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Sources of paralytic shellfish toxin accumulation variability in the Pacific oyster *Crassostrea gigas*

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Abstract :

This study was designed to assess the contribution of feeding behavior to inter-individual variability of paralytic shellfish toxin (PST) accumulation in the Pacific oyster *Crassostrea gigas*. For this purpose 42 oysters were exposed for 2 days to non-toxic algae and then for 2 other days to the PST producer *Alexandrium minutum*. Individual clearance rate (CR) of oysters was continuously monitored over the 4 days using an ecophysiological measurement system. Comparison of CR values when exposed to toxic and non toxic algae allowed to estimate a clearance rate inhibition index (CRII). Toxin concentration of oysters was quantified at the end of the experiment. These data allowed to estimate the toxin accumulation efficiency (TAE) as the ratio of toxin accumulated on toxin consumed. Changes of clearance rate during the experiment indicated that all individuals stopped feeding immediately after being exposed to *A. minutum* for at least 7 h. This fast response likely corresponded to a behavioral mechanism of avoidance rather to a toxin-induced response. Individuals also showed high inter-variability in their recovery of filtration after this period. Most of the inter-individual variability (78%) in PST accumulation in *C. gigas* could be explained by the consumption of *A. minutum* cells, thus emphasizing the importance of the feeding behavior in accumulation. Based on the toxin concentration in their tissues, oysters were clustered in 3 groups showing contrasted patterns of PST accumulation: the high accumulation group was characterized by high feeding rates both on non-toxic and toxic diet and subsequently a low CRII and high TAE. Inversely, the low accumulation group was characterized by low filtration rates, high CRII and low TAE. Both filtration capacity and sensitivity of oysters to toxins may account for the differences in their accumulation. The contribution of TAE in PST accumulation is discussed and might result from differences in assimilation and detoxification abilities among individuals.

Highlights

► Facing exposure to *A. minutum*, individual clearance rates of oysters were measured. ► Algal consumption explains variability in paralytic shellfish toxin accumulation. ► Three phenotypes were identified on the basis of their accumulation potential. ► Phenotypes differed in their feeding rates. ► Phenotypes also differed in their sensitivity to toxins and accumulation efficiency.

Keywords : *Alexandrium minutum*, Paralytic shellfish poisoning, Accumulation, Clearance rate, Feeding behavior, Pacific oyster

30 1. Introduction

31 Historically, the French oyster culture has faced successive crises that threatened the cultured species
32 and thus the industry (Buestel et al., 2009). The Pacific oyster, *Crassostrea gigas* was introduced in the
33 1970s from Japan and Canada into French farming areas to allow the conservation of oyster production
34 (Grizel and Héral, 1991). Following its import, *C. gigas* became the most cultivated bivalve in France, but
35 also worldwide (i.e. 4.8 millions of tons worldwide in 2013, FAO, 2015). Oyster aquaculture, however,
36 is vulnerable to global warming (Rahel and Olden, 2008) and other associates phenomena such as disease
37 epidemics (Gouletquer et al., 1998; Petton et al., 2015), biological invasions (Stachowicz et al., 2002) or
38 harmful algal blooms (HAB; Moore et al., 2008).

39 The increasing number of HAB occurrences (Van Dolah, 2000; Anderson et al., 2002) has recently been
40 related to warming of Atlantic and Pacific oceans (Gobler et al., 2017). These events can be responsible for
41 amnesic, neurotoxic, diarrhetic or paralytic shellfish poisoning (PSP), among others, thus raising sanitary,
42 social and economic problems. In 2005, total annual costs of HAB were estimated to ca. 813 million \$
43 for Europe (Hoagland and Scatasta, 2006). Amongst dinoflagellates, the ubiquitous and hazardous genus
44 *Alexandrium* can produce saxitoxin (STX; Persich et al., 2006; Anderson et al., 2012), and other potent
45 paralytic shellfish toxins (PST) derivatives from STX. By accumulating toxins in their tissues, filter-feeders
46 can become toxic for consumers (animals or humans, e.g. Bond and Medcof, 1958; Nisbet, 1983; Kwong
47 et al., 2006). PSP in humans can induce numbness, tingling up to paralysis or even death (McFarren et al.,
48 1961).

49 Low environmental concentrations in *Alexandrium minutum* can result in significant accumulation. For
50 instance, environmental concentrations ranging between 9 and 140 cells mL⁻¹ during three weeks were
51 sufficient to induce paralytic shellfish toxin accumulation in *C. gigas* above the sanitary threshold (80 µg
52 equivalent STX 100 g⁻¹; REPHY, 2015) in the bay of Brest during summer 2015. In France, a concentra-
53 tion of *Alexandrium* sp. in seawater above the alert threshold (10 cells mL⁻¹) triggers the quantification of
54 toxin concentration within bivalve tissues which results determine if shellfish harvest has to be closed by

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55 the legal authorities. This decision may sometimes be controversial since the toxin accumulation can vary
56 with the site (Cembella et al., 1994), the bivalve species (Sagou et al., 2005), the individual and/or the organ
57 where toxins are quantified (Kwong et al., 2006). Individual size, seston concentration and its volume-
58 specific toxin concentration have been identified as main sources of variability in mussel PST accumulation
59 (*Mytilus galloprovincialis* ; Moroño et al., 2001). Many studies compared behavior and physiology of dif-
60 ferent bivalve species to explain inter-species variability (Marsden and Shumway, 1993; Contreras et al.,
61 2012; Marsden et al., 2015). Bricelj et al. (1996) showed that the feeding response of different bivalve
62 species was correlated to the animal sensitivity to toxins and to the algal toxicity. Bivalve sensitivity to tox-
63 ins was defined after observations of neurological (Twarog et al., 1972), physiological (Bricelj et al., 1990;
64 Contreras et al., 2012), and behavioral responses (Shumway and Cucci, 1987; Gainey et al., 1988; Bricelj
65 et al., 1996). Under similar experimental conditions PST concentrations in bivalve tissues were shown to
66 vary among individuals by a factor up to 5000 (Mat et al., 2013), indicating a huge inter-individual variabil-
67 ity. Nevertheless the mechanisms explaining this variability remained poorly understood making tricky any
68 prediction of accumulated toxins with modeling approach. Oysters exposed to *A. minutum* (Bougrier et al.,
69 2003) showed a positive relationship between feeding time activity (percent of total time spent in active fil-
70 tration) and their toxin concentration. These results suggest that the variability in toxin accumulation might
71 also be explained by the variability in feeding behavior of *C. gigas*. In this context, it can be hypothesized
72 that (1) inter-individual variability in the clearance rate while feeding on toxic algae (i.e. filtration capacity)
73 is responsible for the variability in toxin accumulation. Nevertheless, Haberkorn et al. (2011) was not able
74 to show any link between oyster valve behavior during acclimation (oyster fed non-toxic algae) and concen-
75 tration of toxins accumulated after a subsequent exposure, but rather showed that during the exposure to *A.*
76 *minutum*, some oysters tend to increase their valve-opening time and strongly accumulate (also observed in
77 Mat et al., in prep.). Thus an additional hypothesis is that (2) behavioral inter-individual variability facing
78 an exposure to *A. minutum* is responsible for inter-individual variability in toxin accumulation. Indeed, it
79 can be hypothesized that when facing an exposure to *A. minutum* some oysters will reduce their clearance
80 rate and will accumulate less toxin, while others will maintain filtration activity and will accumulate more.
81 The present study was designed to further explore the relationship between feeding behavior and toxin ac-
82 cumulation and to test *i*) if there is a link between feeding on non-toxic algae prior *A. minutum* exposure and
83 PST accumulation, *ii*) how much feeding on toxic algae contributes to the variability in PST accumulation.
84 For this purpose oysters' clearance rate fed 2 days on non-toxic algae and then exposed to *A. minutum* for 2

85 more days were monitored.

86 2. Material and methods

87 2.1. Biological material

88 *Oysters*. Ten-months old diploid *C. gigas* oysters (N=42) (shell length = 32.7 mm \pm SD 3.1; total wet
89 mass = 4.3 g \pm SD 0.7; wet flesh mass = 1.0 g \pm SD 0.2 and 0.2 g in dry flesh mass \pm SD 0.05) were
90 used in this experiment. They originated from a cohort of specific-pathogen free oysters produced and
91 reared according to a standardized protocol (Petton et al., 2013, 2015) in Ifremer experimental facilities at
92 Argenton (Brittany, France). They were born in August 2014 from 60 wild broodstock genitors collected in
93 Marennes-Oléron (see Petton et al., 2013). During the whole rearing cycle, oysters were fed *ad libitum* on a
94 mixture of *Tisochrysis lutea* and *Chaetoceros muelleri* and were never exposed to any harmful algal bloom.

95 *Algae cultures*. *T. lutea* (CCAP 927/14) and *C. muelleri* (CCAP 1010/3) were used as the main non-toxic
96 food for oysters. They were cultured with continuous light in separated 300-L cylinders enriched with
97 Conway medium (Walne et al., 1970), and with silicium for *C. muelleri*. The dinoflagellate *Alexandrium*
98 *minutum* (RCC4876, strain Daoulas 1257, isolated in the bay of Brest) was used as the paralytic shellfish
99 toxin (PST) producer. This strain produced only PST toxins, *i.e.* no extracellular compounds responsible
100 for any allelopathic effects (Castrec et al, in prep.), at a concentration of 52.8 fg STX equivalent cell⁻¹
101 (quantified by HPLC at Ifremer Nantes "Laboratoire des phycotoxines", according to Guéguen et al., 2011,
102 protocol). This strain of *A. minutum* was cultured at 21°C in 300-L cylinders of filtered seawater enriched
103 with L1 medium (Guillard and Hargraves, 1993) under continuous light. The culture of *A. minutum* was
104 sampled during the exponential growth phase and diluted for further exposure of oysters to PST. Algal con-
105 centrations of the 3 algal species were monitored daily using a Beckman Coulter Multisizer 3 and expressed
106 in number of cells per milliliter and cell volume (μm^3) per milliliter .

107 2.2. Experimental setup and data collection

108 *Ecophysiological measurement system*. The COSA measurement system (fully described in Aguirre-Velarde
109 et al., 2018) allowed to monitor individual clearance rates (Fig. 1) and was similar to previous automatic de-
110 vices (Savina and Pouvreau, 2004; Flye-Sainte-Marie et al., 2007). The system was composed of 8 identical
111 vices (Savina and Pouvreau, 2004; Flye-Sainte-Marie et al., 2007). The system was composed of 8 identical
112 0.54-L flow-through acrylic chambers supplied with algal mix pumped from a mixing tank. Each chamber

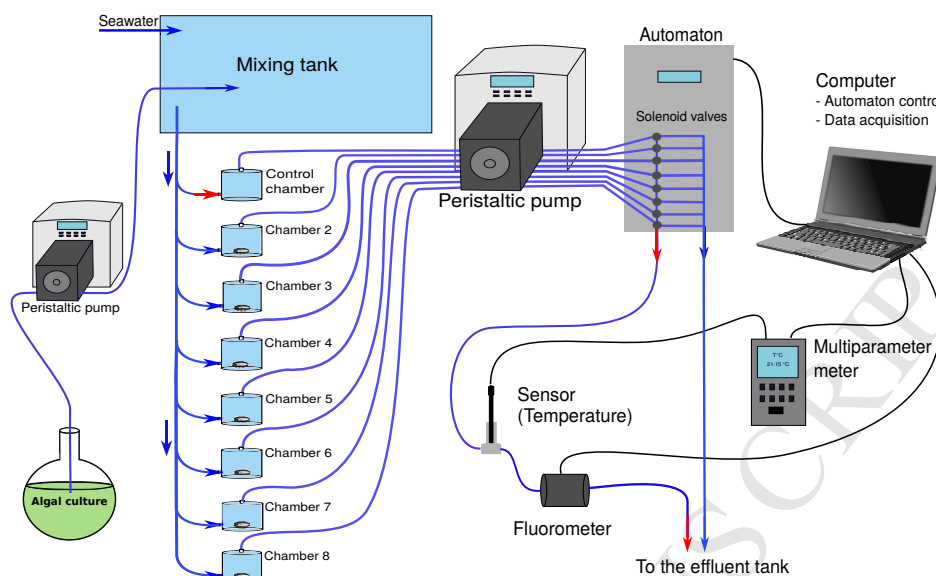


Figure 1: The COSA measurement system. Blue lines indicate the hydraulic circuit and black lines data connections. The control chamber (without oyster) is used as a reference of seawater passing through all chambers. Chambers 2 to 8 contain one oyster each. A peristaltic pump allows the circulation of the water throughout each chamber at a constant flow rate. The computer-controlled automaton controls the water outflowing from any chamber either to a measurement circuit (temperature sensor and fluorometer) or directly to the effluent tank. Chambers 2 to 8 are measured sequentially for 15 min every 3.5h; between each measurement on a chamber containing an oyster the control chamber is measured for 15 min. A computer allows to control the automaton, log and visualize in real-time acquired data.

113 contained one single oyster, except one empty control chamber (Figure 1). Flow rate in the chamber was
 114 adjusted to 40 mL min^{-1} by means of 2 peristaltic pumps (Masterflex L/S 7551, Cole Parmer, USA). The
 115 seawater temperature ($^{\circ}\text{C}$) and the fluorescence (FFU) were measured for 15 minutes in the outflow of each
 116 chamber by a WTW multiparameter meter (WTW Multi 3430) and a fluorometer (WETstar chlorophyll,
 117 WETLABS, Philomat, USA). Calibration lines obtained from cell counts allowed to recalculate microalgal
 118 concentrations from fluorescence. These instruments were connected to a computer that allowed the visu-
 119 alization and acquisition of high frequency time series data. The fluorescence of the water outflowing from
 120 chambers 2 to 8 (containing oysters) was monitored sequentially for 15-min cycle; between each chamber
 121 containing an oyster, the control chamber (chamber 1) was also measured for 15 min. This protocol allowed
 122 the monitoring of each chamber every 3.5 h. All water effluents were treated with chlorine.

123 *Experimental design.* During 4 days, 7 oysters were monitored individually in the flow-through chambers
 124 under controlled conditions. Seawater and ambient air temperatures were maintained at 21°C . During day 1

125 and day 2, oysters were fed on a 50/50 algal mixture of *T. lutea* and *C. muelleri* (Tiso/Chaeto). The exposure
 126 to *A. minutum* was performed on day 3 and day 4. This trial was repeated 6 times so that a total of 42 oysters
 127 were monitored over the whole experiment. The concentration of Tiso/Chaeto mixture was adjusted as a
 128 function of the fluorescence in the control chamber thus resulting in algae concentration ranging between
 129 16 000 and 24 000 cells mL⁻¹ due to the variability of fluorescence properties of the algae culture. For each
 130 exposure trial, *A. minutum* was distributed at different levels of concentration ranging from a mean of 650
 131 cells mL⁻¹ for the lowest to 1800 cells mL⁻¹ for the highest exposure concentration.
 132 The system was stopped daily during two hours for cleaning to prevent the development of a biofilm within
 133 the circuit. Oysters were removed from their chambers and maintained in 1 µm filtered seawater. The entire
 134 circuit (chambers included) was washed with a stabilized mixture of peracetic acid and hydrogen peroxide
 135 (Hydrogent) and rinsed with hot freshwater and then with filtered seawater.

136 *Final biometry and toxin quantification.* At the end of each 4-days trial, oyster tissues were dissected,
 137 weighed (wet mass, g) and stored at -80°C until toxin quantification.

138 PST were quantified individually in total oyster body tissues using ELISA PSP kit developed by Abraxis
 139 (see methods in Lassudrie et al., 2015a,b). For this purpose, oyster tissues were mixed (1:1, w:v) in 0.1
 140 M HCl solution, grounded (Fastprep-24 5G homogenizer) and boiled for 5 minutes at 100°C in order to
 141 acid-hydrolyse PST analogs into saxitoxins (STX). The samples were then disposed in the Abraxis ELISA
 142 PSP kit and toxin concentrations were quantified by spectrophotometry and expressed in µg of STX for 100
 143 g of total flesh mass.

144 2.3. Data analysis

145 *Clearance rates.* Individual clearance rates (CR_{oyst} , L h⁻¹ ind⁻¹) corresponding to the volume of exhaled
 146 water cleared of particles per unit time, were calculated from the fluorescence data recorded during the last
 147 7 minutes of each measurement period (in order to allow a full water renewal on the sensors). According to
 148 Hildreth and Crisp (1976) formula:

$$149 \quad CR_{oyst} = F_R \frac{Fluo_{cont} - Fluo_{oyst}}{Fluo_{oyst}}$$

150 where, F_R is the flowrate throughout the chamber (L h⁻¹), $Fluo_{cont}$ the average fluorescence of the control
 151 chamber measured before and after the chamber, and $Fluo_{oyst}$ the average fluorescence of chamber 2 to 8
 152 containing one oyster each.

153 In order to correct these rates from variations in individual size between chambers, individual clearance
 154 rates were standardized to a standard size of 1 g in flesh wet mass using Bayne et al. (1987) formula:

$$155 \quad CR_s = \left(\frac{W_s}{W_{oyst}} \right)^b \times CR_{oyst}$$

156 where CR_s was the clearance rate corrected for an individual of a standard mass W_s (i.e. 1 g of wet mass),
 157 W_{oyst} the wet mass of the monitored oyster, CR_{oyst} the measured clearance rate of the oyster and b was the
 158 allometric coefficient equal to $\frac{2}{3}$ according to Pouvreau et al. (1999).

159 *Clearance rate inhibition index.* For each individual, standardized clearance rates measured during days
 160 1 and 2 ($CR_{Snon\ toxic}$) and standardized clearance rates at day 4 with toxic algae ($CR_{S\ toxic}$) were used to
 161 compute a clearance rate inhibition index ($CRII$) allowing to quantify the inhibition of clearance rate due
 162 to *A. minutum*. It was calculated as $CRII = 1 - \frac{CR_{S\ toxic}}{CR_{Snon\ toxic}}$

163 *Statistics and clustering.* Statistical analyses were performed using the R software (R Core Team, 2016).
 164 Type II linear regressions with ranged major axis method were applied to adjust linear relations between the
 165 number of algal cells consumed and the toxin concentration (two variables measured with error) by using
 166 the R package "lmodel2" (Legendre, 2014).

167 Because the concentration of *A. minutum* varied among experiments, the 42 oysters were clustered
 168 according to their ratio between the toxin concentration after exposure and the quantity of *A. minutum*
 169 cells delivered during exposure. Three accumulation groups could be easily distinguished on the basis of
 170 this ratio, thus corroborating previous observations (Bouillot, 2017; Mat et al., in prep.). A hierarchical
 171 clustering function was applied on this ratio with the Ward's method to segregate individuals into three
 172 groups.

173 In order to compare individual CR prior and during the exposure to *A. minutum* among the 3 clusters,
 174 linear mixed-effect models were adjusted. Tukey post-hoc tests were applied to distinguish groups.

175 3. Results

176 3.1. Toxin accumulation in oyster tissues

177 After the 2-d exposure to *A. minutum*, all oysters accumulated toxins in their tissues at concentrations
 178 varying between 6 and 173 μg of STX per 100 g of wet flesh. Among them, half of the individuals exhibited
 179 toxins above the sanitary threshold of 80 μg of STX per 100 g of wet flesh and no mortality was observed.

180 The ratio of the lowest to the highest concentration of toxins within each 4-d trial (i.e. for 7 oysters) varied
 181 from 2.1 to 8.5, indicating a strong inter-individual variability in toxin accumulation. In most experiments,
 182 three accumulation groups were easily distinguishable which corroborated previous observations (Bouillot,
 183 2017; Mat et al., in prep.). Based on the ratio of the concentration of toxins to the number of *A. minutum*
 184 cells distributed, the 42 oysters were clustered into 3 groups using a hierarchical clustering function. This
 185 allowed to assign 10 oysters (24 %), 21 oysters (50%) and 11 oysters (26 %), respectively to the low,
 186 intermediate and high toxin accumulation groups.

187 3.2. Temporal evolution of oyster clearance rates

188 Standardized clearance rate measurements indicated that oyster filtration activity almost stopped just
 189 after the exposure to *A. minutum* for a period of ≈ 7 h (Fig. 2). Then a recovery was observed for some
 190 individuals, this tendency being more visible 24h after the beginning of the exposure. Nevertheless, filtra-
 191 tion activity did not recover to values observed with non-toxic algae. Pseudo-faeces production was only
 192 exceptionally observed during the experiments.

193 When fed on non-toxic algae (days 1 and 2), the average CR for a standard oyster of 1 g ($CR_{Snon-toxic}$)
 194 was significantly higher for the high accumulation group compared to the low one. But no significant
 195 differences were observed between the low and intermediate groups nor between the high and intermediate
 196 groups (Tab. 1). After the early phase of CR inhibition, at the beginning of the exposure phase to *A.*
 197 *minutum*, mean individual standardized CR differed significantly among the 3 accumulation groups, with
 198 respectively 0.33 L h^{-1} , 1.06 L h^{-1} and 2.24 L h^{-1} for low, intermediate and high accumulation groups (Tab.
 199 1).

Table 1: Results of the Tukey test performed on linear mixed-effects models in order to compare clearance rates of oysters before (day 2 only) and during (day 4 only) exposure to *A. minutum* for the three accumulation clusters (*, p-values < 0.05 and *** p-values < 0.001).

Accumulation clusters	Before exposure (day 2)				During exposure (day 4)			
	Estimate	Std. Error	z value	p-value	Estimate	Std. Error	z value	p-value
Low - Intermediate	0.7154	0.2760	2.592	0.028*	0.7232	0.2494	2.899	0.011*
Low - High	1.3546	0.3241	4.179	$<10^{-4}$ ***	1.8765	0.2927	6.412	$<10^{-9}$ ***
Intermediate - High	0.6392	0.2852	2.242	0.075	1.1533	0.2583	4.465	$<10^{-4}$ ***

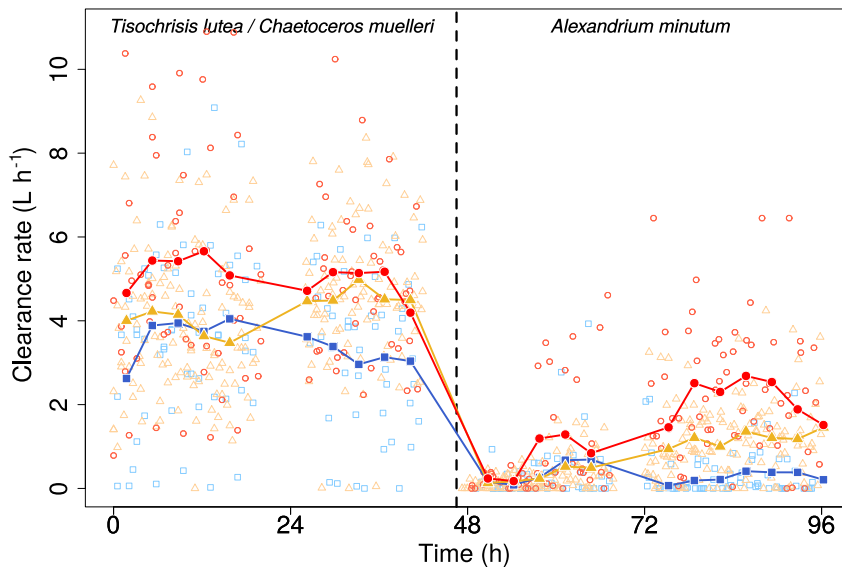


Figure 2: Evolution of standardized clearance rates over the 4 experimental days for all experiments. The first 2 days, oysters were exposed to a mix of *T. lutea* and *C. muelleri*, followed by a 2-day exposure to *A. minutum*. Empty markers correspond to all individual measurements performed on: \square the low accumulation cluster, \triangle the intermediate accumulation cluster and \circ the high accumulation cluster. Filled markers correspond to the average values for each acquisition cycle (3.5h) of each accumulation cluster: \blacksquare low, \blacktriangle intermediate and \bullet high accumulation clusters.

3.3. Inhibition of oyster clearance rate when exposed to *A. minutum*

There was a significant inverse relationship between clearance rate inhibition index (*CRII*) and the concentration of toxins in oyster tissues (Spearman's $\rho = -0.69$, $p\text{-value} = 1.16 \cdot 10^{-6}$; Fig. 3). *CRII* differed significantly between accumulation groups (Wilcoxon test, $p\text{-values} < 0.05$) with mean values of 0.86, 0.71 and 0.53 respectively in the low, intermediate and high accumulation groups.

3.4. Relationship between oyster algal consumption and toxin accumulation

Algal consumption rates ($\text{cell g}^{-1} \text{d}^{-1}$) were calculated from unstandardized clearance rates, algal concentrations and individual oyster wet mass and allowed to take into account the different algal concentrations delivered. The correlation between these values and the final toxin concentration was thus evaluated (Fig. 4 and 5). A strong and significant relationship could be observed between the total number of *A. minutum* cells consumed during the exposure and the final toxin concentration with a R^2 of 0.78 (Fig. 4). Daily relationships are shown in Figure 5.

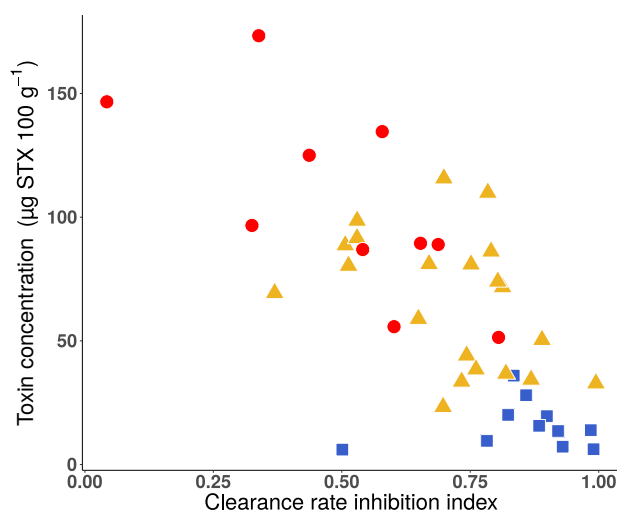


Figure 3: Individual clearance rate inhibition index (*CRII*) as a function of final toxin accumulation. Symbols refer to the different accumulation groups: ■ low, ▲ intermediate and ● high accumulation clusters. Spearman's rho was calculated from these data resulting in an inverse correlation equal to -0.69 ($p\text{-value} = 1.15 \cdot 10^{-6}$).

212 No correlation was found between the toxin concentration in oyster tissues at day 4 and their algae
 213 consumption at days 1 and 2 (Fig. 5 a and b), suggesting that the filtration of oysters on non-toxic algae was
 214 likely not related to their accumulation capacity. Conversely, the toxin concentrations in oyster tissues were
 215 significantly correlated with the number of cells they consumed on day 3 ($R^2=0.29$) and on day 4 ($R^2=0.81$)
 216 (Fig. 5 c and d). This indicated that the number of cells consumed by oysters on the second (and last) day
 217 of exposure to *A. minutum* contributed to the majority of the toxins that have been accumulated in oyster
 218 tissues.

219 3.5. Oyster tissues toxin concentration and toxins consumed

220 Toxin amount consumed by oysters was estimated on the basis of the number of *A. minutum* cells con-
 221 sumed and the STX content of each *A. minutum* cell ($52.8 \text{ fg STX eq. cell}^{-1}$, see section 2.1). The ratio
 222 between the final toxin content and the amount of toxin consumed was calculated for each individual and
 223 compared between accumulation clusters (Fig. 6). Such a ratio provides an indication of the toxin accu-
 224 mulation efficiency (TAE; see e.g. Bougrier et al., 2003; Mafra et al., 2010), which may depend on various
 225 processes i.e. pre-ingestion selection, toxin assimilation but also toxin depuration. This ratio significantly
 226 differed between clusters (Wilcoxon tests; $p<0.01$). The low accumulation cluster had the lowest ratio as
 227 the high accumulation cluster had the highest.

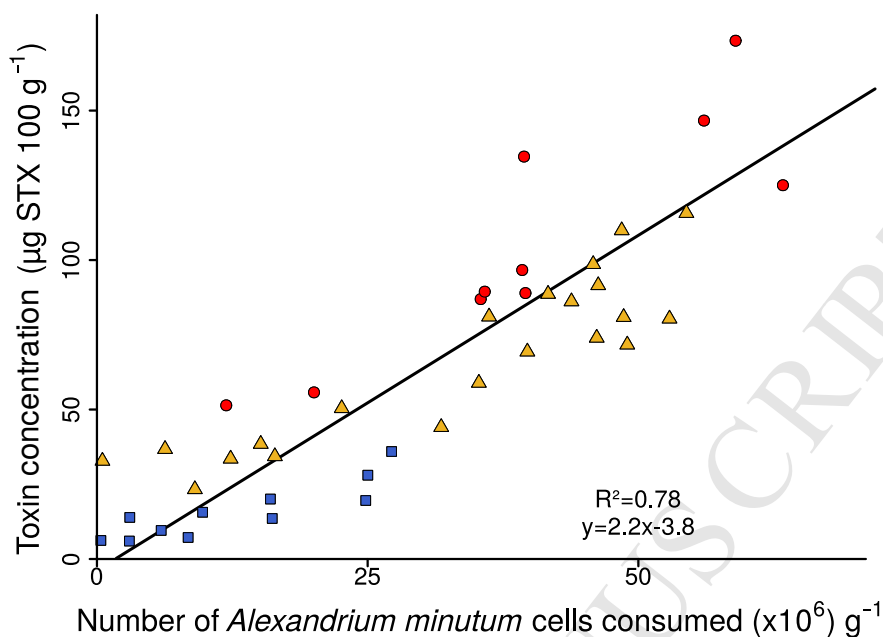


Figure 4: Individual toxin concentration in oyster tissues at day 4 ($\mu\text{g STX } 100 \text{ g}^{-1}$) against the cumulated number of *A. minutum* cells consumed by oysters (g^{-1} of wet mass) over all trials of the experiment. The line corresponds to the adjusted type II regression. Symbols refer to the different accumulation clusters: ■ low, ▲ intermediate and ● high accumulation clusters.

228 4. Discussion

229 4.1. Feeding behavior during an exposure to *A. minutum* drives toxin accumulation

230 Previous results clearly emphasize the importance of inter-individual variability in toxin accumulation
 231 by *C. gigas*. Laboratory experiments showed that oysters exposed to similar concentration of *A. minutum*
 232 exhibited a variability in toxin accumulation up to a factor 5000 (Mat et al., 2013). The aim of this study
 233 was to test if feeding behavior could be responsible for variability in toxin accumulation as hypothesized by
 234 Bougrier et al. (2003) and Haberkorn et al. (2011). Our results emphasized a high inter-individual variability
 235 in clearance rates of both non-toxic and toxic algae although all individuals used for this experiment came
 236 from the same cohort and were reared under the same conditions. Similar to Bougrier et al. (2003) a close
 237 correlation between the number of *A. minutum* cells consumed by oysters and the final concentration of
 238 toxin in their tissues was observed (Fig. 4). Our results thus showed that inter-individual variability in
 239 harmful algal consumption during an exposure to *A. minutum* explained 78 % of the variability in final
 240 toxin accumulation.

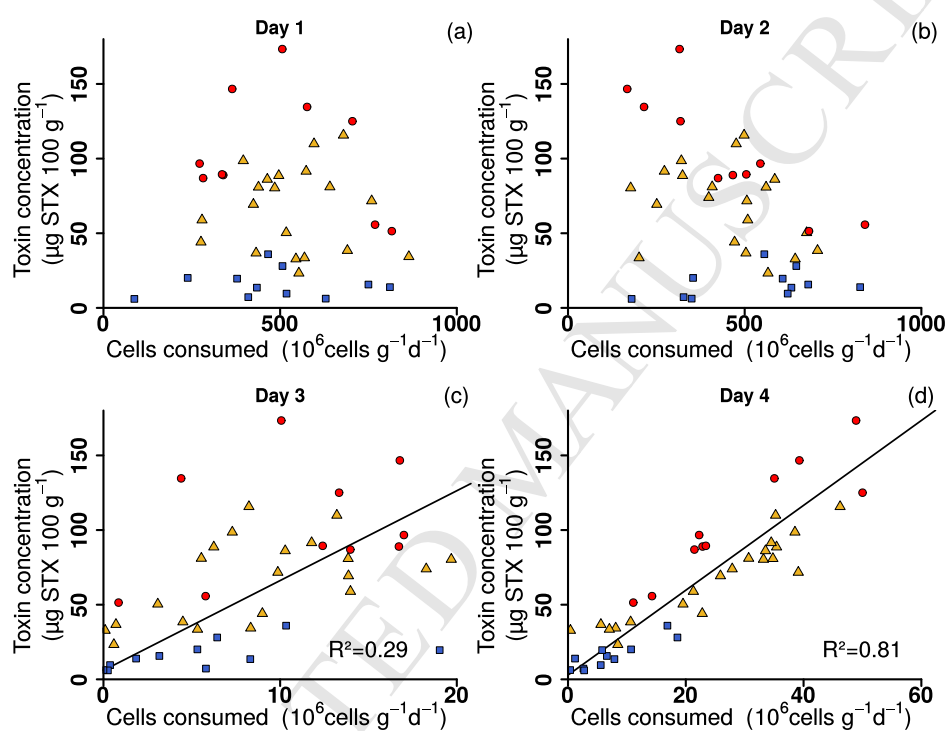


Figure 5: Individual toxin concentration in oyster tissues at the end of the exposure (day 4, $\mu\text{g STX } 100 \text{ g}^{-1}$) against the daily algae consumption of oysters (number of cells g^{-1}) for all trials. Lines indicate the adjusted type II regression models (when significant). Oysters were fed *T. lutea* and *C. muelleri* during days 1 (a) and 2 (b) and *A. minutum* during days 3 (c) and 4 (d). Symbols refer to the different accumulation clusters: ■ low, ▲ intermediate and ● high accumulation clusters.

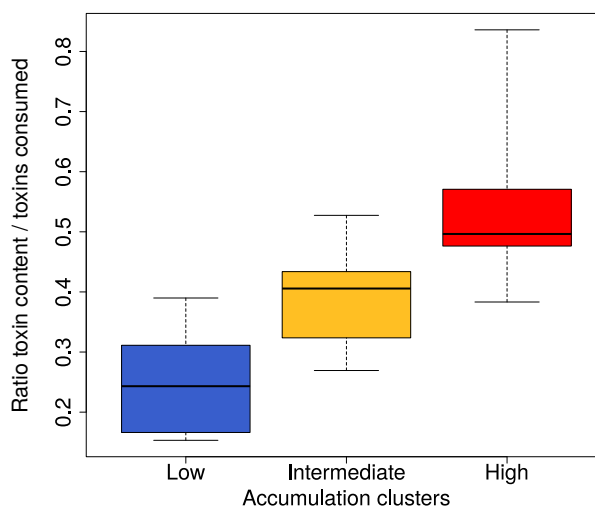


Figure 6: Ratio between final toxin content and toxins consumed for the three accumulation clusters. Low, intermediate and high accumulation clusters are composed of 10, 19 and 9 oysters respectively. Four aberrant values were removed from the dataset. Horizontal lines correspond to the median, boxes to 50 % of the variability and error bars to the minimum and maximum values.

241 4.2. Initial feeding response to *A. minutum* exposure

242 Despite the high inter-individual variability in CR_s , the 42 individuals exhibited the same reaction to
 243 *A. minutum* exposure: they all reduced or even stopped their filtration activity for at least 7 hours (Fig. 2).
 244 This reduction was followed by a partial recovery during which inter-individual variability was high. Such
 245 a two-phase response has already been described in *C. gigas* exposed to *Alexandrium catenella* (Dupuy and
 246 Sparks, 1968).

247 Although not observed in all species (Leverone et al., 2007; Hégarret et al., 2007; Contreras et al., 2012)
 248 this inhibition in feeding activity immediately after an exposure to PSP-causing dinoflagellates seems to be
 249 a general pattern in the genus *Crassostrea* (e.g. Shumway and Cucci, 1987; Gainey et al., 1988; Wildish
 250 et al., 1998; Laabir et al., 2007). Several mechanisms may explain this immediate initial response: a direct
 251 impact of the toxin on gills (Medler and Silverman, 2001) and muscles (Hégarret et al., 2007) decreasing
 252 filtration activity, or a behavioral inhibition of feeding activity allowing avoidance of poor quality or toxic
 253 seston (Lassus et al., 1999, 2004). The first hypothesis is unlikely because it would imply a delayed response
 254 (4-5 days in *C. virginica* exposed to PST; Hégarret et al., 2007), but a behavioral modification was rather
 255 immediate as also observed by Tran et al. (2010). A partial recovery of filtration occurred in most of the
 256 oysters (Fig. 3) after less than 24h and oysters that accumulated more toxins were also those that filtrated

257 more (Fig. 4). These two observations are additional elements against the toxin effect hypothesis. Wildish
258 et al. (1998) did not observe any differences in short-term responses of *C. gigas* exposed to toxic and
259 non-toxic *Alexandrium* sp. and also hypothesized that PSP toxins were not directly involved. A behavioral
260 avoidance mechanism of oysters was the most plausible explanation in our experiment. Pre-ingestive sorting
261 is a well known strategy to avoid low-quality particles (Ward et al., 1998; Mafra et al., 2009) but it is unlikely
262 that this phenomenon occurred because (1) pseudo-faeces production was only exceptionally observed and
263 (2) pseudo-faeces production does not imply reduction of clearance rate as observed. Under sub-optimal
264 condition, bivalves can adapt the filtration activity by reducing valve gape, retracting mantle edge (see
265 review in Jørgensen, 1996) decreasing ctenidia transport velocity (Ward et al., 2003). Facing a change
266 in diet quality (size, shape, nutritive quality, species...) like shifting from forage algae to *A. minutum* it is
267 likely that such a phenomenon occurs. Valve closure of oysters when exposed to *Alexandrium* sp. have been
268 previously observed (Shumway et al., 1985; Tran et al., 2010).

269 4.3. Mechanisms behind the behavioral variability of oysters in response to *A. minutum*

270 One interesting observation is the high inter-individual variability in the recovery of filtration activity in
271 the second phase of the exposure. The three accumulation clusters exhibited significantly different clearance
272 rates on the second day of exposure to *A. minutum* (Fig. 2 ; Tab. 1). This high inter-individual variability
273 is also emphasized by the highly variable clearance rate inhibition index (CRII) that ranges from close to
274 0 for oysters in the high accumulation cluster from close to 1 for oysters in the low accumulation cluster
275 (Fig. 3). High clearance rate inhibition index values were inversely related to low toxin accumulation .
276 Thus variability of the clearance rate inhibition in the reaction to *A. minutum* seems to play a major role
277 in the variability of the toxin accumulation. In other words, when facing an exposure to *A. minutum* all
278 oysters reduce their filtration activity, some less than others, thus leading to an important variability in toxin
279 accumulation. This variability might be explained by two non-mutually exclusive hypotheses.

280 The first one would be that inter-individual variability in standardized clearance rate during exposure
281 is linked to inter-individual variability in filtration capacity estimated as the standardized clearance rate on
282 non-toxic algae. When fed non-toxic algae, individuals displayed variable levels of clearance rates (Fig. 2)
283 that might be interpreted as phenotypic variability in filtration capacity. Our results show that the hierarchy
284 of clearance rates of the different accumulation clusters remain identical before and during exposure. The
285 level of feeding on non-toxic algae might thus constitute a first basis to predict the feeding response of

286 oysters facing *A. minutum*. However the tendency is not that clear since non-toxic food consumed does not
287 significantly explain the final toxin concentration (Fig. 5 a and b). Variability in filtration capacity might
288 contribute to the observed variability facing *A. minutum* nevertheless the relative reduction in clearance rate
289 (CRII) observed is variable between individuals.

290 The second hypothesis would be that individuals present an inter-individual variability in their sensitiv-
291 ity facing *A. minutum*, either linked to behavioral differences facing *A. minutum* or linked to differences in
292 sensitivity to the toxin itself. Interspecific differences in sensitivity, estimated through block of nerve action
293 potential (Twarog et al., 1972), have been associated with differences in toxin accumulation: the most sen-
294 sitive species tend to accumulate less (see review of Bricelj and Shumway, 1998). Such a pattern has also
295 been observed at an intraspecific scale in *Mya arenaria* (Bricelj et al., 2005). Because a part of sensitivity to
296 STX has been observed to have a genetic basis (sodium channel polymorphism; Kontis and Goldin, 1993;
297 Bricelj et al., 1996), it might differ between individuals. It can be thus hypothesized that some individu-
298 als are more sensitive to the toxin, that their clearance rate is more inhibited and that they subsequently
299 accumulate less toxins. Our results, however clearly show that within a single oyster population there is
300 an important inter-individual variability in the level of inhibition of the clearance rate (at day 4) which is
301 significantly linked to the toxin concentration (Fig. 3). The mechanisms behind the variability of clearance
302 rate inhibition after recovery of feeding (day 4) remains to be identified.

303 4.4. Toxin accumulation efficiency

304 Toxin accumulation efficiency (TAE) is generally taken as [cumulative toxin ingested/ toxin incorpor-
305 ated in tissues] \times 100 and has been used for inter-species comparisons (see e.g. Bricelj et al., 1990; Bricelj
306 and Shumway, 1998). Although our experimental design was different from the one of Bougrier et al.
307 (2003) we found an average TAE of the same order of magnitude (35% present study ; 20-23% in Bougrier
308 et al., 2003). Moreover, the mean TAE calculated before might be more likely close to 30% since the ELISA
309 method used to measure the toxin concentration is known to overestimate with an approximate 1.2 factor
310 compared to HPLC (Lassudrie, pers. comm.). These values are close to those obtained for *Mercenaria*
311 *mercenaria* (35-40%, Bricelj et al., 1991) or *Pecten maximus* (30%, Bougrier et al., 2003) but lower than
312 those observed for mussels (72% to 96% in *Mytilus californianus*, Dupuy and Sparks, 1968; 50% in *Perna*
313 *viridis*, Wisessang et al., 1991; 78% in *Mytilus edulis*, Bricelj et al., 1990). Relating these values to Twarog
314 et al. (1972)'s ranking of sensitivity to STX tends to indicate that species presenting a high TAE are less
315 sensitive and, as discussed above, tend to accumulate more (Bricelj and Shumway, 1998).

316 These results are the first ones to emphasize intra-specific variations of TAE, which significantly differed
317 between accumulation clusters (Fig. 6). The high accumulation group had a TAE twice as high as the low
318 accumulation one. Interpretation of the variations of TAE is not straightforward, because total toxin burden
319 is the sum of toxin content of two compartments: (1) undigested toxins that remains in the digestive tract
320 and (2) assimilated toxin within body tissues (Bricelj et al., 1990; Lassus et al., 2007). Variations of TAE
321 may thus be linked to variations in inputs and/or outputs of these compartments. Because consumption was
322 estimated from clearance rate, pseudo-feces production could affect the ingestion and thus the estimation of
323 TAE; but it is unlikely because pseudo-feces production was only punctually observed. Lassus et al. (2007)
324 modeled the PST accumulation kinetics in *C. gigas* in the Thau lagoon by taking into account two depuration
325 ways: (1) a mechanical one, via the egestion of undigested toxins (called "excretion" in Lassus et al., 2007)
326 which is a fast pathway and considered as the major one; (2) metabolic elimination (biotransformation;
327 related to ammonia excretion according to Navarro and Contreras, 2010) of assimilated toxins which is a
328 slower and minor pathway.

329 Two mechanisms might explain the different observed values of TAE. Firstly, high TAE values might be
330 associated with high food (and toxin) assimilation and therefore reducing the amount of egestable toxins.
331 According to Lassus et al. (2007), these assimilated toxins would be less efficiently eliminated. Secondly,
332 the metabolic elimination pathway could be saturated due to the high concentrations of toxins. Thus indi-
333 viduals with high concentrations of PST could reach a maximum toxin elimination rate and subsequently
334 detoxify lower in relation to the amount of toxins. Further experimental work is needed to better understand
335 the relative contribution of the assimilation and detoxification on the variations of TAE.

336 4.5. Applications for aquaculture

337 Further analyses on the three accumulation clusters would be needed to characterize if these differences
338 in phenotype have a genetic basis. A heritable genetic basis of the accumulation of okadaic acid (another
339 phycotoxin) has been shown in *Mytilus galloprovincialis* (Pino-Querido et al., 2015). If PST accumulation
340 in *C. gigas* had a genetic and heritable basis, low PST accumulation oysters may be obtained by selective
341 breeding. Nevertheless, such a selection would imply the selection of oysters also presenting a low filtration
342 activity that might be associated with a low growth potential thus increasing the production time. Oyster
343 farmers try to reduce production times by working with fast-growing oysters (i.e. triploids, selected fast-
344 growing families). It is likely that such a selection would also select for oysters presenting high clearance

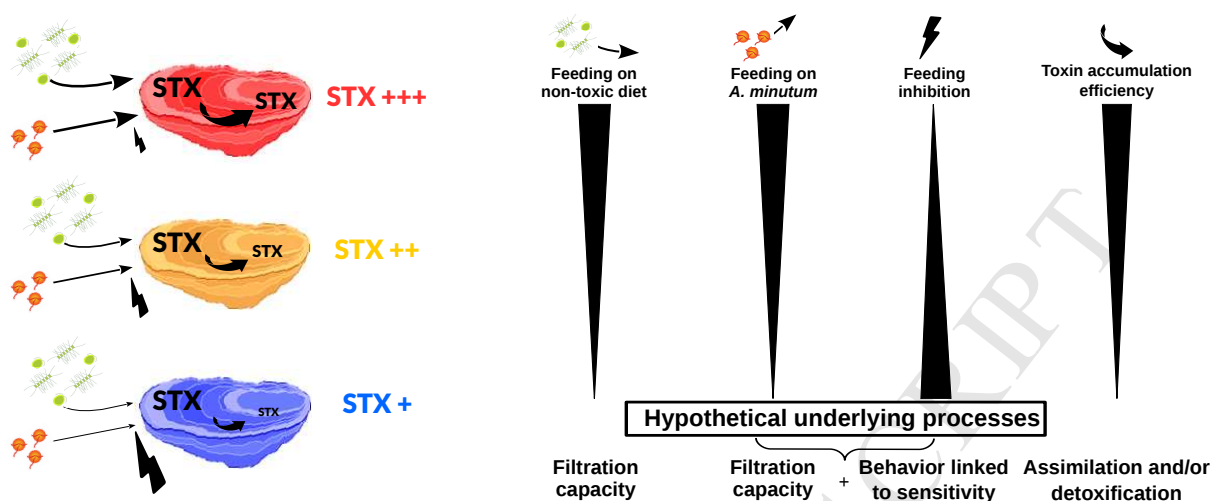


Figure 7: Scheme showing the three different accumulation "phenotypes" of oysters (red: high accumulation; yellow: intermediate accumulation; blue: low accumulation) identified in this study, their level of feeding on non-toxic and toxic algae, feeding inhibition when exposed to toxic algae and toxin accumulation efficiency. Hypothetical processes explaining these observations are also indicated. Note that STX refers to the saxitoxin and its derivatives.

345 rates and subsequently high PST accumulation potential. In both cases selection might thus not be beneficial
 346 for aquaculture.

347 This study provides new insights to improve sampling and analysis methodology used by national net-
 348 works for phytoplankton monitoring (e.g. REPHY in France). Indeed, to properly consider the actual
 349 accumulation of oysters in the field, the sample size (number of oysters) should take into account the high
 350 inter-individual variability in accumulation. Because of this high variability, measurements of toxin con-
 351 centration in oyster pools should be handled with care.

352 5. Conclusion

353 This study clearly highlights the contribution of feeding in toxin accumulation of oysters. Indeed, 78%
 354 of the inter-individual variability in toxin accumulation can be explained by the oyster filtration behavior
 355 during the exposure to *A. minutum*. Even if all the observed oysters exhibited the same primary response to
 356 this harmful algae (i.e. strong to total inhibition of filtration activity) they differed in their level of filtration
 357 recovery. Our results show that this behavior is connected to the filtration capacity, since oysters filtering
 358 the most on non-toxic algae were also the ones filtering the most on *A. minutum*. The present study cannot
 359 conclude on the underlying mechanisms leading to this inter-individual variability; however, it allows to link

360 those ones to different phenotypes. As summarized in Fig. 7, three phenotypes could thus be observed which
361 differed in (1) the filtration before and during exposure to *A. minutum*, (2) the clearance rate inhibition, (3)
362 the toxin accumulation efficiency. Moreover, in each of these processes, clusters responded following the
363 same gradation; oysters from the high accumulation cluster, for example, showed high filtration on both
364 non-toxic algae and *A. minutum* (1), a low clearance rate inhibition (2), and a high toxin accumulation
365 efficiency (3).

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