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Distributions of three *Alexandrium* species and their toxins across a salinity gradient suggest an increasing impact of GDA producing *A. pseudogonyaulax* in shallow brackish waters of Northern Europe

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ABSTRACT

Blooms of *Alexandrium* spp. are a well-known phenomenon in Northern European waters. While *A. tamarense/catenella,* and *A. pseudogonyaulax* have been reported from marine waters, high densities of *A. ostenfeldii* are mainly observed at lower salinities in North Sea estuaries and the Baltic Sea, suggesting salinity as a driver of *Alexandrium* species composition and toxin distribution. To investigate this relationship, an oceanographic expedition through a natural salinity gradient was conducted in June 2016 along the coasts of Denmark. Besides hydrographic data, phytoplankton and sediment samples were collected for analyses of *Alexandrium* spp. cell and cyst abundances, for toxin measurement and cell isolation. Plankton data revealed the predominance of *A. pseudogonyaulax* at all transect stations while *A. ostenfeldii* and *A. catenella* generally contributed a minor fraction to the *Alexandrium* community. High abundances of *A. pseudogonyaulax* in the shallow enclosed Limfjord were accompanied by high amounts of goniodomin A (GDA). This toxin was also detected at low abundances along with *A. pseudogonyaulax* in the North Sea and the Kattegat. Genetic and morphological characterization of established strains showed high similarity of the Northern European population to distant geographic populations. Despite low cell abundances of *A. ostenfeldii*, different profiles of cycloimines were measured in the North Sea and in the Limfjord. This field survey revealed that salinity alone does not determine *Alexandrium* species and toxin distribution, but emphasizes the importance of habitat conditions such as proximity to seed banks, shelter, and high nutrient concentrations. The results show that *A. pseudogonyaulax* has become a prominent member of the *Alexandrium* spp. community over the past decade in the study area. Analyses of long term monitoring data from the Limfjord confirmed a recent shift to *A. pseudogonyaulax* dominance. Cyst and toxin records of the species in Kiel Bight suggest a spreading potential into the brackish Baltic Sea, which might lead to an expansion of blooms under future climate conditions.

1. Introduction

Species of the genus *Alexandrium* are prominent toxin producers and widely distributed in Northern European waters (Moestrup and Hansen, 1988; Touzet et al., 2008; Brown et al., 2010; Hakanen et al., 2012). Their various potent toxins accumulate in fish and shellfish (Anderson et al., 2012) and are therefore of major concern to the aquaculture industry in the region. Toxicities caused by blooms of *Alexandrium* spp. have been reported from different coasts of Northern Europe since the 1970s (Ayres, 1975, Tangen, 1983; Bresnan et al., 2008). Due to their impact on the shellfish industry, toxic *Alexandrium* species have been monitored extensively in marine areas. However, occurrences are

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increasingly reported also from brackish waters of coastal inlets and the Baltic Sea (Kremp et al., 2009; van de Waal et al., 2015). The *Alexandrium* community in the area mainly consists of cold-water adapted *A. tamarense sensu* John et al. (2014) or *A. catenella* sensu Fraga et al. (2015) respectively, co-occurring with *A. ostenfeldii*, and forming blooms during spring (Moestrup and Hansen, 1988; John et al., 2003). In recent years, *A. pseudogonyaulax* and warm-water populations of *A. ostenfeldii* have increasingly been encountered in summer phytoplankton (Dittami et al., 2013; Hakanen et al., 2012; Wasmund et al., 2015).

Alexandrium catenella and *A. tamarense* represent the former *A. tamarense/fundyense/catenella* complex in northern European waters. *A. tamarense* is non-toxic (Higman et al., 2001; Collins et al., 2009), and paralytic shellfish toxicities in the North Sea area thus seem to be caused by *A. catenella* (Brown et al., 2010; John et al., 2014). Both, *A. tamarense* and *A. catenella* mostly occur at higher latitudes and are generally associated with lower water temperatures, compared to the other species of the complex. Nevertheless, different responses of the two co-occurring species to temperature changes may influence the distribution and relative contribution of each species in different locations (Eckford-Soper et al., 2016).

Alexandrium ostenfeldii, a major producer of neurotoxic cycloimines (Cembella et al., 2000) has been encountered together with *A. tamarense/fundyense/catenella* in spring phytoplankton communities of the Northern European waters (Moestrup and Hansen, 1988; Touzet et al., 2008). Bloom formation of the species was not reported in the area until a decade ago when warm water populations started to expand in the Baltic Sea (Kremp et al., 2009) and the coasts of The Netherlands (van de Waal et al., 2015). These populations have the demonstrated ability to produce both, cycloimines and paralytic shellfish toxins (PST) (van de Waal et al., 2015; Harju et al., 2016). PST production was also reported for a brackish water adapted strain from estuarine Limfjord (Hansen et al., 1992) suggesting a role of salinity for PST production in *A. ostenfeldii*. Experimental studies, however, have not substantiated this hypothesis so far (Suikkanen et al., 2013a).

Alexandrium pseudogonyaulax is a large dinoflagellate with a global distribution, originally described from a Mediterranean Lagoon (Biecheler, 1952). In Europe, this species is commonly reported from the Mediterranean Sea (Montresor, 1995; Bravo et al., 2006; Zmerli Triki et al., 2014), but records from northern Europe also exist (Balech, 1995; Nehring, 1997). The species prefers coastal “interior” habitats (Balech, 1995) and can be associated with brackish salinities. New occurrences and high biomasses of the species are being increasingly reported from northern temperate waters (Klein et al., 2010; Dittami et al., 2013; Wasmund et al., 2015, 2017). *A. pseudogonyaulax* has been shown to produce goniodomin A (GDA) (Murakami et al., 1988; Zmerli Triki et al., 2016), a macrolide polyether with toxic properties (Hsia et al., 2006). In experiments, the species affected higher trophic levels and food web transfer efficiency negatively (Blanda et al., 2016), indicating a potential effect on ecosystem function.

The recent observations of high *A. ostenfeldii* and *A. pseudogonyaulax* abundances in Northern Europe at brackish salinities and high water temperature suggest that the composition of *Alexandrium* species may change with climatic conditions in the area. Consequences of climate change include the freshening of coastal surface waters due to increased river run-off and higher sea surface temperatures (SST). As shown by long term monitoring data, such changes of SST affect the phytoplankton community and enhance toxic algal blooms in both, the North Sea and the Baltic Sea (Hinder et al., 2012; Suikkanen et al., 2013b). For *Alexandrium,* experimental data indicate that locally and seasonally adapted populations could respond sensitively to the respective changes of environmental conditions (Brown et al., 2010; Østergaard-Jensen and Moestrup, 1997). As a variety of toxins are produced by the *Alexandrium* community (Anderson et al., 2012) changes in *Alexandrium* species composition are expected to affect toxicities at the coasts of Northern Europe.

In this study, we use a natural gradient of different salinity and temperature regimes to investigate the effects of climate-sensitive and stable habitat conditions on three *Alexandrium* species and the composition of their respective toxins in Northern European waters. A transect survey was conducted in June 2016 along the coast of Denmark starting in marine conditions (Salinity 34) of the German Bight (Stations 1–6), crossing through shallow brackish and temperature influenced Limfjord (Stations 14–28) to the Kattegat (Stations 29–38) and Great Belt (Stations 39–41) and ending in Kiel Bight, Baltic Sea (Stations 42–44, Salinity 14). Also, time series of *Alexandrium* spp. monitoring data were analysed from the Limfjord, where the temperature has increased significantly over the past decade (Hansen, 2018), to substantiate climate-related changes in *Alexandrium* species and toxin distribution.

2. Material and methods

2.1. Field sampling

2.1.1. Hydrographic parameters

An oceanographic expedition was performed along the Danish coasts and the Limfjord in June 2016 on R/V Uthörn (Fig. 1) starting from Bremerhaven Germany, continuing along the Danish west coast to the entrance of the Limfjord. Several parts of the Limfjord were sampled during daily excursions from Løgstør. After that, the expedition continued from Løgstør to the Kattegat, Great Belt and Kiel Bight. A total of 44 stations were sampled (Fig. 1). At each station, CTD profiles were conducted using a Seabird 'sbe19plus V2' CTD (Sea-Bird Electronics Inc., Seattle, WA, USA) attached to a sampling rosette (6 × 4 L Niskin bottles). The CTD was equipped with an additional fluorescence sensor (SCUFA Fluorometer, Turner Design, USA). Data acquisition was carried out via CTD-client onboard (Seasave V 7.22.2); post-processing was done with SBE data processing 7.22.5. Temperature was corrected to ITS-90 (Preston-Thomas 1990). CTD data are available at Pangaea database (Krock et al., 2017). Water samples were collected from specific depths during the upcast of the instrument, relating to the chlorophyll–*a* (chl-*a*) fluorescence profiles during the downcast and the deep chl-*a* maxima (DCM).

Water samples were taken simultaneously with the CTD at discrete depths, depending on the chl-*a* maxima seen from the downcast. Chlorophyll–*a* concentrations were determined from sampled water (500–2000 mL) filtered onto Whatman GF/F filters (nominal pore size of 0.7 µm) and analysed using a fluorometer (Arar and Collins, 1997). Chlorophyll-*a* concentrations were used to calibrate the fluorescence values of the SCUFA fluorometer on the CTD.

2.1.2. Plankton sampling

At each station one or two vertical net tows were taken from the water column using a 20 µm phytoplankton net (438-030, Hydro-Bios, Kiel, Germany). The depth of the hauls corresponded to the total water depth. Net tow concentrates were collected into plastic containers and diluted to a defined volume (usually 1 L) with filtered seawater. 50 mL aliquots were then taken for cell isolations, and 18 mL aliquots were fixed with paraformaldehyde (1% final concentration) for microscopic analyses of phytoplankton. The remaining net tow volume was size-fractionated over a filter array to collect the > 200, > 50 and > 20 µm fractions. Plankton of each size fraction was rinsed with filtered seawater into 50 mL centrifugation tubes and adjusted to 45 mL. Of each size fraction three 15 mL aliquots for determination of hydrophilic (1) and lipophilic phycotoxins (2) and for DNA analysis (3) were transferred into 15 mL centrifuge tubes and centrifuged for 15 min at 3220 x g and 4 °C. After removal of supernatants DNA samples were frozen and stored at −20 °C until analysis. Cell pellets for analysis of hydrophilic and lipophilic phycotoxins were re-suspended in 1 mL 0.03 M acetic acid or methanol, respectively, and transferred to FastPrep tubes containing 0.9 g lysing matrix D (Thermo-Savant, Illkirch, France). Samples

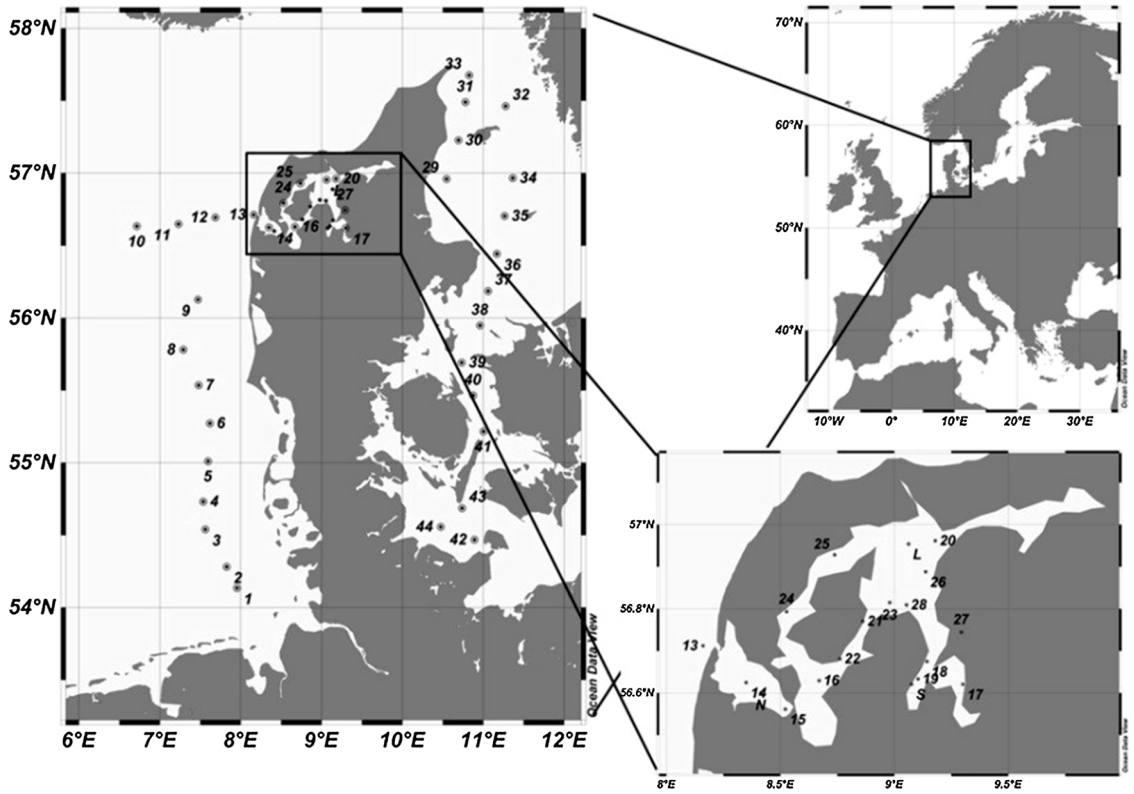

Fig. 1. Study area and distribution of stations sampled during the cruise in Danish coastal waters. Stations 1–13 represent the North Sea transect, Stations 14–28 were located in Limfjord, and Stations 29–44 in the Kattegat-Belt Sea area of the Baltic Sea. Capital letters denote stations of the long term monitoring program in Nissum Bredning (N), Løgstør Bredning (L), and Skive Fjord (S).

were homogenized by reciprocal shaking for 45 s at maximal speed (6.5 m s^{-1}) in a FastPrep instrument (Thermo-Savant, Illkirch, France) and subsequently centrifuged for 15 min (10 °C, 16,100 x g). Supernatants were transferred into spin filters (Ultra-free, Millipore, Eschborn, Germany) and filtered by centrifugation for 30 s at 10 °C and 5000 x g. Filtrates were transferred to HPLC vials and stored at −20 °C until analysis.

2.1.3. Sediment samples

In addition to water samples, sediment was collected for the determination of *Alexandrium* spp. resting cyst concentrations along the transect using an Ekman grab sampler. From intact pieces of surface sediment, quantitative subsamples of the uppermost 0–3 cm were collected using open plastic syringes and transferred into 50 mL centrifuge tubes. These were wrapped in aluminium foil and stored in the cold (+4 °C) and dark until further processing. Enrichment and isolation of the dinoflagellate cyst fraction before counting followed the protocol of Bolch (1997) and included sonication, cleaning and size fractionation of 3 mL sediment aliquots to capture the 20 to 100 µm fraction of the sediment which contains *Alexandrium* spp. cysts. Density gradient centrifugation was performed to concentrate and separate the organic cyst fraction from other sediment particles. The resulting material was resuspended in filtered seawater and adjusted to a defined volume for quantification of cysts.

2.2. Toxin measurements

Samples were tested for paralytic shellfish toxins (PST) by ion pair chromatography, post-column derivatization and fluorescence detection as previously described (Krock et al., 2007). As positive controls external standards of C1/2, GTX1/4, GTX2/3, B1, NEO and STX (Certified Reference Material programme of IMB-NRC, Halifax, NS, Canada) were run.

Lipophilic toxins were determined as described by Krock et al. (2008) with addition of for transitions for goniodomins: *m/z* 786.5 → 733.5 for GDA and *m/z* 722.5 → 719.5 for GDB, respectively. Calibration solutions for GDA (10, 50, 100, 500 and 1000 pg μL^{1}) were prepared and used to generate an external calibration curve. External calibration was used to quantify GDA/B concentration.

Cycloimines including spirolides and gymnodimines were analysed as described in Martens et al. (2017).

2.3. Determination of Alexandrium spp. cell and cyst abundances

2.3.1. Microscopy

From selected stations, 1 mL of paraformaldehyde fixed net-tow samples were stained with a drop of Calcofluor-white, settled in a small sediment chamber, and analysed at 200–640 x magnification with an Axiovert 200 (Zeiss) epifluorescence inverted microscope and UV excitation. Cells of *Alexandrium* spp. were identified and counted based on cell shape and plate details. Whenever the ventral area of an *Alexandrium* cell with the first apical plate and the ventral pore was visible, cells were identified at the species level.

2.3.2. qPCR assays

Plankton net tow samples were analysed for the presence and quantity of *A. catenella*, *A. ostenfeldii* and *A. pseudogonyaulax* 28S rDNA genes. The > 200 µM size fraction was excluded from analysis. Samples were prepared essentially as described by Garneau et al. (2011). *Alexandrium pseudogonyaulax* -specific primers (5′-CTTGGTAAGATTGCT GCG-3′, 5′-CACCCGCAAGCATTTCAC-3′) were designed for this study, *A. ostenfeldii*-specific (5′-TTGCGTCCACTTGTGGG-3′, 5′- GCAAACACA TGCATTCCAAT-3′) and *A. catenella*-specific (5′-GTGTGTGTCAGGGCT TGT-3′, 5′- TGTGTCTGGTGTATCTGTTTTTGT-3′) primers were modified from Savela et al. (2016) and Toebe et al. (2013), respectively. Assay design was carried out following the “Minimum Information for

Publication of Quantitative Real-Time PCR Experiments" guidelines (Bustin et al., 2009). Standards for each assay were prepared, purified and quantified as described previously by Savela et al. (2016), using the PCR reaction and thermal cycling conditions described below for each assay. Standards were produced from the following strains: X-LF-17-D12, X-LF-12-F6, X-LF-19-E10 for *A. pseudogonyaulax*, *A. catenella* and *A. ostenfeldii* assays, respectively. All analyses were performed in triplicate on a Bio-Rad CXF96 real-time PCR instrument. Prior to analysis, sample lysates were diluted 1:100 in molecular biology grade water to avoid PCR inhibition from detergents in the lysis buffer and any inhibitory compounds in the samples. Reactions contained 1X SsoAdvanced Universal SYBR Green Supermix (Bio-Rad), either 0.3 μM (*A. pseudogonyaulax*) or 0.5 μM (*A. catenella*, *A. ostenfeldii*) of forward and reverse primers (Integrated DNA Technologies) and 2 μL of 1:100 diluted sample lysate in a 10 μL volume. Thermal cycling was carried out as follows: 3 min at 98 °C, 40 cycles of 15 s. at 98 °C and 60 s. at either 56 °C (*A. catenella*, *A. pseudogonyaulax*) or 60 °C (*A. ostenfeldii*) with data acquisition after each cycle, followed by the generation of a melting profile by incrementally (0.5 °C) increasing the temperature from 65 °C to 95 °C. Data analysis, including automatic C_q determination, was carried out using CFX Manager 3.2 software (Bio-Rad). Results from reactions in which the C_q deviated for more than one cycle from their respective replicates were excluded from analysis. Further details, including assay properties and primer specificity are described in Supplementary data.

2.3.3. Quantification of cysts from processed sediment samples

Concentrations of *Alexandrium* resting cysts were analyzed using a Leica DMI3000 B inverted microscope. Three mL of the prepared cyst suspension was placed into an Utermöhl chamber and allowed to settle. The entire chamber was then screened at 200 x magnification, and cysts of *A. catenella*, *A. ostenfeldii* and *A. pseudogonyaulax* were counted. While the first species can be recognized with confidence based on the typical oval-shaped appearance, size and colour were used according to the criteria of Bravo et al. (2006) and Kremp et al. (2009) to distinguish between the round and morphologically similar cysts of *A. ostenfeldii* and *A. pseudogonyaulax* Cysts of *A. ostenfeldii* are typically 35–40 μm in size and have dark pigmentation while the size of *A. pseudogonyaulax* cysts is > 40 μm and cysts are lightly pigmented.

2.4. Statistical analyses of field data

The non-parametric Spearman's rank correlation was used to examine correlations between the cell numbers of *Alexandrium* spp., 28S rDNA copy numbers of *A. pseudogonyaulax*, cyst numbers of *A. ostenfeldii*, *A. pseudogonyaulax* and *A. catenella*, concentrations of GDA and spirolides, as well as total station depth, mean salinity and temperature. Salinity and temperature were averaged over the same depth as the net tows used to take plankton and toxin samples at each station. A non-parametric method was used because the data were not normally distributed. The analysis was performed using the R software (R Core Team 2016).

2.5. Characterization of Alexandrium spp strains

2.5.1. Isolation

At 4 stations in the North Sea and Limfjord (Table 3) a number of single *Alexandrium* spp. cells were isolated from live net tow samples for morphological, molecular and toxigenic characterization as described in Tillmann et al. (2014).

2.5.2. Species confirmation of strains by PCR

To confirm strain identity and for 28S rDNA copy number estimation, genomic DNA was extracted from a known number of cells of strains listed in Table 3 with a DNeasy Plant Mini Kit (Qiagen) according to the instructions supplied by the manufacturer.. Cell lysis was carried out using a battery-operated motorized pestle (Kontes Glass Company). DNA yield and quality were measured spectrophotometrically (NanoDrop ND-1000) and samples were stored at −70 °C until further analysis. Amplification of the 28S rDNA gene (D1-D3) was performed in reactions containing 0.5 μM D1R and D3B primers (Scholin et al., 1994), manufactured by biomers.net), 0.2 mM dNTPs (Bioline), 1X Phire HotStart buffer and 0.5 μL Phire HotStart DNA polymerase (Thermo Scientific) and 1 ng of template DNA in a total volume of 25 μL. Thermal cycling was carried out on a C1000 instrument (Bio-Rad) as follows: 98 °C 60 s. followed by 30 cycles of 98 °C 10 s, 50 °C 60 s. and 72 °C 60 s. with a final extension of 72 °C for 5 min. Amplification was confirmed on a 1.5% w/v agarose gel stained with 1X SYBR Safe dye (Thermo Scientific). The PCR products were subsequently excised and purified using a QIAquick Gel Extraction kit (Qiagen) following the manufacturer's protocol. All samples were submitted to Macrogen Inc. for sequencing. The resulting sequences were quality checked, edited to remove primers and aligned using UGENE 1.27.0 (Okonechnikov et al., 2012). A BLAST search (Altschul et al., 1997) was carried out to identify the obtained partial 28S rDNA gene sequences. Sequences for the strains listed in Table 3 have been submitted to GenBank under accession numbers MK441695-MK441705. Genomic 28S rDNA gene copy numbers were estimated by analysing one ng of gDNA from each isolate (with the exception of X-LF-12-C1) using the qPCR methods described above. Before analysis, DNA concentrations were determined using a QuantIt PicoGreen dsDNA kit (Thermo Fisher Scientific) according to manufacturer instructions.

2.5.3. Toxin profiles

Clonal isolates were grown in 200 mL Erlenmeyer flasks under standard culture conditions using K-medium (Keller et al., 1987), prepared from sterile-filtered natural seawater, at 15 °C, a photon flux density of 50 μmol m^{-2} s^{-1}, and a 16:8 h light:dark photocycle. Salinity was 34 for North Sea strains and adjusted to 15 for strains originating from the Limfjord. and were harvested by centrifugation (Eppendorf 5810R, Hamburg, Germany) at 3220 x g for 10 min. Cell pellets were extracted with 500 μL methanol by reciprocal shaking at 6.5 m/s with 0.9 g lysing matrix D (Thermo Savant, Illkirch, France) in a Bio101 FastPrep instrument (Thermo Savant, Illkirch, France) for 45 s. Extracts were then centrifuged (Eppendorf 5415 R, Hamburg, Germany) at 16,100 × g at 4 °C for 15 min. Each supernatant was transferred to a 0.45-mm pore-size spin-filter (Millipore Ultrafree, Eschborn, Germany) and centrifuged for 30 s at 800 × g, and the resulting filtrate being transferred into an autosampler vial for analysis as described above for field samples.

2.6. Analysis of long-term Alexandrium spp. Monitoring data from Limfjorden

Phytoplankton data for three stations situated in separate Limfjord basins (Nissum Bredning,N, Løgstør Bredning, L, and Skive Fjord, S Table S3) were extracted from the database for the Danish National Aquatic Monitoring and Assessment Program (DNAMAP), which is maintained by the Institute of Bioscience, Aarhus University, Denmark. Samples from the three stations had been analyzed for phytoplankton community composition, abundance and cell volume following the guidelines of Kaas and Markager (1998) since the late nineteen eighties. Details on the phytoplankton part of DNAMAP can be found in (Jakobsen et al., 2015). Generally, the monitoring stations have been visited one to four times per month since 1987. However, not all stations were continuously monitored over the entire period and the station in Nissum Bredning was discontinued in 2009.

For analyses of the DNAMAP phytoplankton data, cell concentrations were log transformed, because data were not normally distributed. After the annual and monthly means were estimated, data were back-transformed. Annual summer means (May through September), as well as monthly means, were established for the period

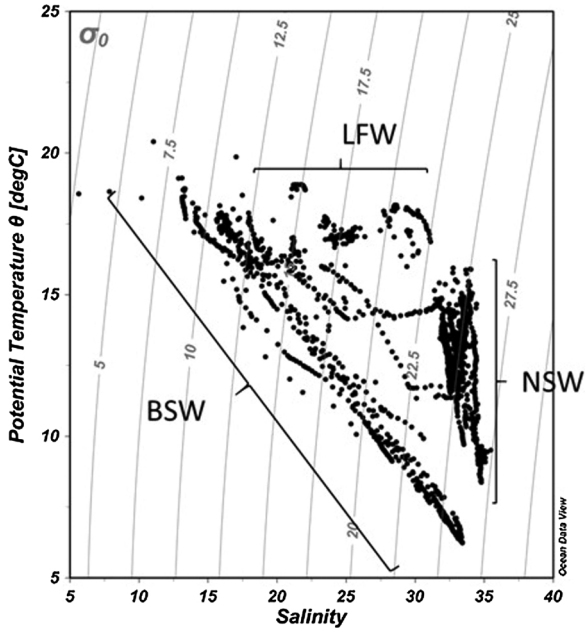

Fig. 2. Temperature and salinity conditions in the study area in June 2016, showing spatial separation of the three major sampled water masses: NSW = North Sea water; LFW = Limfjord water; BSW = Baltic Sea water.

from 1987 to 2015 for (1) all *Alexandrium* spp. observed excluding *A. pseudogonyaulax* and (2) for *A. pseudogonyaulax* only.

3. Results

3.1. Hydrographic conditions

The studied transect represented three distinct water masses, which could be identified based on salinity, temperature and density data (Fig. 2): The North Sea Water (NSW, St. 1–13) was characterized by high salinities and density, and low temperature (Fig. 2, Table 1). The Baltic Sea Water (BSW, St. 29–44) had relatively low average salinity, density and temperature despite strong gradients and wide ranges. In the Limfjord waters (LFW, St. 14–28) density and salinity were similar to the BSW, but the mean temperature was notably higher than in the two other water masses.

Depth profiles of hydrographic parameters, however, revealed more complex conditions (Fig. 3): Salinity and temperature data showed that both NSW and BSW were characterized by a strong stratification regime. In the Baltic Sea, a steep halocline at approximately 10 m depth separated saline and cold deep water from warm and brackish surface water. The stratification regime of NSW was dominated by temperature. The shallow Limfjord, in contrast, had a well-mixed water column.

Surface salinity decreased continuously from marine conditions in the North Sea (mean 33.3) to brackish salinities (mean 24) in the Baltic Sea where surface salinities dropped steeply across the Belt Sea from > 30 at the entrance of the Kattegat down to a minimum of 5.6 in Kiel Bight (Table 1, Fig. 3). Salinity in the Limfjord (mean 26.1) was influenced by NSW entering the system through the narrow western boundary, and riverine freshwater discharging into the fjord. Temperatures in the shallow Limfjord and the stratified Baltic surface waters were several degrees higher than in the North Sea (Fig. 3, Table 1). High primary production as represented by Chl-a fluorescence was observed in the Limfjord (Fig. 3).

3.2. Distribution of Alexandrium spp. cells and cysts

3.2.1. Cell abundances of Alexandrium spp.

Microscopic analyses showed that *Alexandrium* spp. were present at 28 of the 34 analysed transect stations (Fig. 4A). Morphological analyses of calcofluor stained cells identified *A. pseudogonyaulax*, *A. ostenfeldii* and *A. catenella* in the samples, the latter, however, was only detected at two North Sea stations. The majority of encountered *Alexandrium* spp. cells were *A. pseudogonyaulax*. Generally, cell abundances were 10–100 times lower at the North Sea and Baltic Sea stations compared to the Limfjord, where maximum abundances of 100×10^3 - 200×10^3 cells $NT^{-1}\,m^{-1}$ were measured. These abundances correspond to approximately 1–1.5 $\times 10^3$ cells L^{-1} across the entire water column. Highest abundances occurred at St. 25 in the central Limfjord and at St. 17–19 and 27 in the shallow brackish Skive Fjord basin.

Dominance of *A. pseudogonyaulax* was confirmed by qPCR results (Supplementary Table S4): copy numbers of *A. pseudogonyaulax* 28S rDNA correlated significantly with *Alexandrium* spp. cell counts. Molecular identification revealed presence of *A. ostenfeldii* at stations 17, 18 and 20 in the central Limfjord and in the Skive Fjord basin (Table S4). However, 28S rDNA copy numbers remained below reliable quantification levels. *A. catenella* was identified from 10 of the 44 analyzed stations by qPCR. These were mostly located in the North Sea and the western parts of the Limfjord. Quantifiable, but low amounts, compared to *A. pseudogonyaulax*, occurred at St. 11 and 12 in the North Sea. At all other positive stations, only traces were detected. The high abundance of *A. pseudogonyaulax,* quantified by 28S rDNA copy numbers, correlated with low depth, low salinity and high temperature (Spearman's rank correlation, $p < 0.02$ for all) as shown in Table 2.

3.2.2. Distribution of resting cysts

Resting cysts of *A. pseudogonyaulax, A. ostenfeldii* and *A. catenella* were detected in all studied sea areas (Fig. 4B–D). Their distribution was patchy in the North Sea and restricted to the Kattegat area in the Baltic Sea (Stations 29–39) except *A. ostenfeldii*, which was also prevalent in Kiel Bight (Station 42) sediments. Similar to cell distribution in plankton samples, cysts occurred more frequently in the Limfjord sediments and generally had higher concentrations here compared to the North Sea and Baltic Sea. Like cell concentrations, highest cyst concentrations were found in Skive Fjord (S). In contrast to cell abundances, cyst numbers of *A. pseudogonyaulax* and *A. ostenfeldii* were in the same range. *A. catenella* cysts were less abundant in the Limfjord than cysts of the two other *Alexandrium* species. In the Baltic Sea, *A. catenella* cysts were only seen at St. 31, one of the northernmost marine stations sampled in the Kattegat. Generally, in stations where cysts accumulated, cyst concentrations of all three species were high (Fig. 4). For *A. ostenfeldii* and *A. pseudogonyaulax* cysts, a negative correlation was found between depth and cyst concentrations (Table 2, $p < 0.05$).

Table 1
Mean and standard deviation of major oceanographic variables depth, salinity, temperature and density characterizing the different water masses studied during the survey: North Sea, Limfjord and Baltic Sea. Values in brackets represent measured ranges.

	n Stations	depth	Salinity	Temperature	Density
North Sea	13	27.35 ± 7.20	33.3 ± 0.9 (30.2 ; 35.5)	12.48 ± 1.79 (8.38 ; 16.54)	25.13 ± 0.90 (21.94 ; 27.41)
Limfjord	15	9.11 ± 2.6	26.07 ± 2.47 (17.71 ; 32.71)	17.38 ± 0.56 (14.20 ; 18.89)	18.59 ± 1.87 (12.47 ; 24.18)
Baltic Sea	16	26.10 ± 13.4	24.49 ± 7.45 (5.53 ; 33.68)	13.47 ± 3.93 (6.24 ; 20.41)	18.13 ± 6.33 (2.78 ; 26.27)

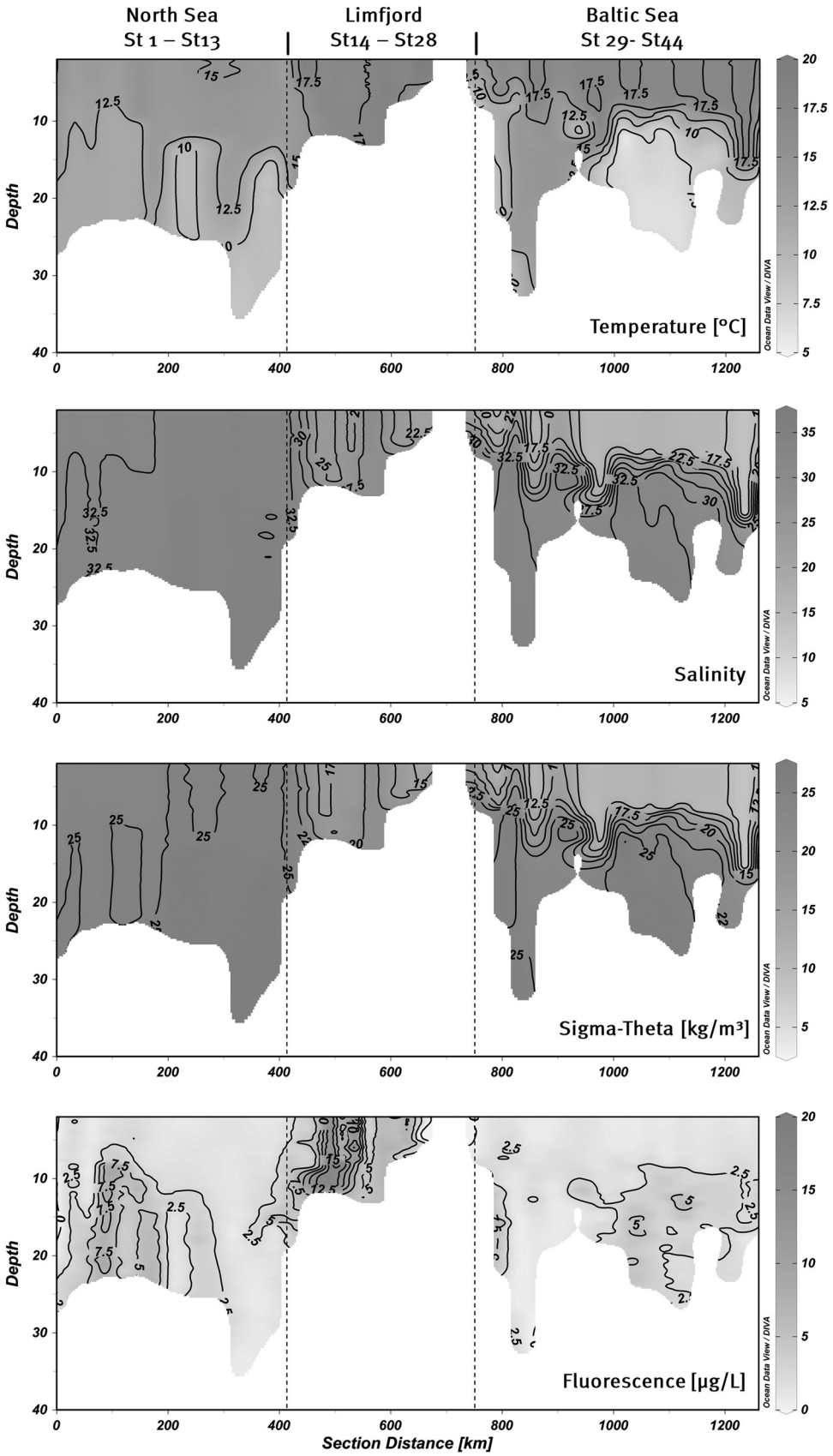

Fig. 3. Depth profiles of salinity, density, temperature (°C) and Chl*a* fluorescence (µg L^{-1}) across the sampled transect. Profiles start at Station 1 in the German Bight /North Sea and end at Station 44 in Kiel Bight, Baltic Sea.

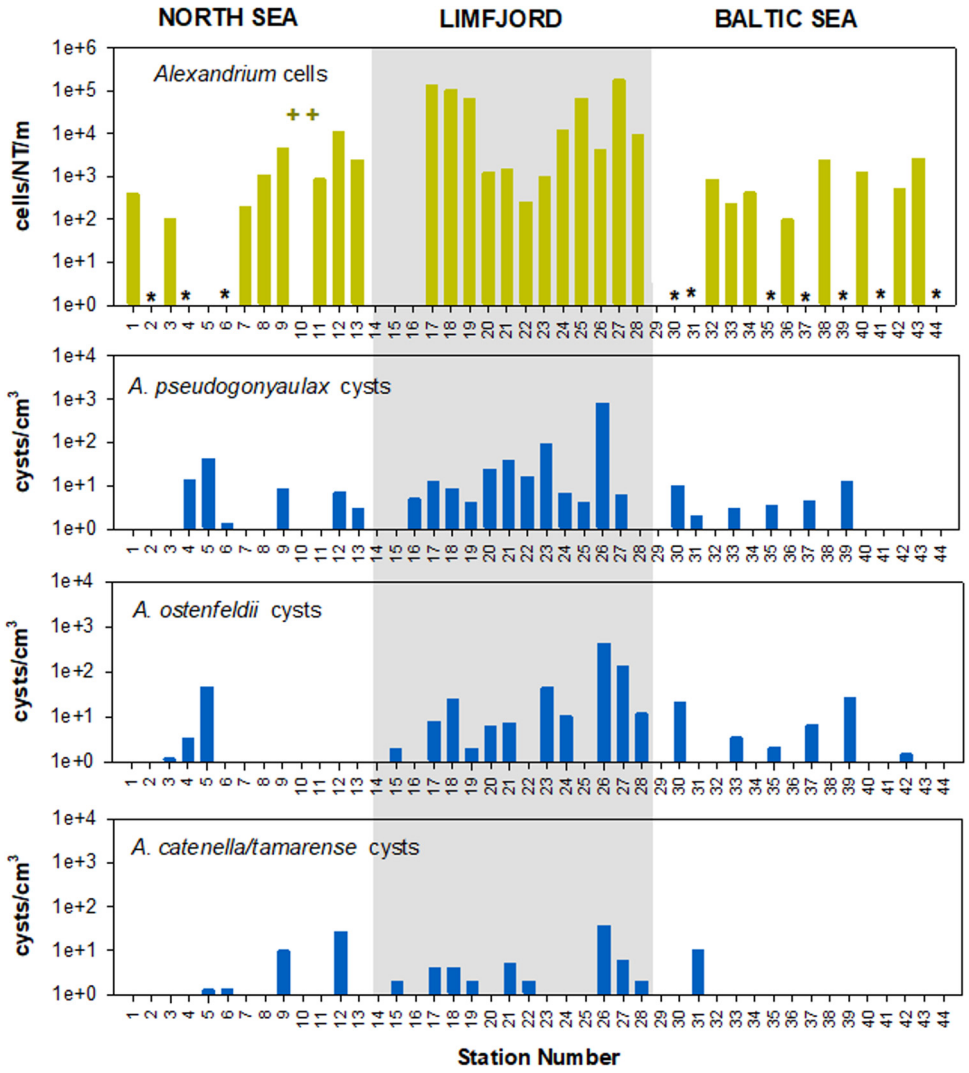

Fig. 4. *Alexandrium* spp. cells and cysts across the North Sea – Baltic Sea transect. Cell numbers were calculated from net tows integrating the water column (A). Cyst concentrations of *A. pseudogonyaulax* (B), *A. ostenfeldii* (C) and *A. catenella* (D) at the sediment surface. Stations indicated with an asterisk were not analyzed for cell abundances. Stations marked with a green cross represent the presence of *A. catenella* (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

3.3. Toxin distribution

3.3.1. Goniodomins

Goniodomin A was the most abundant toxin measured in the study area at the time of the survey. It was detected in all three investigated water bodies: North Sea, Limfjord and Baltic Sea (Fig. 5). Though GDA was detected at most sampled stations in the 20–50 µm plankton fraction, amounts of this toxin varied widely throughout the studied transect. High levels of GDA were found in the Limfjord (up to 590 ng $NT^{-1}\,m^{-1}$), whereas GDA in the North Sea and the Kattegat remained mostly below 100 ng $NT^{-1}\,m^{-1}$ (Fig. 5). GDA was not detected in the German Bight but appeared at the Stations between Sylt Island and the western entrance of the Limfjord (Stations 6–13). While in the Kattegat hardly any GDA was detected, abundances comparable to the North Sea were measured further south in the Great Belt and Kiel Bight area (Stations 39–44). GDA concentrations were positively correlated with both *Alexandrium* spp cell numbers and 28S copy numbers of *A. pseudogonyaulax* ($p < 0.001$ for both, Table 2).

Table 2
Spearman's rank correlation, r_s values. Significant correlations ($p < 0.05$) in bold. A pse = *A. pseudogonyaulax*; A. ost = *A.ostenfeldii*, A. cat = *A. catenella*.

	Depth	Salinity	Temperature	*A.pse* 28S rDNA copies	*A.ost* cysts	*A.pse* cysts	*A.cat* cysts	GDA
Salinity	**0.38**							
Temperature	**−0.75**	**−0.48**						
***A.pse* 28S rDNA copies**	**−0.41**	**−0.41**	**0.41**					
***A.ost* cysts**	**−0.31**	−0.15	0.08	0.14				
***A.pse* cysts**	−0.26	0.21	0.05	0.19	**0.62**			
***A.cat* cysts**	−0.22	0.22	0.18	0.21	**0.40**	**0.56**		
GDA	−0.11	−0.30	0.14	**0.73**	−0.04	−0.06	0.05	
SPX total	−0.12	**0.53**	0.00	0.08	0.11	0.28	**0.50**	0.00

Table 3
Characterization of representative *Alexandrium* spp. isolates established from net samples at different stations of the survey. Species, location (Station number, North Sea/Limfjord/Baltic Sea, coordinates), toxin composition, GenBank accession number.

Species	Strain	Origin Station	Length (µm) Mean ± SD Min-max	Width (µm) Mean ± SD Min-max	l/w ratio Mean ± SD	N	Accession number	Toxins
A. pseudogonyaulax	X-LF-12-B4	12	37.0 ± 2.9 31.3–43.3	35.7 ± 3.5 28.4–44.7	1.04 ± 0.05	50	MK441698	GDA
A. pseudogonyaulax	X-LF-12-D1	12	39.6 ± 3.5 33.4-52.2	37.4 ± 4.4 31.3-50.8	1.06 ± 0.05	50	MK441699	GDA/B
A. pseudogonyaulax	X-LF-19-D12	19	35.9 ± 2.8 30.3–44.1	34.7 ± 2.6 29.1–39.9	1.03 ± 0.05	47	MK441702	GDA
A. pseudogonyaulax	X-LF-19-F5	19	35.4 ± 5.2 27.7–50.6	35.4 ± 5.3 26.6–49.5	1.00 ± 0.06	50	MK441703	GDA/B
A. pseudogonyaulax	X-LF-17-H10	17	37.5 ± 5.6 28.6-51.6	35.2 ± 5.9 25.1-48.1	1.07 ± 0.06	53	MK441701	GDA/B
A. pseudogonyaulax	X-LF-17-D12	17	39.7 ± 4.5 30.7–50.9	38.0 ± 5.1 28.8–49.7	1.05 ± 0.07	52	MK441700	GDA/B
A. pseudogonyaulax	X-LF-27-G4	27	–	–	–	–	MK441704	GDA/B
A. catenella	X-LF-12-F9	12	29.4 ± 1.9 26.4-39.4	30.4 ± 1.5 27.5-34.8	0.97 ± 0.05	54	MK441695	PST
A. catenella	X-LF-12-F6	12	–	–	–	–	MK441696	PST
A. ostenfeldii	X-LF-19-E10	19	35.6 ± 5.7 23.3-51.2	34.3 ± 5.8 22.5-49.5	1.04 ± 0.05	103	MK441697	SPX, GYM
A. ostenfeldii	X-LF-19-F10	19	38.3 ± 5.4 26.4-52.6	37.8 ± 5.0 25.8-49.1	1.01 ± 0.04	104	–	SPX, GYM
A. tamutum	X-LF-12-C1	12	27.1 ± 2.6 21.2–32.4	25.9 ± 2.5 21.0–30.0	1.05 ± 0.04	40	MK441705	ND

3.3.2. Cycloimines

Spirolides (SPX) were present in most samples of the 20–50 µm plankton fraction, collected along the transect (Fig. 6). Their abundances, however, were several magnitudes lower than GDA at most of the stations. SPX profiles consisted of 4 different compounds: spirolide G (SPX-G), 13-desmethyl spirolide C (SPX1), 13,19didesmeSPX-C and 20meSPX-G which contributed differently to the profiles depending on the water body. In the North Sea and the Kattegat areas, 20meSPX-G dominated the profile, followed by SPX1, 13,19didesmeSPX-C and traces of SPX-C. The SPX composition was reverse in the Limfjord with SPX-G being the dominant fraction and low levels of 20-meSPX-G. Spirolide abundances were generally higher in the Limfjord compared to the North Sea and the Baltic Sea. In the Baltic Sea, south of the Kattegat (Belt Sea and Kiel Bight, Stations 39–44) spirolides occurred at low abundances, hardly ever exceeding 10 pg NT^{-1} m^{-1}. Gymnodimines (GYM) were absent from North Sea samples but occurred in the eastern Limfjord basin (Skive Fjord, S) together with SPX-G, SPX1 and 13,19didesmeSPX-C. Although no significant correlation was found between SPX and *A. ostenfeldii* cyst abundances, SPX distribution was related to salinity ($p < 0.001$, Table 2).

3.3.3. PST

PST was only detected at stations 9, 11 and 12 in the North Sea. PST abundances at these three stations were generally low, but increased from station 9 to 12 and consisted of C1/2 (13.9, 15.2 and 21.5 ng $NT^{-1}\,m^{-1}$), GTX2/3 (7.7, 9.3 and 10.3 ng $NT^{-1}\,m^{-1}$), NEO (nd, 7.5 and 8.8 ng $NT^{-1}\,m^{-1}$) and STX (6.0, 6.9 and 8.2 ng $NT^{-1}\,m^{-1}$).

3.4. Characteristics of Alexandrium spp. strains

Identities of *Alexandrium* spp. strains isolated at St. 12 (North Sea), 17, 19 and 27 (Skive Fjord, Limfjord, S in Fig. 1) confirmed the distribution of the different species in field samples. From the Limfjord stations, the majority of strains were identified as *A. pseudongonyaulax,* and only two strains of *A. ostenfeldii* were established from Station 19 in the strongly freshwater influenced innermost bay of Skive Fjord. The marine Station 12 yielded two *A. pseudogonyaulax* strains, two saxitoxin-producing *A. catenella* (previously *A. tamarense* complex group 1) strains and one non-toxic *A. tamutum* strain (Table 3)

All Limfjord A. pseudogonyaulax strains had nearly identical 28S rDNA sequences (> 99% identity), and were likewise genetically indistinguishable from global A. pseudogonyaulax strains, including a previously sequenced strain from Oslofjord (JF521638). The extremely low sequence variation among the few available 28S rDNA sequences did not provide phylogenetic resolution. The 28S rDNA genomic copy number in *A. pseudogonyaulax* strains was estimated to vary between 81 000 and 703 000 per genome. The successfully analysed sequence of *A. ostenfeldii* strain X-LF-19-E10 (Accession number MK441697) was identical to 28S rDNA sequences from strains K0289 (AJ535356) and CCMP1773 (JF521636), which were both obtained from the same area 30 years ago. The genome of X-LF-19-E10 contained an estimated 2500 copies of the 28S rDNA gene, comparable to previous observations from

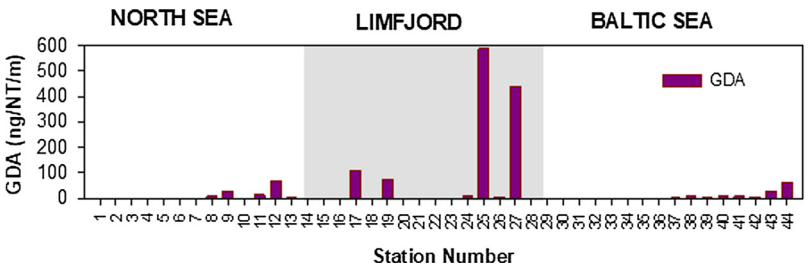

Fig. 5. Abundances of goniodomin A (GDA) measured in the 20–50 µm size fraction of vertical net tows along the studied transect. Note that the scale is linear and lower values may not be visible. GDA abundances were normalized to net tow per meter of water column (NT m^{-1}).

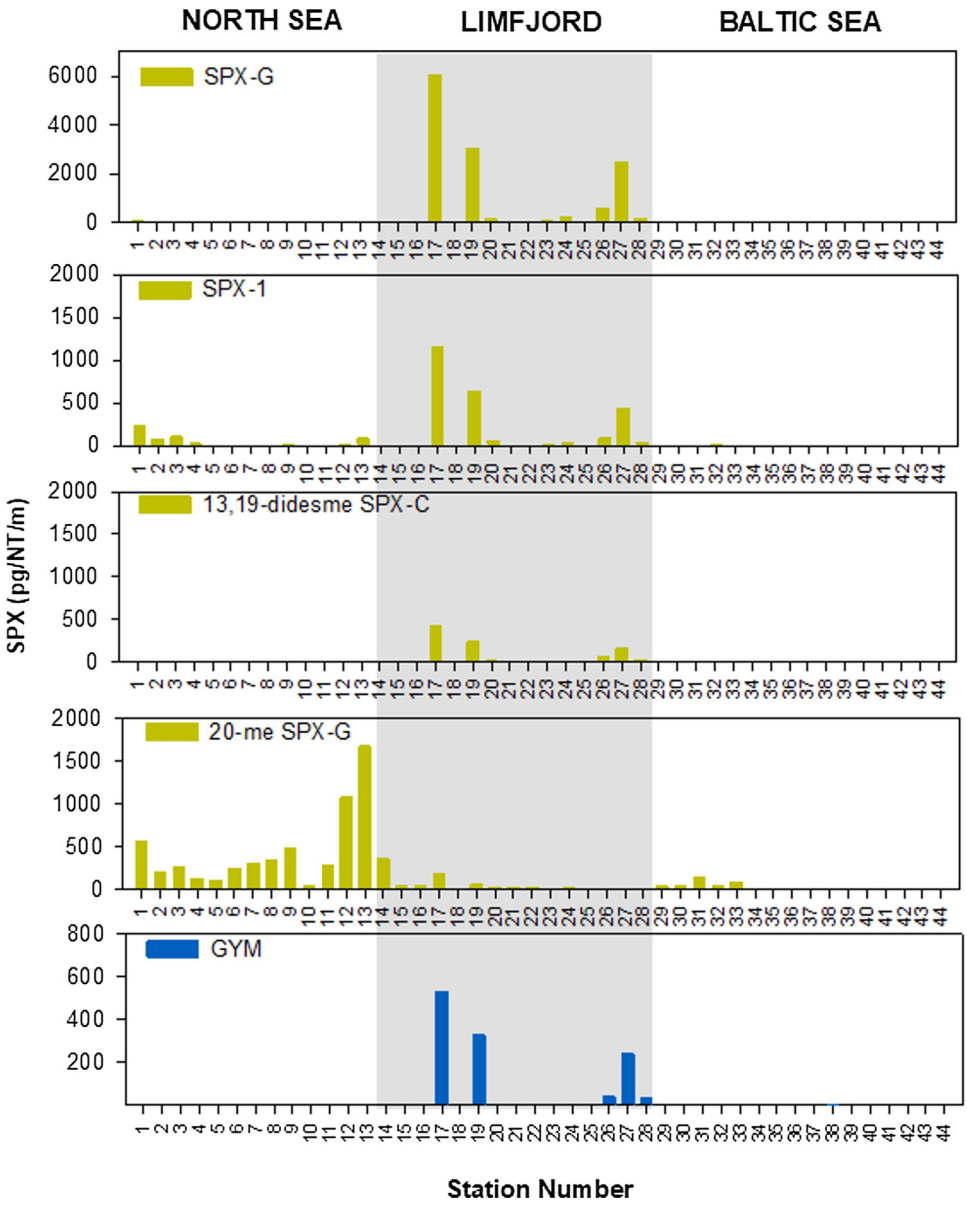

Fig. 6. Abundances of cycloimines measured in the 20–50 µm size fraction of vertical net tows along the studied transect.

Baltic Sea *A. ostenfeldii* (Savela et al., 2016). The 28S rDNA copy numbers of the two *A. catenella* varied from 6900 to 43 000 per genome, and the sequences closely matched strains assigned to the *A. tamarense* complex Group I, including strains isolated from the east coast of North America, Scottish and English waters (Fig. S3)

Size ranges of *A. pseudogonyaulax* were large (Table 3, Fig. 7A–J) although average cell sizes were slightly lower than typical for the species, overlapping with *A. ostenfeldii*. Cell shapes ranged from slightly compressed to round (Fig. 7A, B, F, G). The compressed appearance typical for the species was only found in a few strains. Growing cultures of all strains contained characteristic division cysts (Fig. 7H). The plate patterns visualized by calcofluor staining were typical for *A. pseudogonyaulax* (Fig. 7C–E and I, J): The first apical plate (1′) was disconnected from the pore (po) plate (Fig. 7C and I) and the large sp plate was laterally compressed (Fig. 7D and J). *A. catenella* cells of the examined strain Z-LF-12-F9 (Fig. 7K–M) were relatively small (Table 3) and in exponentially growing cultures often appeared in couplets (Fig. 7L) or 4-cell chains. A ventral pore was present on the right margin of the first apical plate (Fig. 7M). The two *Alexandrium ostenfeldii* strains from the Limfjord St. 19 were in the same size range as *A. pseudogonyaulax* (Table 3) and were mostly round (Fig. 7N). Patterns and shapes of their thecal plates were as described earlier of the Limfjord strains: the 1′ plates were narrow and had a large ventral pore and cells predominantly contained door-latch shaped s.a. plates (Fig. 7O).

The toxin composition of *A. pseudogonyaulax* strains is extensively presented in Krock et al. (2018). The isolates characterized here contained predominantly goniodomin A (GDA), but goniodomin B (GDB) was also detected in all cultures from both water bodies. Other toxins (PST, spirolides or gymnodimines) could not be detected. *A. catenella* cultures had complex PST profiles, containing C1/C2, GTX2/3, GTX1/4, B1, NEO and STX but the cellular amounts were low and PST composition varied among the two established strains from station 12 (Fig. 8). *A. ostenfeldii* strains, including CCMP1773 isolated from the same area in 1987 by Hansen et al. (1992), did not produce PST, but spirolides and gymnodimines A (GYM A) (Fig. 8). While the two new isolates (X-LF-19-E10 and F10) contained mainly SPX-G, CCMP1773 had 13,19-didesme SPX-C besides SPX-1. Cell quotas varied among the investigated strains.

From North Sea station 12 one strain of *A. tamutum* was established.

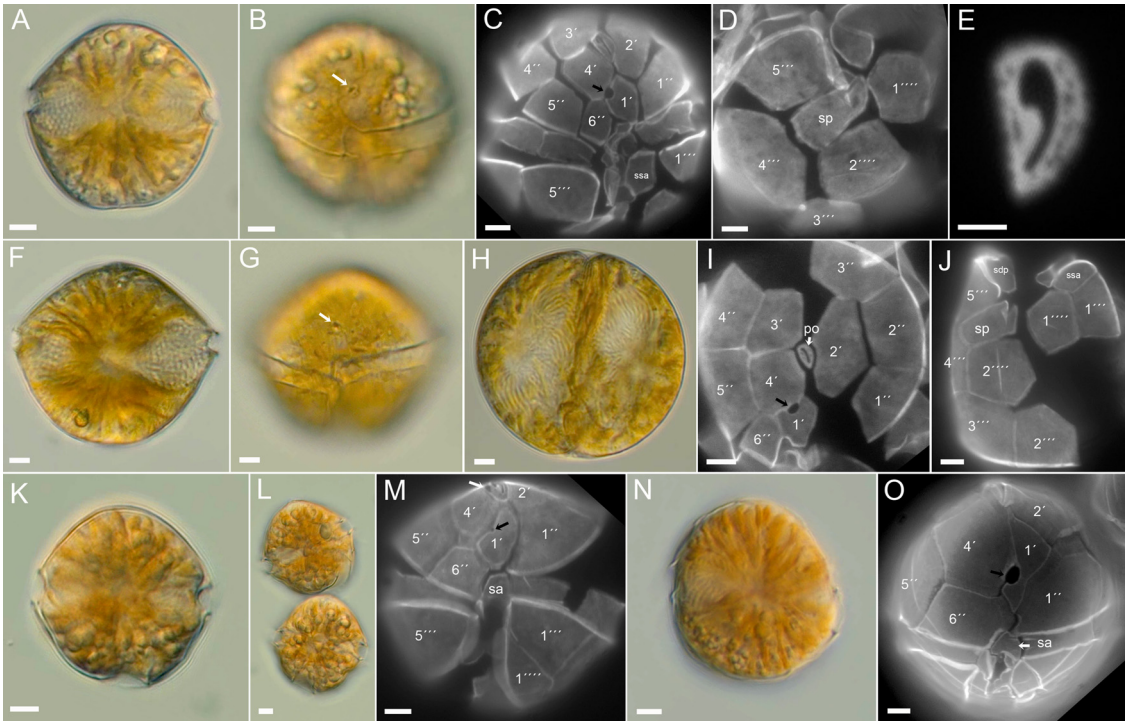

Fig. 7. Morphology of *A. pseudogonyaulax* strains X-LF-17-H10 (A–E) and X-LF-19-F5 (F–J) isolated from Limfjord, and *Alexandrium catenella* X-LF-9-F9 (K–M) from the North Sea, and *A. ostenfeldii* X-LF-19-E10 (N–O from Limfjord). Scale bar = 5 µm (except E = 2 µm).

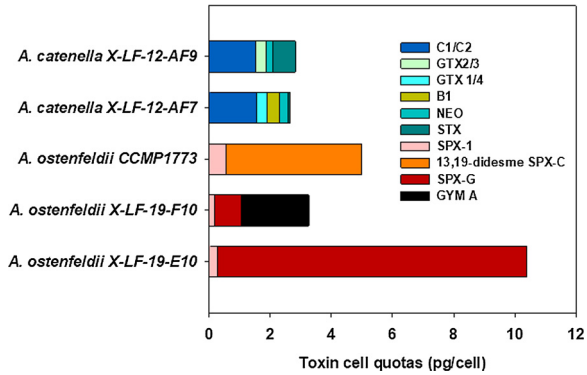

Fig. 8. Toxin profiles of *A. catenella* (PST) and *A. ostenfeldii* (cycloimines) isolates.

The identity of the strain, suggested by morphological characters was confirmed by 28S rDNA sequence data. Cells of this strain were distinctly smaller than the other three species. No toxins were detected in this strain.

3.5. Long-term seasonal and annual dynamics of Alexandrium spp. and A. pseudogonyaulax in Limfjorden

The monitoring data document that *Alexandrium* spp. have been present in the Limfjord since 1987 at least (Fig. 9A). The highest annual summer (May – September) means of approx. 1×10^3 cells L^{-1} was measured in 1987. Typically, abundances, ranged between 0.2 and 0.4×10^3 cells L^{-1} until 2009 after which *Alexandrium* spp. (excluding *A. pseudogonyaulax*) disappeared from the Limfjord monitoring samples (Fig. 9A). In the summer of 2007, *A. pseudogonyaulax* was observed for the first time in monitoring samples at abundances of 0.1×10^3 cells L^{-1}. In the following two years, peak abundances of this species reached $3.0–3.5 \times 10^3$ cells L^{-1} and afterwards the annual summer mean declined and reached 0.7×10^3 cells L^{-1} in 2015.

The monthly mean cell concentrations of *Alexandrium* spp. excluding *A. pseudogonyaulax* during 1987–2007 increased from zero in February to around 0.4×10^3 cells L^{-1} in April (Fig. 9B). From April and until November *Alexandrium* spp. concentration was constant at ~0.4×10^3 cells L^{-1}. The highest concentrations were typically recorded between July and September. The monthly mean of *A. pseudogonyaulax* (Fig. 9C) during 2007–2015 was low in April and May but increased drastically to values between of 3.5 and 5×10^3 cells L^{-1} in June through September where-after abundance dropped to 0.2×10^3 cells L^{-1} in October and was not detected during winter (Fig. 9C). *Alexandrium* spp. had a wider temporal distribution than *A. pseudogonyaulax*, and highest monthly means were 10 fold higher for *A. pseudogonyaulax* compared to *Alexandrium* spp.

4. Discussion

4.1. Low salinity, high temperature and proximity to seed "banks" favor Alexandrium pseudogonyaulax in the Limfjord, Denmark

The observed differences in *Alexandrium* abundances and toxins along the studied transect coincide with the boundaries separating the three major water masses. Differences were particularly pronounced between the shallow well-mixed brackish Limfjord and the adjacent deep and stratified sea areas. Throughout the Limfjord high abundances of goniodomin A (GDA) and *A. pseudogonyaulax* cell and DNA copy numbers were encountered which significantly correlated with high temperature, low salinities and low depth prevailing in the fjord system at the time of sampling. *Alexandrium pseudogonyaulax* has previously been mostly reported from warm water environments (Biecheler, 1952; Honsell et al., 1992; Montresor, 1995; Bravo et al., 2006; Zmerli Triki et al., 2014) and thus the high water temperature found in the shallow sheltered Limfjord basins at the time of sampling likely favoured growth of this species here. Being an estuarine species, which often seems to be associated with decreased salinities (Balech, 1995), *A. pseudogonyaulax* can be expected to tolerate the brackish conditions in the Limfjord very well. In Bizerte lagoon, Tunisia, where the species has

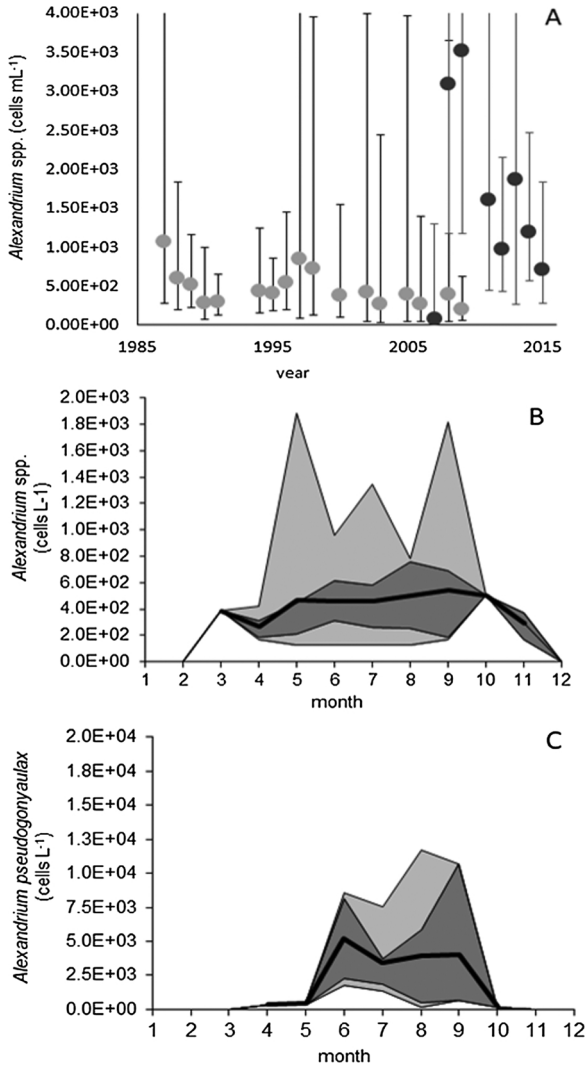

Fig. 9. A) Summer abundances (cells L^{-1}) of *Alexandrium* spp and *Alexandrium pseudogonyaulax* since 1987 at the DNAMAP monitoring stations in Limfjorden. Grey circles represent *Alexandrium* spp. and **black** circles are abundances of *Alexandrium pseudogonyaulax*. Error bars are standard errors of the mean. Due to data being lognormal distributed, the "positive" error bars are higher than the "negative" error bars. B) Monthly mean concentrations (cells L^{-1}) of *Alexandrium* spp. excluding *Alexandrium pseudogonyaulax* of the three DNAMAP monitoring stations in Limfjorden. Full **black** line is the mean for the period 1987–2007, Light grey displays the variation, and dark grey are the upper and lower quartiles. C) Monthly mean concentrations (cells L^{-1}) *Alexandrium pseudogonyaulax* of the three DNAMAP monitoring stations in Limfjorden. Full **black** line is the mean for the period 2007 – 2015. Light grey display the variation, and dark grey are the upper and lower quartiles.

been studied extensively, a wide salinity tolerance span was detected (Zmerli-Triki et al. 2015).

North Atlantic *Alexandrium catenella* generally prefers cooler water (Etheridge and Roesler, 2005; Eckford-Soper et al., 2016), which may explain why this species was only detectable in colder North Sea water and not in the Limfjord or the Baltic Sea in this survey. With regard to salinity, many *Alexandrium* species prefer estuarine environments and have optimal growth rates at around 25–30 psu (Etheridge and Roesler, 2005; Bill et al., 2016). Depending on the environments they are adapted to and even thrive at salinities < 15 psu (Suikkanen et al., 2013a, Martens et al., 2016). Also for *A. ostenfeldii* from the Limfjord wide salinity tolerance spans matching the conditions of this water body have been confirmed previously, as well as a preference for warm water (Østergaard-Jensen and Moestrup, 1997). It is unclear why *A. ostenfeldii* was so much less abundant than *A. pseudogonyaulax* when Limfjord was sampled, despite so similar temperature and salinity adaptations. It could be speculated that the general increase of temperature in the system (Hansen, 2018) opened a niche for the invasion of *A. pseudogonyaulax* at the cost of *A. ostenfeldii*. However, it needs to be noted that this study covered only a very short period that may have been insufficient for a comprehensive understanding of the situation.

In this study, *Alexandrium* cell and 28S rDNA copy numbers were negatively correlated with water depth, i.e. highest values were associated with low depth. This observation concurs with data on the distribution of *Alexandrium* cells in Danish waters as determined by the national algal monitoring program (Hans H. Jakobsen, personal communication, Table S3). The shallow depth of the Limfjord system provides an important advantage for *Alexandrium* compared to the North Sea and the Baltic Sea- proximity to seed banks due to relatively low water depth. Like most *Alexandrium* species, also the three species studied here form resistant resting cysts to survive unfavourable conditions (Anderson, 1998; Montresor, 1995; MacKenzie et al., 1996). Their resting cysts were present in all three water bodies, but at notably higher concentrations in the Limfjord. The significant correlation between cyst concentrations of the three *Alexandrium* species suggests that cyst accumulation hot spots exist in the different sampled Limfjord basins. Particularly high cyst numbers were found in the shallow and sheltered Skive Fjord basin (max depth 8 m), where strong benthic-pelagic coupling has been documented previously (Krause-Jensen et al., 2012). For *Alexandrium*, a shallow water column in a sheltered water body provides optimal conditions for recruitment from the "seed bank" represented by cyst accumulations: light can penetrate to the bottom and stimulate germination (Zmerli Triki et al., 2015). Having access to light, germinated cells, in turn, can resume growth immediately and have a better chance to form blooms than cells germinated in deep water. Thus, sizeable cyst deposits together with low water depth should support successful recruitment and bloom formation in the Limfjord.

Although the high cyst concentrations in the separate Limfjord basins are likely a result of accumulation due to local bloom retention, the major current patterns prevailing at the Danish coasts might promote cyst deposition in the fjord. Northwards flowing water masses carrying *Alexandrium* cells from the English Channel and the southern North Sea (Aure et al., 1998; Gyllencreutz et al., 2006) up the Danish west coast during spring are pushed eastwards in the area of Jutland due to dominating westerly winds (Møller, 1996). As this interplay between the major current and wind directions control the water exchange in the Limfjord, and inflowing *Alexandrium* cells may conclude their life cycles here. The resulting resting cysts are deposited in bottom sediments of the Limfjord, where they add to the *Alexandrium* seed banks.

Abundances of *A. ostenfeldii* cysts as identified here where generally in the same range as *A. pseudogonyaulax* and again it remains unclear why proximity to dense cyst deposits should only favour *A. pseudogonyaulax*, but not *A. ostenfeldii* despite their similar habitat preferences and effective germination strategies at higher water temperature and in the presence of light (Østergaard-Jensen and Moestrup, 1997; Gu, 2011, Zmerli Triki et al., 2015). It is possible that *A. ostenfeldii* cyst abundances were overestimated because the generally smaller cysts of *A. ostenfeldii* overlap to some extent with the generally larger cysts of *A. pseudogonyaulax*. Limfjord *A. pseudogonyaulax* cells seem to be generally smaller than the species' average size (see below) thus the overlap might have been higher than expected. Alternatively, *A. ostenfeldii* cyst deposits could represent old stocks reminiscent of the period before 2009 when *A. ostenfeldii* abundances declined in monitoring observations (Fig. 9).

The characteristically elongated cysts of *Alexandrium catenella* where found in low abundances at many stations in the North Sea and in the Limfjord confirming that a seed stock for toxic blooms of this species is present here. In the Baltic Sea, these cysts were only recorded at one of the northernmost marine influenced stations in Kattegat. This

is in line with the preference for higher salinities as compared to *A. ostenfeldii* and *pseudogonyaulax*.

4.2. High goniodomin abundances and specific cycloimine profiles, but no PST in the Limfjord

The distribution pattern of the three *Alexandrium* species in the plankton was reflected by toxin patterns. Goniodomin abundances mirror cell abundance patterns with highest values in Skive Fjord where also highest cell numbers of *A. pseudogonyaulax* were encountered. Cycloimines, on the other hand, were found at many stations where cell and DNA copy numbers of *A. ostenfeldii* remained below detection level. This discrepancy may reflect sensitivity differences between both toxin groups. Whereas cycloimines are detected very sensitively by mass spectrometry due to the easy ionization of the imino function, pure polyketides without any preferred ionization site such as goniodomins are detected with much less sensivity.

In this study, the cycloimine data provided interesting additional information on *A. ostenfeldii* distribution. Cycloimines were present in all three water bodies, at nearly all stations, indicating that also *A. ostenfeldii* occurred throughout the entire transect. The cycloimine composition was unique in the Limfjord. While in the North Sea and Baltic Sea 20me-SPX-G were dominant and no GYM A was detected (except for Stn 38 in the Baltic Sea), Limfjord samples had diverse spirolide profiles containing significant proportions of all measured spirolides and GYM A, but no 20meSPX-G. The pattern was consistent at all stations with measurable amounts of cycloimines, suggesting that the Limfjord population of *A. ostenfeldii* has evolved specific toxin profiles or that the specific prevailing conditions affect toxin profiles.

Salinity is one factor to consider in this context as the condition that changed considerably along the transect and salinity was positively correlated with total amounts of SPX. Nevertheless, relating the toxin composition to salinity is not straightforward. The observed differences between the North Sea and the Limfjord were not the same as in the Baltic Sea. The northern stations of the Baltic Sea had the same brackish salinities as the Limfjord, but cycloimine patterns were similar to those observed in marine waters of the North Sea. Toxin profiles are generally strain-specific and stable in their composition, but the relative proportion of the different derivatives can vary at different salinities in *Alexandrium ostenfeldii* (Suikkanen et al., 2013a). One could thus hypothesize that salinity directly influenced the amounts of specific derivatives along the salinity gradient, but again, the observed differences are not consistent along the sampled gradient. Martens et al. (2017) found a large variability of cycloimine profiles and cell quotas among single strains isolated from a brackish water population of *A. ostenfeldii*. It is possible that other conditions than salinity, e.g. competition or grazer regimes, lead to the selection of genotypes with consistently different toxin profiles in the Limfjord. Such environmental geno- and/or phenotype selection has recently been documented for diatoms (Godhe et al., 2016; Sjöqvist and Kremp, 2016).

PSTs were only found on rare occasions in plankton field samples at three North Sea stations. The concentrations of *A. catenella* in North Sea water were too low for more frequent PST detection. Interestingly the PST profiles at the three stations resembled the toxin profile of *A. catenella* strain X-LF-12-AF9 isolated from St. 12 containing C1/2, GTX2/3, NEO and STX, whereas the other strain (X-LF-12-AF7) isolated from the same station produced C1/2, B1 and GTX1/4 as major PST variants. This is a clear evidence for different strains contributing in different amounts to a field population of a given species.

The absence of PST in the Limfjord samples, where these toxins have been documented earlier in connection with *A. ostenfeldii* (Hansen et al., 1992) was somewhat unexpected. The PST profiles reported by these authors for a strain isolated from Limfjord (CCMP1773) were however quite unusual, consisting of > 90% B2. They were never confirmed by later analyses (Krock, unpublished). Generally, brackish water populations of *A. ostenfeldii* can produce PST together with spirolides and/or gymnodimines (Kremp et al., 2009; van de Waal et al., 2015; Harju et al., 2016), but this ability seems to be ribotype specific. Both new Limfjord strains and other strains representing the *A. ostenfeldii* Limfjord ribotype (Kremp et al., 2014) were never found to produce PST, and so far all investigated members lack sxt genes (Suikkanen et al., 2013a).

4.3. Alexandrium pseudogonyaulax, a toxic bloom former expanding in N-European waters?

The predominance of *Alexandrium pseudogonyaulax* and goniodomins in the *Alexandrium* community of the three investigated water bodies is an unexpected result of this survey. In previous reports, this species has not raised much attention compared to other *Alexandrium* species in the area (e.g. Larsen and Moestrup, 1989), although it has been occasionally listed as present in past cyst and plankton records from the North Sea (Balech, 1995; Nehring, 1997). Being quite a rare background species in the area, its North European populations were never characterized properly.

Here we provide, for the first time, documentation of North European strains. While the plate morphology largely agrees with the characterization provided by Balech (1995), the Limfjord and North Sea strains are more variable in size and shape. The cells are generally smaller and not as wide and compressed as typical for *A. pseudogonyaulax* described from other locations, though variation among the isolates exists. A large range of sizes and varying shapes of cells in cultured strains might in part be due to the presence of gametes (smaller and round, see Montresor, 1995) and other stages of the sexual cycle. In some of the cultured strains nuclear cyclosis, a clear sign of sexual reproduction was observed. Vegetative cell division of the Limfjord cultures was as previously described, in division cysts (Montresor, 1995), however para-tabulate resting cysts (Montresor, 1993) were never seen in sediments and/or cultures: Cysts found in the sediment here, lacked para-tabulation similar to in other regions (Bravo et al., 2006). This may be due to the brackish salinities in the area: processes and other surface structures of dinoflagellate resting cysts are often reduced in low salinity environments (Mertens et al., 2011). Unfortunately, the 28S sequence data generated here for the isolated strains were not able to resolve relationships to other geographic populations, e.g. from Asia, New Zealand (Gu et al., 2013) or North Africa (Zmerli Triki et al., 2014). This could suggest that the species has spread from a geographically distant source population relatively recently, but firm conclusions cannot be made due to the limited availability of sequence data.

The wide distribution and predominance of *A. pseudogonyaulax* in the *Alexandrium* community shown here for the investigated North Sea-Limfjord-Baltic Sea transect indicate an ongoing expansion of the species in northern Europe. This is corroborated by the clear change observed in 2007–2008 in the composition of the *Alexandrium* spp. community in the Limfjord long term monitoring data. For a yet unclear reason, *A. pseudogonyaulax* suddenly replaced other *Alexandrium* spp. in the early summer phytoplankton community with cell concentrations exceeding *Alexandrium* spp. by one order of magnitude. An explanation for this shift is not directly evident. It could be assumed that specific hydrographic changes observed over the past two decades in the Limfjord (Carstensen et al., 2013) including an increase in temperature, might play a role here. However, as explained above, physical habitat conditions seem to be equally tolerated by *A. ostenfeldii* and *A. pseudogonyaulax*. Both species are reported to phagocytize other organisms (Gribble et al., 2005; Jeong et al., 2010) and should respond similarly to changed nutrient conditions. Nevertheless, *A. pseudogonyaulax* has a particularly effective “food” acquisition strategy that is unique among mixotrophic protists: it produces toxic mucus, which traps and concentrates protist prey cells (Blossom et al., 2012, 2017). This feeding mechanism increases the growth of the species and provides a clear competitive advantage.

It could be assumed that through this mechanism *A. pseudogonyaulax* benefits specifically from high phytoplankton productivity fueled by climate enhanced river discharge from the catchment area (Blanda et al., 2016). Besides providing suitable temperature conditions, climate change may thus indirectly favour *A. pseudogonyaulax* in brackish waters affected by high riverine inflow. Possibly such a mechanism is also responsible for the recently observed high biomass occurrences of the species in summer phytoplankton of the western and southern Baltic Sea (Wasmund et al., 2015, 2017).

5. Conclusions

This field survey revealed that salinity alone does not determine abundances and composition of *Alexandrium* species and their toxins, but emphasizes the importance of habitat conditions such as proximity to seed banks, shelter, and high nutrient concentrations. The results show that *A. pseudogonyaulax* is now a prominent member of the *Alexandrium* spp. community. Analyses of long term monitoring data from Limfjord confirmed a recent shift to *A. pseudogonyaulax* dominance in *Alexandrium* spp. Our results and interpretations support the initially suspected expansion of *A. pseudogonyaulax* in Northern European waters. Cyst and toxin records of the species in Kiel Bight suggest a spreading potential into the brackish Baltic Sea, where it might expand in the future and form blooms when climate warming continues. At the same time our data indicate that *A. pseudogonyaulax* has the potential to cause problems in Scandinavian waters since it produces GDA. This toxin can accumulate in marine shellfish and fish and lead to invertebrate mortalities with potentially severe consequences for aquaculture and the shellfish industries in the area.

Author contributions

The original idea was developed by AK, BK, PJH and UT. AK coordinated the activities, analyzed cyst samples and wrote the manuscript draft, to wich all coauthors contributed. PJH coordinated the field campaign in Limfjorden and participated in material collection. UT collected material and performed taxonomic analyses, HS developed and performed the qPCR assay for quantification of *Alexandrium* spp, S.S. participated the cruise and performed statistical analyses, DV and FB measured and analyzed hydrography, HHJ analyzed long term data. BK organized and lead the research cruise and took charge of the toxin analyses.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.hal.2019.101622.

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