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Toxin profile change in vegetative cells and pellicle cysts of *Alexandrium fundyense* after gut passage in the eastern oyster *Crassostrea virginica*

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ABSTRACT: Vegetative cells and pellicle cysts of the toxic dinoflagellate *Alexandrium fundyense* Balech were fed to the eastern oyster *Crassostrea virginica* Gmelin under controlled conditions. Paralytic shellfish toxins (PSTs) were measured in vegetative cells and pellicle cysts prior to feeding and directly after passage through the oyster alimentary canal and defecation as intact cells. Oysters fed with vegetative cells and those fed with pellicle cysts accumulated toxins. One experimental treatment tested for direct uptake of toxins from the water (oysters and *A. fundyense* cells were separated by a screen); PSTs were not accumulated from the water by the oysters. There were no significant changes in total, per-cell toxicity after passage through the oyster alimentary canal, suggesting limited transfer of toxins from intact cells to the oysters. However, there were statistically significant changes in the toxin composition of cells following gut passage. Vegetative cells and pellicle cysts from feces had increased amounts of saxitoxin (STX) and decreased amounts of gonyautoxin 4 (GTX4) per cell, compared to amounts prior to gut passage. Following gut passage, pellicle cysts showed better survival in the feces than vegetative cells, which is consistent with the view of pellicle-cyst formation as a successful survival strategy against adverse conditions.

KEY WORDS: *Alexandrium fundyense* · *Crassostrea virginica* · Dinoflagellate · Toxin · PST · Pellicle cyst

INTRODUCTION

The dinoflagellate *Alexandrium fundyense* is one of several microalgae that produce paralytic shellfish toxins (PSTs), i.e. saxitoxin and its derivatives (>50 compounds with different toxicities). PSTs are perhydropurine alkaloids that share a tricyclic carbon backbone but differ in their functional groups at 4 positions (Schantz 1960, Wiese et al. 2010). The transfer and accumulation of PSTs into commercially harvested shellfish soft tissues is a global human-health issue (Bricelj & Shumway 1998, Anderson et al. 2002, Wang 2008) that is managed by toxin monitoring programs and harvesting bans when toxin limits are exceeded. When *Alexandrium* spp. vegetative cells are filtered by oysters and other mollusks, some cells can pass through the bivalve digestive system alive. The proportion of cells that survive gut passage is variable, depending upon such factors as concentration, species, and health status of the shellfish, but it can be considerable (Laabir & Gentien 1999 and references therein, Garcés et al. 2002, Persson et al. 2006, Hégaret et al. 2007, 2008). At some point during the capture and ingestion process by shellfish, *Alexandrium* spp. cells

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cast off the thecae and flagella, and enter a protected stage known as the 'pellicle cyst' (also termed 'temporary' or 'ecdysal'). Dinoflagellates form pellicle cysts quickly when they encounter unfavorable conditions, e.g. shaking of culture flasks, transfer of cells into new nutrient media, or change in temperature. Pellicle cyst transformation can protect the dinoflagellates from exposure to harmful substances or conditions (Garcés 2001, Tillmann et al. 2007) as well as from parasite infection (Toth et al. 2004). When the environment again becomes favorable for vegetative growth, the cells form new thecae and flagella, swim again, and continue to grow by binary fission. These cells can swim again in <24 h if put into a growth medium (unpubl. obs., this experiment).

It is usually assumed that PSTs remain within dinoflagellate cells until they are digested, but it is difficult to measure PSTs that may be released by cells into salt water because salt interferes with the analysis. It is possible to use activated charcoal columns to collect the toxins, as has been done in some studies with bivalves (Sekiguchi et al. 2001, Suzuki et al. 2003, Asakawa et al. 2006). Lefebvre et al. (2008) measured the extracellular toxin levels in both field samples and laboratory cultures by receptor binding assay (RBA) and enzyme-linked immunosorbent assay (ELISA). This study showed that extracellular PSTs were present during blooms of *Alexandrium* spp., and in culture media. In many experiments with PST-producing dinoflagellates, data indicate that PSTs were released into the medium (e.g. Flynn et al. 1996, Wang et al. 2002, Kim et al. 2005). For dinoflagellates that produce hydrophobic toxins associated with DSP (diarrhoeic shellfish poisoning), toxins dissolved in the water are measured with the so-called 'SPATT (solid phase adsorption toxin tracking) bags' filled with a polymeric resin that adsorbs hydrophobic compounds (MacKenzie et al. 2004, Fux et al. 2008, 2009). During DSP-producing blooms, significant amounts of DSP toxins are dissolved in the seawater (MacKenzie et al. 2004). In the case of freshwater cyanobacteria that produce PSTs, the toxins are released into the water (Filho Ada et al. 2008, Kayal et al. 2008), and this can be interpreted as a chemical defense strategy. Okadaic acid, which is produced by the dinoflagellate *Prorocentrum lima*, is also released into the extracellular medium (Rausch de Traubenberg & Morlaix 1995). The ability of oysters to sense the presence of toxic algae and quickly cease feeding (e.g. Bardouil et al. 1993, Wildish et al. 1998) suggests that PSTs could be expelled as a defense or an allelopathic substance by *Alexandrium* spp. in the marine environment. Hwang et al. (2007) showed that PSTs, specifically gonyautoxins (GTX) 1 to 4, attracted snails that acquire this toxin through feeding and use it as a defense strategy.

It is theoretically possible for toxic dinoflagellates to release toxins during gut passage in oysters without being digested. If so, this could cause toxicity in oysters without eliminating ingested dinoflagellate cells. Egested living cells could continue to divide and form new toxins again.

This study explored cellular PSTs, both in terms of total toxicity per cell and toxin composition, in *Alexandrium fundyense* cells before and immediately after oyster feeding on intact cells and pellicle cysts, and also investigated the possibility of direct uptake of PSTs from the water.

MATERIALS AND METHODS

Cultures and culturing conditions

We used *Alexandrium fundyense* strain BF-2 (length: 32.2 ± 2.3 µm SE, width: 30.7 ± 2.6 µm) from the NOAA/NMFS Milford Microalgal Culture Collection (isolated from the Gulf of Maine). This strain was cultured in *f*/2 medium with no silicate added at 16°C, 14 h light:10 h dark cycle, and 100 µE m^{-2} s^{-1} photosynthetically active radiation (PAR). Cells in the early stationary phase were used for the oyster-exposure experiment.

Alexandrium fundyense pellicle cysts were produced by vigorous shaking of a portion of the same culture as used for vegetative-cell treatments. The subculture was vortexed (Vortex-Genie 2, Scientific Industries) for 2 min and then rinsed on a 20 µm screen with filtered seawater; thereafter, the volume was reconstituted with filtered seawater. The cells were confirmed to be intact immobile pellicle cysts by light microscopy. Pellicle cysts are also commonly called 'temporary' or 'ecdysal' cysts, and here we use 'temp' for the pellicle cysts in tables and figures since the pellicle cyst is a short temporary state for this species.

Oysters

Oysters *Crassostrea virginica* from Long Island Sound (mean shell length: 83 ± 5 mm) were obtained from Fishers Island Shellfish Farm, Fishers Island, New York. The oysters were kept in the laboratory for ~1 wk in running, unfiltered seawater until experimental use. Temperature was increased by 2°C d^{-1} from 12 to 18°C. Before the experiment, oysters were brushed, washed and placed in 0.1 µm filtered seawater for 3 d in the room where the experiment was later to be performed. Oysters were fed with one ration of *Tetraselmis striata* (Plat-P) immediately after transfer, but were starved thereafter until the start of the experiment. Water was changed daily.

Table 1. Experimental treatments

Treatment	Cells	Live oyster
Alex	Vegetative cells	Yes
Temp	Pellicle (= temporary) cysts	Yes
Screen	Vegetative cells, separated from the oyster by a screen	Yes
Control	Vegetative cells	No

Experimental design

Experimental treatments (Table 1) were established in 1 l polypropylene beakers. Each treatment had 5 replicates, and each replicate was 1 beaker that contained 1 oyster (or empty oyster shell, to account for physical trapping of *Alexandrium* cells by the shell itself), and 1 l of 0.1 µm filtered seawater. Unfed oysters were maintained in filtered seawater as a control for oyster soft-tissue toxin content.

The experiment was conducted in a constant-temperature room at 18°C with 35 µE m^{-2} s^{-1} PAR artificial illumination (room lights) from above (as measured inside the beakers before the experiment). A single, high-concentration dose of cells was used to optimize the number of undigested cells in the feces, because these were the study material. A short total time for the experiment was necessary because aeration or stirring could not be used (the feces needed to be identifiable and intact for picking). A cell number that was high enough to yield a sufficient quantity of intact cells in feces samples for toxin measurements was needed, while avoiding the risk of a valve-closure response at high cell numbers. A balance between these needs was achieved through experience from earlier experiments with oysters and *Alexandrium fundyense* (Persson et al. 2006, A. Persson et al. unpubl. data).

For vegetative-cell treatments, 5 samples were collected at the start of the experiment, and 4 were collected for the pellicle-cyst treatment, for toxin analysis. For the enumeration of cells, 5 samples were taken from each culture before the start of the experiment. Then, the experiment was started by the addition of 16 ml of cultured vegetative cells or pellicle cysts to each beaker. This procedure yielded 670 000 cells $beaker^{-1}$ for vegetative cells and 595 000 cells $beaker^{-1}$ for pellicle cysts.

The oysters were allowed to feed for 23 h. Oysters in the treatment wherein cells and oysters were separated by screens were given 5 ml of a *Nannochloropsis* sp. (UTEX2341) culture (1×10^7 cells ml^{-1}) after 20 h to determine if the oysters were actively filtering. *Nannochloropsis* sp. are small (2 – 4 µm) cells that have a bright green color. They are not digested by oysters, pass quickly through the alimentary canal, and are visible in feces. Appearance of bright green feces is confirmation that oysters are actively filtering.

Termination of the experiment

Feces produced in each beaker were carefully picked with a pipette and saved in a beaker in a refrigerator (4°C). The next day, they were pooled with the rest of the feces from the same oyster (see below), and rinsed on a 20 µm screen with filtered seawater to remove broken cells and debris, leaving only intact pellicle cysts. The sample was rinsed into a 100 ml volumetric flask with filtered seawater and brought to volume. Pseudofeces were not found (and not expected at this cell density given our earlier experience from studies using the same algal and oyster species; Persson et al. 2006, A. Persson et al. unpubl.).

Oysters in the treatment wherein cells and oysters were separated by screens were carefully removed from the water before filtering it to ensure that oysters were not in direct contact with any cells at any time. The beaker water (without feces) was rinsed with filtered seawater onto a 20 µm sieve, and the container and the live oyster, or empty oyster shell, were also rinsed with filtered seawater onto the sieve. Then the material on the sieve was transferred to a 100 or 200 ml volumetric flask, and brought to volume. Samples were taken for cell counts and for toxin measurement according to the description below. Samples for cell counts were taken from the volumetric flasks (3 to 4 ml) and preserved with iodine crystals. Cells were counted in a Sedgewick-Rafter counting cell with a light microscope.

Samples for toxin measurements were gently filtered onto 25 mm GF/F. The filters were put in Eppendorf vials and directly frozen with lids open to dry the filters. Then 1 ml of 0.05 M acetic acid was added to each Eppendorf vial. The sample was frozen, thawed, and shaken 4×. After the last freezing, the samples were thawed and aspirated with a 1 ml syringe. Special filters with very little dead volume had been made; a 1 ml pipette-tip cut off to fit on the syringe had a small piece of GF/F inserted inside the tip. The sample was pressed with the syringe through the filter in the tip into a HPLC vial which was then crimp sealed. Samples from the control and screen treatments were divided into two; one part was extracted with acetic acid as described above, and the other was extracted with 0.1 N HCl. This was done to calculate the concentrations of C toxins in the sample (see below).

At the end of the experiment, after oysters were rinsed, they were placed in new, clean beakers with 0.5 l of filtered seawater. To each beaker, 5 ml of *Nannochloropsis* sp. culture (as described above) were

added to monitor progress through the oyster digestive systems. The oysters were left in the beakers (in the same culturing room as above) overnight. All material in the beakers was rinsed and sieved thoroughly with filtered seawater, as above, to remove *Nannochloropsis* cells and fecal components. Feces samples were saved for toxicity measurements and *Alexandrium* cell counts.

Oysters were kept dry in a refrigerator (4°C) overnight, and they were then 'shucked' to extract soft tissues, which were then rinsed in running, cold tap water. The soft-tissue mass was weighed, homogenized with a tissue homogenizer and divided into two. One part was extracted with boiling 0.1 N HCl for 5 min according to the protocol of Oshima (1995); the other part was extracted with 0.05 M acetic acid without boiling. The extracted soft-tissue samples were frozen (–80°C). Oyster tissue samples were transferred into 15 ml centrifuge tubes and thawed, and the original sample volume restored with distilled water. Samples were vortexed, then centrifuged at 1000× *g*, and the supernatant was filtered into HPLC vials as described above.

Toxin analysis. Samples for toxin analysis were shipped frozen to the Department of Marine Ecology at the Sven Lovén Center for Marine Research, Tjärnö, Sweden, and stored at –20°C until sample analysis.

Paralytic shellfish toxins were analyzed using HPLC with fluorescence detection (HPLC-FD) according to Asp et al. (2004). HPLC analyses were carried out on a Hitachi-7000 system equipped with a RP8 Column (Genesis C8, Vymac 4 µm, 150 mm, inner diameter = 3 mm). PST standards (saxitoxin, decarbamoylsaxitoxin, neosaxitoxin, and gonyautoxin GTX1 to 4) were purchased from NRC-CNRC, Halifax, Nova Scotia, Canada. The samples contained both N-sulfocarbamoyl toxins (C toxins) and carbamates. As C toxins are not retained in the HPLC method, a hydrolyzed sample (0.1 N HCl) was analyzed to calculate the concentration of C-toxins based on the increase in corresponding carbamates, compared to non-hydrolyzed samples extracted with 0.05 M acetic acid. GTX1 to 4 were resolved and analyzed using isocratic elution with Eluent A: 2 mM sodiumheptanesulfonate in 10 mM ammonium phosphate buffer (pH 7.1). Saxitoxin, neosaxitoxin, and dc-saxitoxin were analyzed using isocratic elution with Eluent B: 2 mM sodiumheptanesulfonate in 30 mM ammonium phosphate buffer (pH 7.1): acetonitrile (96:4). After the separation, toxins were oxidized with 7 mM periodic acid in 50 mM sodium phosphate buffer (pH 9.0, 0.2 ml min^{-1}) in a PEEK capillary (10 m, 80°C). The oxidation was terminated with 0.5 M acetic acid (0.2 ml min^{-1}) before fluorescence detection at λ_{ex} = 330 nm, and λ_{em} = 390 nm.

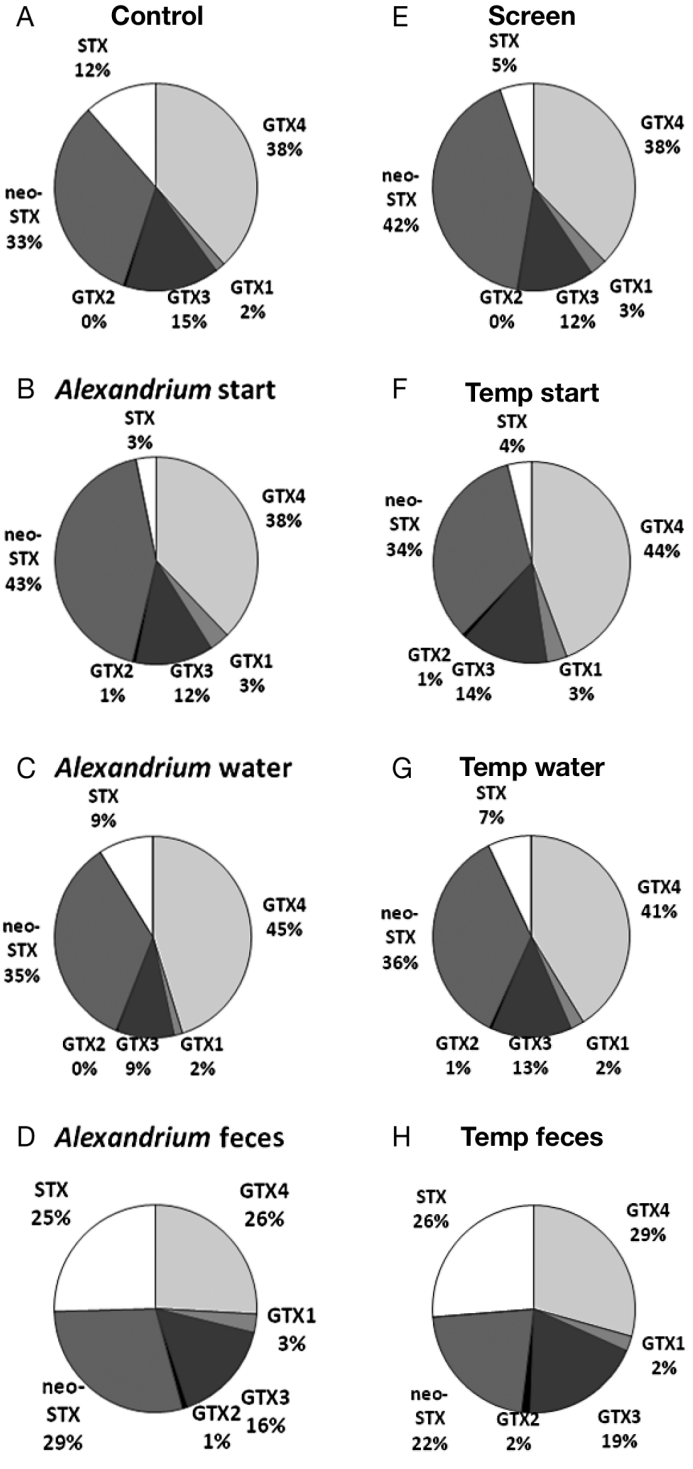

Fig. 1. *Alexandrium fundyense*. Proportional changes in toxin profile before and after oyster gut passage. C toxins are not included since they were measured only in the control and screen treatments. (A) No-oyster controls; (E) screen treatments: cells were separated from the oysters by a 20 µm screen; (B, F) start: vegetative cells and pellicle cysts at the beginning of the experiment; (C, D) *Alexandrium*: vegetative cell treatments; (G, H) temp: pellicle (temporary) cyst treatments. Water: cells not in feces at the end of the experiment; feces: cysts found in oyster feces; GTX: gonyautoxins, STX: saxitoxin

RESULTS

Oysters actively filtered both vegetative cells and pellicle cysts of *Alexandrium fundyense* from suspension, producing feces containing mainly pellicle cysts. There were significant changes in toxin composition during transport through the oyster digestive system (Fig. 1); intact cells from feces samples had a significantly different PST composition compared to all other samples. The principal toxin in uningested cells (start, control, water and screen) was GTX4, followed by neosaxitoxin (neoSTX) and GTX3 (Figs. 1 & 2). C toxins were determined for control and screen treatments, and the principal C toxin was C2 (see below). The levels of saxitoxin (STX) were significantly higher ($p < 0.01$), and the GTX4 toxin level was significantly lower ($p < 0.04$) in feces samples; the change was the same for cells that were initially vegetative cells as for those that were initially pellicle cysts.

The measurements of toxicity in oyster soft tissues were done without internal toxin standards and additional cleaning steps, and are therefore only qualitative. Impurities in these samples resulted in difficulties in identification and quantification of PSTs. The presence of GTX4 and neoSTX in soft tissues from oysters fed with vegetative *Alexandrium fundyense*, and oysters fed with pellicle cysts was evident, whereas no PSTs accumulated in the oyster soft tissues in the screen treatment. Toxin levels were ~0.03 µmol GTX4 g^{-1} and 0.15 µmol neoSTX g^{-1} soft tissue for oysters feeding on vegetative cells or pellicle cysts. All *A. fundyense* cells in the pellicle-cyst treatment (temp) were immobile pellicle cysts at the start of the experiment. However, there were numerous swimming cells near the upper end of the beakers, toward the brighter side, at the end of the experiment (Fig. 3). The feces contained many empty thecae and membranes/pellicles that had surrounded the pellicle cysts; these appeared to have germinated, i.e. they were not digested or destroyed, but left by cells returning to the vegetative stage (Fig. 4). This was especially apparent in the pellicle-cyst treatment. In feces from the vegetative-cell treatment, there were some clumps of cells

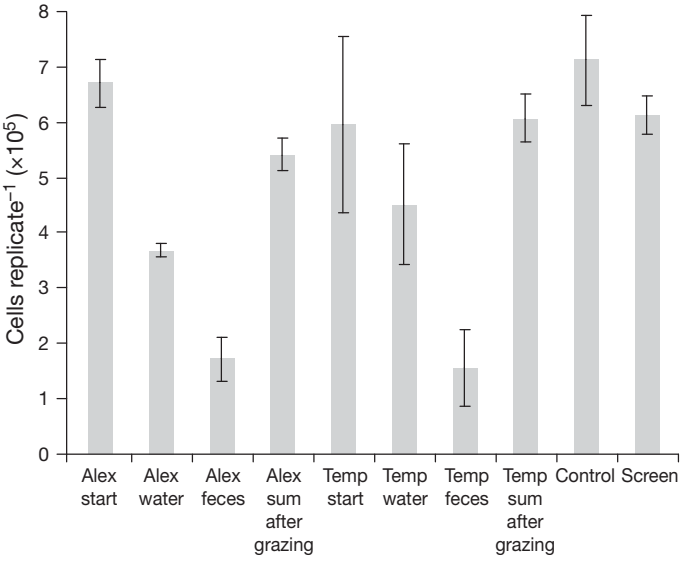

Fig. 3. *Alexandrium fundyense*. Cell numbers (total number of cells per 1 l beaker). Bars: average number of cells in beakers in each treatment. Error bars: SD. (See Fig. 1 for treatment explanations)

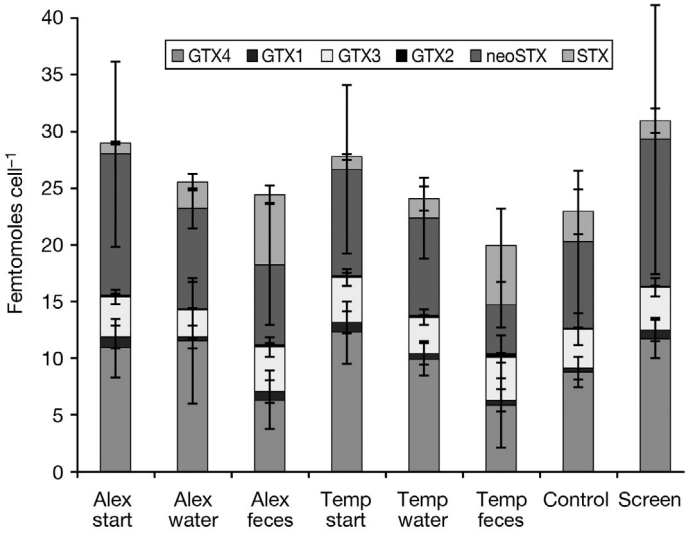

Fig. 2. *Alexandrium fundyense*. Toxicity in the different treatments. C toxins are not included since they were measured only in the control and screen treatments. Error bars: SD. (See Fig. 1 for treatment explanations)

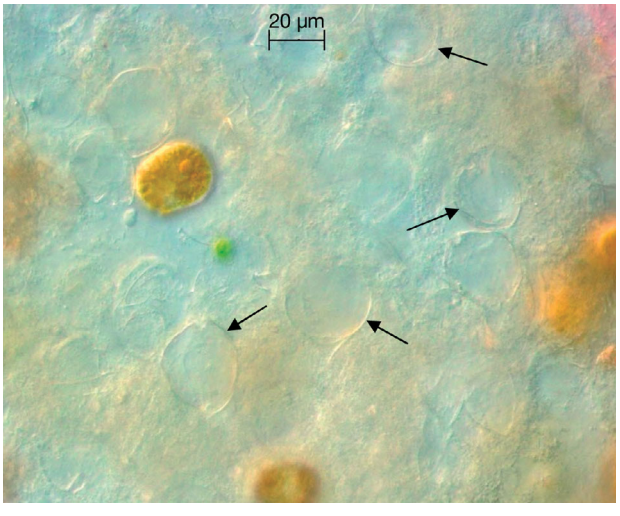

Fig. 4. Oyster feces from pellicle cyst treatment, with empty thecae and pellicle membranes (arrows)

and some cells that did not appear to be alive, in addition to the living pellicle cysts that were the main component in the feces samples (Fig. 5). Toxin analyses were performed on rinsed samples containing intact cells and no broken cells or half-digested material. Compared to the numbers at the start, the sums of *A. fundyense* cells present after filter-feeding (feces + water) were significantly lower for the vegetative cell treatment, but not for the pellicle cyst treatment. The mean digestion of cells was 19% in the vegetative cell treatment ($p = 0.004$), but no digestion could be shown by statistical analysis of cell numbers in the pellicle cyst treatment ($p = 0.88$) (Fig. 3), although the accumulation of toxins in oyster soft tissues indicates that digestion took place. Vegetative cells in the water were swimming along the upper edge of the beaker near the brightest side. In beakers containing screens, *A. fundyense* cells swam up along the screen, or were caught by surface tension near the screen.

The results from the feces are based on the oysters that defecated sufficient material for analysis: 3 replicates from the vegetative (Alex) treatment and 4 from the pellicle cyst (temp) treatment. It was difficult to ascertain if the oysters in the screen treatment were actively filtering, as they could not produce biodeposits without food. The valves appeared to be closed, so *Nannochloropsis* sp. culture was added 3 h before the end of the experiment. After this, 2 oysters defecated, but only very little. All oysters in the experiment filtered and defecated after the experiment, when placed in clean containers and fed with *Nannochloropsis* sp., hence they were all assumed to be in a fully healthy state.

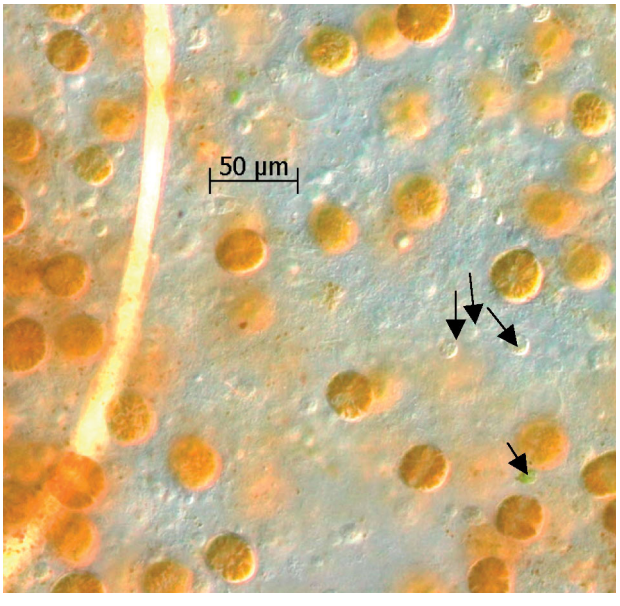

Fig. 5. Oyster feces from the *Alexandrium* (vegetative cell) treatment before rinsing. Intact but immobile living cells of *Alexandrium* (pellicle cysts) can be seen, as well as small *Nannochloropsis* cells (arrows) and debris

Screen treatments and no-oyster controls were extracted both with HCl and acetic acid, which provided information about the C toxins through calculation of the increase in corresponding carbamates in hydrolyzed compared to non-hydrolyzed samples. *Alexandrium fundyense* cells in the control treatments contained (mean ± SD) 12.0 ± 4.2 fmol C2 toxin cell^{-1}, and cells in the screen treatment contained on average 14.7 ± 6.2 fmol C2 toxin cell^{-1}. C1 was present in trace amounts, but C3 and C4 were not detected.

DISCUSSION

The toxin content per *Alexandrium fundyense* cell did not decrease significantly during passage through the oyster digestive system, but there were significant changes in toxin composition. Thus, *A. fundyense* cells that survived gut passage did not release much toxin prior to being egested. The change in toxin composition in *A. fundyense* cells during gut passage cannot be attributed to digestion because total toxicity did not decrease, and the cells were still alive. Our results show that toxin transformations can take place within living dinoflagellate cells before digestion, thus before assimilation of toxins into bivalve tissues. There was significantly less GTX4 and significantly more STX in *A. fundyense* cells recovered from feces than in cells before gut passage, which is consistent with the descriptions of toxin transformations (from source *Alexandrium* cells to bivalve soft tissues) reported in the literature (e.g. Bricelj & Shumway 1998, Li 2004, Jaime et al. 2007); these reports concluded that STX can be formed directly from neosaxitoxin and, through other intermediate forms, also from GTX4 and C toxins. The BF-2 strain of *A. fundyense* used in the present study contained C2 toxins which, with GTX3 as an intermediate, can also form STX. It should be noted that the toxin transformations seen here are from less toxic to more toxic forms, which has implications for the interpretation of the biological relevance of these toxin transformations. The transformation to more toxic forms has also been shown in scallops (Cembella et al. 1994) and in surf clams (Shumway et al. 1994). A passive change in the internal pH in living cells resulting from the lower pH of the surrounding digestive fluids is a possible explanation, and the subject will be studied further. Discovery of these toxin transformations within *A. fundyense* cells experiencing the extraordinarily stressful conditions within the digestive system of an oyster also adds to the evidence that *Alexandrium* spp. toxin

composition is not an exact 'fingerprint' for a species, but is rather variable depending on environmental factors affecting the cell. There are many reports of changes in toxin profile in different conditions or environments (Ravn et al. 1995, Ciminiello et al. 2000, Hamasaki et al. 2001, Parker et al. 2002, Teegarden et al. 2003, Etheridge et al. 2004, Kim et al. 2005), although other papers argue that the toxin profile can be considered as a fingerprint (e.g. MacKenzie et al. 1996, Ichimi et al. 2001). Information about the biological significance of the different toxin forms is incomplete, but it cannot be ruled out that changes to more toxic forms that occur in living cells in a threatening situation might have a biological cause. The information in the literature on what affects toxicity is very diverse: temperature, salinity, light, N and P limitation (e.g. Anderson et al. 1990), life stage (Cembella et al. 1990), division cycle (Taroncher-Oldenburg et al. 1999) and shock (Ravn et al. 1995) can affect *Alexandrium* spp. toxicity levels and/or profiles. The presence of grazers induces a higher toxin production in *A. minutum* (Selander et al. 2006, Bergkvist et al. 2008).

The oyster soft tissues in our experiment contained GTX4 and neosaxitoxin, which are the 2 dominating forms of toxin in the dinoflagellates, but not STX. Transformation of toxin forms occurs both by enzymatic processes and by spontaneous chemical reactions with pH change (Bricelj & Shumway 1998, Li 2004, Jaime et al. 2007). These transformations probably occur as part of digestive or metabolic processes. Jaime et al. (2007) showed toxin transformation *in vitro* with toxin extracts from *Alexandrium fundyense* and different shellfish tissues. Asakawa et al. (1995) showed different toxin profiles in *A. tamarense* and the bivalves that ingested them. Cembella et al. (1993) suggested that toxin conversions might be particularly significant during the early stages of digestion when dinoflagellate cells are still intact and metabolically active. It is clear that changes in toxin structure occur during digestion, and also within cells that survive gut passage. It is possible that some of the swimming cells in the beakers might have passed the oysters and returned to the mobile phase, considering that the feces contained empty thecae and pellicle membranes (that would likely be destroyed if they were empty during gut passage; Fig. 4). It is unknown if these cells would maintain the changed toxin profile for any length of time, as we do not know the proportion of these cells among the swimming cells. Laabir et al. (2007) showed changed toxin profiles in cultures of *Alexandrium catanella* and *A. minutum* that were established from pellicle cysts collected from molluscan feces.

The digested cells caused accumulation of toxins in the oyster soft tissues, from both vegetative cells and pellicle cysts. No direct uptake of PSTs from the water was shown to occur. As oysters seemed to close their shells, thus restricting exposure of soft tissues to water in the beakers containing *Alexandrium fundyense* cells separated from the oysters by a screen, we cannot exclude the possibility that direct PST uptake from the water can occur in nature or in other experiments. Oysters (*Crassostrea* spp.) are more sensitive to neurotoxins than many other bivalves (Twarog et al. 1972, Shumway et al. 1990). In most studies, the PST content of the medium was not measured. In a few studies with bivalves, a column with activated charcoal was used to adsorb dissolved PSTs for subsequent elution and analysis (Sekiguchi et al. 2001, Suzuki et al. 2003, Asakawa et al. 2006). A recent study by Lefebvre et al. (2008) using a receptor-binding assay (RBA) and an enzyme-linked immunosorbent assay (ELISA) found extracellular PSTs in both field samples and laboratory cultures. Activated charcoal is used to remove saxitoxin in freshwater treatments (Falconer et al. 1989, Orr et al. 2004), but this treatment is not 100% effective (Newcombe & Nicholson 2002, 2004, Orr et al. 2004). Thus, although seldom measured, extracellular PSTs are present both in laboratory cultures in experiments, and in the field.

Fewer pellicle cysts than vegetative cells were digested; this is consistent with the view that the pellicle cyst is a protective stage. Pellicle cysts cannot be penetrated by the nuclear stain SybrGreen, unlike vegetative cells (pers. obs.). It appears that when dinoflagellate cells form pellicle cysts, they quickly close many ports of communication or intake of substances, and this protects them from digestive fluids, albeit incompletely.

CONCLUSIONS

PSTs were not released from cells of *Alexandrium fundyense* that survived passage through the oyster (*Crassostrea virginica*) digestive system. However, there was a significant change in toxin profile from less toxic to more toxic forms within the cells, suggesting that toxin form change is a biological defense reaction. Formation of pellicle cysts provides some protection against digestion, and dinoflagellates that survive passage through bivalves do so through the formation of pellicle cysts.

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