Harmful Algae 49 (2015) 94–104

Contents lists available at ScienceDirect

Harmful Algae

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

Characterization of multiple isolates from an *Alexandrium ostenfeldii* bloom in The Netherlands

Dedmer B. Van de Waal [a,*], Urban Tillmann [b], Helge Martens [b], Bernd Krock [b], Yvonne van Scheppingen [c], Uwe John [b]

[a] Department of Aquatic Ecology, Netherlands Institute of Ecology, PO Box 50, 6700 AB Wageningen, The Netherlands
[b] Alfred Wegener Institute Helmholtz Centre for Polar and Marine Research, Am Handelshafen 12, 27570 Bremerhaven, Germany
[c] Waterschap Scheldestromen, PO Box 1000, 4330 ZW Middelburg, The Netherlands

ARTICLE INFO

Article history:
Received 23 December 2014
Received in revised form 21 August 2015
Accepted 21 August 2015
Available online 25 September 2015

Keywords:
Harmful algal blooms
PSP toxins
Spirolide
Gymnodimine
Lytic activity
Allelochemical potency

ABSTRACT

Alexandrium ostenfeldii is an emerging harmful algal bloom species forming a global threat to coastal marine ecosystems, with consequences for fisheries and shellfish production. The Oosterschelde estuary is a shallow, macrotidal and mesotrophic estuary in the southwest of The Netherlands with large stocks of mussels, oysters, and cockles. These shellfish stocks were threatened by a recent *A. ostenfeldii* bloom in the Ouwerkerkse Kreek, which is a brackish water creek discharging water into the Oosterschelde. Little is yet known about the characteristics of the *A. ostenfeldii* population in this creek. We therefore isolated 20 clones during an *A. ostenfeldii* bloom in 2013, and characterized these clones on their growth and toxin profile in their exponential growth phase. The cyclic imines were identified by comparison of *A. ostenfeldii* extracts with the retention time and CID spectra of standard solutions, or with published CID spectra. We furthermore assessed the allelochemical potency and phylogeny of a selection of 10–12 clones. Morphology and molecular phylogeny showed that all clones belong to Group 1 of *A. ostenfeldii*. All clones showed comparable growth rates of on average $0.22 \pm 0.03\ d^{-1}$. During exponential growth, they all produced a unique combination of paralytic shellfish poisoning toxins, spirolides and gymnodimines, of which particularly the latter showed a high intra-specific variability, with a 25-fold difference between clones with the lowest and highest cell quota. Furthermore, the selected 12 clones showed high allelopathic potencies with EC_{50} values based on lysis assays against the cryptophyte *Rhodomonas salina* between 212 and 525 *A. ostenfeldii* cells mL^{-1}. Lytic activities were lower for cell extracts, indicating an important extracellular role of these compounds. A high intra-specific variability may add to the success of genotypically diverse *A. ostenfeldii* blooms, and make populations resilient to changes in environmental and climatic conditions.

1. Introduction

Harmful algal blooms are a global threat to coastal marine ecosystems, with consequences for fisheries and shellfish production (Anderson et al., 2002; Heisler et al., 2008; Wang, 2008). *Alexandrium* is among the most common bloom forming toxic dinoflagellate genera and is generally held responsible for the outbreak of paralytic shellfish poisoning (PSP; Anderson et al., 2012). Besides PSP toxins, some *Alexandrium* species are known to produce other toxins including different spirolides (SPX) or gymnodimines (Cembella, 2003; Van Wagoner et al., 2011; Borkman et al., 2012; Kremp et al., 2014). These species belong to different phylogenetic groups of *Alexandrium ostenfeldii* as recently defined by Kremp et al. (2014), including *Alexandrium peruvianum*. Most *A. ostenfeldii* strains have been shown to produce SPX, some strains also produce detectable amounts of PSP toxins, while only a few strains were reported to combine PSP toxin, SPX and 12-methylgymnodimine production (Cembella, 2003; Borkman et al., 2012; Kremp et al., 2014).

PSP toxins are a group of neurotoxic compounds, including saxitoxin (STX), neosaxitoxin (NEO), gonyautoxins (GTX), and their N-sulfocarbamoyl variants the B- and C-toxins (Shimizu, 1996; Cembella, 1998). STX is highly toxic with an LD_{50} value (i.p. mice) of 8 $\mu g\ kg^{-1}$ body weight (Wiberg and Stephenson, 1960). The addition of a sulfate group at the C-11 position forms GTX, thereby reducing the toxicity by up to 40%. A further addition of a sulfonyl

* Corresponding author. Tel.: +31 317 473 553.
E-mail address: d.vandewaal@nioo.knaw.nl (D.B. Van de Waal).

http://dx.doi.org/10.1016/j.hal.2015.08.002
1568-9883/

group at the carbamoyl group forms C-toxins, which exhibit a 99% lower toxicity as compared to STX (Wiese et al., 2010). SPX and gymnodimines are fast acting highly toxic neurotoxins, with LD_{50} values (i.p. mice) down to 6.9 and 96 μg kg^{-1} body weight, respectively (Munday et al., 2004, 2012). Besides toxicity, various *Alexandrium* species were shown to have allelopathic potencies toward grazers and other phytoplankton species (Tillmann and John, 2002; Tillmann et al., 2007, 2008; John et al., 2015).

Alexandrium ostenfeldii is globally distributed in brackish and marine environments, and isolates have been characterized from locations worldwide (Kremp et al., 2014). *A. ostenfeldii* is historically seen as background bloomer (Cembella et al., 2000; John et al., 2003), however, dense *A. ostenfeldii* blooms have been reported recently in the Narragansett Bay and the New River Estuary in the U.S. east coast (Borkman et al., 2012; Tomas et al., 2012), the Baltic Sea coast of Finland (Hakanen et al., 2012), along the Adriatic coast of Italy (Ciminiello et al., 2006), and recently in a creek of the Oosterschelde estuary in the Southwest of The Netherlands (Burson et al., 2014). This Oosterschelde estuary is a shallow, macrotidal and mesotrophic estuary with large stocks of mussels, oysters, and cockles (Fig. 1A and B; Troost et al., 2010; van Broekhoven et al., 2014). Because of potential contamination of these shellfish with phytoplankton toxins, The Netherlands has a regular monitoring program for toxic compounds in shellfish, as well as for the occurrence of harmful algal species in the Oosterschelde estuary (van der Fels-Klerx et al., 2011). A recent dense *A. ostenfeldii* bloom in a creek discharging water into the Oosterschelde threatened the shellfish stocks, and was terminated by the addition of hydrogen peroxide (Burson et al., 2014). The bloom, however, recurred in 2013 and reached population densities of up to 4500 cells mL^{-1} (Fig. 1C). Little is yet known about the characteristics of the *A. ostenfeldii* population in the creek. We therefore isolated a number of *A. ostenfeldii* clones during the bloom in 2013, and characterized the bloom population in terms of growth, morphology, phylogeny, toxin composition, and lytic activity.

2. Material and methods

2.1. Field sampling

The Ouwerkerkse Kreek is a small brackish water creek in the province of Zeeland, Southwest of The Netherlands (Fig. 1A and B; See also Burson et al., 2014). The inlet of the creek is connected with ditches that drain the agricultural lands in the area, and water from the creek is regularly discharged into the Oosterschelde estuary via a pumping station. The phytoplankton population in the creek was monitored for *Alexandrium ostenfeldii*, and weekly samples were taken for cell counts during the bloom. An integrated water sample was taken of the upper 1 m of the water column at location 'a' (Fig. 1B), and a 1 L sub sample was fixed with Lugol's iodine solution (Lugol) to a final concentration of 1%. *A. ostenfeldii* cells were counted in a Sedgewick chamber on an inverted microscope (Olympus Vanox, Hamburg, Germany). Isolates of *A. ostenfeldii* cells were sampled at the onset of the bloom on 16 July 2013 from locations 'a' and 'b' in the creek (Fig. 1B).

2.2. Isolation

Cells of *Alexandrium ostenfeldii* were picked from small droplets in a petridish using a Pasteur pipette or a 10 μL mirco-pipette. Individual cells were cleaned five times in 2 μL droplets of sterile medium consisting of filtered and diluted North Sea water with a salinity of about 10, or in sterilized artificial brackish water medium with a salinity of about 10 (Appendix A). Both media contained nutrients corresponding to 50% of K-medium (Keller et al., 1987). *A. ostenfeldii* cells were subsequently grown in 100 μL medium mixed in a 1:1 ratio with sterile filtered water from the creek (0.2 μm membrane filter) in microplate wells. Clones OKNL1-10 were subsequently cultured in artificial brackish water medium (Appendix A), and clones ONNL11-22 in diluted North Sea water medium.

2.3. Culturing of clones

Twenty of the successfully isolated clones were grown in 250 mL Erlenmeyer flasks at 15 °C under an incident light intensity of 100 μmol photons m^{-2} s^{-1} at a light-dark cycle of 16:8. After acclimation to these growth conditions (i.e. >5 generations), cells were transferred and growth was monitored by cell counts performed every second day. At mid-exponential phase (~6000 cells mL^{-1}) cultures were harvested for analysis of toxins and for cell size measurements. Cultures were counted again two days later, confirming that cell sampling two days before was still during the exponential phase. For toxin sampling, 15 mL samples were taken for extraction of SPX and gymnodimines, and 50 mL samples each were taken for PSP toxin and DNA extraction. Samples were centrifuged at 6800 × *g* for 15 min (SL16, Thermo Scientific, Waltham, USA) and after removal of the supernatant the pellets were stored at −20 °C.

2.4. Cell counts and measurements

Cell densities of *Alexandrium ostenfeldii* cultures were determined by using sedimentation chambers for settling 0.2–1 mL of culture suspension, and subareas with at least 400 cells were counted with an inverted microscope (20X, Zeiss Axiovert 40C). Observation and documentation of live and fixed cells was carried

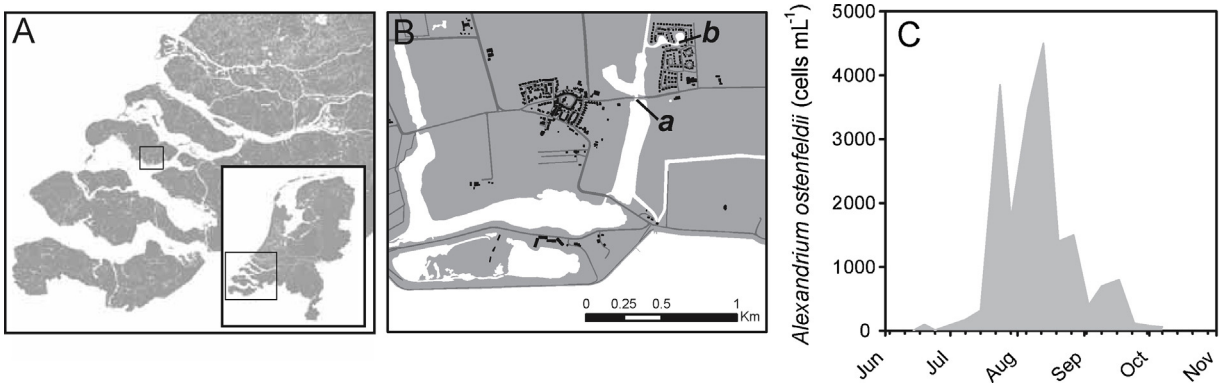

Fig. 1. Overview of the sampling location and the bloom dynamics of *Alexandrium ostenfeldii* in Rhine-Muesse-Scheldt delta, Southwest of The Netherlands (A). With the specific sampling locations 'a' and 'b' in the Ouwerkerkse Kreek (B), and the *A. ostenfeldii* population densities during the 2013 bloom sampled from location 'a' (C).