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Viability, growth and toxicity of *Alexandrium catenella* and *Alexandrium minutum* (Dinophyceae) following ingestion and gut passage in the oyster *Crassostrea gigas*

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Abstract – Adult oysters *Crassostrea gigas* were experimentally fed with *Alexandrium catenella* and *Alexandrium minutum* which are responsible for recurrent toxic blooms in French coastal waters. *C. gigas* produced faeces and pseudofaeces containing intact and viable temporary pellicular cysts of these two Paralytic toxin producing species. When incubated in favourable conditions, these pellicular cysts were able to germinate at high rates (between 74 and 94%) and the resulting vegetative cells divided with growth rates close to the non-ingested cells (control). The toxin profile of the vegetative cells originated from the germinated temporary cysts was analyzed by liquid chromatography/fluorescence detection. Total toxin content of newly germinated cells was lower than that of cultured cells. Besides, cell contents of C2, B1, B2 and dcGTX3 toxins featured some changes. Our results suggest that the increased spreading of toxic dinoflagellates through the transfer of shellfish from contaminated towards pristine coastal areas cannot be ruled out. We also suggest that pellicular cysts and newly germinated cells could represent a potential way for the transfer of paralytic toxins toward the higher trophic levels.

Key words: *Alexandrium catenella* / *Alexandrium minutum* / Cysts / Oysters / Paralytic Shellfish Poisoning

Résumé – Viabilité, croissance et toxicité d'*Alexandrium catenella* et *Alexandrium minutum* (Dinophyceae) après leur ingestion et leur transit stomacal chez l'huître creuse *Crassostrea gigas*. Des huîtres creuses adultes *Crassostrea gigas* ont été alimentées en laboratoire avec *Alexandrium catenella* et *Alexandrium minutum*, deux dinoflagellés responsables de la bioaccumulation de toxines paralysantes dans les mollusques cultivés dans les eaux côtières françaises. *C. gigas* a produit des fèces et des pseudofèces contenant des kystes pelliculaires intacts et viables des deux dinoflagellés testés. Une fois replacés dans des conditions de culture favorables, ces kystes temporaires ont germé dans des proportions élevées (entre 74 et 94 %) et les cellules végétatives résultantes se sont divisées avec des taux de croissance proches de ceux des cellules non ingérées (témoins). Le profil toxique des cellules végétatives, issues des kystes ayant germé, a été analysé par chromatographie liquide/fluorescence. La quantité totale de toxines dans les cellules végétatives nouvellement formées était plus faible que dans les cellules non ingérées. Par ailleurs, les contenus cellulaires en toxines C2, B1, B2, et dcGTX3 ont montré des changements. Nos résultats suggèrent que l'extension des efflorescences de dinoflagellés toxiques pourrait être causée par des transferts de mollusques bivalves depuis des zones contaminées vers des zones vierges. Il est suggéré, par ailleurs, que les kystes pelliculaires et les cellules végétatives issues de ces kystes peuvent participer au transfert des toxines paralysantes dans les niveaux trophiques supérieurs.

1 Introduction

Increased outspreading of toxic dinoflagellates (Hallegraeff 1993) or diatoms in coastal areas could be related to the transfer of shellfish species from contaminated towards pristine areas. Certain mollusc species can keep intact

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and viable phytoplankton cells in their digestive tract. As a result, when faecal pellets are discharged in a safe area, temporary pellicular cysts released from these pellets can potentially lead to the proliferation of algal populations resulting from excystement process (Carriker 1992; Laabir and Gentien 1999; Imada et al. 2001). Scarratt et al. (1993) and Bricelj et al. (1993) demonstrated the capacity of the dinoflagellates *Alexandrium tamarense* and *Alexandrium fundyense* to survive

the gut passage in some bivalve molluscs including *Mytilus edulis*. Laabir and Gentien (1999) showed that the dinoflagellates *A. tamarense*, *A. minutum* and *Scrippsiella trochoidea* survived the gut passage in the oyster *Crassostrea gigas*, and most cells contained in their faeces were intact and viable. Bauder and Cembella (2000) showed that, when fed with the epibenthic dinoflagellate *Prorocentrum lima* a diarrhetic toxins producer, the Bay scallop *Argopecten irradians* ejected faecal ribbons with viable *P. lima* cells capable of cell division. Shumway and Cucci (1987) reported that *Alexandrium tamarense* was filtered and rejected in pseudofaeces of several bivalve molluscs. However, as documented in many studies, the effects of toxic dinoflagellates on feeding and behaviour of bivalve molluscs are species-specific and depend on the sampling location of the latter (Shumway 1990 and references therein).

No studies have investigated whether dinoflagellate cells remained toxic after their gut passage. Besides, despite the pseudofaeces could contain high amounts of filtered microalgae, no attempt have been made yet to quantify the germination of the pellicular cysts found in faecal material nor to measure the growth rate of the vegetative cells originating from these cysts.

This study focus on the following not yet addressed questions: (1) Are *A. catenella* and *A. minutum* able to remain viable after passing through the digestive tract of *C. gigas*? (2) To what extent the pellicular, temporary cysts embedded in faeces and pseudofaeces biodeposits can remain viable and can germinate into new vegetative cells? (3) What are the characteristics of growth and toxicity of the new populations resulting from the revived dinoflagellate cells?

Target species are the dinoflagellates *Alexandrium catenella* and *Alexandrium minutum* which are often responsible for paralytic shellfish poisoning (PSP) outbreaks in temperate coastal waters throughout the world. Since 1988, blooms of *Alexandrium minutum* Halim have occurred sporadically along the North Brittany Coast of France, causing an accumulation of paralytic toxins in bivalves (essentially mussels and oysters) in contaminated areas (Lassus et al. 2004). *Alexandrium catenella* was responsible for a bloom in Thau lagoon (French Mediterranean coast) for the first time during the Autumn of 1998. Cells concentration was higher than 85 000 cells L^{-1} and toxicity up to 852 μgSTX eq 100 g^{-1} of mussel meat according to AOAC method used for PSP detection by mouse test. Thereafter similar toxic episodes were observed regularly in Thau lagoon in Spring and/or Autumn with negative effect (shellfish market ban, public health risk) on the shellfish industry (essentially *C. gigas*) which represents a very important economic and social resource in the studied area.

2 Methods

2.1 Biological materials and algal culture methods

The oysters (*C. gigas*) were collected in Thau lagoon (France) during spring in 2004 and 2005. Adults were purged in filtered (10 μm) seawater (salinity: 35-39 psu) at a constant temperature of 20 ± 1 °C and water changed at least once during the 24 h acclimation period without feeding. Before the

experiments, animals were placed in 0.2 μm filtered sea water for 24 h to clear the gut contents.

The experiments were carried out with three algal strains maintained in the "Ecosystèmes lagunaires" (Montpellier) and IFREMER (Nantes) laboratories. The *A. catenella* (ACT03) strain was isolated from Thau waters during a bloom in autumn 2003. The *A. minutum* (AM89BM) strain was isolated in Morlaix Bay, Brittany, France, in 1989. The diatom *Thalassiosira weissflogii* was used as control algae for feeding experiments. Algal cells were grown in batch cultures in ESAW (enrichment solution artificial seawater) medium (Harrison et al. 1980) at 20 °C and salinity 38 psu, with a 12 h:12 h photoperiod and under a light intensity of 100 μmol photons m^{-2} s^{-1}. For *T. weissflogii* silica was added to the medium. For the oyster feeding experiments, the micro-algal cultures were harvested in the exponential growth phase.

Cell concentrations in cultures and feeding experiments were determined from cell counts in a Nageotte chamber under a photonic microscope. Microalgae growth rates were calculated according to Guillard (1973).

2.2 Feeding experiments

To detect any effect of the toxic dinoflagellates on feeding behaviour of *C. gigas*, the clearance rate (CR) of incubated oysters was measured according to Coughlan's (1969) method, i.e. by determining the decrease in micro-algal cell concentrations due to oyster filtration over time. Oyster clearance rate was calculated using the following equation:

$$CR = Ln(N_0/N_t) \, M/t$$

where N_0 is the initial concentration of the microalgae in the incubation medium, N_t the concentration after t hours, M the total volume of the experimental water, and t the time after the start of the experiment.

For each experiment, batches of three oysters were exposed to the tested microalgae. Each bivalve was placed individually at the bottom of a transparent beaker containing 3 L of filtered sea water, then fed with the microalgae at a defined concentration. Mean concentrations of dinoflagellate species in the beakers were in the range of observed *in situ* densities during bloom conditions in Thau lagoon (1×10^6 cells L^{-1} for *A. catenella*) and Penzé river (2×10^6 cells L^{-1} for *A. minutum*). *T. weissflogii* was offered to oysters with equivalent concentrations of 2×10^6 cells L^{-1}. No aeration was used during feeding experiments with dinoflagellates (Sullivan et al. 2003). In the beakers with *T. weissflogii*, the water was gently stirred periodically to avoid sedimentation of diatom cells.

After 6 h and 24 h, all the faeces and pseudofaeces were separately removed with a glass Pasteur pipette and placed on different sieves. Then, these pellets were gently washed with filtered sea water until no live, swimming cells remained in the water as checked under an inverted microscope. The algal cells were not counted in the pellets. For clearance rate measurements, immediately after removing the faeces and pseudofaeces, the experimental containers were gently homogenated and three samples of 5 ml were taken for counting the algal cells under the microscope.

Undisrupted faeces and pseudofaeces were placed in microplate wells filled with ESAW medium, and incubated under our usual culture conditions as above. After a 24 h-incubation, the faecal material was observed using an inverted microscope to determine if temporary cysts had been able to germinate and had produced free motile vegetative cells in the medium.

For germination rate measurements, temporary cysts were sampled randomly and separately from disrupted faeces and pseudofaeces. Up to 100 cysts were incubated in microplate wells filled with ESAW medium and under our usual culture conditions for 48 h. At the end of this incubation period, the number of revived cells and empty thecae were determined using an inverted microscope.

For growth rate measurements, a large number of *A. catenella* and *A. minutum* pellicular cysts were isolated randomly from disrupted faeces and pseudofaeces and carefully watched. Then, cysts were incubated in microplates placed in favourable culture conditions (see above). 24 h later, a sufficient number of revived cells were taken carefully with a Pasteur pipette and batch cultures were established in dishes filled with ESAW medium and exposed to standard culture conditions. Each day during two weeks, cell concentration was determined. All experiments were conducted in triplicates.

Data were expressed as means ± SD and statistical analyses were performed with Student's *t*-test. Clearance rate of oysters exposed to *T. weissflogii* was defined as the control CR.

2.3 Chemical analysis by liquid chromatography/ fluorescence detection (LC/FD)

Toxin contents and toxin profiles of cells germinated from pellicular cyst were compared to those of not ingested vegetative cells (control) in the same growth phase. Triplicates of 10 ml batch culture (cell concentration was up to 10^4 cells ml^{-1}) were taken during exponential and stationary growth phases for *A. catenella* and only during exponential phase for *A. minutum*. After centrifugation (3000 g, 8 min, 4 °C), cell concentrates were suspended in 1 ml of 0.1 N acetic acid. To release the cell toxins, the samples were frozen (−20 °C)-thawn then sonicated for 5 min in a waterbath for three times, and centrifuged at 17 000 g for 10 min at 4 °C. The supernatants were used for the subsequent LC/FD PSP toxin analyses, which were performed using the method of Oshima (1995) with slight modifications. Toxins were separated by reverse chromatography using a C8 column (5 μm Develosil, 4.6 mm i.d. × 250 mm) with a flow rate of 0.8 ml min^{-1}. Eluent pH and/or column temperature were calibrated to optimise the separation of some gonyautoxins (dc-DTX3/B1/dc-GTX-2). Toxins were quantified using certified standards provided by CNRC-Halifax-Canada. B2 and C2-toxins were detected and quantified indirectly after acid hydrolysis (HCL 0.4 N at 97 °C for 5 min) (Masselin et al. 2001).

3 Results

Mean clearance rates after 6 h and 24 h were significantly lower (Student *t*-test, $p < 0.05$) for oysters *C. gigas* fed with *A. catenella* (0.34 ± 0.01 and 0.116 ± 0.003 L h^{-1}) and *A. minutum*
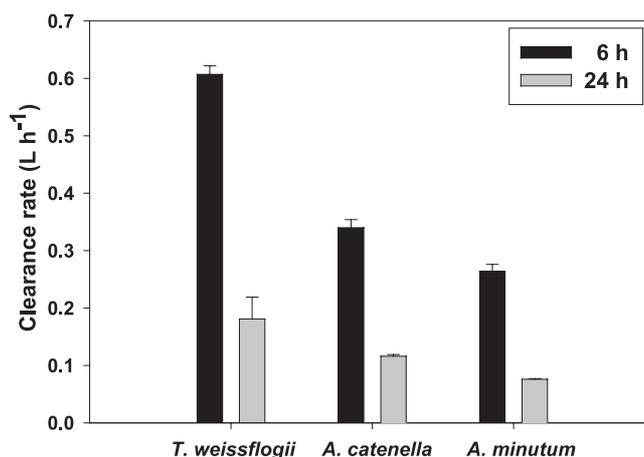
Fig. 1. Mean 6 h- and 24-h clearance rates and standard errors of *Crassostrea gigas* when fed with different monoalgal diets: the diatom *Thalassiosira weissflogii* (control) and two toxic dinoflagellate species, *Alexandrium minutum* and *Alexandrium catenella.*

(0.26 ± 0.01 and 0.07 ± 0.001 L h^{-1}) than those fed with *T. weissflogii* (0.61 ± 0.01 and 0.18 ± 0.03 L h^{-1}) (Fig. 1).

Two hours after the beginning of the experiment, *T. weissflogii* cells were completely digested in the faeces. In contrast, *A. catenella* and *A. minutum* cells have been transformed in temporary cysts, i.e. retaining their thecae but non motile, and were packed in the faecal ribbons (Fig. 2). Pseudofaeces also contained many temporary cysts of both dinoflagellates species (Fig. 2). When oysters were fed *T. weissflogii,* pseudofaeces contained intact cells of this diatom. After the 24-hour incubation period in favourable culture conditions, in all microplate wells containing undisrupted faeces and pseudofaeces, a large number of vegetative motile cells were observed, indicating that pellicular cysts either contained in the faeces with a membrane or trapped in pseudofaeces dense mucus could germinate and give free-swimming cells.

After incubation in favourable conditions, the *A. catenella* pellicular cysts isolated from faecal and pseudofaecal material showed high proportions of revived cells ($74 \pm 3\%$ and $89 \pm 8\%$, respectively); these proportions were higher in *A. minutum* cysts with $94 \pm 1\%$ and $93 \pm 6\%$, respectively, of revived cells (Fig. 3). Germination of temporary cysts occurred in majority on the second day of incubation for the two dinoflagellates.

The revived cells were isolated and incubated in new culture medium following the previously described culture conditions (cf. methods). Cells of both *Alexandrium* spp. divided normally and the measured growth rate for *A. catenella* was 0.29 ± 0.15 divisions day^{-1} for cells originating from the faeces and 0.3 ± 0.1 divisions day^{-1} for those found in the pseudofaeces (Fig. 4). For *A. minutum*, the growth rate was 0.45 ± 0.05 divisions day^{-1} for the faeces and 0.54 ± 0.02 divisions day^{-1} for the pseudofaeces (Fig. 4). These rates were close to growth rate (0.53 ± 0.19 divisions day^{-1}) of cells not ingested by *C. gigas* (control).

The toxin profiles were similar in the two *Alexandrium* strains. Both of them contained carbamate toxins (STX, neo-STX and GTX-1,-2,-3,-4), N-sulfocarbamoyl toxins (B-1,-2,C-1,-2,-3,-4) and decarbamoyl toxins (dcSTX and
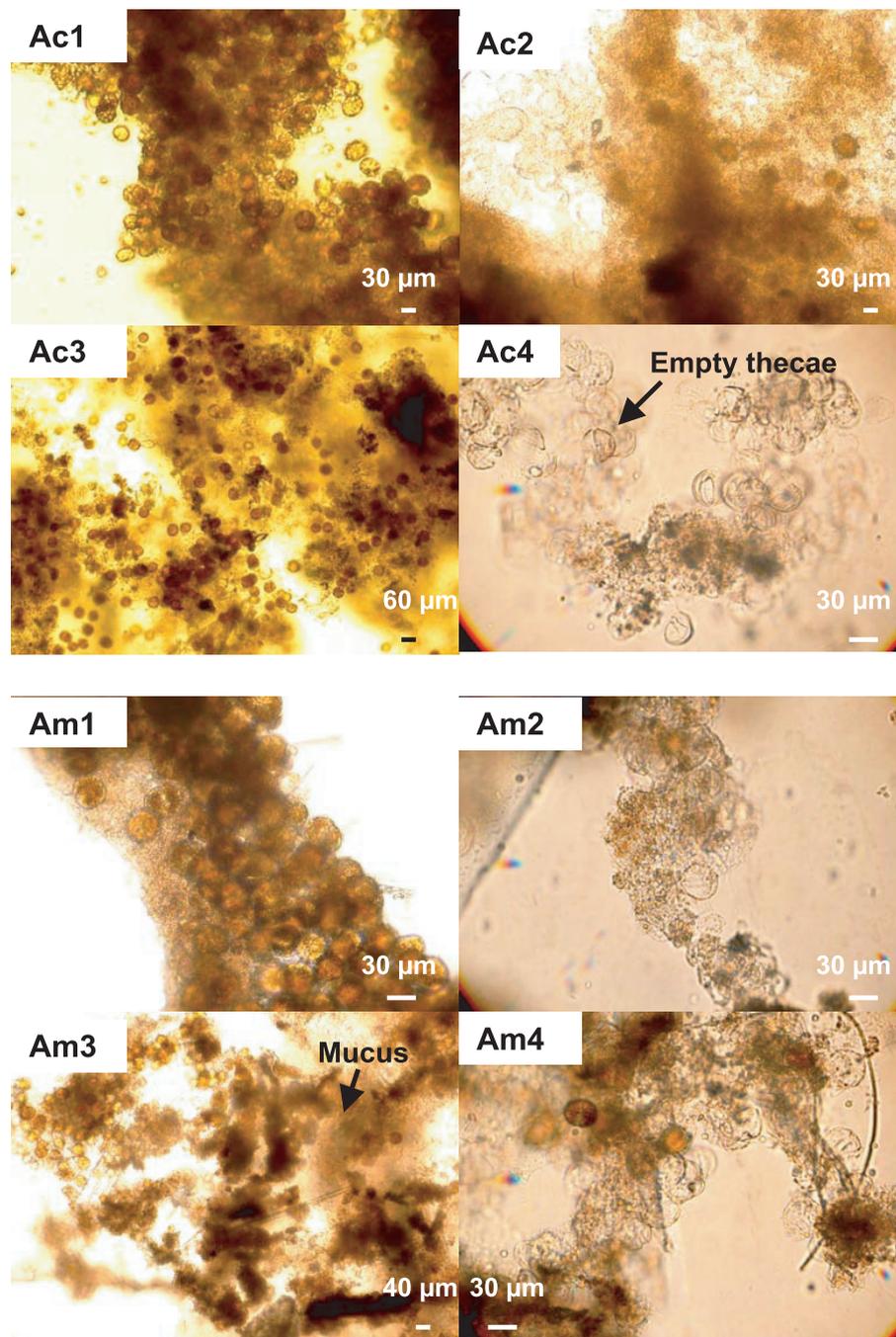
Fig. 2. Light microscope photographs of faeces (Ac1, Am1) and pseudofaeces (Ac3, Am3) produced by *Crassostrea gigas* fed with the dinoflagellates *Alexandrium catenella* (Ac) (35 μm in mean diameter) and *Alexandrium minutum* (Am) (22 μm in mean diameter). The faecal material was made of viable temporary cysts. Empty thecae of the *Alexandrium* species were observed in the undisrupted faecal material at the end of the 24-h incubation time (ESAW medium) in faeces (Ac2, Am2) and pseudofaeces (Ac4, Am4) of both dinoflagellate species.

dc-GTX-2,-3). In cultured *A. catenella* cells, the toxin content varied over the culture cycle, with the highest values obtained in the stationary phase (Fig. 5). However there were no noticeable changes in toxin profile within all examined growth phases. The following toxins were found in decreasing order: C2, GTX4, B1, B2, GTX3, C1, Neo-STX, dcSTX, STX, GTX1, GTX2, with dcGTX2 and 3 present as trace amounts. Toxin contents in cells revived from temporary cysts showed

a substantial decrease of C2 toxin in exponential phase when compared with that of cells in the control experiment (Fig. 5), and did not contain B1/B2 toxins in stationary phase. The total toxin content of these cells decreased by approximately 30% during their exponential growth phase. For *A. minutum*, toxins were only analyzed during the exponential growth phase and showed the dominance of C1, C2, dcGTX3 and GTX3 toxins (Fig. 6). The overall toxin content of the germinated cells of
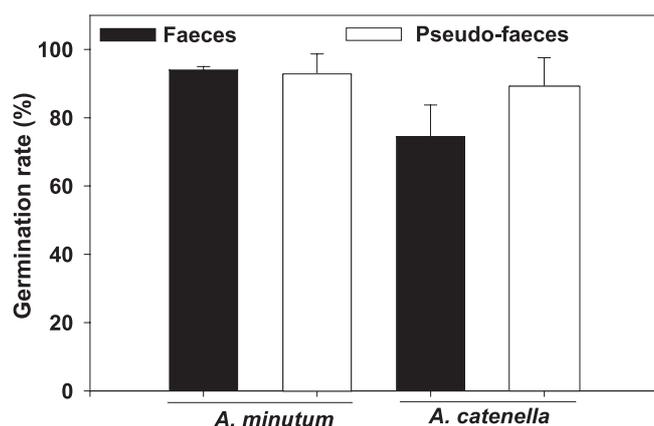
Fig. 3. Maximum germination rate of temporary pellicular cysts sampled from faeces and pseudofaeces produced by *Crassostrea gigas* individuals fed with monoalgal diets of the dinoflagellates *Alexandrium catenella* and *Alexandrium minutum*. These rates were measured after 48 h incubation period of the temporary cysts in favourable conditions.

Fig. 4. Growth rate of non-ingested vegetative cells (control) and of germinating cells issued from *Alexandrium catenella* and *Alexandrium minutum* temporary cysts found in the faeces and pseudofaeces of *Crassostrea gigas* fed with these two dinoflagellates.

A. minutum slightly decreased due to lower concentrations of C1, C2 and dcGTX3 (Fig. 6).

4 Discussion

In this study, reduction of filtration rate was observed in *C. gigas* fed with either *A. catenella* or *A. minutum*, in agreement with previous observations (Bardouil et al. 1996; Laabir and Gentien 1999). Cells of these two dinoflagellates were rejected as temporary cysts in the faecal ribbons and occupied an important volume of the faecal material produced by *C. gigas*. 24 hours after incubation of these produced pellicular cysts in ESAW medium at light they germinated and formed new vegetative cells. The temporary cysts produced in our experiments kept their theca which was slightly detached and then completely abandoned at the time of the revivification of these cells sampled in both faeces and pseudofaeces (Fig. 2). Dinoflagellate cells are exposed during their gut transit to important chemical (acidic pH) and mechanical constrains which promote the formation of temporary cysts. We observed that almost all of the cells rejected in pseudofaeces, and embedded in the oyster mucus were transformed into temporary cysts (Fig. 2). Marasovic (1993) showed that *Gonyaulax polyedra* could overcome adverse environmental conditions by forming pellicular cysts. This cellular type is produced when one or more physicochemical conditions change drastically.

Our study clearly demonstrated that *A. catenella* cells have the capacity to survive gut passage in *C. gigas*, are able to germinate in high proportions (>74%) and have a growth rate close to control cells. Similarly, almost all *A. catenella* cells released in the pseudofaeces remained viable and were able to revive. In our experiments, *C. gigas* kept on filtrating *A. catenella* cells over 24 hours. During a bloom, this behaviour could generate an important production of faeces and pseudofaeces containing viable toxic *A. catenella* cells. These cells can be transferred via the biodeposits on top of sediments and constitute a seeding stock cells. Even if the survival time of
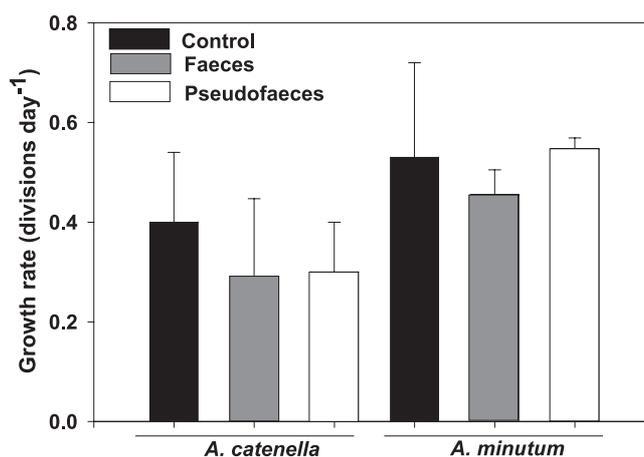
pellicular cysts is much lower than that of resting cysts (Garcés et al. 2002), the displacement of the sediment with the pellicular cysts could potentially lead to the contamination of the water column in pristine areas when environmental conditions become favourable to their germination.

As proposed by Shumway and Cucci (1987) for *Protogonyaulax tamarensis*, here the production of faeces and pseudofaeces with intact *A. catenella* or *A. minutum* cells could be considered as a selection against these toxic dinoflagellates, avoiding by this process the accumulation of toxins in the digestive gland and potential subsequent mortality of the oysters. However, Laabir and Gentien (1999) showed that non toxic thecate dinoflagellates were found intact and viable in faeces of *C. gigas*, suggesting that toxicity is not the main factor allowing cells to pass intact through the gut. Rather, the cellulosic thecae are likely to play a crucial role in resistance to adverse conditions experienced during gut transit.

Our toxin analysis revealed that the major component of *A. catenella* (ACT03 strain) are carbamate toxins and GTXs particularly C-2 and GTX-4 which features high intrinsic toxicity whereas STX and NeoSTX are absent. Similar toxin profiles are exhibited by two other strains isolated from Thau lagoon during the 1998 toxic bloom (ATTL01 and ATTL02: Lilly et al. 2002). However, in vitro studies demonstrated that cell toxin content depends on culture conditions, especially the concentrations in dissolved inorganic nutrients (Siu et al. 1997).

The cells originating from revivified pellicular cysts of *A. catenella* had toxin profile similar to control cells but were less toxic and that C-2 toxin decreased drastically during the exponential phase whereas B-1 and B-2 decreased during the stationary phase. It is not clear whether toxin production and biosynthetic pathway in the newly formed vegetative cells have been affected by the gut transit. When considering the decrease in the total toxin content, we can suppose that the newly formed cells redirected their metabolites toward somatic growth and division and therefore synthesized a lower content of toxins. Toxin analysis of *A. minutum* germinated cells corroborates these suggestions. Previous finding showed
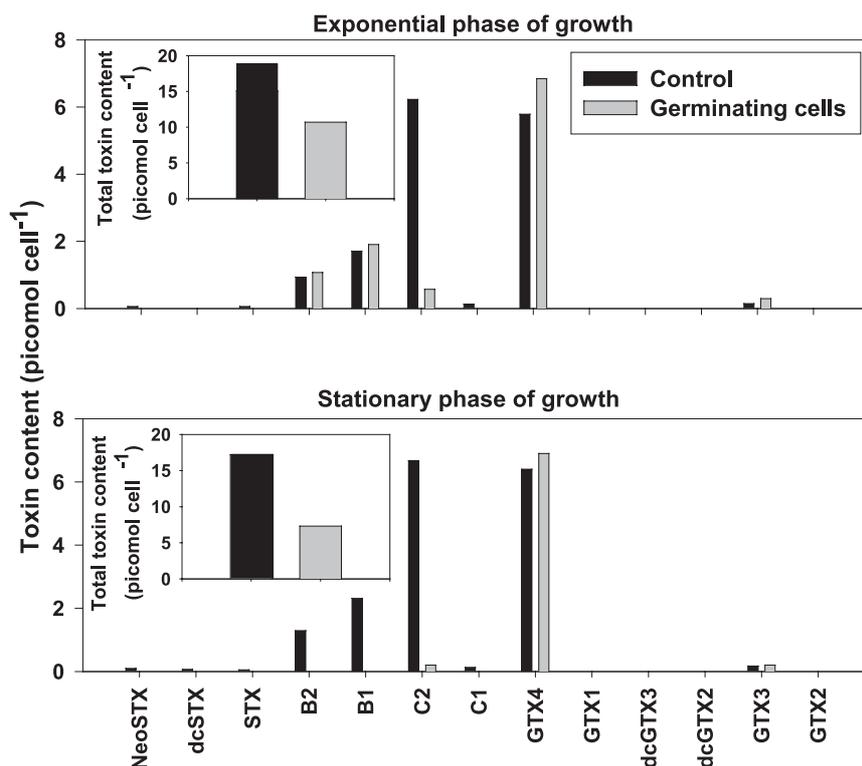
Fig. 5. Toxin profile and total toxin content of *Alexandrium catenella* measured during both exponential and stationary growth phases of non ingested cells (control) and of cells which germinated from temporary cysts found in the faeces of *Crassostrea gigas*.
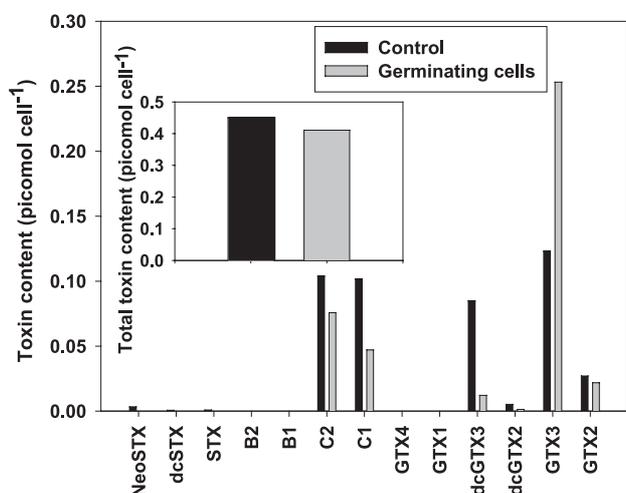
Fig. 6. Toxin profile and total toxin content of *Alexandrium minutum* measured during exponential growth phase of the non-ingested cells (control) and of germinating cells from pellicular cysts obtained in the faeces of *Crassostrea gigas*.

that *Gymnodinium catenatum* resting cyst-germinated cultures from lab-germinated cysts had significantly different paralytic toxin contents (Negri et al. 2001).

The proven toxicity of the germinated cells issued from the pellicular cysts indicates that these dinoflagellates keep their toxic potential after the gut transit or when they are rejected in the pseudofaeces. These cells can be ingested by the predators colonizing the water column (copepods) or the

sediment (macrobenthos including mollusca and polychaeta) which would represent a transfer of toxins at the higher trophic levels.

The ability of *C. gigas* oysters to keep viable dinoflagellate temporary cysts in their digestive tract can potentially help the proliferation of algal populations originating from germinated cysts. Hence, dinoflagellate cells trapped in the faecal material as pellicular cysts could potentially play a role in bloom maintenance after germination process. Similarly, it was suggested that naturally produced temporary cysts of *Alexandrium taylori* could participate bloom maintenance (Garcés et al. 2002).

The time needed for the complete elimination of toxic cells from the digestive tract of targeted molluscs remains to be determined. It is important to determine how the internal cleaning of molluscs could be effective in eliminating pellicular cysts in the digestive tract and therefore decreasing the potential geographic spreading of the toxic algae. Similarly to what was found for *Pfiesteria piscicida* (Shumway et al. 2002), our data clearly showed that *C. gigas* oysters cannot be used as biocontrol agents against *A. catenella* blooms. Conversely they are potential vectors of *A. catenella* if transported from a contaminated area into pristine waters, and a such transfer could extend the bloom duration through germination of pellicular cysts contained in the faecal material. Finally, our results further stress the necessity to regulate the geographic transfer of bivalve shellfish stocks (Van den Bergh et al. 2002).

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