavior, the frequency of valve  
290 micro-closures was positively correlated to the percentage of *A. minutum* added, at a constant  
291 total microalgae concentration. Tran et al., (2010), previously suggested that such behavior  
292 could be related to an avoidance response to minimize contact with harmful cells and/or  
293 effects of toxins released during digestion of *A. minutum* cells. This behavior could also help  
294 the animal to rapidly test the ambient water content (back and forth water movements across

295 the mantle border and in the pallial cavity). The present report demonstrates that, in the  
296 present experimental conditions, this was associated to the concentration of *A. minutum* in the  
297 water and not to the PSP accumulation in the animal. Similar increases of valve micro-  
298 closures, correlated with increased concentrations of harmful microalgae, have been observed  
299 by Basti et al. (2009) in *R. philippinarum* exposed to *Heterocapsa circularisquama*. The  
300 absence of PSP toxin production by *H. circularisquama* demonstrated that the presence of this  
301 toxin is not a prerequisite to induce an increase of micro-closures.

302

303 Relationship with toxin concentration in the digestive gland

304 In *C. gigas*, 90 % of PSP toxins accumulate in the digestive gland (Guéguen et al.,  
305 2008; Lassus et al., 2007) explaining why PSPs were only analyzed in this organ. Present data  
306 showed that valve opening duration was dose-related to the concentration of PSP in the  
307 digestive gland. A comparison with previous reports shows that a positive relationship  
308 between feeding time and tissue PSP concentration was reported in *C. gigas* and *Pecten*  
309 *maximus* exposed to *A. minutum* (Bougrier et al., 2003). In this study, feeding time activity  
310 was indirectly evaluated by measuring the decrease in toxic cell density at the outlets of  
311 experimental tanks and biodeposit production. Although mean oyster responses to microalgal  
312 treatments and toxin accumulation are well documented in the literature, it is known that  
313 response intensity at the individual level is quite variable, from no change at all to very  
314 important and dramatic changes (see for examples Bricelj and Shumway, 1998; Lassus et al.,  
315 2007). This is also similar to PSP accumulation in *C. gigas*: numerous animals remained in  
316 the low range of the global distribution data while a minority accumulated the largest amounts  
317 (present report; Fig. 6 in Lassus et al., 2007). The present report demonstrates that this inter-  
318 individual variability of bioaccumulation was positively correlated to the opening duration  
319 during the exposure period (Fig. 4B, 4D) but not before it (Fig. 5A, 5C). Was the presence of  
320 larger quantities of PSP in the digestive gland responsible for the longer opening duration  
321 during exposure? Or, alternatively, was the longer opening duration, and incidentally a larger  
322 clearance value, responsible for a larger toxin bioaccumulation?

323

324 The hen and egg problem

325 One must recall first that with *A. minutum* we are dealing with a paralyzing toxin, that  
326 we measured it in the digestive gland following a 5 day exposure period and that its  
327 distribution in the whole animal was presumably not time limited. Was the presence of larger  
328 quantities of PSP in the digestive gland responsible for the longer opening duration? Among

329 the presently known physiological and behavioral alterations induced by PSP, are (i)  
330 alterations of action potential transmission by blocking sodium conductance in nerve fibers  
331 (Narahashi and Moore, 1968) and (ii) absence of response to mechanical stimulation of the  
332 gills and adductor muscle in gaping *Crassostrea virginica* (Hégaret et al., 2007). In the  
333 softshell clam *Mya arenaria*, Bricelj et al. (2005) reported muscle paralysis induced by  
334 paralytic shellfish toxin and in *C. gigas*, Haberkorn et al. (2010) described important  
335 morphological alterations of the adductor muscle. Physiological impacts could explain the  
336 larger opening durations. Alternatively, Tran et al. (2010) suggested muscular fibers of gills  
337 (Medler and Silverman, 2001) could be targets of PSP toxins. This could lead to a decrease in  
338 pumping efficiency in terms of volume of water ventilated per unit of time. Thus, a reduction  
339 of filtration efficiency during exposure to *A. minutum* would be compensated for by longer  
340 ventilatory periods in order to fulfill the animal's O<sub>2</sub>-requirements and/or nutritional needs.

341         Was the longer opening duration, and a larger filtered volume of water, responsible for  
342 a larger toxin bioaccumulation? Feeding behavior was hypothesized by Bricelj and Shumway  
343 (1998) to be one of the parameters responsible for inter-individual variation in toxin  
344 accumulation. The present results show that in *C. gigas* there was no relationship between  
345 acclimation opening duration and contamination levels (Fig. 5A, 5C) nor between acclimation  
346 opening duration and animal reactivity (measured as test – acclimation opening duration) to  
347 *A. minutum*. On the contrary, present results suggest the existence of an inter-individual  
348 reactivity that may reflect differences in sensitivity to PSPs or to any other compounds or  
349 characteristics associated with *A. minutum*. In that view, oysters accumulating the largest  
350 amounts of toxins could be considered less sensitive in comparison to oysters accumulating  
351 fewer toxins. This hypothesis has already been formulated by Bricelj and Shumway (1998)  
352 for different bivalve species. Bivalve species possessing low nerve sensitivity to PSP (e.g. *M.*  
353 *edulis*) were found to readily feed on toxic cells and thus accumulate high levels of toxins  
354 (Bricelj and Shumway, 1998). In contrast, oysters (e.g. *C. virginica*) described as PSP-  
355 sensitive species accumulated less PSP (Bricelj and Shumway, 1998). Differences in toxin  
356 accumulation (up to five times), observed between different populations of the same clam  
357 species, *M. arenaria*, were related to intra-species variability, in terms of PSP sensitivity. A  
358 natural mutation of a single amino acid residue decreases affinity (1,000-fold) of the  
359 saxitoxin-binding site in the sodium channel pore. This mutation was found to be responsible  
360 for the difference in nerve sensitivity between the two populations of *M. arenaria* exposed to  
361 PSP-producing *Alexandrium fundyense* (Bricelj et al., 2005). Furthermore, the present results  
362 underscore the possibility of finding differences in sensitivity to PSPs within an oyster stock

363 (intra-population). Less-sensitive oysters would open their valves longer and would consume  
364 more toxic dinoflagellates. This is of course speculation but opens exciting research directions  
365 for the future.

366 To go further in understanding relationships between oysters behavior, toxin  
367 accumulation and *A. minutum* concentration in water, it would be interesting to use the same  
368 experimental design, but by maintaining constant *A. minutum* concentration during the whole  
369 exposure period. To better understand if behavioral changes were because of PSP content,  
370 oysters could be exposed to PSP producer *A. minutum* strain and non-PSP producer *A.*  
371 *minutum* strain.

372

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373

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## 481 **Figure captions**

482

483 Fig. 1: Total microalgae concentrations measured in the inflow (black bars) and outflow (grey  
484 bars) of experimental tanks, for different experimental conditions, during experiments 1 (A)  
485 and 2 (B). Mean  $\pm$  confidence interval,  $n = 8$  samples per condition. Letters (a, b, c and d)  
486 represent 4 homogenous groups, identified using multiple range comparison tests whenever  
487 ANOVA was significant.

488

489 Fig. 2: Experiment 1. Dose-response behavior at various concentrations of *A. minutum* in the  
490 ambient water. Frequency of micro-closures, expressed in number per hour, in 8 different  
491 experimental tanks running in parallel (4 conditions and 2 replicates). Acc. (48 h of  
492 acclimation), Exp. (48 h of exposure) and Rec. (48 h of recovery). Mean  $\pm$  confidence  
493 interval,  $n = 4$  oysters per tank. \* significantly different from Acc., \*\* significantly different  
494 from Exp.

495

496 Fig. 3. Experiment 1, mean dose-response behavior. Mean frequency of valve micro-closures  
497 in oysters *C. gigas* exposed to different *A. minutum* concentrations in 8 different experimental  
498 tanks running in parallel (4 conditions and 2 replicates). Mean  $\pm$  confidence interval,  $n = 4$   
499 oysters per tank. Letters (a, b, c and d) represent 4 homogenous groups, identified using  
500 multiple range comparison tests whenever ANOVA was significant.

501

502 Fig. 4: Experiment 2, change of toxin contents in oysters, expressed as  $\mu\text{g STX eq. } 100 \text{ g}^{-1}$   
503 digestive gland wet weight (DGWW), either per individual oyster (A, B) or experimental tank  
504 (C, D): toxin content as a function of (A and C) concentration of *A. minutum* in the inflow  
505 water and of (B and D) valve-opening duration. Five days of exposure duration; A, B,  $n = 24$   
506 oysters; C, D,  $n = 6$  tanks, mean  $\pm$  confidence interval.

507

508 Fig. 5: Experiment 2, change of toxin contents in oysters, expressed as  $\mu\text{g STX eq. } 100 \text{ g}^{-1}$   
509 digestive gland wet weight (DGWW), either per individual oyster (A, B) or experimental tank  
510 (C, D): toxin content as a function of (A and C) opening duration during acclimation period  
511 and of (B and D) difference of valve-opening duration between Exposure and Acclimation  
512 period (Exp – Acc). Five days of exposure duration; A, B,  $n = 24$  oysters; C, D,  $n = 6$  tanks,  
513 mean  $\pm$  confidence interval.

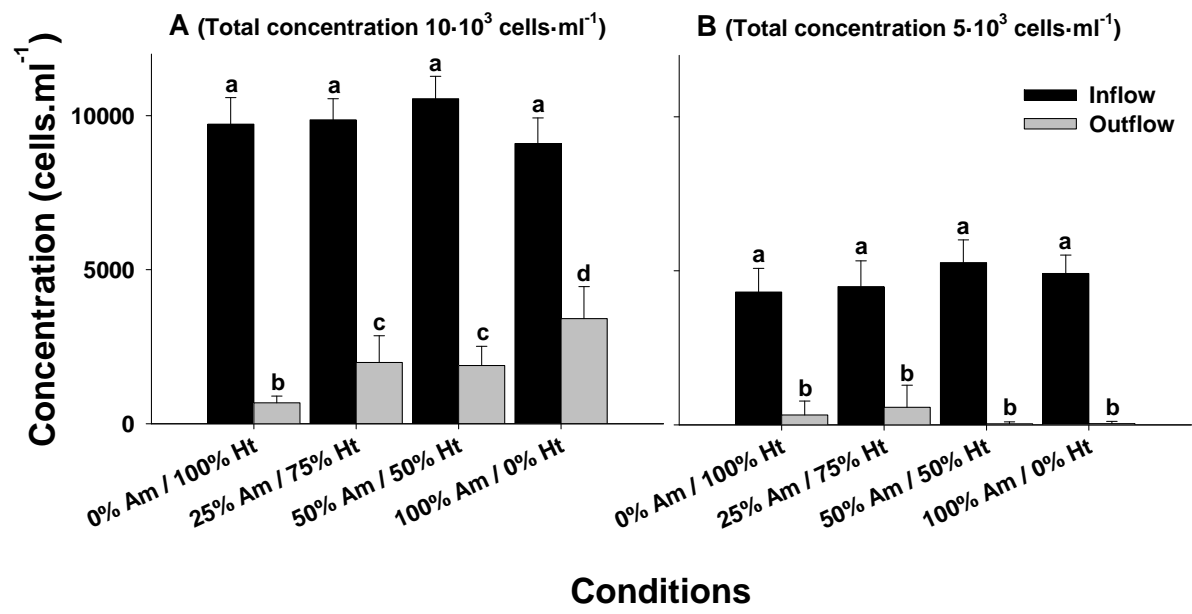


Figure 1

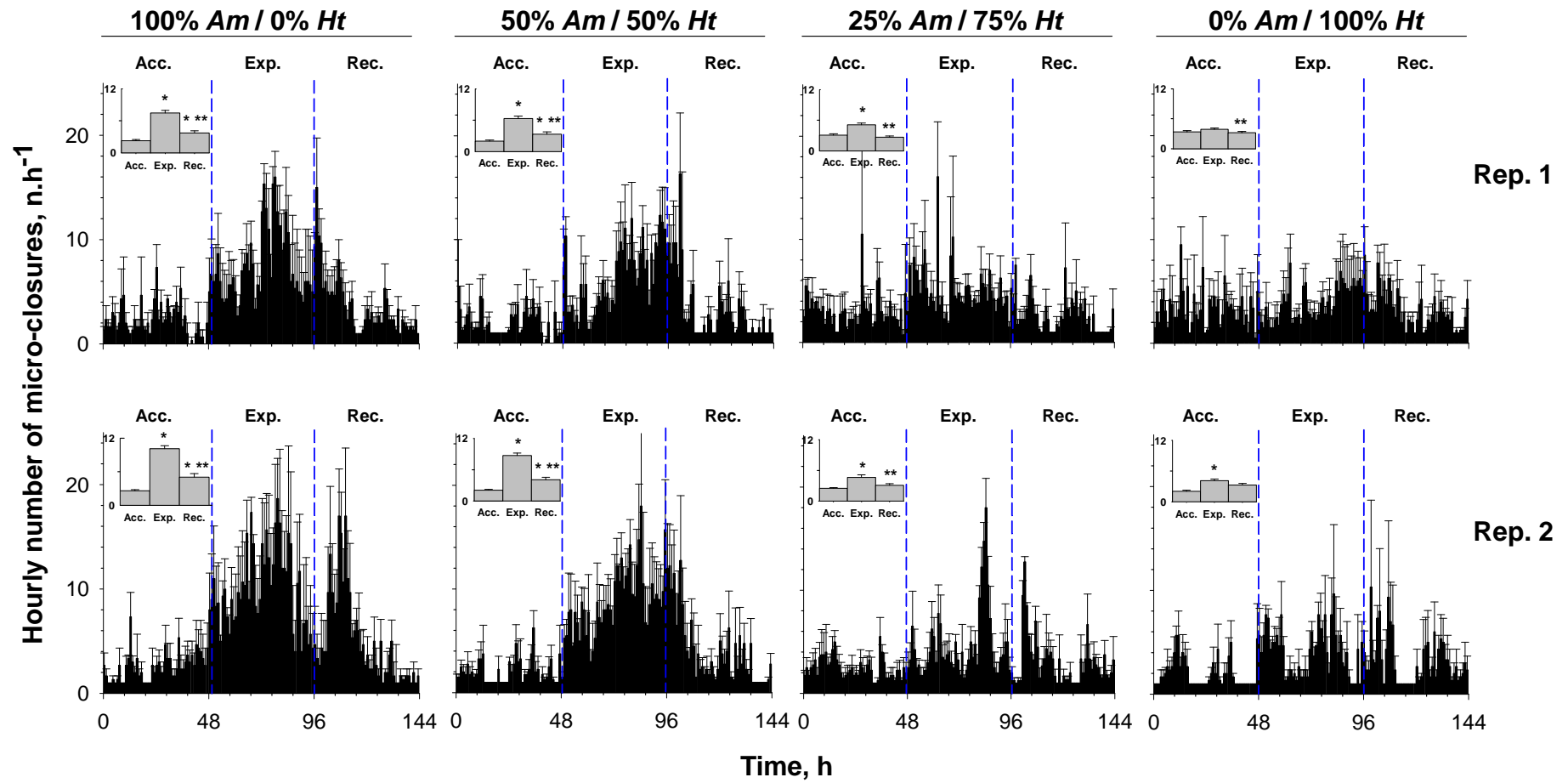


Figure 2

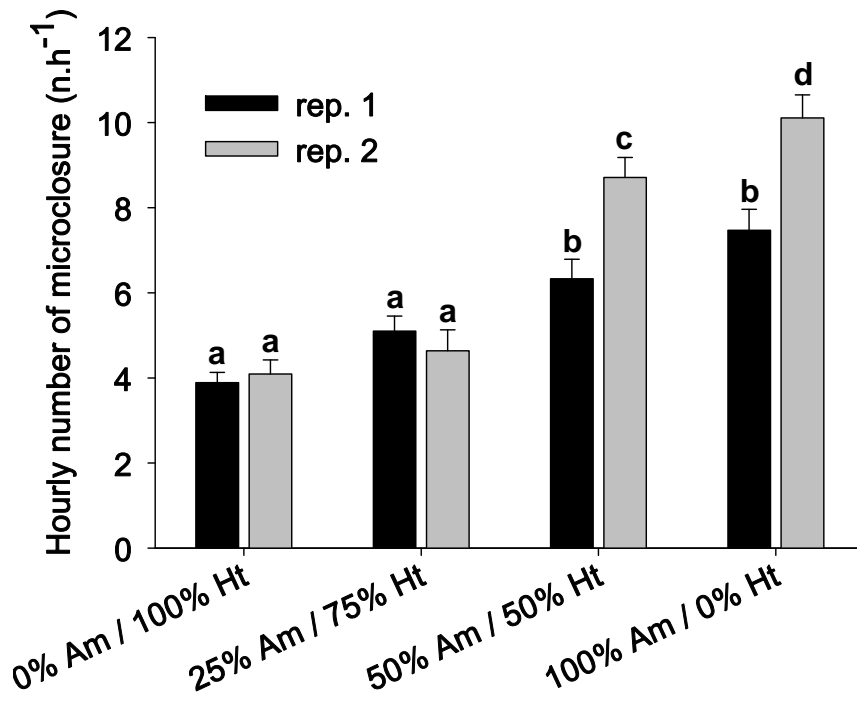


Figure 3

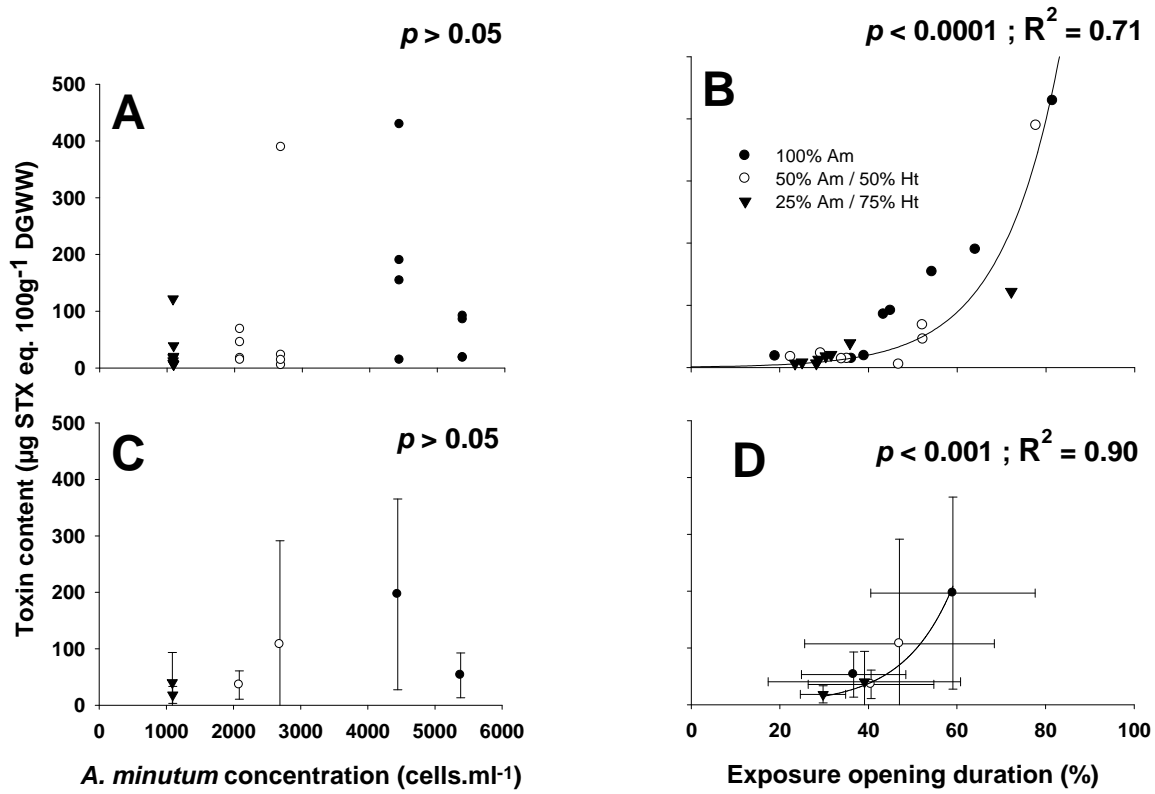


Figure 4



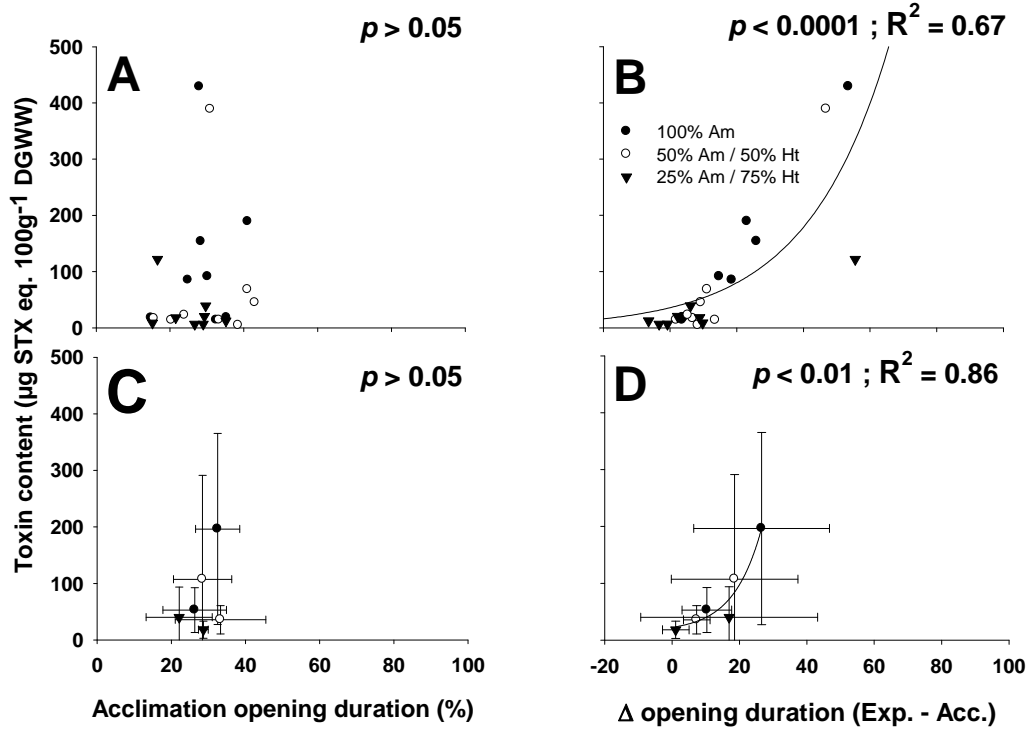


Figure 5