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# Toxin content differs between life stages of *Alexandrium fundyense* (Dinophyceae)

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ABSTRACT

Different life stages of two mating-compatible clones of the paralytic shellfish toxin (PST)-producing dinoflagellate *Alexandrium fundyense* Balech were separated using a combination of techniques; culturing and sampling methods were used to separate vegetative cells and gametes, and sorting flow cytometry was used to separate zygotes. PST profiles were significantly different between life stages; the two gonyautoxins GTX1 and 2 were present in vegetative and senescent cells, but disappeared from gametes and zygotes. Toxin-profile changes were shown to occur very quickly in both strains when pellicle cyst formation was induced by shaking (four minutes) followed by rinsing on a screen. These pellicle cysts produced from exponentially-growing, vegetative cells lost GTX1 and 2 completely. Rapid toxin epimerization of GTX1 to GTX4 and GTX2 to GTX3 is one possible explanation, although the biological advantage of this remains unclear. Another possible explanation is that during the mating phase of a bloom or when cells are disturbed, GTX1 and GTX2 are released into the surrounding water. It may be advantageous for a dinoflagellate bloom to be surrounded by free toxins in the water.



## 1. Introduction

Dinoflagellates of the genus *Alexandrium* are well known for causing toxic blooms and have been studied extensively (e.g. Anderson et al., 2008, 2012 and references therein). Blooms of *Alexandrium* species can cover vast areas, and effects upon human societies are substantial, consisting of both health and economic issues (e.g. Anderson et al., 2012). Toxin content or profile of *Alexandrium* spp. dinoflagellates is known to be affected by environmental factors such as temperature, salinity, light, nutrient composition (e.g. Anderson et al., 1990a), or the presence of grazers (Selander et al., 2006). Also, stage in the cell-division cycle has been shown to influence toxin profile and content (Taroncher-Oldenburg et al., 1999). Many studies present measurements of toxin content over time after placement in N-deficient media. When used in dinoflagellate life-cycle studies, or for the purpose of resting-cyst production, such media are called "encystment media" because they induce a shift in life stage to gamete formation and lead to sexual reproduction. In many of the toxin studies, the data show a very sudden decrease in per-cell toxin content 5–8 days after placement of cultures in N-deficient media (e.g. Anderson et al., 1990b; Flynn and Flynn, 1995; Flynn et al., 1995; MacIntyre et al., 1997; Wang and Hsieh, 2001; Wang et al., 2002; Li, 2004; Kim et al., 2005). This toxin loss is coincident with gamete formation that occurs in low-nitrogen medium, regardless of the researcher's intention for using it. When observations of the motility of cells are made, gamete formation can be identified by a typical "mating behavior" or "dance" with cells accumulating densely in spots (petri dishes), lines, stripes, descending plumes (flasks) or layers (salinity-stratified columns and in the field) (Persson et al., 2008; personal observations and submitted manuscript).

The present study was undertaken to describe the toxin contents of the different life stages of *Alexandrium fundyense*, with measurements conducted on pure preparations of each life stage. We also describe the methods developed to separate these life stages: culturing and sampling techniques were used to separate vegetative cells and gametes, and sorting flow cytometry was used to separate zygotes from other life stages. Toxin analyses using standard methods were performed to detect any changes in toxin content and profile as *A. fundyense* cultures transitioned through life-history stages.

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## 2. Materials and methods

### 2.1. Cultures

The experiment was performed at the NOAA Milford Laboratory using two sexually-compatible, clonal strains of *A. fundyense*, both isolated as single vegetative cells from the Gulf of Maine; BF-2 (NOAA/NMFS Milford Microalgal Culture Collection) and 38-3 (Woods Hole Oceanographic Institution; Brosnahan et al., 2010). The different life stages were isolated from cultures using a combination of techniques as outlined below for each stage. After separation, cells were harvested using centrifugation (Clay Adams Dynac Centrifuge) 10 min at 1060 × *g*, (20 °C), the supernatant was discarded, and cells were frozen at −80 °C. Samples were transported frozen to the Department of Marine Sciences, University of Connecticut, Groton, Connecticut, where toxins were extracted and then quantified using HPLC.

### 2.2. Vegetative cells

The two strains of *A. fundyense* were grown in f/2-enriched Milford Harbor water in a lighted bio-incubator at 20 °C, 300 μmol photons $m^{-2}$ $s^{-1}$, 14:10 light:dark (L:D) in 500 mL Erlenmeyer flasks containing 250 mL of culture. Four days before start of the experiment, for each of the strains, two flasks with exponentially-growing cells were added together in a 2800 mL Fernbach flask containing 1250 mL fresh f/2 medium, thus making one, 1750 mL culture in exponential growth for each strain. These cultures provided a common origin of each strain for all the following treatments. At the start of the experiment, to reduce transfer of old medium into experimental media, old medium was removed carefully with a sterile pipette with the tip covered with a piece of sterilized, 20 μm plankton screen. The initial volumes and concentrations in these "starting cultures" were 750 mL of $125 \pm 5 \times 10^3$ cells $mL^{-1}$ for BF-2 and 1000 mL of $84 \pm 11 \times 10^3$ cells $mL^{-1}$ for 38-3. Three replicate samples from the same culture of each strain were used for toxin measurements (10 mL samples from BF-2 and 5 mL samples from 38-3; Table 1).

### 2.3. Pellicle cysts

Exponentially-growing cells from the above cultures were vortexed (Vortex-Genie 2, Scientific Industries) for 4 min and rinsed on a 20 μm screen with filtered seawater according to Smith et al. (2011). The minimum time needed to induce pellicle formation in the strains was used. The cells were confirmed to be intact, immobile, pellicle cysts by light microscopy, and no broken cells were seen. Three replicate preparations from each strain were used for toxin measurements. Cell numbers and sample volumes for all treatments are given in Table 1.

### 2.4. Gametes

Cultures for sampling of gametes of each strain were established by adding 84 mL of the BF-2 starting culture to 216 mL encystment medium (f/2 without N), and 44 mL of the 38-3 starting culture to 256 mL encystment medium in tall petri dishes (80 mm high, 100 mm diameter, thus containing 300 mL culture). Five replicates of each strain were kept separately to yield measurements from each specific strain. Earlier work in which swimming behavior was studied in detail (submitted manuscript and unpublished observations) had shown that the presence of a compatible strain is not necessary for gamete formation or gamete behavior to occur.

Many earlier detailed observations had repeatedly shown that gametes of the BF-2 and 38-3 strains assemble in lines and descending plumes after 2–4 days in encystment medium when kept in Erlenmeyer flasks, beakers, or tall petri dishes (and in spots, that are short descending plumes, when kept in shallow petri dishes). Gametes swam in circles and contacted other cells frequently, and were almost entirely single cells. Also, gametes did not display either positive or negative phototaxis; whereas, vegetative cells were strongly phototactic (personal observations, also phototactic behavior of vegetative cells is well known, e.g. MacIntyre et al., 1997). Gametes were harvested by picking cells densely assembled in patterns with a Pasteur pipette (looking through a magnifying glass) after 4 days in encystment medium. The cultures were kept on a white table in a constant light (24 h) at 20 °C. The light was 100 μmol photons $m^{-2}$ $s^{-1}$ from the side, which induced vegetative cells to separate from gametes by differences in phototactic behavior. Sample volume was noted, and subsamples from these were taken for microscope cell counts (Table 1).

**Table 1**
Summary of cell counts and sample volumes (±standard deviation).

| Life stage | Cells ($10^3$ sample$^{-1}$) | Replicates (no.) | Sample volume (mL) |
|---|---|---|---|
| 38-3 vegetative | 84 ± 11 | 3 | 5.0 ± 0.0 |
| BF-2 vegetative | 125 ± 5 | 3 | 10.0 ± 0.0 |
| 38-3 gamete | 48 ± 20 | 5 | 2.9 ± 0.8 |
| BF-2 gamete | 69 ± 15 | 5 | 3.2 ± 0.7 |
| 38-3 pellicle cyst | 138 ± 4 | 3 | 10.0 ± 0.0 |
| BF-2 pellicle cyst | 102 ± 5 | 3 | 10.0 ± 0.0 |
| Zygote | 79 ± 51 | 4 | Not measured, counts by cytometer |
| 38-3 stationary | 166 ± 75 | 5 | 5.0 ± 0.8 |
| BF-2 stationary | 242 ± 64 | 5 | 4.8 ± 0.4 |
| BF-2 vegetative* | 153 ± 48 | 5 | 5.6 ± 2.2 |
| Mixed stages | 244 ± 27 | 3 | 9.7 ± 0.7 |
| Senescent cells | 623 ± 40 | 5 | 13.5 ± 0.5 |

* Vegetative cells in gamete containers, separated from gametes by phototactic behavior.

### 2.5. Vegetative cells from gamete cultures

As described above for gametes, the vegetative cells separated from gametes in the containers under the conditions given (cultures kept in tall petri dishes containing 300 mL of culture in encystment medium on a white table in constant light, 100 μmol photons $m^{-2}$ $s^{-1}$ from the side, at 20 °C). For BF-2, enough vegetative cells assembled at the bottom of the dishes on the lightest side to enable separate sampling from these locations. Sampling was done after 4 days in encystment medium with a Pasteur pipette, as for gametes, from the five replicates (Table 1). For 38-3, the vegetative cells were more spread out; therefore, separate sampling of enough cells in the same containers as gametes was not possible.

### 2.6. Zygotes

Zygotes were produced in 1 L baking dishes and in 1 L cones according to the description in Smith and Persson (2004). Baking dishes were placed in a lighted bio-incubator at 20 °C, 300 μmol photons $m^{-2}$;$s^{-1}$, 14:10 L:D and cones at 20 °C, 100 μmol photons $m^{-2}$ $s^{-1}$, in a 24 h lighted culture room. Encystment medium (f/2 without N) was used, and the initial cell density was 375 cells $mL^{-1}$ of the BF-2 starting culture (30 mL) and 260 cells $mL^{-1}$ (15.5 mL) of the 38-3 starting culture, added together in 1 L of medium per dish or cone ($6.35 \times 10^3$ cells per container; preliminary cell counts by flow cytometry indicated equal numbers of each strain). There were three replicates of each experimental treatment. As clean samples of zygotes are difficult