PSP-producing dinoflagellate *Alexandrium minutum* induces valve microclosures in the mussel *Mytilus galloprovincialis*

Luc A. Comeau[1*], Jose M.F. Babarro[2], Pilar Riobo[2], Michael Scarratt[3], Michel Starr[3], and Réjean Tremblay[4]

[1] Fisheries and Oceans Canada, Gulf Fisheries Centre, Science Branch, P.O. Box 5030, Moncton, New Brunswick, E1C 9B6, Canada

[2] Instituto de Investigaciones Marinas, CSIC, Eduardo Cabello 6, 36208 Vigo, Spain

[3] Fisheries and Oceans Canada, Institut Maurice-Lamontagne, P.O. Box 1000, Mont Joli, Québec, G5H 3Z4, Canada

[4] Institut des Sciences de la Mer (ISMER), Université du Québec à Rimouski (UQAR), 310 allée des Ursulines, CP 3300, Rimouski, Québec G5L 3A1, Canada

[*] Corresponding author. Tel.: +1 506 851 2723; fax: +1 506 851 7732.

E-mail address: luc.comeau@dfo-mpo.gc.ca (L.A. Comeau)

Abstract

The saxitoxin-producing dinoflagellate *Alexandrium minutum* is a well-known microalga that causes paralytic shellfish poisoning (PSP) in many coastal regions of the world. In this study, we measured the valve movements of cultivated mussels *Mytilus galloprovincialis* feeding on toxic *A. minutum* (n = 29 mussels, shell length = 67.1 ± 3.2 mm, $\bar{x}$ ± SD) or a morphologically-similar, but toxin-free phytoplankton, *Heterocapsa triquetra* (n = 24 mussels, shell length = 68.3 ± 2.9 mm). Phytoplankton inoculations were conducted in three sequential "pulses" intended to increase microalgal cell concentrations in a stepwise manner up to ~5,000 cells l^{-1} over a 9-h period. *M. galloprovincialis* was connected to a non-invasive valvometry apparatus that concurrently measured the magnitude of valve openness once every 0.1 sec. It was found that *M. galloprovincialis* tended to keep its valves open over the course of the experiment, regardless of the phytoplankton species present in water. Standard valve opening metrics, such as the opening duration and opening amplitude, were not significantly affected by the species of phytoplankton. However, the frequency of brief and partial valve closure (microclosures) was significantly influenced by phytoplankton species ($P < 0.01$). *M. galloprovincialis* subjected to toxic *A. minutum* exhibited 20.3 ± 0.4 ($\bar{x}$ ± SEM) microclosures per 3-h pulse period, whereas those exposed to the control *H. triquetra* exhibited 7.9 ± 0.4 ($\bar{x}$ ± SEM) microclosures. This response was detectable over the 3h following the first inoculation pulse that provided a phytoplankton concentration of 1,000 cells l^{-1}. Our findings are consistent with growing evidence that bivalves are sensitive to very low concentrations of harmful microalgae. Deploying *in situ* valvometry sensors with real-time monitoring capabilities may provide an early warning of harmful algal blooms.

Keywords: *Mytilus galloprovincialis*; *Alexandrium minutum*; harmful algae; valve gape

1. Introduction

Harmful algal blooms (HABs) are caused by noxious and/or toxic microalgae, which are present in nearly all aquatic environments (Kudela et al., 2015). Organisms that ingest such microalgae can accumulate toxins and transfer them up the food web. Human consumption of marine bivalves contaminated with potent neurotoxins can result in various paralytic or amnesic poisoning syndromes, causing illness or even death (Bates et al., 1998; Etheridge, 2010; Lefebvre and Robertson, 2010). Globally, there are troubling signs that the magnitude and frequency of HABs may be increasing due to anthropogenic factors such as global climate change (Hallegraeff, 2010; McKibben et al., 2017; Wells et al., 2015) and eutrophication (Anderson et al., 2002; Heisler et al., 2008; but see e.g. Davidson et al., 2014). Concomitantly, the increasing use of coastal waters for bivalve aquaculture is expected to amplify HAB related economic (Lassus et al., 2016; Matsuyama and Shumway, 2009) and public health (Grattan et al., 2016) impacts.

The published literature contains sparse, but growing, experimental evidence that certain bivalves respond rapidly (~1-h) to toxic microalgae by modifying their gaping behavior (Haberkorn et al., 2011; Shumway and Cucci, 1987; Tran et al., 2010; Tran et al., 2015). In parallel, new engineering initiatives are transforming laboratory biosensors into field-deployable instruments (Ballesta-Artero et al., 2017; Garcia-March et al., 2016; Miller and Dowd, 2017), with some devices integrating real-time monitoring capabilities in conformity with the concept of biological early-warning systems (Andrewartha et al., 2015; Borcherding, 2006; Kramer and Foekema, 2001). The MolluSCAN eye, for instance, is a highly specialized valvometry system

that automatically transfers real-time data on valve movements of sentinel bivalves through a mobile network (Andrade et al., 2016), allowing land-based servers to scan for abnormal behavior. Such systems could potentially provide an early warning by alerting stakeholders on the timing and location of developing HABs. While *in situ* biosensors might not replace conventional field sampling and toxin analyses, they could optimize the allocation of costly diagnostic resources.

Sporadic occurrences of HABs are particularly challenging for the bivalve aquaculture industry. In Galicia (NW Spain), for instance, the cultivation of mussels (*Mytilus galloprovincialis*) is carried out at a scale of 267,000 tons per year, representing about 40% of the European mussel production and 15% of the world's production (Gosling, 2015). Although Galicia is the second highest mussel producer in the world, a considerable portion its culture sites can remain closed for extended periods because of HABs (Álvarez-Salgado et al., 2008). In 2013, for instance, 38% of the mussel production area was closed to harvesting over a period of 7 months (http://www.intecmar.gal). While HABs are recurrent in this area, they remain unpredictable with respect to timing, location and magnitude (Bravo et al., 2010).

The present study aimed to obtain insights on the behavioral response of *M. galloprovincialis* exposed to a saxitoxin-producing dinoflagellate, *Alexandrium minutum*, which is a well-known causing agent of paralytic shellfish poisoning (PSP) in the Galician Rías (Spain) and many other coastal regions of the world. Specifically, *M. galloprovincialis* was challenged with different concentrations of *A. minutum* cells for short durations (hours) under controlled laboratory conditions. Valve movements were recorded using high frequency valvometry devices intended

for laboratory work, since real-time data transmission capabilities (e.g., MolluSCAN eye) were not required for this particular experiment. By confirming the behavioural sensitivity of *M. galloprovincialis* to low *A. minutum* concentrations in the water, our results are expected to support the use of bivalves as early warning systems.

2. Methods

2.1. Phytoplankton

The toxin-producing *A. minutum* VGO1079 (17–29 µm) (Baiona, Spain) was obtained from the Culture Collection of the Instituto Español de Oceanografía in Vigo (IEO), while the toxin-free *Heterocapsa triquetra* RCC4813 (20–30 µm) (Brittany, France) was obtained from the Roscoff Culture Collection. Both species were cultured from January to February, 2017. As previously described by Tran et al. (2010), these two microalgae species are morphologically similar in size and shape, thus eliminating any potential confounding effects on bivalve behavior. Both cultures were maintained at IEO de Vigo in medium "L1" without silicates (Guillard and Hargraves, 1993). The temperature was maintained at 19ºC and salinity at 34 psu. Irradiance was set at 165 µmol photons m^{-2} s^{-1} and followed a 12:12 light:dark (L:D) cycle. Toxin standard solutions for GTX4, GTX1, GTX2, GTX3, dcGTX2, dcGTX3, neoSTX, dcSTX, and STX were supplied by the National Research Council of Canada (NRC Institute for Marine Biosciences, Halifax, NS, Canada). Based on HPLC analysis with post column derivatization (Rourke et al., 2008), *A. minutum* VGO1079 contained GTX4 (62%), GTX1 (9%), GTX3 (23%) and GTX2 (6%). The

dominance of GTX4 is consistent with *A. minutum* natural population outbreaks in Galician Rías (Franco et al., 1994), including a recent outbreak in 2018 (P. Riobo, Instituto de Investigaciones Marinas, unpublished data). Using specific toxicity conversion factors provided by Oshima (1995), the toxicity was estimated at 176 fg saxitoxin equivalent (STX eq.) $cell^{-1}$.

2.2. Mytilus galloprovincialis sampling and acclimation

In January 2017, *M. galloprovincialis* (shell length 67.7 ± 3.1 mm, $\bar{x}$ ± SD) were collected from a commercial culture raft (42° 36.02′ N, 8° 49.587′ W) in Ría Arousa, Spain. Specimens were collected carefully to avoid damaging the byssus gland or foot. *M. galloprovincialis* samples were transported to the neighboring Instituto de Investigaciones Marinas in Vigo (IIM-CSIC), where they were held in 19-l tanks located inside a temperature-regulated cold room. Tanks were continuously supplied with filtered (10 µm) seawater delivered at a stable temperature (14.6 ± 0.3 ºC, $\bar{x}$ ± SD) and salinity (~35 g l^{-1}) over the course of the study. These conditions are consistent with those recorded *in situ* during a toxic *A. minutum* bloom in NW of the Iberian Peninsula (Bravo et al., 2010). As in Babarro and Fernández-Reiriz (2010), the seawater was supplemented with a mixture of microalgae (Tahitian *Isochrysis* aff. *galbana*, T-ISO) and sediment collected from the seafloor below the mussel culture rafts (40:60 microalgae/sediment, by weight). Particulate material load was maintained at 1.0 mg l^{-1} with an organic content percentage of 50%, simulating mean food availability in the Galician Rías (Babarro et al., 2000).

In February 2017, following a 4-week acclimation period to holding conditions, 24 mussels were connected to a non-invasive valvometry system described in Nagai et al. (2006). A coated Hall

element sensor (HW-300a, Asahi Kasei, Japan) was attached to one valve using cyanoacrylate glue. A small magnet (4.8 mm diameter × 0.8 mm height) was then glued to the other valve so that it was located on the opposite side of the Hall sensor. The magnet and the Hall element weighed 0.1 and 0.5 g, respectively, and were both positioned at the posterior end of the animal, at the point farthest from the umbo where gaping is maximum. The magnetic field (flux density) between the Hall sensor and magnet was a function of the gap between the two valves. This field was recorded in the form of output voltage by dynamic strain recording devices (DC-204R, Tokyo Sokki Kenkyujo Co., Japan). Wired-mussels were fixed to glass supports (Figure 1) using a two-part adhesive consisting of an epoxy resin and a hardener (Araldite, Ceys, Barcelona, Spain). Each glass support offered a favorable substrate for byssus attachment (Babarro and Comeau, 2014) and accommodated four wired-mussels. The mussels were fixed to prevent the movement and clustering of individuals, which would have led to signal (flux density) distortion from sensor assembly sets interacting with one another. Finally, following a curing process of approximately 24 h, mussels were distributed amongst six large (120 l) circular tanks inside the temperature-regulated room, with one glass support holding four mussels in each tank. Mussels were not fed and were left to acclimate in the large tanks for four days before starting the experiment. A mild, but constant, air supply was injected in the lower portion of tanks to promote oxygenation and water mixing.

2.3. Mytilus galloprovincialis exposure to toxic microalgae

DC-204R recorders were switched on and set to measure the valve opening of each mussel once every 0.1 s. Three randomly-selected tanks were inoculated with the PSP-producing *A. minutum*

and three other tanks were inoculated with the toxin-free *H. triquetra*. Inoculations in each tank were conducted in three sequential "pulses" intended to increase cell concentrations in a stepwise manner over a 9-h period. Pulse 1 instantaneously raised concentrations from 0 to 1000 cells l^{-1}; pulse 2 and 3 were administered three and six hours later, respectively, with the intent of further increasing concentrations to approximately 3000 and 5000 cells l^{-1}, respectively.

At the end of the 9-h experiment, the adductor muscle of each *M. galloprovincialis* was severed, and small calibration wedges (1–6 mm in height) were maneuvered between the two valves at the point farthest from the umbo, that is at the posterior edge of the animal where gaping is maximum. By adjusting the height of the wedge and recording the resulting voltage, we obtained the relationships between voltage and wedge height (i.e., valve opening amplitude), which was determined to be statistically significant ($r^2 > 0.93$) for all mussels. Each sensor on each mussel was calibrated in this manner. Precision was evaluated by repeating the measurements 50 times on wedges of known dimensions. These tests were conducted using four distinct Hall element assemblies. The overall precision (coefficient of variation) was 2.8 ± 2.0 % ($\bar{x}$ ± SD).

Over an interval of 3 weeks (February 20—March 7, 2017) the entire experiment was replicated three times using a new set of 24 *M. galloprovincialis* in each experiment, thus building up the population size to 72 individuals distributed amongst 18 tank replicates. Population size, however, was ultimately reduced due to unanticipated technical issues. Water infiltrated some Hall sensors and short-circuited communications to the data loggers, resulting in voltage values that were continually aberrant. In addition, in three tanks, a milky coloration of water signaled the presence of mussel gametes; these tanks were excluded from the analyses because spawning

affects bivalve behavior (Bernard et al., 2016). Considering these issues, the final sample size was 53 individuals distributed amongst 15 tanks. Of the 53 individuals, 29 were exposed to toxic *A. minutum* and 24 to *H. triquetra*.

2.4. Modelling of algae concentration and toxin content

Cell depletion rates in ambient water were modeled by taking into account known phytoplankton inoculation pulses and estimated *M. galloprovincialis* clearance rates. In more details, using the clearance rate allometric relationship (3.92×10^{-3} × shell length$^{1.72}$) reported in Filgueira et al. (2008), it was calculated that the four mussels in each of the experimental tank filtered approximately 22 l h^{-1} and that they would require approximately 6.4 h to filter the entire tank volume (140 l). Noting this filtration capacity and the non-linear nature of particle depletion in static systems holding bivalves (Jørgensen, 1900; Riisgård et al., 2011), the reduction in cell numbers over time was defined as $-0.256\times\ln(t)-0.4832$, where t represents the elapsed time in hours from an inoculation point. The projected cell balance or number of ungrazed cells remaining in the tank at the end of each inoculation phase (3 h) was added to the known inoculation value that followed. For model validation, actual cell counts were performed at three time points, specifically 1, 4, and 9 h after the onset of the experiment. Duplicate water samples (10 l) were extracted from each tank and immediately filtered (10 μm) to concentrate cells, which were then preserved in Lugol and later counted within a Sedgewick-rafter chamber mounted onto an inverted microscope.

Unfortunately HPLC technical issues prevented the measurements of toxins in mussel tissues. Therefore toxin accumulation in mussels was estimated by considering *A. minutum* toxicity (176 fg STX eq. cell^{-1}) and the projected rate of cell depletion inside the experimental tanks (above). It was assumed that depleted *A. minutum* cells were ingested as opposed to being rejected as pseudofeces. This assumption is reasonable considering that cell concentrations in our experiment were below the threshold of pseudofeces production (Figueiras et al., 2002). It is nonetheless recognized that toxin accumulation in bivalves is a complex process, with several physiological factors potentially modulating toxin uptake and depuration rates (Cusson et al., 2005; Guéguen et al., 2012; Haberkorn et al., 2010; Hermabessiere et al., 2016; M.Y. Li et al., 2005). In our study, toxin accumulation was simply estimated as cell toxicity multiplied by the number of cells presumably ingested at a given time point. The intention was to provide a rough estimate of the toxin load, expressed as µg STX eq. 100 g^{-1} of wet tissue weight. Tissues were removed from the shells and drained for 5 min before weighing; the average tissue weight was 6.43 g per individual mussel.

2.5. Statistics

Measured concentrations of *A. minutum* and *H. triquetra* were compared using a non-parametric test (Mann-Whitney U test), because data transformations failed to stabilize variance.

Three valve gaping metrics were produced to quantity *M. galloprovincialis* behavior. Opening duration (OD) was computed as the percentage of time an individual mussel had its valves opened over a 3-h period following a phytoplankton pulse. Opening amplitude (OA) was

calculated as the absolute distance (in mm) separating to the two valves at the point farthest from the umbo where gaping is maximum. Differences between successive OA (ΔOA) recorded over the 0.1 s intervals were also computed in an attempt to detect rapid contractions of the adductor muscle or microclosures (Micro). The total number of microclosures over the 3-h period was determined for each *M. galloprovincialis* individual.

A mixed-model analysis of variance design (Sahai and Ageel, 2012) was developed to test the effects of phytoplankton species (Phyto [i = 1,2]), phytoplankton cell concentration/time at the onset of pulses (ConcT [j=1 to 3]), and their interactions on behavior metrics (OD, OA, Micro). Phytoplankton cell concentration and time cannot be identified as separate individual factors because inoculations were conducted through time. Replicate experiments (Exp [k=1 to 3]) and replicate tanks (Tank [l=1-3]) were set as random factors. Shapiro-Wilks was applied to the unstandardized residuals of the model to verify normality. Micro data were log 1 transformed to meet the assumption of homoscedasticity. Levene's test was used to verify homogeneity of variance.

$$\text{Behavior (OD, OA or Micro)}_{ijklm} = \mu + \text{Phyto}_i + \text{ConcT}_j + \text{Phyto} \times \text{ConcT}_{ij} + \text{Exp}_k(\text{Phyto}_i\ \text{ConcT}_j) + \text{Tank}_l(\text{Phyto}_i\ \text{ConcT}_j\ \text{Exp}_k) + \text{Error}_{ijklm}$$

All valvometry and statistical analyses were performed in SPSS v. 20 (IBM SPSS Inc., Chicago). Statistical significance for all tests was set at 0.05.

3. Results

3.1. Phytoplankton

The phytoplankton clearance model output indicated three sequential pulses increasing in magnitude over time, with each pulse being noticeably grazed down over a short period of time (Figure 2a). The estimated cell concentrations ranged from 200 (end of pulse 1) to 5700 (start of pulse 3) cells l^{-1}. Intermittent cell counts over the course of the experiments (hours 1, 4, and 9) were consistent with the model outcome. No phytoplankton species effect was found at any of the recurrent sampling points (Mann–Whitney, $P > 0.05$), suggesting that mussels were non-selectively clearing *H. triquetra* and *A. minutum* cells from the water. The toxin accumulation model suggested a stepwise increase in toxicity, with a maximal toxicity of 0.63 µg STX eq. 100 g^{-1} of wet mussel tissues (Figure 2b).

3.2. Mytilus galloprovincialis behavior

M. galloprovincialis tended to keep the valves open in all treatment groups. When considering the entire population (n = 53 individuals), OD was 90.1 ± 1.8 percent of the time ($\bar{x}$ ± SEM) and OA was 3.3 ± 0.2 mm ($\bar{x}$ ± SEM).

Phytoplankton concentration/time (ConcT) had no significant effect on any of the behavior metrics (Table 1, Figure 3). Similarly, phytoplankton species (Phyto) had no effect on OD or OA. Only a replicate effect ($P < 0.01$) indicated varying OD across the experiments.

A visual inspection of time series revealed no acute responses immediately following phytoplankton inoculations; however, brief and partial closures (Figure 4a) appeared erratically over the entire time series. These microclosures were apparent when differencing the OA time-series (Figure 4b). They were tagged as closures > 1 mm s^{-1} (measured over a time span of 0.1 sec). ANOVA indicated a significant phytoplankton species effect ($P < 0.01$) on the number of microclosures (Micro). This result was robust, as there were no interaction effects with phytoplankton concentration/time or experimental replicates. *M. galloprovincialis* exposed to toxic *A. minutum* exhibited 20.3 ± 0.4 ($\overline{x}$ ± SEM) microclosures over a 3-h period, whereas those exposed to the control *H. triquetra* exhibited 7.9 ± 0.4 ($\overline{x}$ ± SEM) microclosures (Figure 3c).

4. Discussion

Bivalves are globally ubiquitous and their behavioral sensitivity to pollutants, such as crude oil and heavy metals, has been well documented (Davenport and Manley, 2009; Galtsoff, 1964; Redmond et al., 2017; Tran et al., 2004; Tran et al., 2003). Here we provide the first evidence that a toxin-producing dinoflagellate (*A. minutum*) induces a behavioral response in a commercially-important mytilid bivalve (*M. galloprovincialis*). Our close monitoring of valve movements under controlled conditions revealed that *A. minutum* induces a series of short and incomplete valve closure reactions, which are referred to as microclosures. This brief closure and reopening of the shell correspond to minor contractions of the adductor muscle, and have long been interpreted as a response to irritating substances entering the pallial cavity (Galtsoff, 1964). The frequency of microclosures in bivalves generally increases with the concentration of toxic algae in the water (Basti et al., 2009; Haberkorn et al., 2011), suggesting that microclosures are

somehow linked to an avoidance response. In our study, *M. galloprovincialis* promptly responded to the presence of toxic *A. minutum* in the water. Within hours of exposure, the occurrence of microclosures increased nearly threefold on average, possibly as a result of *A. minutum* making contact with external organs (gills, labial palps, mantle) or releasing bioactive extracellular compounds (BECs) in the surrounding seawater. Castrec et al. (2018) demonstrated that BECs significantly increase the frequency of microclosures in the Pacific oyster *Crassostrea gigas*. Similarly, Borcier et al. (2017) concluded that BECs alter the escape behavior of the great scallop *Pecten maximus*. Not all *A. minutum* strains produce BECs and we did not assess the production of BECs by the strain utilized in the current study. However, regardless of the underlying mechanism at play, *M. galloprovincialis*' sensitivity to *A. minutum* was notable because the behavioral response began after the first inoculation pulse and therefore at low cell concentrations (<1000 cells l^{-1}). The lack of a "concentration effect" in our results may be related to the low variation of algae concentration (× 5) and a confounding "time effect", whereby bivalves were predominately adjusting their behavior in response to changing ambient conditions over time.

Similar behavioral responses to toxic microalgae have been reported for the Pacific oyster *Crassostrea gigas* (Haberkorn et al., 2011; Mat et al., 2016; Tran et al., 2010; Tran et al., 2015), the Akoya pearl oyster *Pinctada fucata* (Nagai et al., 2006), the Great scallop *Pecten maximus* (Coquereau et al., 2016), the Northern scallop *Argopecten purpuratus* (Hégaret et al., 2012), and the Manila clam *Ruditapes philippinarum* (Basti et al., 2009). As in the case of *M. galloprovincialis* (current study), relatively low concentrations of toxic dinoflagellates (~ 500–1200 cells l^{-1}) heightened the frequency of microclosures in *R. philippinarum* (Basti et al., 2009)

and *C. gigas* (Haberkorn et al., 2011). The persistence of such concentrations over time might contaminate shellfish populations, resulting in the mandatory closures of commercial harvesting activities (Blasco et al., 2003). Cell abundances might reach astonishing levels during bloom events. In Galicia Spain, for instance, the saxitoxin-producing dinoflagelletes *A. minutum* and *Gymnodinium catenatum* attain concentrations of 1.0×10^6 and 6.3×10^5 cells l^{-1}, respectively (Bravo et al., 2010).

Once bivalves ingest harmful microalgae, the release of neurotoxins might lead to the paralysis of the adductor muscle and impact behavioral metrics, such as OA and OD, until depuration is completed over a 4–5-day period (Haberkorn et al., 2011; Tran et al., 2010). No such effects were detected over our relatively short (9-h) experiment. Phytoplankton cell counts indicated that *M. galloprovincialis* effectively cleared toxic *A. minutum* cells from the ambient water; however, the projected level of toxicity in *M. galloprovincialis* tissues over the course of the experiment was extremely low (< 0.62 ug STX eq. 100 g^{-1} of wet tissues) and was possibly insufficient to modulate OA and OD. In most countries, the PSP regulatory limit for the marketing of live bivalves intended for consumption has been set to 80 µg STX eq 100 g^{-1} of wet flesh (Fernández, 2000). Based on this information the toxin loads in *M. galloprovincialis* were approximately 130 times (80/0.62) below the PSP regulatory limit. Given this magnitude, it is doubtful that a more elaborate modelling scheme or actual measurements of toxin content would have altered the concluding perspective of the current study. Therefore, in the case of *M. galloprovincialis*, it seems that the heightened frequency of microclosures might serve as an early warning of the accumulation of toxins in the tissues of *M. galloprovincialis*.

Our laboratory work suggests that there is merit in pursuing field investigations under developing PSP conditions, such that behavioral responses could be validated under natural conditions. *M. galloprovincialis* might prove an adequate sentinel, considering that it displays behavioral sensitivity that is well below the PSP regulatory limit. Raft-cultivated *M. galloprovincialis* might be particularly suitable for such monitoring of water quality. In Galicia, *M. galloprovincialis* is suspended from over 3000 large floating rafts that are anchored in numerous estuarine embayments (Rías) along a 1200 km stretch of coastline along the Iberian Peninsula. These large rafts (~ 500 m^2) are good platforms for deploying sentinels and supporting electronics. Such devices were recently deployed outside an HAB event (Comeau et al., 2018), showing that valve closures are infrequent and unsynchronized in raft-cultured *M. galloprovincialis*; thus, the population is constantly interacting with its environment. These characteristics are essential for bivalves to serve as biosensors and early-warning systems.

5. Conclusions

M. galloprovincialis showed a clear response to low concentrations of toxic dinoflagellates (<1000 cells l^{-1}), by heightening the frequency of valve microclosures, which are detectable using high frequency valvometry. The rapid nature of the behavioral response suggested that microclosures occur in response to toxic cells or their extracellular compounds irritating external organs. This interpretation is consistent with the growing literature relating to the behavioral sensitivity of bivalves exposed to toxic microalgae under laboratory conditions. Deploying *in situ* valvometry sensors in recurrent HAB areas represents the next step for validating the utility of bivalves as early warning systems.

Acknowledgements

The Galway Statement on Atlantic Ocean Cooperation motivated this collaborative project. Financial support was provided by Fisheries and Oceans Canada. In-kind support was provided by the Spanish Instituto de Investigaciones Marinas in Vigo (IIM-CSIC), the Instituto Españo de Oceanografía (IEO) de Vigo, and the Institut des Sciences de la Mer (ISMER) in Rimouski, Québec. The authors gratefully acknowledge E. Silva Caride and Jean-Bruno Nadalini for providing valuable technical assistance in the laboratory.

References

Álvarez-Salgado, X.A., Labarta, U., Fernández-Reiriz, M.J., Figueiras, F.G., Rosón, G., Piedracoba, S., Filgueira, R., Cabanas, J.M., 2008. Renewal time and the impact of harmful algal blooms on the extensive mussel raft culture of the Iberian coastal upwelling system (SW Europe). Harmful Algae 7, 849–855.

Anderson, D.M., Glibert, P.M., Burkholder, J.M., 2002. Harmful algal blooms and eutrophication: nutrient sources, composition, and consequences. Estuaries 25, 704–726.

Andrade, H., Massabuau, J.-C., Cochrane, S., Ciret, P., Tran, D., Sow, M., Camus, L., 2016. High Frequency Non-invasive (HFNI) bio-sensors as a potential tool for marine monitoring and assessments. Front. Mar. Sci. 3: 187. doi:10.3389/fmars.2016.00187.

Andrewartha, S.J., Elliott, N.G., McCulloch, J.W., Frappell, P.B., 2015. Aquaculture Sentinels: Smart-farming with Biosensor Equipped Stock. J. Aquac. Res. Development 6: 393. doi:10.4172/2155-9546.1000393.

Babarro, J.M.F., Fernández-Reiriz, M.J., 2010. Secretion of byssal threads in *Mytilus galloprovincialis*: quantitative and qualitative values after spawning stress. J. Comp. Physiol. B 180, 95–104.

Babarro, J.M.F., Comeau, L.A., 2014. Byssus attachment strength of two mytilids in mono-specific and mixed-species mussel beds. Biofouling 30, 975–985.

Babarro, J.M.F., Fernández-Reiriz, M.J., Labarta, U., 2000. Feeding behaviour of seed mussel *Mytilus galloprovincialis*: environmental parameters and seed origin. J. Shellfish Res. 145, 204–213.

Ballesta-Artero, I., Witbaard, R., Carroll, M.L., van der Meer, J., 2017. Environmental factors regulating gaping activity of the bivalve *Arctica islandica* in Northern Norway. Mar. Biol. 164, 116.

Basti, L., Nagai, K., Shimasaki, Y., Oshima, Y., Honjo, T., Segawa, S., 2009. Effects of the toxic dinoflagellate *Heterocapsa circularisquama* on the valve movement behaviour of the Manila clam *Ruditapes philippinarum*. Aquaculture 291, 41–47.

Bates, S.S., Garrison, D.L., Horner, R.A., 1998. Bloom dynamics and physiology of domoic-acid-producing *Pseudo-nitzschia* species. in: Anderson, D., Cembella, A., Hallegraeff, G. (Eds.), Physiological ecology of harmful algal blooms. Springer-Verlag, Heidelberg, pp. 267–292.

Bernard, I., Massabuau, J.C., Ciret, P., Sow, M., Sottolichio, A., Pouvreau, S., Tran, D., 2016. In situ spawning in a marine broadcast spawner, the Pacific oyster *Crassostrea gigas*: timing and environmental triggers. Limnol. Oceanogr. 61, 635-647.

Blasco, D., Levasseur, M., Bonneau, E., Gelinas, R., Packard, T.T., 2003. Patterns of paralytic shellfish toxicity in the St. Lawrence region in relationship with the abundance and distribution of *Alexandrium tamarense*. Sci. Mar. 67, 261-278.

Borcherding, J., 2006. Ten years of practical experience with the Dreissena-Monitor, a biological early warning system for continuous water quality monitoring. Hydrobiologia 566, 417–426.

Borcier, E., Morvezen, R., Boudry, P., Miner, P., Charrier, G., Laroche, J., Hegaret, H., 2017. Effects of bioactive extracellular compounds and paralytic shellfish toxins produced by *Alexandrium minutum* on growth and behaviour of juvenile great scallops *Pecten maximus*. Aquat. Toxicol. 184, 142–154.

Bravo, I., Santiago, F., Figueroa, R., Pazos, Y., Massanet, A., Ramilo, I., 2010. Bloom dynamics and life cycle strategies of two toxic dinoflagellates in a coastal upwelling system (NW Iberian Peninsula). Deep Sea Res. Part 2 Top. Stud. Oceanogr. 57, 222–234.

Castrec, J., Soudant, P., Payton, L., Tran, D., Miner, P., Lambert, C., Le Goïc, N., Huvet, A., Quillien, V., Boullot, F., Amzil, Z., Hégaret, H., Fabioux, C., 2018. Bioactive extracellular compounds produced by the dinoflagellate *Alexandrium minutum* are highly detrimental for oysters. Aquat. Toxicol. 199, 188–198.

Comeau, L.A., Babarro, J.M.F., Longa, A., Padin, X.A., 2018. Valve-gaping behavior of raft-cultivated mussels in the Ría de Arousa, Spain. Aquaculture Reports 9, 68–73.

Coquereau, L., Jolivet, A., Hégaret, H., Chauvaud, L., 2016. Short-term behavioural responses of the great scallop *Pecten maximus* exposed to the toxic alga Alexandrium minutum measured by accelerometry and passive acoustics. PLoS ONE 11, e0160935.

Cusson, M., Tremblay, R., Daigle, G., Roussy, M., 2005. Modeling the depuration potential of blue mussels (*Mytilus* spp.) in response to thermal shock. Aquaculture 250, 183–193.

Davenport, J., Manley, A., 2009. The detection of heightened sea-water copper concentrations by the mussel *Mytilus edulis*. J. Mar. Biol. Assoc. U. K. 58, 843–850.

Davidson, K., Gowen, R.J., Harrison, P.J., Fleming, L.E., Hoagland, P., Moschonas, G., 2014. Anthropogenic nutrients and harmful algae in coastal waters. J Environ Manage 146, 206–216.

Díaz, P., Reguera, B., Ruiz-Villarreal, M., Pazos, Y., Velo-Suárez, L., Berger, H., Sourisseau, M., 2013. Climate variability and oceanographic settings associated with interannual variability in the initiation of *Dinophysis acuminata* blooms. Marine Drugs 11, 2964–2961.

Etheridge, S.M., 2010. Paralytic shellfish poisoning: seafood safety and human health perspectives. Toxicon 56, 108–122.

Fernández, M.L., 2000. Regulations for marine microalgal toxins: towards harmonization of methods and limits. S. Afr. J. Anim. Sci. 22, 339–346.

Figueiras, F.G., Labarta, U., Fernández-Reiriz, M.J., 2002. Coastal upwelling, primary production and mussel growth in the Rías Baixas of Galicia. Hydrobiologia 484, 121–131.

Filgueira, R., Labarta, U., Fernández-Reiriz, M.J., 2008. Effect of condition index on allometric relationships of clearance rate in *Mytilus galloprovincialis* Lamarck, 1819. Rev. Biol. Mar. Oceanogr. 43, 391–398.

Franco, J.M., Fernández, P., Reguera, B., 1994. Toxin profiles of natural populations and cultures of*Alexandrium minutum* Halim from Galician (Spain) coastal waters. J. Appl. Phycol. 6, 275–279.

Galtsoff, P.S., 1964. The American oyster, *Crassostrea virginica* Gmelin. U.S. Fish and Wildlife Serv., Fish. Bull. 64, 1–480.

Garcia-March, J.R., Jiménez, S., Sanchis, M.A., Monleon, S., Lees, J., Surge, D., Tena-Medialdea, J., 2016. In situ biomonitoring shows seasonal patterns and environmentally mediated gaping activity in the bivalve, *Pinna nobilis*. Mar. Biol. 163, 29.

Gosling, E., 2015. Marine bivalve molluscs, second edition, John Wiley & Sons Ltd, Chichester UK.

Grattan, L.M., Holobaugh, S., Morris, J.G., 2016. Harmful algal blooms and public health. Harmful Algae 57, 2–8.

Guéguen, M., Baron, R., Bardouil, M., Haberkorn, H., Soudant, P., Truquet, P., Lassus, P., 2012. Influence of *Crassostrea gigas* (Thunberg) sexual maturation stage and ploidy on uptake of paralytic phycotoxins. Toxicon 60, 40–43.

Guillard, R.R.L., Hargraves, P.E., 1993. *Stichochrysis immobilis* is a diatom, not a chrysophyte. Phycologia 32, 234–236.

Haberkorn, H., Tran, D., Massabuau, J.-C., Ciret, P., Savar, V., Soudant, P., 2011. 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*. Mar. Pollut. Bull. 62, 1191–1197.

Haberkorn, H., Lambert, C., Le Goïc, N., Moal, J., Suquet, M., Guéguen, M., Sunila, I., Soudant, P., 2010. Effects of *Alexandrium minutum* exposure on nutrition-related processes and reproductive output in oysters *Crassostrea gigas*. Harmful Algae 9, 427–439.

Hallegraeff, G.M., 2010. Ocean climate change, phytoplankton community responses, and harmful algal blooms: a formidable predictive challenge. J. Phycol. 46, 220–235.

Hégaret, H., Brokordt, K.B., Gaymer, C.F., Lohrmann, K.B., García, C., Varela, D., 2012. Effects of the toxic dinoflagellate *Alexandrium catenella* on histopathogical and escape responses of the Northern scallop *Argopecten purpuratus*. Harmful Algae 18, 74–83.

Heisler, J., Glibert, P., Burkholder, J., Anderson, D., Cochlan, W., Dennison, W., Gobler, C., Dortch, Q., Heil, C., Humphries, E., Lewitus, A., Magnien, R., Marshall, H., Sellner, K., Stockwell, D., Stoecker, D., Suddleson, M., 2008. Eutrophication and harmful algal blooms: a scientific consensus. Harmful Algae 8, 3–13.

Hermabessiere, L., Fabioux, C., Lassudrie, M., Boullot, F., Long, M., Lambert, C., Le Goïc, N., Gouriou, J., Le Gac, M., Chapelle, A., Soudant, P., Hégaret, H., 2016. Influence of

gametogenesis pattern and sex on paralytic shellfish toxin levels in triploid Pacific oyster *Crassostrea gigas* exposed to a natural bloom of *Alexandrium minutum*. Aquaculture 455, 118–124.

Jørgensen, C., 1900. Bivalve filter feeding: hydrodynamics, bioenergetics, physiology and ecology. Olsen & Olsen, Fredensborg, Denmark, p 140.

Kramer, K.J.M., Foekema, E.M., 2001. The "Musselmonitor®" as Biological Early Warning System. in: Butterworth, F.M., Gunatilaka, A., Gonsebatt, M.E. (Eds.), Biomonitors and Biomarkers as Indicators of Environmental Change 2: A Handbook. Springer US, Boston, MA, pp. 59–87.

Kudela, R.M., Berdalet, E., Bernard, S., Burford, M., Fernand, L., Lu, S., Roy, S., Tester, P., Usup, G., Magnien, R., Anderson, D.M., Cembella, A., Chinain, M., Hallegraeff, G., Reguera, B., Zingone, A., Enevoldsen, H., Urban, E., 2015. Harmful Algal Blooms. A Scientific Summary for Policy Makers. IOC/UNESCO, Paris (IOC/INF-1320) http://unesdoc.unesco.org/images/0023/002334/233419e.pdf.

Lassus, P., Chomérat, N., Nézan, E., Hess, P., 2016. Toxic and harmful microalgae of the World Ocean - Micro-algues toxiques et nuisibles de l'océan mondial. IOC Manuals and Guides 68 (English/French), Intl. Society for the Study of Harmful Algae (ISSHA) / Intergovernmental Oceanographic Commission of UNESCO (IOC), Copenhagen, Denmark, 523 pp, 2016.

Lefebvre, K.A., Robertson, A., 2010. Domoic acid and human exposure risks: A review. Toxicon 56, 218–230.

M.Y. Li, A., Yu, P., Hsieh, D., Wang, W.-X., Wu, R., Lam, P., 2005. Uptake and depuration of paralytic shellfish toxins in the green-lipped mussel, *Perna viridis*: A dynamic model.

Mat, A.M., Perrigault, M., Massabuau, J.-C., Tran, D., 2016. Role and expression of cry1 in the adductor muscle of the oyster *Crassostrea gigas* during daily and tidal valve activity rhythms. Chronobiol. Int. 33, 949–963.

Matsuyama, Y., Shumway, S., 2009. Impacts of harmful algal blooms on shellfisheries aquaculture.

McKibben, S.M., Peterson, W., Wood, A.M., Trainer, V.L., Hunter, M., White, A.E., 2017. Climatic regulation of the neurotoxin domoic acid. PNAS 114, 239–244.

Miller, L.P., Dowd, W.W., 2017. Multimodal in situ datalogging quantifies inter-individual variation in thermal experience and persistent origin effects on gaping behavior among intertidal mussels (*Mytilus californianus*). J. Exp. Biol. 220, 4305–4319.

Nagai, K., Honjo, T., Go, J., Yamashita, H., Seok Jin, O., 2006. Detecting the shellfish killer *Heterocapsa circularisquama* (Dinophyceae) by measuring bivalve valve activity with a Hall element sensor. Aquaculture 255, 395–401.

Oshima, Y., 1995. Post-column derivatization HPLC methods for paralytic shellfish poisons. in: Hallegraeff, G.M., Anderson, D.M., Cembella, A.D. (Eds.), Manual on harmful marine microalgae. UNESCO, IOC (Intergovnt Oceanogr Comm) Manuals and Guides No 33, Rome, p 81–94.

Redmond, K., Berry, M., Pampanin, D., Andersen, O., 2017. Valve gape behaviour of mussels (*Mytilus edulis*) exposed to dispersed crude oil as an environmental monitoring endpoint.

Riisgård, H.U., Hoffmann Jørgensen, B., Lundgreen, K., Storti, F., Walther, J.H., Meyer, K.E., Larsen, P.S., 2011. The exhalant jet of mussels *Mytilus edulis*. Mar. Ecol. Prog. Ser. 437, 147–164.

Rourke, W.A., Murphy, C.J., Pitcher, G., van de Riet, J.M., Burns, B.G., Thomas, K.M., Quilliam, M.A., 2008. Rapid postcolumn methodology for determination of paralytic shellfish toxins in shellfish tissue. J. AOAC Int. 91, 589–597.

Sahai, H., Ageel, M.I., 2012. The analysis of variance: fixed, random and mixed models. Birkhäuser, Boston.

Shumway, S.E., Cucci, T.L., 1987. The effects of the toxic dinoflagellate *Protogonyaulax tamarensis* on the feeding and behaviour of bivalve molluscs. Aquat. Toxicol. 10, 9–27.

Tran, D., Fournier, E., Durrieu, G., Massabuau, J.-C., 2004. Copper detection in the Asiatic clam Corbicula fluminea: optimum valve closure response. Aquat. Toxicol. 66, 333–343.

Tran, D., Ciret, P., Ciutat, A., Durrieu, G., Massabuau, J.-C., 2003. Estimation of potential and limits of bivalve closure response to detect contaminants: application to cadmium. Environ. Toxicol. Chem. 22, 914–920.

Tran, D., Haberkorn, H., Soudant, P., Ciret, P., Massabuau, J.-C., 2010. Behavioral responses of *Crassostrea gigas* exposed to the harmful algae *Alexandrium minutum*. Aquaculture 98, 338–345.

Tran, D., Ciutat, A., Mat, A., Massabuau, J.-C., Hégaret, H., Lambert, C., Le Goic, N., Soudant, P., 2015. The toxic dinoflagellate *Alexandrium minutum* disrupts daily rhythmic activities at gene transcription, physiological and behavioral levels in the oyster *Crassostrea gigas*. Aquat. Toxicol. 158, 41–49.

Wells, M.L., Trainer, V.L., Smayda, T.J., Karlson, B.S.O., Trick, C.G., Kudela, R.M., Ishikawa, A., Bernard, S., Wulff, A., Anderson, D.M., Cochlan, W.P., 2015. Harmful algal blooms and climate change: Learning from the past and present to forecast the future. Harmful Algae 49, 68–93.

Table 1. Mixed model ANOVAs testing for the effect of phytoplankton species (Phyto) and phytoplankton concentration/time (ConcT) on various parameters of *M. galloprovincialis*, including valve opening duration (OD), opening amplitude (OA), and the number of microclosures (Micro) over a 3-h period. Experiment (Exp) and tank (Tank) replicates were declared random effects in the model. Bold values highlight statistical significance. Micro was log 1 transformed to stabilize variance.

Source		OD		OA		Micro	
	df	MS	*P*	MS	*P*	MS	*P*
Phyto	1	157.18	0.89	4.27	0.30	3.67	**<0.01**
Conc	2	2295.52	0.75	1.75	0.63	0.94	0.12
Phyto×Conc	2	72.90	0.99	0.30	0.92	0.26	0.50
Exp (Phyto×Conc)	12	7670.34	**<0.01**	3.65	0.06	0.36	0.29
Tank (Phyto×Conc×Exp)	27	2022.21	0.11	1.78	0.75	0.28	0.63
Error	114	1431.23		2.23		0.32	

Figure legends

Figure 1. Photo of 24 *M. galloprovincialis* individuals equipped with Hall sensors and magnets. Glass supports allowed byssus fixation.

Figure 2. Summary of experimental design: (a) Phytoplankton cell concentration within experimental tanks over the course of the experiment; solid line represents cell concentrations modeled from the added quantity of phytoplankton and assumed clearance rates; bars represent measured cell concentrations ($\overline{x} \pm$ SD, pooled replicates) relating to *H. triquetra* (open bars) and *A. minutum* (solid bars) at hours 1, 4, and 9. (b) Modeled toxin content in *M. galloprovincialis* exposed to *A. minutum*, expressed as µg STX eq. 100 g^{-1} of wet tissue (WT) weight.

Figure 3. Mean ($\overline{x} \pm$ SEM) a) valve opening duration (OD), b) valve opening amplitude (OA) and c) number of microclosures (Micro) exhibited by *M. galloprovincialis* as a function of the concentration of *H. triquetra* (open bars) or *A. minutum* (solid bars). Peak phytoplankton concentrations provided on the x-axis represent estimates at the onset of pulses. It should be noted that time is a confounding factor as inoculations were performed over time. Behavior metrics on the y-axis were calculated for each individual over a 3-h period that followed peak concentrations, and were then averaged at the group level.

Figure 4. a) Valve opening amplitude (OA) of a single mussel (*M. galloprovincialis*) as a function of time. b) Differences between consecutive measurements (ΔOA) that were highlighted by the microclosures of the mussel. In this example, the two peaks that fell below the dotted reference line (closure > 1 mm per sec) indicate the occurrence of two distinct microclosures over a short time period (~ 4 minutes).

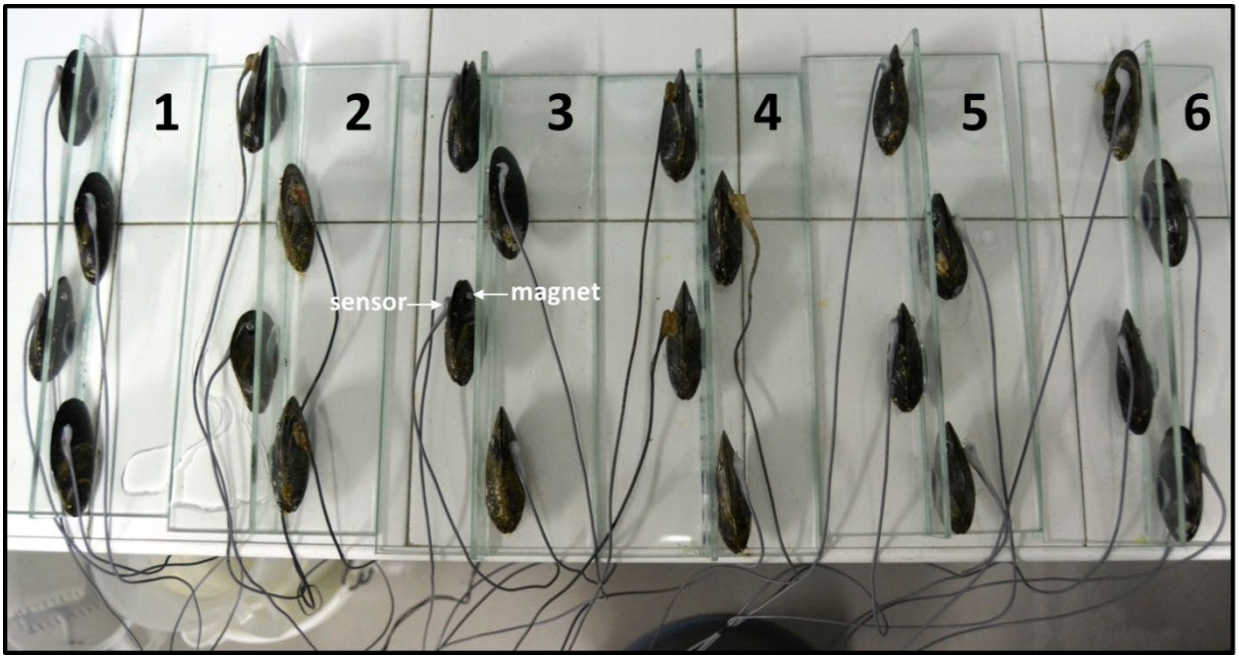

Figure 1

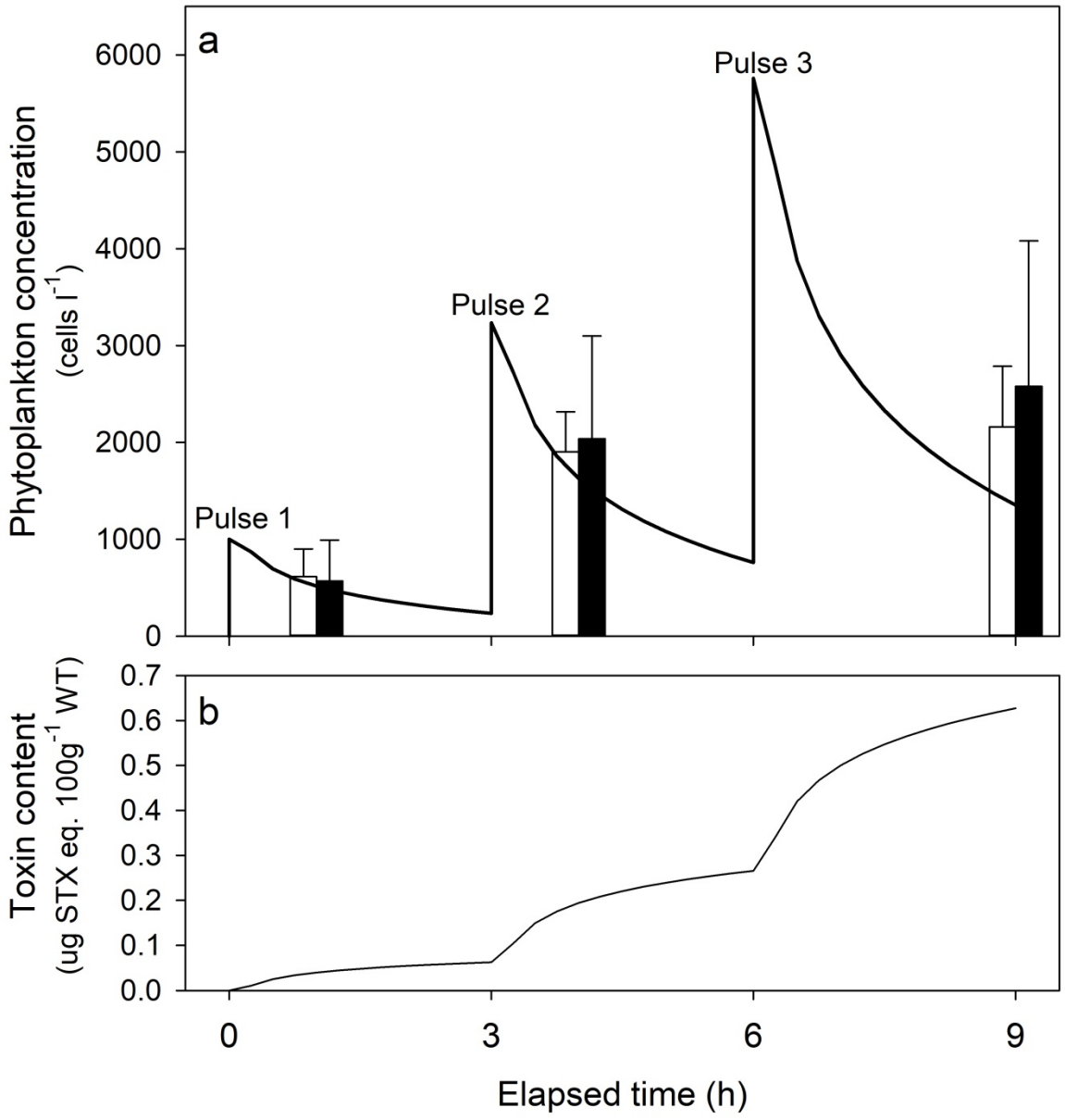
a
b
Pulse 1
Pulse 2
Pulse 3
Phytoplankton concentration
(cells l-1)
Toxin content
(ug STX eq. 100g-1 WT)
Elapsed time (h)
0
1000
2000
3000
4000
5000
6000
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0
3
6
9

Figure 2

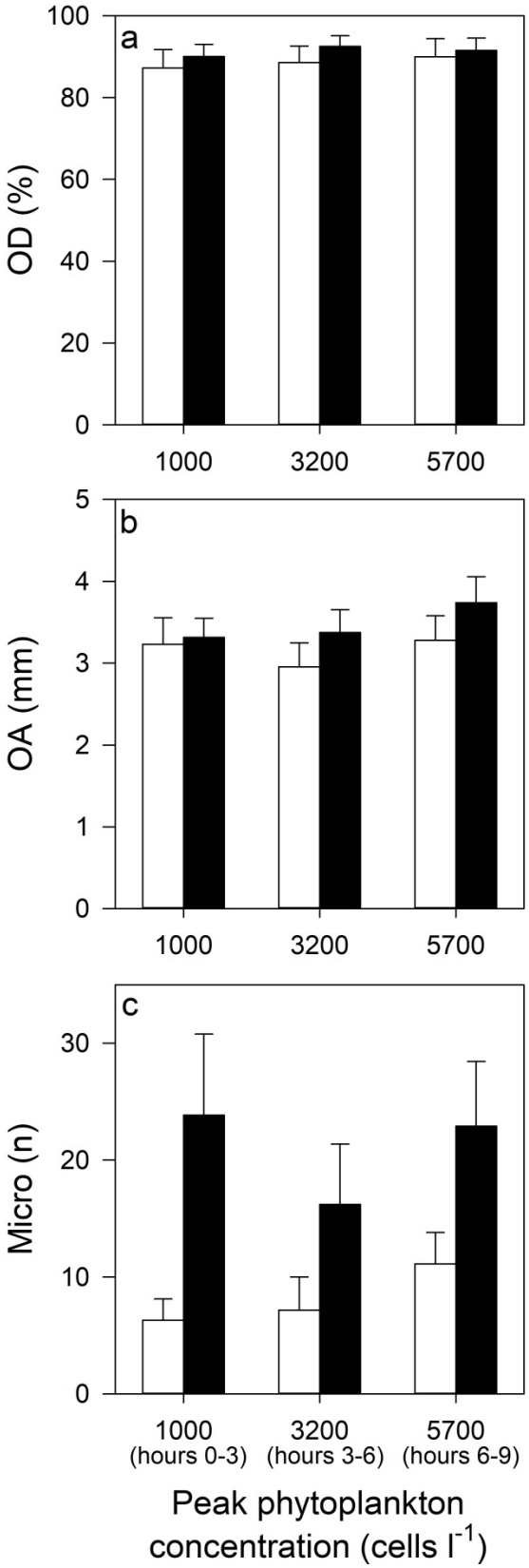
a
OD (%)
100
80
60
40
20
0
1000
3200
5700
b
OA (mm)
5
4
3
2
1
0
1000
3200
5700
c
Micro (n)
30
20
10
0
1000
(hours 0-3)
3200
(hours 3-6)
5700
(hours 6-9)
Peak phytoplankton
concentration (cells l-1)

Figure 3

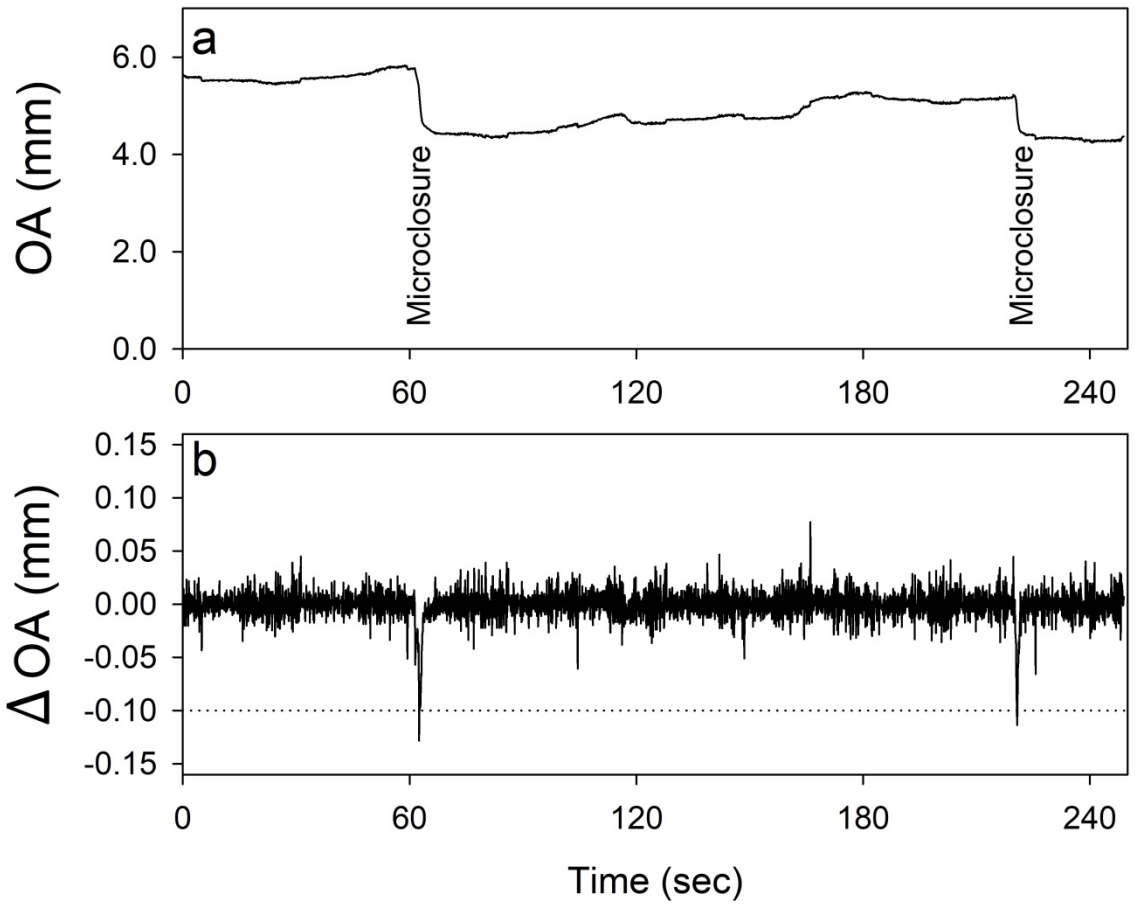
a
6.0
4.0
2.0
0.0
OA (mm)
Microclosure
Microclosure
0
60
120
180
240
b
0.15
0.10
0.05
0.00
-0.05
-0.10
-0.15
Δ OA (mm)
0
60
120
180
240
Time (sec)

Figure 4