Harmful Algae 26 (2013) 52–59

Contents lists available at SciVerse ScienceDirect

Harmful Algae

journal homepage: www.elsevier.com/locate/hal

# Paralytic shellfish toxins or spirolides? The role of environmental and genetic factors in toxin production of the *Alexandrium ostenfeldii* complex

Sanna Suikkanen [a,*], Anke Kremp [a], Henna Hautala [b], Bernd Krock [c]

[a] Finnish Environment Institute, Marine Research Centre, FI-00251 Helsinki, Finland
[b] Division of Biotechnology, Department of Biochemistry and Food Chemistry, University of Turku, FI-20520 Turku, Finland
[c] Alfred Wegener Institute for Polar and Marine Research, D-27570 Bremerhaven, Germany

ARTICLE INFO

*Article history:*
Received 30 November 2012
Received in revised form 4 April 2013
Accepted 4 April 2013

*Keywords:*
*Alexandrium*
Dinoflagellate
Paralytic shellfish toxin
Spirolide
Saxitoxin gene
Toxin profile

ABSTRACT

Dinoflagellates of the *Alexandrium ostenfeldii* complex (*A. ostenfeldii*, *A. peruvianum*) are capable of producing different types of neurotoxins: paralytic shellfish toxins (PSTs), spirolides and gymnodimines, depending on the strain and its geographic origin. While Atlantic and Mediterranean strains have been reported to produce spirolides, strains originating from the brackish Baltic Sea produce PSTs. Some North Sea, USA and New Zealand strains contain both toxins. Causes for such intraspecific variability in toxin production are unknown. We investigated whether salinity affects toxin production and growth rate of 5 *A. ostenfeldii*/*peruvianum* strains with brackish water (Baltic Sea) or oceanic (NE Atlantic) origin. The strains were grown until stationary phase at 7 salinities (6–35), and their growth and toxin production was monitored. Presence of saxitoxin (STX) genes (*sxtA1* and *sxtA4* motifs) in each strain was also analyzed. Salinity significantly affected both growth rate and toxicity of the individual strains but did not change their major toxin profile. The two Baltic Sea strains exhibited growth at salinities 6–25 and consistently produced gonyautoxin (GTX) 2, GTX3 and STX. The two North Sea strains grew at salinities 20–35 and produced mainly 20-methyl spirolide G (20mG), whereas the strain originating from the northern coast of Ireland was able to grow at salinities 15–35, only producing 13-desmethyl spirolide C (13dmC). The effects of salinity on total cellular toxin concentration and distribution of toxin analogs were strain-specific. Both saxitoxin gene motifs were present in the Baltic Sea strains, whereas the 2 North Sea strains lacked *sxtA4*, and the Irish strain lacked both motifs. Thus *sxtA4* only seems to be specific for PST producing strains. The results show that toxin profiles of *A. ostenfeldii*/*peruvianum* strains are predetermined and the production of either spirolides or PSTs cannot be induced by salinity changes. However, changes in salinity may lead to changed growth rates, total cellular toxin concentrations as well as relative distribution of the different PST and spirolide analogs, thus affecting the actual toxicity of *A. ostenfeldii*/*peruvianum* populations.



## 1. Introduction

Toxic marine dinoflagellates of the *Alexandrium ostenfeldii* complex (Balech, 1995) are widely distributed in coastal waters around the globe, occurring in the northern Atlantic as well as the Pacific Ocean, the Mediterranean and the brackish Baltic Sea. The two morphospecies *A. ostenfeldii* (Paulsen) Balech and Tangen and *Alexandrium peruvianum* (Balech and Mendiola) Balech and Tangen belonging to the complex are defined by subtle differences in plate structure, which do not conform to phylogenetic relationships. Hence, species boundaries have remained unclear. Both are capable of producing different types of neurotoxins: paralytic shellfish toxins (PSTs), spirolides and/or gymnodimines. PSTs are a group of heat-stable, water-soluble alkaloids with close to 60 analogs, including saxitoxin, which is one of the most potent natural neurotoxins known (Wiese et al., 2010). In the aquatic environment, PSTs are mainly produced by marine eukaryotic dinoflagellates of the genera *Alexandrium, Gymnodinium* and *Pyrodinium*, and by at least 5 genera of freshwater prokaryotic cyanobacteria. Paralytic shellfish poisoning events with contaminated seafood and human intoxications are widespread and well-studied (Anderson et al., 2012). Saxitoxin synthesis genes have recently been characterized in cyanobacteria (Kellmann et al., 2008) and dinoflagellates (Stüken et al., 2011; Hackett et al., 2013).

Spirolides and gymnodimines are fast-acting macrocyclic polyether toxins produced by *Alexandrium ostenfeldii/peruvianum*

* Corresponding author. Tel.: +358 400 148866.
*E-mail address:* sanna.suikkanen@ymparisto.fi (S. Suikkanen).

1568-9883/$ – see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.hal.2013.04.001

(Cembella et al., 2000; Franco et al., 2006; Van Wagoner et al., 2011), gymnodimines also by *Karenia selliformis* A. J. Haywood, K. A. Steidinger and L. MacKenzie (Kharrat et al., 2008). Although there are no reports of negative human health effects related to spirolides or gymnodimines, they have been found to accumulate in shellfish along the North Atlantic, Mediterranean and New Zealand coasts (Aasen et al., 2005; Villar González et al., 2006; Kharrat et al., 2008; Medhioub et al., 2011; Pistocchi et al., 2012). Biosynthesis genes for these toxins have not yet been confirmed.

Individual strains of *Alexandrium ostenfeldii/peruvianum* usually produce either spirolides or PSTs. Causes for such variability in toxin profile are unknown, but salinity may play a role since toxin profiles of brackish water (salinity <30) strains clearly differ from those of strains isolated from oceanic salinities (>30). More specifically, *A. ostenfeldii/peruvianum* strains isolated from the northern Atlantic coast of Canada and USA (Cembella et al., 2000, 2001; Gribble et al., 2005), Ireland (Touzet et al., 2008) and the Mediterranean (Ciminiello et al., 2006; Franco et al., 2006) produce spirolides, whereas strains originating from Malaysia and the brackish water Baltic Sea produce PSTs (Lim et al., 2005; Kremp et al., 2009). Furthermore, strains originating from an intermediate salinity, i.e. the Danish Straits connecting the Baltic Sea to the North Sea, as well as a rivermouth in the eastern USA, contain both toxin types (Hansen et al., 1992; MacKinnon et al., 2004; Otero et al., 2010; Tomas et al., 2012). On the other hand, some *A. ostenfeldii* strains originating from oceanic salinities, i.e. from coastal New Zealand and Scotland, also produce both PSTs and spirolides (Mackenzie et al., 1996; Brown et al., 2010; Beuzenberg et al., 2012).

In past experimental studies, it has been proposed that PST composition is a stable characteristic, fixed genetically for each clonal strain of *Alexandrium* spp., but that significant shifts may occur under changes in growth regime, e.g. inorganic nutrient limitation, temperature, irradiance, salinity or depending on growth phase (Boczar et al., 1988; Anderson et al., 1990; Hwang and Lu, 2000; Etheridge and Roesler, 2005; Anderson et al., 2012). Previous studies on effects of environmental factors on *Alexandrium ostenfeldii/peruvianum* toxin content and profile have mostly concentrated on spirolides, and it has been found that total toxin present in batch cultures can be affected by e.g. light, the amount of spirolides increasing with cell concentration but cell spirolide quota and profile remaining constant despite environmental changes (John et al., 2001; Maclean et al., 2003). On the other hand, in other studies (Otero et al., 2010; Medhioub et al., 2011; Tatters et al., 2012), salinity, culture media, photoperiod and nutrient limitation have been found to affect cellular spirolide toxicity and toxin profile of *A. ostenfeldii/peruvianum*, and it was suggested that even the production of either spirolides or PSP toxins by *A. ostenfeldii* might be determined by salinity, temperature or nutrients (Otero et al., 2010).

In this study we investigated whether individual *Alexandrium ostenfeldii/peruvianum* strains are capable of producing both PSTs and spirolides, depending on salinity. We hypothesized that low salinity conditions will induce PSTs in oceanic spirolide producers and high salinities will lead to spirolide production in brackish water strains containing PSTs. To test this, batch culture experiments were performed with strains originating from the NE Atlantic and the Baltic Sea at salinities between 6 and 35, and growth rate, toxin production and composition was measured. To substantiate the genetic basis for PST production, strains were furthermore analyzed for presence of saxitoxin genes.

## 2. Material and methods

### 2.1. Batch culture experiments

Growth and toxin production of five *Alexandrium ostenfeldii* and *Alexandrium peruvianum* strains originating from the Baltic Sea and NE Atlantic (Table 1) were monitored in batch cultures set up along a gradient of seven salinities (6, 10, 15, 20, 25, 30 and 35). Growth media with the different salinities were prepared by adding artificial sea salt (Tropic Marin® Pro-Reef Sea Salt, Dr. Biener, Germany) to 0.45 μm filtered (Sartobran 300 sterile capsule filters, Sartorius Stedim Biotech, Germany), autoclaved Baltic Sea water (salinity 6), letting the salt dissolve for 2 h, and filtering the solution once more through 0.45 μm. Salinity of the media was measured using a conductivity meter (TetraCon 325, Cond 3210 SET 1, WTW, Germany). Finally, F/2 nutrients (excluding silicate) were added according to Guillard (1975). The Baltic Sea strains were initially grown in a salinity of 6, and the NE Atlantic strains in a salinity of 30, at 16 °C, in ca. 60 μmol photons $m^{-2}$ $s^{-1}$, and in a light:dark cycle of 12L:12D.

The strains were stepwise acclimated (max. five salinity units at a time) to the different salinities: ca. 1000 exponentially growing cells $mL^{-1}$ were inoculated to the next salinity and grown for ca. 4 weeks until exponential phase (>5000 cells $mL^{-1}$). Then triplicate experimental cultures were inoculated at 500 cells $mL^{-1}$ and their growth was monitored with fluorescence measurements (Varian Cary Eclipse fluorescence spectrophotometer, excitation 440 nm, emission 680 nm) every 2–3 days. The cultures were grown in 250 mL tissue culture flasks with an experimental volume of 200 mL, at 16 °C, 60 μmol photons $m^{-2}$ $s^{-1}$ and a 12:12 light:dark cycle. Samples for toxin analysis were taken both in exponential and stationary growth phases ($V = 2 \times 40$ mL for spirolide and PST analyses, respectively). In addition, samples (V = 1.2 mL) were taken and preserved with a drop of acid Lugol solution for cell counts and length measurements on every toxin sampling occasion. Cell number was counted and mean cell length ($n = 30$/treatment) was measured in Sedgewick-Rafter cells ($V = 1$ mL) with an inverted microscope (Leica DMI 300B). The duration of the experiments varied between 50 and 78 days, depending on the growth rate of each strain.

Growth rates, $k$, defined as doublings per day, were calculated based on the longest period of exponential growth, using the equation $k = \log_2(N_t/N_0)/\Delta t$, where $N$ = cell number and $t$ = time (Wood et al., 2005). The interval of exponential growth was determined from growth curves established for each experimental culture replicate.

For toxin analysis cells were concentrated by centrifugation, first 15 min at 4000 rpm (Heraeus Sepatech Megafuge 2.0), followed by 5 min at 10,000 × *g* (IEC MicroCL 21R, Thermo Electron Corporation), and subsequent removal of the supernatant.

**Table 1**
*Alexandrium ostenfeldii/peruvianum* strains used in the study.

| Strain code | Morphotype | Geographic origin | Native salinity | Toxins produced | Isolated by |
|---|---|---|---|---|---|
| AOF 0927 | *A. ostenfeldii* | Föglö, Åland (Baltic Sea) | 6 | GTX2/3, STX | A. Kremp |
| AOVA 30 | *A. ostenfeldii* | Gotland, Sweden (Baltic Sea) | 6 | GTX2/3, STX | A. Kremp |
| NCH 85 | *A. ostenfeldii* | Skagerrak (North Sea, NE Atlantic) | 30 | 20mG, 13dmC | T. Alpermann |
| S06/013/01 | *A. ostenfeldii* | E Scotland (North Sea, NE Atlantic) | 35 | 20mG, 13dmC (and STX, NEO; Brown et al., 2010) | L. Brown |
| LS A06 | *A. peruvianum* | N Ireland (Lough Swilly, NE Atlantic) | 30 | 13 dmC | N. Touzet |