

Relationship between valve activity, microalgae concentration in the water and toxin accumulation in the digestive gland of the Pacific oyster *Crassostrea gigas* exposed to *Alexandrium minutum*

Hansy Haberkorn^a, Damien Tran^b, Jean-Charles Massabuau^b, Pierre Ciret^b, Véronique Savar^c and Philippe Soudant^{a,*}

^a Laboratoire des Sciences de l'Environnement Marin, Institut Universitaire Européen de la Mer, Université de Bretagne Occidentale, Place Copernic, Technopôle Brest-Iroise, 29280 Plouzané, France

^b Université Bordeaux 1, CNRS, UMR 5805 EPOC, Place du Dr Peyneau, 33120, Arcachon, France

^c IFREMER Centre de Nantes, Laboratoire Phycotoxines, BP 21105, 44311 Nantes, France

*: Corresponding author : Philippe Soudant, email address : philippe.soudant@univ-brest.fr

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 four microalgae mixtures at constant total concentrations of 10×10^3 cells ml^{-1} (experiment-1) and 5×10^3 cells ml^{-1} (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 10×10^3 cells ml^{-1} , the microalgal ingestion decreased ($p < 0.05$) with increasing $[A.m.]_w$ but not at 5×10^3 cells ml^{-1} ($p > 0.05$). 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.

Highlights : ► Evaluation of behavioural responses of the Pacific oyster *C. gigas* to *A. minutum* exposure. ► Are behavioural changes related to concentration of harmful microalgae in water and/or to toxin concentration in oyster digestive gland? ► A dose-response relationship between the frequency of micro-closures and the concentration of *A. minutum* in the ambient water. ► A dose-response relationship between opening-duration and the PSP concentration in the digestive gland. ► Analysis at the individual level suggest that oysters exhibiting different sensitivity to PSPs exist within an oyster stock.

Keywords: Harmful algae; Oysters; Behavioral responses; Algae concentration; Toxin accumulation

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-populational 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 ± 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 ± 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 μm). 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·ml⁻¹ and $5 \cdot 10^3$
127 cells·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·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·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·ml⁻¹), ii) 50% *A. minutum*- 50% *H. triquetra* ($5 \cdot 10^3$ cells·ml⁻¹ of *A.*
140 *minutum* and $5 \cdot 10^3$ cells·ml⁻¹ of *H. triquetra*), iii) 25% *A. minutum*- 75% *H. triquetra* ($2.5 \cdot 10^3$
141 cells·ml⁻¹ of *A. minutum* and $7.5 \cdot 10^3$ cells·ml⁻¹ of *H. triquetra*) and iv) 100% *H. triquetra*
142 ($1 \cdot 10^4$ cells·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·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·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·ml⁻¹), ii) 50% *A. minutum*- 50% *H.*
154 *triquetra* ($2.5 \cdot 10^3$ cells·ml⁻¹ of *A. minutum* and $2.5 \cdot 10^3$ cells·ml⁻¹ of *H. triquetra*), iii) 25% *A.*

155 *minutum*- 75% *H. triquetra* ($1.25 \cdot 10^3$ cells.ml⁻¹ of *A. minutum* and $3.75 \cdot 10^3$ cells.ml⁻¹ of *H.*
156 *triquetra*) and iv) 100% *H. triquetra* ($5 \cdot 10^3$ cells.ml⁻¹). Each tank received a continuous flow
157 of 12.5 L.day⁻¹ 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 “Dangoumeau”
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 (≈ 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⁻¹ without *A. minutum*,
221 oysters consumed 92.5 ± 4.6 % of the microalgae supplied in the tanks (Fig. 1A). This
222 percentage decreased with increasing ratio of *A. minutum* added (79.5 ± 9.9 % at 25% *A.m./75*
223 % *H.t.*; 81.7 ± 6.8 % at 50% *A.m./50* % *H.t.* and 48.8 ± 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⁻¹,
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⁻¹ and $5 \cdot 10^3$ cells·ml⁻¹), with the aim to experimentally manipulate oyster
279 feeding activity. Analysis of experiment 1 ($10 \cdot 10^3$ cells·ml⁻¹) 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⁻¹ 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⁻¹. 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 are 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 O₂-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

372 **Acknowledgments**

373

374 We thank Marielle Guéguen and Patrick Lassus, for their advices and technical assistance.

375 This study was carried out with the financial support of the National Research Agency

376 (ANR), "MODECOPHY" project 06SEST23 (2006-2009) and of the Brittany Region,

377 "EPHYTOX" project. We also thank Aquitaine Region and the SME "EUKREA

378 Electromatique" for financial support.

379

379 References

- 380
381 Bardouil, M., Bohec, M., Cormerais, M., Bougrier, S., Lassus, P., 1993. Experimental study
382 of the effects of a toxic microalgal diet on feeding of the oyster *Crassostrea gigas*
383 Thunberg. J. Shellfish Res. 12, 417–422.
- 384 Basti, L., Nagai, K., Shimasaki, Y., Oshima, Y., Honjo, T., Segawa, S., 2009. Effects of the
385 toxic dinoflagellate *Heterocapsa circularisquama* on the valve movement behaviour
386 of the Manila clam *Ruditapes philippinarum*. Aquaculture 291, 41–47.
- 387 Belin, C., Raffin, B., 1998. Les espèces phytoplanctoniques toxiques et nuisibles sur le littoral
388 français de 1984 à 1995, résultats du REPHY (Réseau de surveillance du
389 phytoplancton et des phycotoxines). REPHY report. Ifremer, France, p. 143.
- 390 Bougrier, S., Lassus, P., Bardouil, M., Masselin, P., Truquet, P., 2003. Paralytic shellfish
391 poison accumulation yields and feeding timeactivity in the Pacific oyster (*Crassostrea*
392 *gigas*) and king scallop (*Pecten maximus*). Aquat. Living Resour. 16, 347–352.
- 393 Bricelj, V.M., Lee, J.H., Cembella, A.D., Anderson, D.M., 1990. Uptake kinetics of paralytic
394 shellfish toxins from the dinoflagellate *Alexandrium fundyense* in the mussel *Mytilus*
395 *edulis*. Mar. Ecol. Prog. Ser. 63, 177–188.
- 396 Bricelj, V.M., Shumway, S.E., 1998. Paralytic shellfish toxins in bivalve molluscs:
397 occurrence, transfer kinetics, and biotransformation. Res. Fish. Sci. 6, 315–383.
- 398 Bricelj, V.M., Connell, L., Konoki, K., Mac-Quarrie, S.P., Scheuer, T., Catterall, W.A.,
399 Trainer, V.L., 2005. Sodium channel mutation leading to saxitoxin resistance in clams
400 increases risk of PSP. Nature 434, 763–767.
- 401 Chambon, C., Legeay, A., Durrieu, G., Gonzalez, P., Ciret, P., Massabuau, J.-C., 2007.
402 Influence of the parasite worm *Polydora* sp. on the behavior of the oyster *Crassostrea*
403 *gigas*: a study of the respiratory impact and associated oxidative stress. Mar. Biol.
404 152, 329–338.
- 405 Fernández-Reiriz, M.J., Navarro, J.M., Contreras, A.M., Labarta, U., 2008. Trophic
406 interactions between the toxic dinoflagellate *Alexandrium catenella* and *Mytilus*
407 *chilensis*: Feeding and digestive behaviour to long-term exposure. Aquat. Toxicol. 87,
408 245–251.
- 409 Guéguen, M., Bardouil, M., Baron, R., Lassus, P., Truquet, P., Massardier, J., Amzil, Z.,
410 2008. Detoxification of Pacific oyster *Crassostrea gigas* fed on diets of *Skeletonema*
411 *costatum* with and without silt, following PSP contamination by *Alexandrium*
412 *minutum*. Aquat. Living Resour. 21, 13–20.
- 413 Guillard, R.R.L., Hargraves, P.E., 1993. *Stichochrysis immobilis* is a diatom, not a
414 chrysophyte. Phycologia 32, 234–236.
- 415 Haberkorn, H., Lambert, C., Le Goïc, N., Moal, J., Suquet, M., Guéguen, M., Sunila, I.,
416 Soudant, P., 2010. Effects of *Alexandrium minutum* exposure on nutrition-related
417 processes and reproductive output in oysters *Crassostrea gigas*. Harmful Algae. 9,
418 427–439.
- 419 Hégaret, H., Wikfors, G.H., Soudant, P., Lambert, C., Shumway, S.E., Bérard, J.B., Lassus,
420 P., 2007. Toxic dinoflagellates (*Alexandrium fundyense* and *A. catenella*) have little
421 apparent effect on oyster hemocytes. Mar. Biol. 141, 441–447.
- 422 Lassus, P., Bardouil, M., Beliaeff, B., Masselin, P., Naviner, M., Truquet, P., 1999. Effect of a
423 continuous supply of the toxic dinoflagellate *Alexandrium minutum* Halim on the
424 feeding behavior of the Pacific oyster (*Crassostrea gigas* Thunberg). J. Shellfish Res.
425 18, 211–216.
- 426 Lassus, P., Bardouil, M., Masselin, P., Naviner, M., Truquet, P., 2000. Comparative
427 efficiencies of different non-toxic microalgal diets in detoxification of PSP-
428 contaminated oysters (*Crassostrea gigas* Thunberg). J. Nat. Toxins 9, 1–12.

- 429 Lassus, P., Baron, R., Garen, P., Truquet, P., Masselin, P., Bardouil, M., Leguay, D., Amzil,
430 Z., 2004. Paralytic shellfish poison outbreaks in the Penze estuary: Environmental
431 factors affecting toxin uptake in the oyster, *Crassostrea gigas*. *Aquat. Living Resour.*
432 17, 207–214.
- 433 Lassus, P., Bardouil, M., Baron, R., Bérard, J.B., Masselin, P., Truquet, P., Pitrat, J.P., 2005.
434 Improving detoxification efficiency of PSP-contaminated oysters (*Crassostrea gigas*
435 Thunberg). *Aquaculture Europe*, 3–6.
- 436 Lassus, P., Amzil, Z., Baron, R., Séchet, V., Barillé, L., Abadie, E., Bardouil, M., Sibat, M.,
437 Truquet, P., Bérard, J.B., Guéguen, M., 2007. Modelling the accumulation of PSP
438 toxins in Thau Lagoon oysters (*Crassostrea gigas*) from trials using mixed cultures of
439 *Alexandrium catenella* and *Thalassiosira weissflogii*. *Aquat. Living Resour.* 20, 59–
440 67.
- 441 Li, S.C., Wang, W.X., Hsieh, D., 2001. Feeding and absorption of the toxic dinoflagellate
442 *Alexandrium tamarensis* by two marine bivalves from the South China Sea. *Mar. Biol.*
443 139, 617–624.
- 444 Li, S.C., Wang, W.X., 2001. Radiotracer studies on the feeding of two marine bivalves on the
445 toxic and nontoxic dinoflagellate *Alexandrium tamarensis*. *J. Exp. Mar. Biol. Ecol.*
446 263, 65–75.
- 447 Marie, D., Partensky, F., Vaultot, D., Brussaard, C., 1999. *Current Protocols in Cytometry*,
448 chap. Enumeration of phytoplankton, bacteria, and viruses in marine samples, pp.
449 11.11.11_11.11.15, New York: John Wiley & Sons Inc.
- 450 Medler, S., Silverman, H., 2001. Muscular alteration of gill geometry in vitro: implications
451 for bivalve pumping processes. *Biol. Bull.* 200, 77–86.
- 452 Nagai, K., Honjo, T., Go, J., Yamashita, H., Oh, S.J., 2006. Detecting the shellfish killer
453 *Heterocapsa circularisquama* (Dinophyceae) by measuring bivalve valve activity with
454 a Hall element sensor. *Aquaculture* 255, 395–401.
- 455 Narahashi, T., Moore, J.W., 1968. Neuroactive agents and nerve membrane conductances. *J.*
456 *Gen. Physiol.* 51, 93–101.
- 457 Navarro, J.M., Contreras, A.M., Chaparro, O.R., 2008. Short-term feeding response of the
458 mussel *Mytilus chilensis* exposed to diets containing the toxic dinoflagellate
459 *Alexandrium catenella*. *Rev. Chil. Hist. Nat.* 81, 41–49.
- 460 Oshima, Y., Sugino, K., Itakura, H., Hirota, M., Yasumoto, T., 1990. Comparative studies on
461 paralytic shellfish toxin profile of dinoflagellates and bivalves. In: Graneli, E.,
462 Sundstrom, B., Edler, L., Anderson, D.M. (eds.) *Toxic marine phytoplankton*. Elsevier
463 Science Publishing, New York, p. 391–396.
- 464 Oshima, Y., 1995. Postcolumn derivatization liquid chromatographic method for paralytic
465 shellfish toxins. *J AOAC Int.* 78, 528–532.
- 466 Schmitt, F.G., De Rosa, M., Durrieu, G., Sow, M., Tran, D., P., Ciret, P., Massabuau, J.C.,
467 2011. Statistical study of bivalve high frequency microclosing behavior: scaling
468 properties and shot noise analysis. *Int. J. Bifurcat. Chaos.* in press
- 469 Tran, D., Ciret, P., Ciutat, A., Durrieu, G., Massabuau, J.C., 2003. Estimation of potential and
470 limits of bivalve closure response to detect contaminants: application to cadmium.
471 *Envir. Tox. Chem.* 22, 914–920.
- 472 Tran, D., Haberkorn, H., Soudant, P., Ciret, P., Massabuau, J.C., 2010. Behavioral responses
473 of *Crassostrea gigas* exposed to the harmful algae *Alexandrium minutum*.
474 *Aquaculture*. 298, 338–345.
- 475 Tran, D., Nadau, A., Durrieu, G., Ciret, P., Parisot, J.P., Massabuau, J.C., 2011. Field
476 chronobiology in a molluscan bivalve: how the moon and sun cycles interact to drive
477 oyster activity rhythms. *Chronobiol. Int.* (in press)
- 478 Wildish, D., Lassus, P., Martin, J., Saulnier, A., Bardouil, M., 1998. Effect of the PSP-causing

479 dinoflagellate, *Alexandrium sp.*, on the initial feeding response of *Crassostrea gigas*.
480 Aquat. Living Resour. 11, 35–43.
481

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.

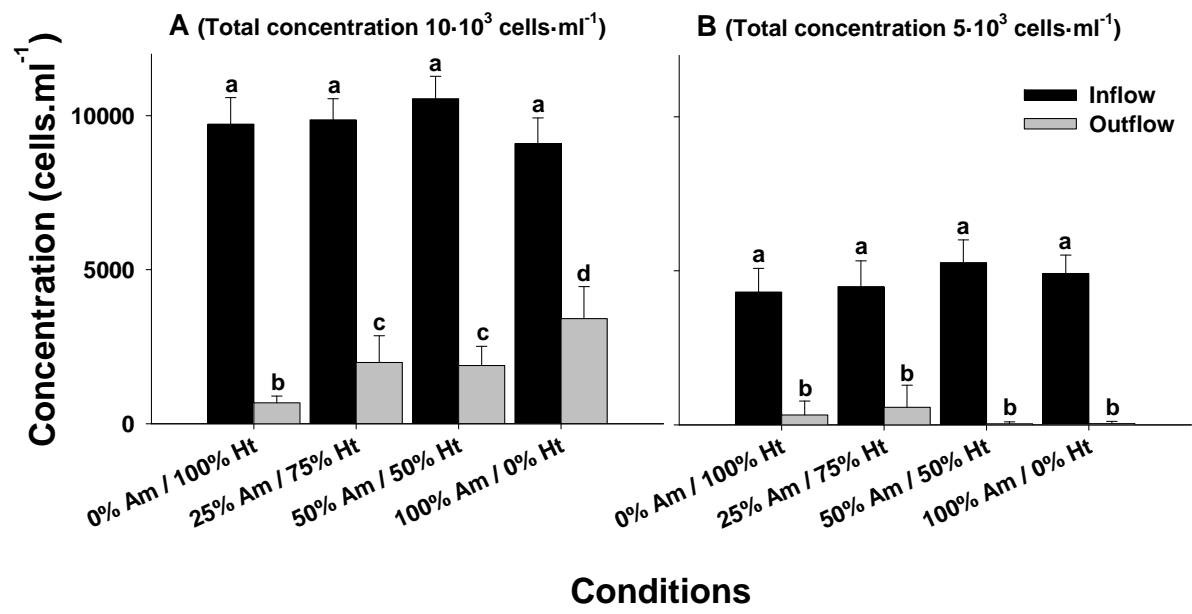


Figure 1

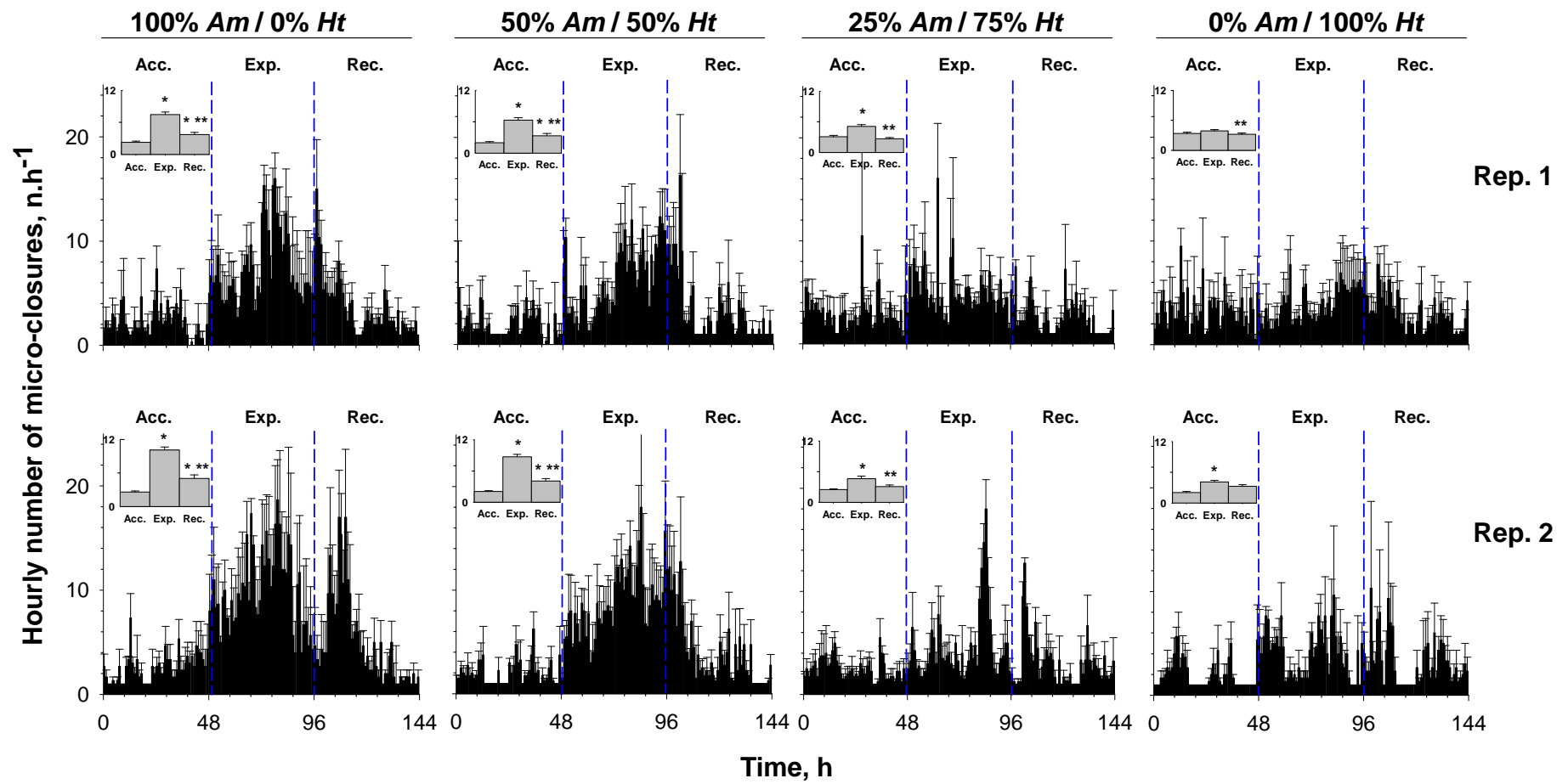


Figure 2

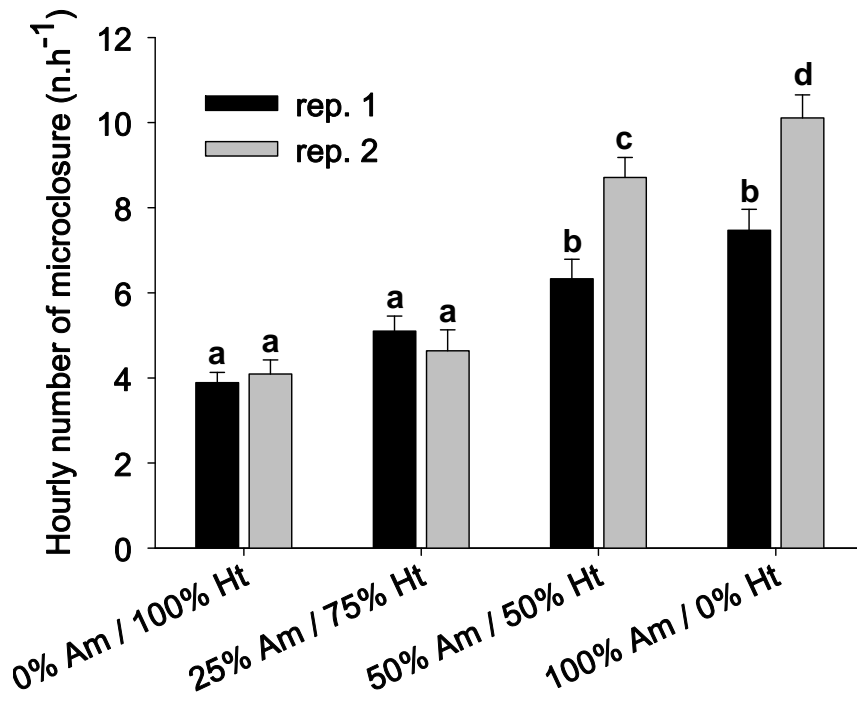


Figure 3

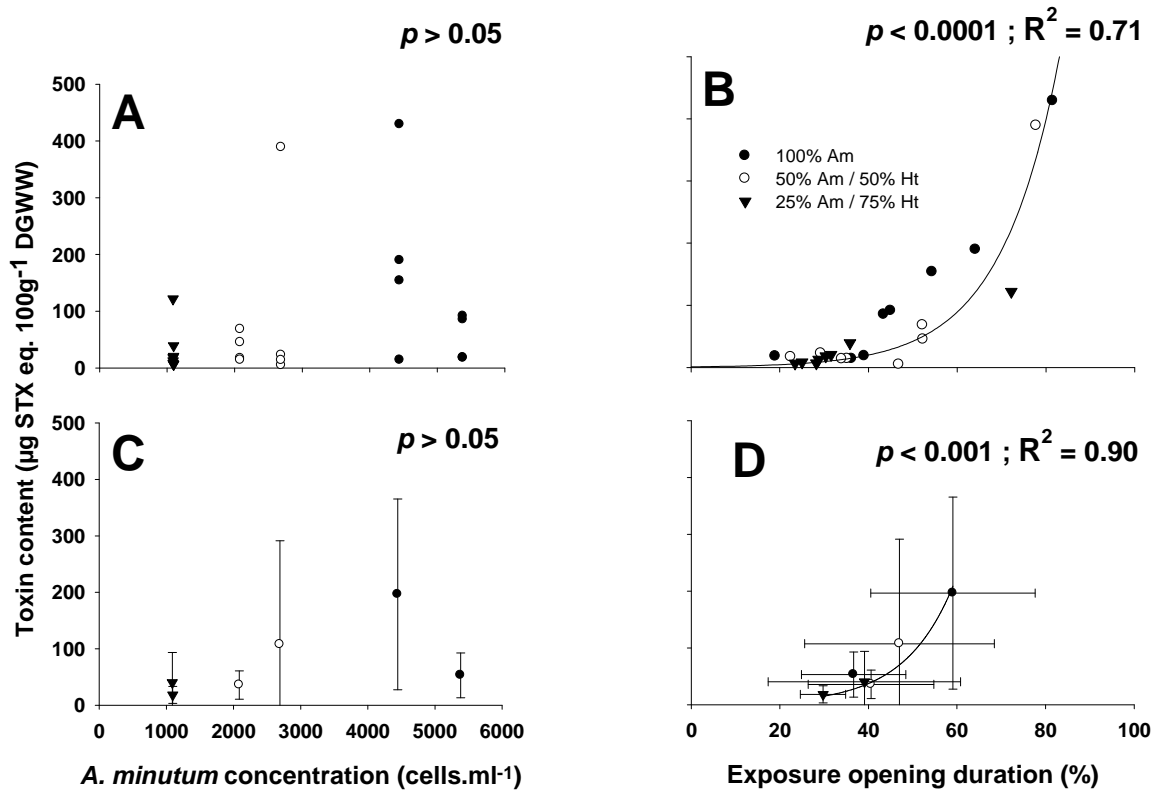


Figure 4

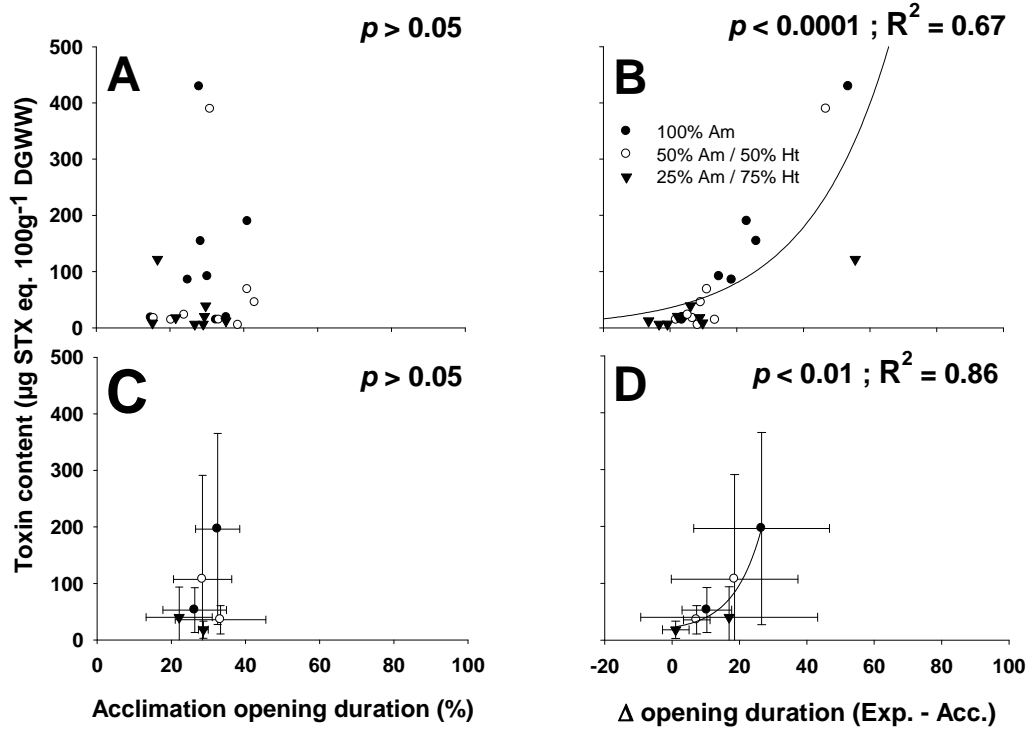


Figure 5