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Quantity of the dinoflagellate *sxtA4* gene and cell density correlates with paralytic shellfish toxin production in *Alexandrium ostenfeldii* blooms

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ABSTRACT

Many marine dinoflagellates, including several species of the genus *Alexandrium*, *Gymnodinium catenatum*, and *Pyrodinium bahamense* are known for their capability to produce paralytic shellfish toxins (PST), which can cause severe, most often food-related poisoning. The recent discovery of the first PST biosynthesis genes has laid the foundation for the development of molecular detection methods for monitoring and study of PST-producing dinoflagellates. In this study, a probe-based qPCR method for the detection and quantification of the *sxtA4* gene present in *Alexandrium* spp. and *Gymnodinium catenatum* was designed. The focus was on *Alexandrium ostenfeldii*, a species which recurrently forms dense toxic blooms in areas within the Baltic Sea. A consistent, positive correlation between the presence of *sxtA4* and PST biosynthesis was observed, and the species was found to maintain PST production with an average of 6 genomic copies of *sxtA4*. In August 2014, *A. ostenfeldii* populations were studied for cell densities, PST production, as well as *sxtA4* and species-specific LSU copy numbers in Föglö, Åland, Finland, where an exceptionally dense bloom, consisting of 6.3×10^6 cells L^{-1}, was observed. Cell concentrations, and copy numbers of both of the target genes were positively correlated with total STX, GTX2, and GTX3 concentrations in the environment, the cell density predicting toxin concentrations with the best accuracy (Spearman's $\rho = 0.93$, $p < 0.01$). The results indicated that all *A. ostenfeldii* cells in the blooms harbored the genetic capability of PST production, making the detection of *sxtA4* a good indicator of toxicity.

1. Introduction

Marine dinoflagellates are known for their ability to produce numerous highly toxic compounds. The best-known are saxitoxins, a group of potent neurotoxins including saxitoxin itself and its closely related structural analogues. These small hydrophilic alkaloids are also known as paralytic shellfish toxins (PST) due to their tendency to accumulate in the food chain, mainly to filter-feeding bivalves which can then act as vectors of PST poisoning. In mammals the toxins act by reversibly blocking voltage-gated sodium channels (Catterall, 1980), thus inhibiting the transmission of neuronal signals. Symptoms are dose-dependent, and range from slight numbness and tingling of face and extremities to total neuromuscular paralysis, respiratory arrest and death (Llewellyn, 2006). The estimated mortality rate of PST poisoning can be as high as 15% (Hallegraeff, 2003), and treatment is limited to supportive care. Most developed countries with commercial shellfish farming have set monitoring programs in place to prevent food-related PST poisoning. Major economic losses are suffered due to the combined public health costs and forced harvesting closures of fish and mussel farms (Hoagland and Scatasta, 2006).

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Species of the dinoflagellate genera *Alexandrium*, *Gymnodinium* and *Pyrodinium* are known to be able to synthesize PSTs, genus *Alexandrium* harboring the majority of the producers known to date (Orr et al., 2011). The species *Alexandrium ostenfeldii*, including *Alexandrium peruvianum* (Kremp et al., 2014), contains nontoxic strains, PST producers (Hansen et al., 1992) and strains able to biosynthesize neurotoxic spirolides (Cembella et al., 2000) as well as gymnodimines (Van Wagoner et al., 2011). Some strains have been reported to produce two or more of these toxin types simultaneously (Hansen et al., 1992; MacKinnon et al., 2006; Van Wagoner et al., 2011; Tomas et al., 2012). The species is encountered worldwide in cold and temperate waters, but usually at a low cell density and as a background species together with other dinoflagellates (Gribble et al., 2005; John et al., 2003). Very high cell density blooms have been observed in the Netherlands (Burson et al., 2014), where cell concentrations reached 5.5×10^6 cells L^{-1}, and in the Baltic Sea, including the Åland Archipelago in Finland where recurrent blooms of *A. ostenfeldii* with cell densities of up to $1–2 \times 10^6$ cells L^{-1} have been recorded since the early 2000s (Kremp et al., 2009).

The genetic background of PST production in dinoflagellates remained elusive for a long time despite extensive research effort. The *sxt* gene cluster was first discovered in cyanobacteria (Kellmann et al., 2008), and 14 core genes necessary for toxin biosynthesis have been identified (Murray et al., 2011a). Paralytic shellfish toxin biosynthesis is initiated by a multi-domain enzyme encoded by the gene *sxtA*, the dinoflagellate counterpart of which has recently been discovered (Stüken et al., 2011). In cyanobacteria, *sxtA* is composed of four distinct domains A1–A4 (Kellmann et al., 2008), a structure mirrored in dinoflagellates. Interestingly, two types of mRNA transcripts in the latter have been identified; one composed on domains A1 to A3, and the other including all four (Stüken et al., 2011). Current research has shown the presence of the *sxtA4* domain to be crucial for PST biosynthesis in cyanobacteria, and to be the single best predicting factor for toxin production in dinoflagellates (Stüken et al., 2011; Murray et al., 2011b; Suikkanen et al., 2013; Orr et al., 2013). These discoveries have opened up new possibilities for the study of PST production in dinoflagellates, making way to development of nucleic acid based detection and quantification methods. These methods could be highly useful in the routine monitoring programs of PST-toxins mitigating the risks posed by the PST producing dinoflagellates to human welfare.

In this study a quantitative PCR method including primers and a probe to detect the *sxtA4* gene in potentially PST-producing dinoflagellates was developed, with the main focus on *Alexandrium ostenfeldii*. An additional method for the quantification of the large ribosomal subunit (LSU) encoding gene was established, and these methods were used to determine the number of genomic *sxtA4* and LSU copy numbers in *A. ostenfeldii* strains. Investigation of the relationship between intracellular PST concentrations, *A. ostenfeldii* cell abundance and target gene copy numbers in the environment was carried out on samples collected at Föglö, Åland Archipelago, Finland.

2. Materials and methods

2.1. Dinoflagellate cultures and environmental samples

The cultured dinoflagellate strains used in this study are listed in Table 1. While representatives of other species were obtained from culture collections, most *Alexandrium ostenfeldii* isolates were established by the authors from water or sediment samples collected in the Baltic Sea and North Atlantic. Detailed information on origin and characteristics of these strains are given in Tahvanainen et al. (2012) and Kremp et al. (2014). The identity of species belonging to the *Alexandrium tamarense* species complex was assigned according to John et al. (2014), except for *A. tamarense* SCCAP K-1471, for which rDNA data conclusively confirming its identity was not available. Generally, strains were grown in 40 mL batch cultures in vented 50 mL polycarbonate tissue culture flasks containing f/2-Si medium (Guillard and Ryther, 1962) adjusted to native salinities at 16 °C on a 12:12 light:dark cycle at 100 μmol photons m^{-2} s^{-1}. Environmental samples were collected in August 2014, at Föglö, Åland Archipelago, located in southwestern Finland, in the northern Baltic Proper. The study area in Föglö has been previously described in detail by Kremp et al. (2009). The sampling stations are located in a shallow, heavily vegetated sound, where the water depth ranges from 0.5 to 1.5 m. Three stations were included in the sampling: sites 3 and 4 as described by Kremp et al. (2009), and an additional station, Lillängö, located between stations 1 and 2 of that study in a coastal bay surrounded by dense reeds where discoloration of water suggested an exceptional density of *A. ostenfeldii*. Sampling was carried out once a day on three subsequent days; duplicate sea water samples were collected manually at the depth of 0–0.5 m for both DNA extraction and toxin analysis. At sites 3 and 4, 20 L samples were concentrated down to approximately 50 mL using a plankton net with a 25 μm mesh size. The concentrated water was further filtered onto 25 mm GF/C filters (Whatman, UK) using disposable 50 mL syringes and sterilizable polycarbonate filter holders (Sartorius Stedim Biotech, Germany). At Lillängö sampling station, due to the high *A. ostenfeldii* cell density, 0.2–1.5 L of unconcentrated water was filtered onto GF/C filters as described above. All filters were stored in a cooled container until moved to a freezer (−20 °C) a maximum of 5 h later. The samples were kept frozen until toxin extraction or preparation of cell lysates for qPCR for 2 and 2–8 months, respectively. Samples for identification and enumeration of dinoflagellate cells were fixed with Lugol's iodine solution on site and counted using sedimentation chambers (Utermöhl, 1958) in an inverted microscope (Zeiss PrimoVert) under 100× magnification.

2.2. DNA extraction and sample preparation for PCR analysis

Two types of templates were used in the PCR experiments: extracted genomic DNA and cell lysates. For gDNA extraction and purification cultured dinoflagellate cells were collected by centrifugation (10 min, $3220 \times g$ at 20 °C, Eppendorf 5810R, Hamburg, Germany). The cell pellets were resuspended in a small volume of supernatant and transferred to microcentrifuge tubes and re-centrifuged (10 min, $16 \times 000 \times g$, RT, Eppendorf 5415D). The cells were homogenized mechanically with a battery-operated motor pestle (Kontes Glass Company, NJ, USA) and genomic DNA was extracted using the Qiagen Plant Mini reagent kit (Qiagen, Germany) according to the instructions provided by the manufacturer, including the recommended centrifugation step after cell lysis. Elution was carried out in two stages to obtain maximum DNA yield (60 μL and 40 μL). The concentration and purity of the extracted DNA was measured spectrophotometrically (Nanodrop ND-1000, Thermo Scientific, A_{260}/A_{280} ranging between 1.5 and 2.7). All purified gDNA was stored at −20 °C until analysis.

Environmental samples retained on GF/C filters were processed essentially as described by Garneau et al. (2011). Briefly, the filters were suspended in 1.5 mL of lysis buffer (100 mM Tris, 40 mM EDTA, pH 8.0, 100 mM NaCl, 1% (v/v) sodium dodecyl sulphate) and 400 mg of 0.5 mm zirconia–silica beads (BioSpec Products, Bartlesville, OK, USA) were added to each tube. Samples were lysed by first vortexing the tubes at maximum speed for 1 min, followed by heating at 70 °C for 5 min. After a total of three vortexing–heating rounds the lysates were centrifuged (10 min, 16 $000 \times g$, RT, Eppendorf 5415D) as described above to remove