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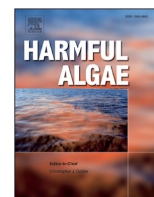

Effects of temperature, salinity and CO_2 concentration on growth and toxin production of the harmful algal bloom species *Alexandrium pseudogonyaulax* (Dinophyceae) from the Danish Limfjord

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A B S T R A C T

The marine dinoflagellate *Alexandrium pseudogonyaulax* is a widely distributed Harmful Algal Bloom (HAB) species that produces the macrocyclic polyketide goniodomin A (GDA). Occurrences in northern European waters are increasing and a spreading of the species along a salinity gradient into the Baltic Sea has been observed. As GDA is suspected to lead to invertebrate mortality, the spreading is of concern for the environment and possibly human health. In order to assess the potential of *A. pseudogonyaulax* to adapt to the environmental conditions in the Baltic Sea and the risk of future harmful blooms of that species, we quantified the influence of bottom-up factors on the growth and toxin content of three strains of *A. pseudogonyaulax* from the Danish Limfjord. Specifically, we exposed these strains to salinities ranging from 5 to 50, temperatures in the range of 10 – 30 °C and to three different CO_2 concentrations of 250, 400 and 1000 µatm. All strains tolerated a broad range of salinities and temperatures, resulting in positive growth rates ranging from 0.06 to 0.33 d^{-1} between temperatures of 12 and 27 °C and between salinities of 10 and 40. The highest cell quotas of GDA were measured at low temperatures. For two strains, GDA amounts were almost unaffected by salinity, while the cell quota of the third strain decreased about 20-fold when salinity increased above 30. Different CO_2 concentrations had no effect on growth or GDA production. In summary, these findings show a high ecological tolerance towards a wide range of temperatures and salinities of the Limfjord population of *A. pseudogonyaulax*, together with distinct intraspecific physiological differences within the population. Our results also suggest that a further spreading into the Baltic Sea might be possible.

1. Introduction

The marine dinoflagellate *Alexandrium pseudogonyaulax* (Dinophyceae) was first described as *Goniodoma pseudogoniaulax* from the Thau Lagoon in the Mediterranean Sea (Biecheler, 1952), and is widely distributed in various areas of the world, including coastal waters of New Zealand and Malaysia, the Gulf of California, the Black Sea, the South China Sea and the Yellow Sea, the Southwestern Mediterranean Sea and the Danish North Sea (Abdullah et al., 2023; Gu et al., 2013; Kremp et al., 2019; MacKenzie et al., 2004; Morquecho and Lechuga-Devéze, 2004; Terenko, 2005; Zmerli Triki et al., 2016). Hence, *Alexandrium pseudogonyaulax* is present from tropical to boreal regions, suggesting tolerance to a wide temperature range. Reports of *A. pseudogonyaulax* from southern Norway are common (Balech, 1995; Dittami et al., 2013; Throndsen et al., 2007), and recent studies revealed that since 2007, *A. pseudogonyaulax* increased in density in the Danish Limfjord, accompanied by a decrease of *A. catenella* and *A. ostenfeldii* in that area (Kremp et al., 2019). The Limfjord is a eutrophic and shallow sound with a maximum depth of 24 m and a mean depth of 4.5 m (Teixeira et al., 2014). With a connection to the North Sea in the West and to the Kattegat in the East, the Limfjord is characterized by a salinity gradient with salinities of 31 (West) and 23 (East) (Hofmeister et al., 2009). *Alexandrium pseudogonyaulax* is seemingly spreading further from the Limfjord along the longitudinal salinity gradient through the Kattegat into less saline waters of the Baltic Sea (Wasmund et al., 2017), reaching areas where a salinity of about 8 has been described (Feistel et al., 2010).

Along with other species of the genus *Alexandrium, A.*

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pseudogonyaulax is regarded as a harmful algal boom (HAB) species, as it produces the macrocyclic polyketide toxin goniodomin A (GDA) (Zmerli Triki et al., 2016). Furthermore, *A. pseudogonyaulax* also produces bioactive extracellular compounds (BECs) (Long et al., 2021; Möller et al., 2024b) which can immobilize and lyse various protistan species (Tillmann and John, 2002), showing the capability of constitutive mixotrophy (Blossom et al., 2012). Another GDA producing species, *Alexandrium monilatum,* has been associated with the death of the veined rapa whelk (*Rapana venosa*) during a bloom event in the York River (Harding et al., 2009). Furthermore, laboratory experiments have shown that the presence of *A. monilatum* altered the food uptake, behaviour and survival of three different species of shellfish (May et al., 2010). However, if the mortality of the marine invertebrates is a result of GDA or other substances is unclear.

Given the negative impact *A. pseudogonyaulax* can have on wildlife, it is important to understand to which extent this species may spread, especially in the light of climate change, which may broaden its habitat range. However, we still miss basic autecological studies defining the temperature and salinity tolerance ranges of local strains of *A. pseudogonyaulax*, and how variations in these environmental parameters influence its toxin production.

The aim of this study was to assess the growth rate of *A. pseudogonyaulax* at a range of temperatures and salinities and at different CO_2 concentrations in order to evaluate the spreading and bloom-forming potential under different conditions. Simultaneously, effects of temperature, salinity and CO_2 on GDA contents of the cells were measured to estimate the impacts of a possible further spreading. To account for intraspecific variability the study was performed using three strains of *A. pseudogonyaulax* recently isolated from the Danish Limfjord.

2. Material and methods

2.1. Isolation and culture

Three *A. pseudogonyaulax* strains (L4-B1, L4-B9 and L2-D2) were isolated from plankton samples taken in the western part of the Limfjord (Denmark, 56°37.8′ N; 8°17.4′ E) on August 17th 2020. They were grown as xenic cultures in half strength K-Medium (K/2) excluding silicate (Keller et al., 1987), slightly modified by replacing the organic phosphorous source with 1.81 µM Na_2HPO_4. The medium was prepared from autoclaved and sterile filtered (0.1 µm, VacuCap filters, Pall Life Sciences, Dreieich, Germany) North Sea water with an original salinity of 31, which was diluted with deionized and purified water (Milli-Q, Millipore, Eschborn, Germany) to a salinity of 25. The pH of the medium was adjusted to 8.0 with 1 M hydrochloric acid. Cultures were kept at 20 °C and a photon flux density of 80–100 µmol m^{-2} s^{-1} in a 16:8 h light-dark photocycle in 70 mL cell culture flasks (TECHNO Plastic Products, Trasadingen, Switzerland).

2.2. Experimental conditions

2.2.1. Temperature experiment

Experiments at different temperatures were performed at 10, 12, 15, 20, 25, 27, and 30 °C in K/2 medium at a salinity of 25 and a photon flux density of 80–100 µmol m^{-2} s^{-1} (ULM-500, Walz, Effeltrich, Germany) that have been shown to be in the range where light saturates growth of *A. pseudogonyaulax* (Möller et al., 2024a). Different growth temperatures were obtained by placing the culture flasks into water baths on a temperature-gradient table. Cultures of *A. pseudogonyaulax* were acclimated at experimental conditions to the different temperatures in steps from the initial temperature of 20 °C. At first *A. pseudogonyaulax* were acclimated to 15 and 25 °C, respectively, and after at least three cell divisions, the 25 °C cultures were further acclimated stepwise to 27 °C and 30 °C while the 15 °C cultures were acclimated stepwise to 12 °C and 10 °C. To estimate growth during acclimation, a subsample of 1 mL was fixed every second or third day with Lugol's iodine at a final concentration of 1 % and cells were counted using an inverted microscope (Zeiss Axiovert 200 M, Zeiss, Göttingen, Germany). If cells did not grow during the acclimation phase, the growth rate was regarded as zero and detailed experiments at these conditions were not performed.

To start the experiment, the acclimated cultures were divided into triplicates with densities of 150–200 cells mL^{-1}. During the experiment, samples for GDA analysis (see 2.3.) were taken at two time points. The first sampling was in the exponential growth phase, and the second sampling was performed when cells were in the stationary growth phase, which was defined when no further increase in cell density was detected for 4 days. At both sampling points, cell sizes were measured using Lugol-fixed subsamples viewed at x160 magnification under an inverted microscope. The diameter of at least 50 cells per replicate was measured using a digital camera (Axiovert, Zeiss) and the Axiovision software (Zeiss). Cell volume was calculated assuming spherical cells. Cell densities were determined every second day by counting Lugol-fixed subsamples (1% final concentration) using an inverted microscope at magnification x100. The volumes of the subsamples varied between 0.25 and 1.5 mL, depending on the estimated cell density, to ensure that at least 350 cells per replicate were counted. Specific growth rates µ (d^{-1}) were calculated, using exponential regression using at least three data points in the exponential growth phase. The range of optimum growth for each tested strain was defined as 75 % of the maximum growth rate and was calculated with the best fitting polynomial regression using the Dynamic Fit Wizard (SigmaPlot, Systat Software Inc., Düsseldorf, Germany) through all data points where growth was observed. Growth at 12 °C was very slow and cell densities remained low for all tested strains, so sampling of the exponential growth phase at cell densities comparable to the other conditions was missed, and GDA and cell size were only measured in the stationary phase.

2.2.2. Salinity experiment

For the salinity experiment, media with different salinities < 31 were prepared by dilution of North Sea water with deionized and purified water (Milli-Q, Millipore, Eschborn, Germany). Salinities > 31 were obtained by the addition of commercial sea salt (Sea Salts S9883, Sigma Aldrich). Both, deionized water and sea salt were added in advance of the medium-preparation filtration step. Before the start of the experiments, cultures were acclimated at salinities of 5, 10, 15, 20, 30, 35, 40, 45 and 50. Therefore, cultures of the original salinity (25) were first transferred to salinities 20 and 30. After at least three cell divisions, subcultures were further transferred stepwise to the next lower or upper salinity, and this procedure was repeated until subcultures acclimated at all salinities were available. When no growth was detected during the acclimation by cell counts of Lugol-fixed subsamples, these conditions were excluded from the experiments. Salinity was measured with a conductivity meter (SB80PC, VWR sympHony Meters, Hannover, Germany). The experiment was performed in triplicates at 20 °C in K/2 medium at a photon flux density of 80–100 µmol m^{-2} s^{-1} with a starting cell density of 150–200 cells mL^{-1}. Cell densities and size were determined as described above (2.2.1.). Cells for GDA analysis were harvested in the exponential and the stationary growth phase except for strain L4-B1 at salinity 10, of which growth was too slow and cell density too low so that sampling the exponential growth phase was missed and toxin samples were only taken in the stationary phase.

2.2.3. CO_2 experiment

Before starting the main experiment, preliminary tests in triplicates with strain L4-B1 revealed that small-scale turbulences due to aeration of the medium had no significant effect on the growth rate (Figure S1).

Experiments were performed in 300 mL cell culture flasks filled with 250 mL of K/2 medium, set to a salinity of 25, at 20 °C and a photon flux density of 80–100 µmol m^{-2} s^{-1} in a 16:8 h light-dark photocycle. For acclimation, one flask per condition and strain was inoculated with a density of 400 cells mL^{-1} and continuously but slightly aerated,

containing 250 (pre-industrial), 400 (present day), and 1000 (Representative Concentration Pathway 8.5 scenario for the year 2100 (Pachauri et al., 2014)) µatm of CO_2 through autoclaved Pasteur pipettes that were pinched through the lids of the culture flasks. Gas mixtures were prepared from CO_2 free air, mixed with pure CO_2 (Air Liquide, Düsseldorf, Germany). To avoid contamination of the cultures, the gas was sterile filtered (0.45 µm, Acro50 filters, Pall Life Sciences, Dreieich, Germany) and washed in deionized water. The cultures were acclimated at the experimental conditions for at least three cell divisions, while growth was determined every second or third day, by counting Lugol-fixed subsamples under an inverted microscope. Acclimated cultures were subsequently divided into triplicates with cell densities of 400 cells mL^{-1} to start the experiment. Samples for GDA analysis and cells size were taken for both, exponential and stationary growth phase as described for the previous experiments. The pH in the cultures was measured when samples for toxin analysis were taken (EcoScan pH 5, EUtech Scientific Engineering, Aachen, Germany). Furthermore, culture flasks containing only medium (abiotic controls) were aerated at the different conditions for 10 days in triplicates to measure the pH uninfluenced by carbon uptake.

2.3. Goniodomin sampling

2.3.1. Cell harvesting and toxin extraction

In the exponential growth phase and the stationary phase, 20–50 mL of culture were transferred into a 50 mL centrifugation tube and centrifuged at 3220 x g and 10 °C for 10 minutes (model 5810R, Eppendorf, Hamburg, Germany). As GDA has been shown to be predominantly intracellular and is not stable in the alkaline conditions of the culture medium (Harris et al., 2023; Hintze, 2021), the supernatants were discarded and the cell pellets were transferred to 2 mL cryovials (Sarstedt micro tube, Nümbrecht, Germany). After another centrifugation step at 16,000 x g for 5 minutes (Eppendorf 5415), the remaining medium was removed and each tube was filled with 0.9 g lysing matrix D (Thermo-Savant, Illkich, France) and 500 µL of UPLC grade methanol. The cells were lysed by reciprocal shaking in a homogenizer (Fast-Prep-24, MP Biomedicals, Eschwege, Germany) at 6.5 m s^{-1} for 45 s. After another centrifugation step at 10 °C and 16,000 x g, the supernatant was transferred to a spin filter with a pore size of 0.45 µm (Millipore, Eschborn, Germany). The samples were spin filtered for 30 s at 845 x g. The filtrate was transferred to autosampler vials and stored at -20 °C until measurements by LC-MS/MS.

2.3.2. LC-MS/MS conditions

Toxin measurements were performed on an Acquity UPLC system, coupled to a Xevo TQ-XS mass spectrometer (Waters, Eschborn, Germany). For the reverse phase chromatography, a Purospher C18 column (100 × 2.1 mm, 2 µm, Merck, Darmstadt, Germany) heated to 40 °C was used. Elution was performed as a binary gradient with eluent A water and eluent B acetonitrile/water (90:10, v:v), both enriched with 6.7 mM NH_4OH, resulting in a pH of 9.5. Initial conditions were 80 % A kept isocratically for 1.5 min, followed by a gradient to 10 % A within 2 min. Conditions were kept constant for 0.5 min and eluent composition was set to 100 % B for 1 min afterwards. Within 0.3 min a return to initial conditions was performed, followed by 3 min column equilibration. Total run time was 8.3 min at a constant flow rate of 0.6 mL min^{-1} and the injection volume was 0.5 µL per sample.

Mass spectrometric measurements were performed in the positive mode of selected reaction monitoring, selecting the ammonium adduct $[M+NH_4]^+$ of GDA (*m/z* 786>139). Further parameters of the mass spectrometer were: Desolvation temperature: 600 °C, desolvation gas flow: 1000 L h^{-1}, cone gas flow: 150 L h^{-1}, cone voltage: 20 V, source temperature: 150 °C, collision gas flow 0.15 mL min^{-1}, and collision energy 40 eV GDA concentrations were quantified against external standards at concentrations of 41.25, 550 and 825 pg µL^{-1} using the software MassLynx (Version 4.2, Waters)

2.4. Statistical analysis

To compare the means of the three different strains, a two-way ANOVA was performed. For pairwise comparisons, the Holm-Sidak post-hoc test with adapted significance levels was performed. To analyse means of different treatments within one strain, a Kruskal-Wallace one-way ANOVA on ranks with a Student-Newman-Kewls test for pairwise comparisons was used. All statistics were performed with SigmaPlot 11 (Systat Software Inc., Düsseldorf, Germany)

3. Results

3.1. Temperature

3.1.1. Growth

All strains of *A. pseudogonyaulax* grew in a temperature range between 12 °C and 27 °C (Fig. 1) and growth versus temperature plots of all strains showed a similar bell shape optimum curve. No growth was observed during the acclimation at 10 °C, but instead cell density decreased by about 50 % within 11 days (strains L4-B1 and L4-B9) or was nearly constant over the same period (strain L2-D2) (Figure S2 a-c). Overall, temperature had significant effects on growth of all tested strains ($p \leq 0.01$) and the thermal response varied between the strains ($p_{L4-B1/L4-B9} \leq 0.05$, $p_{L4-B1/L2-D2} \leq 0.01$, $p_{L4-B9/L2-D2} \leq 0.001$).

The lowest growth rates for all strains at 12 °C ranged from 0.055 ± 0.022 to 0.068 ± 0.005 d^{-1}. The highest growth rates for strains L4-B1 and L2-D2 were measured at 20 °C with 0.334 ± 0.038 and 0.267 ± 0.020 d^{-1} respectively. Strain L4-B9 had slightly increased growth rates above 20 °C, which reached maximum values of about 0.270 d^{-1} at 25 and 30 °C. For strain L4-B1, growth rates at all temperatures outside the maximum at 20 °C were significantly reduced ($p \leq 0.05$). The optimum temperature range for strain L4-B1 was calculated as 16–24 °C. In contrast, strains L2-D2 and L4-B9 had slightly broader optimum temperature ranges from 15–26 °C and, 18–30 °C respectively. While the cell density of strain L4-B1 decreased rapidly during the acclimation at 30 °C (Figure S2 a), strains L4-B9 and L2-D2 had positive growth rates at 30 °C. However, no growth but a decline in cell density of both strains by about 60 % was observed during the acclimation at 32 °C (Figure S2 b-c).

Temperature did not only alter the growth rates of *A. pseudogonyaulax,* but also the maximum cell density of the cultures. At 12 and 27 °C, densities of strain L4-B1 remained below 1000 cells mL^{-1}, whereas at 15 °C the maximum cell density was 2950 cells mL^{-1} (Table S1). For the strains L4-B9 and L2-D2, differences in cell density were less distinct. Both strains reached about 2000 cells mL^{-1} at

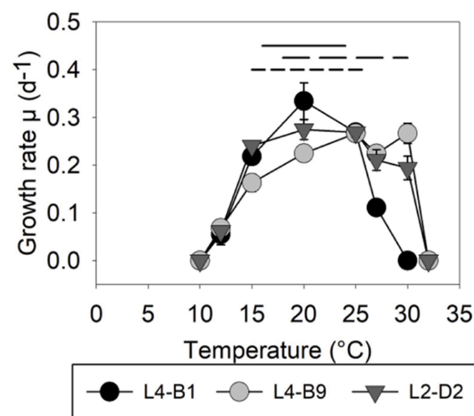

Fig. 1. Growth rates of the three tested strains of *A. pseudogonyaulax* at different temperatures. n=3 ± 1 SD. Horizontal lines show the optimum growth range of the tested strains: solid line=L4-B1, long dashed line=L4-B9, short dashed line=L2-D2.

temperatures above 12 °C and a maximum of 2677 and 4350 cells mL^{-1} at 25 °C for L2-D2 and L4-B9, respectively.

Cell volumes at different temperatures ranged from 13,000 to 47,000 μm^3 (Figure S3 a-c). For all three strains cell volumes significantly increased at 12 °C ($p \leq 0.05$) in the stationary growth phase, but in the exponential growth phase, temperature effects on the cell volumes were minor. A comparison between strains revealed that L4-B9 in the exponential phase had significantly smaller cells compared to the other strains ($p \leq 0.001$).

3.1.2. Goniodomin A

Goniodomin A was detected in all strains at all temperatures. In the exponential growth phase, GDA cell quotas ranged from 6.78 ± 2.13 to 13.84 ± 2.12 pg $cell^{-1}$ at 15 °C for all strains (Fig. 2a). In this growth phase there were significantly higher cell quotas at low temperatures for strain L4-B9 ($p \leq 0.01$) but not for strains L4-B1 and L2-D2. The lowest detected cell quotas were measured at 30 °C with 1.45 ± 0.22 and 0.04 ± 0.01 pg $cell^{-1}$ for strain L4-B9 and L2-D2 respectively and at 27 °C for strain L4-B1 (3.94 ± 1.05 pg $cell^{-1}$). In the stationary growth phase, the highest GDA cell quotas for strains L4-B1 and L2-D2 were measured at 12 °C with 10.66 ± 2.16 and 17.75 ± 2.04 pg $cell^{-1}$, respectively. Strain L4-B9 had the highest GDA cell quota at 15 °C with 15.49 ± 2.80 pg $cell^{-1}$ (Fig. 2b). At higher temperatures the GDA cell quotas decreased significantly in all strains ($p \leq 0.05$). A comparison of the three strains showed that L4-B1 had significantly lower GDA cell quotas at 12 and 15 °C than the other two strains in the stationary growth phase ($p \leq 0.05$). However, no consistent trend to higher GDA cell quotas in the stationary growth phase compared to the exponential growth phase was detected ($p > 0.05$)

To consider differences in cell volume, GDA cell quotas were also normalized to 1000 μm^3 of cell volume (Fig. 2c, d). While temperature had only minor effects on the normalized GDA cell quota of strain L4-B1, strains L4-B9 and L2-D2 had significantly increased GDA cell quota at 15 °C compared to higher temperatures in the stationary growth phase ($p \leq 0.01$).

3.2. Salinity experiment

3.2.1. Growth

During the salinity experiment, growth was observed for all strains between salinity 10 and 40 (Fig. 3). No growth, but a constant decline in cell density in all strains was observed during the acclimation at salinity 5 (Figure S4 a-c). While no growth was detected for L4-B1 during the acclimation at salinity of 45, the two other strains still showed growth rates close to their detected maximum. At an acclimation at salinity 50 however, a decline in cell density was observed (Figure S4 b-c).

The lowest growth rates were detected at a salinity of 10, ranging from 0.111 ± 0.023 to 0.187 ± 0.006 d^{-1} for all strains. The highest growth rate of strain L4-B9 was measured at a salinity of 35 with 0.332 ± 0.025 d^{-1}, while strains L4-B1 and L2-D2 showed the fastest growth at salinity 25 with 0.334 ± 0.038 and 0.275 ± 0.021 d^{-1}, respectively. Strain L2-D2 had a broad optimum salinity range from 10 to 45 even though significant differences in growth rates within this range were detected. In contrast, growth rates of strain L4-B1 decreased significantly at salinities lower and higher than 25 ($p \leq 0.05$). The optimum salinity range of strain L4-B1 was determined to range from salinities 19 to 26. For L4-B9, the optimum range shifted to higher salinities, ranging from 15 to 45.

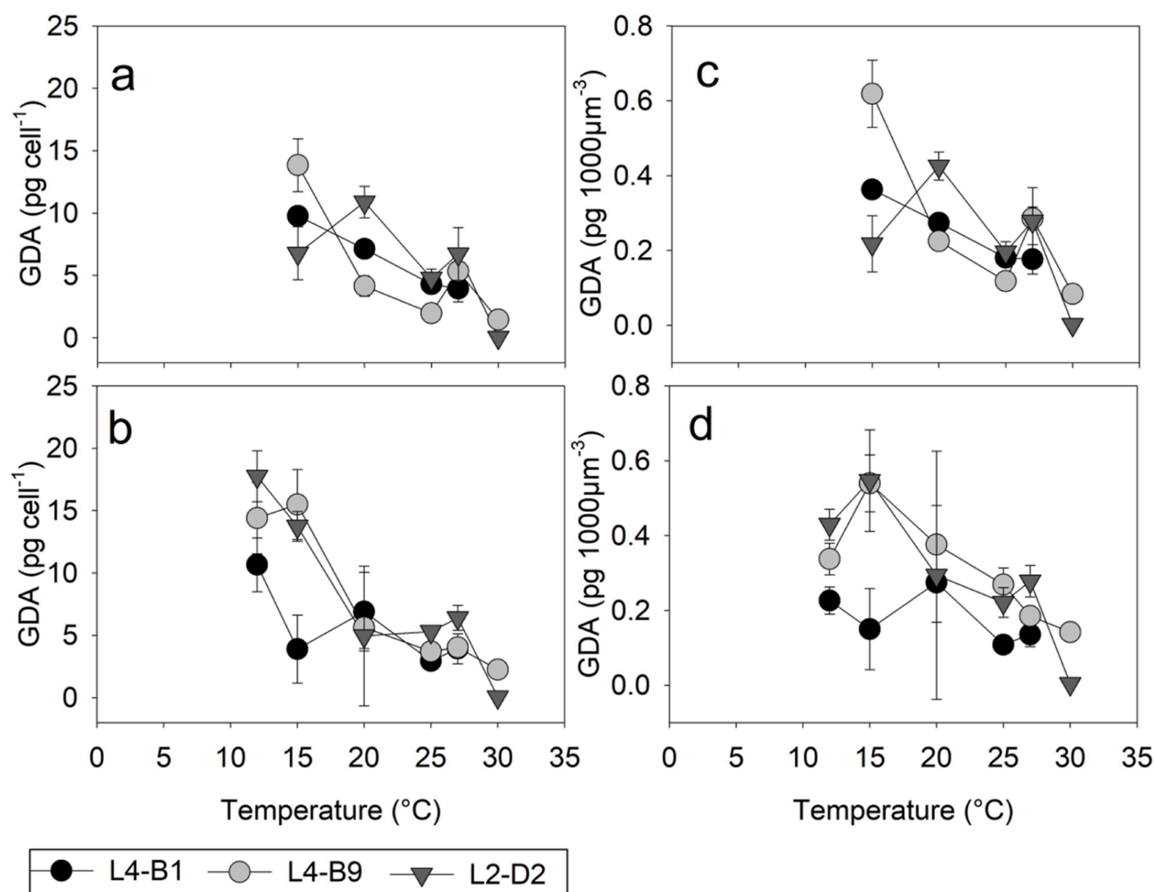

Fig. 2. Goniodomin A cell quotas (a, b) and GDA normalized to cell volume (c, d) of *A. pseudogonyaulax* at different temperatures. a, c=exponential growth phase, b, d=stationary growth phase. At 12 °C samples for toxin analysis were only taken in the stationary growth phase. n=3, ± 1 SD.

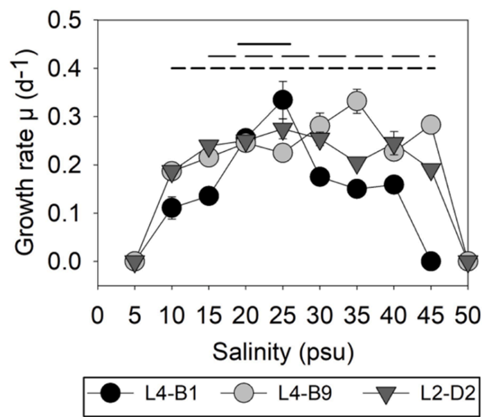

Fig. 3. Growth rates of the three tested strains of *A. pseudogonyaulax* at different salinities. n=3 ± 1 SD. Horizontal lines show the optimum growth range of the tested strains: solid line=L4-B1, long dashed line=L4-B9, short dashed line=L2-D2.

A comparison of the three strains under different salinity treatments revealed significant differences of the growth of strain L4-B1 compared to the remaining strains ($p_{L4\text{-}B1/L4\text{-}B9} \leq 0.001$, $p_{L4\text{-}B1/L2\text{-}D2} \leq 0.001$), but no significance was detected in the comparison of L4-B9 and L2-D2 ($p>0.05$).

Besides growth, salinity also influenced the maximum cell densities (Table S2). Those ranged from 321 to 3933 cells ml^{-1}, with lower values at salinities 10 and 45. While changes in maximum cell density of strain L4-B9 were only minor in a range between salinity 10 and 35, strain L4-B1 had its highest densities at salinities 20 and 25, and L2-D2 had the highest cell densities at salinity 25 to 35.

Salinity had only minor effects on the cell volumes of *A. pseudogonyaulax*. Cell volumes ranged from 16,000 to 31,000 μm^3 in the exponential growth phase and from 13,000 to 28,000 μm^3 in the stationary growth phase (Figure S3 d-f). Cells of the exponential growth phase were overall bigger. For strain L4-B9 differences between the growth phases were significant at all conditions. For strain L2-D2, cell volumes significantly decreased at salinities above 25 in the exponential growth phase. In both growth phases, cells of strain L4-B9 were significantly smaller than cells of the two remaining strains ($p \leq 0.001$).

3.2.2. Goniodomin A

GDA was detected in all strains at all salinities. Similar patterns with lower GDA cell quotas at salinities of 25 and 30 were found for strains L4-B1 and L4-B9, with GDA cell quotas ranging from 3.8 to 12.9 pg $cell^{-1}$ in the exponential growth phase, (Fig. 4a). For both strains, salinity induced changes in exponential phase GDA cell quotas were tested significant ($p \leq 0.01$). For strain L2-D2, GDA cell quotas in exponential growth increased significantly from 5.0 pg $cell^{-1}$ at salinity 10 to 10.9 pg $cell^{-1}$ at salinity 25 (Fig. 4a), and this general pattern was also present for this strain in stationary phase (Fig. 4b). At higher salinities, the GDA cell quota decreased 20-fold to a minimum of 0.29 ± 0.05 and 0.50 ± 0.02 pg $cell^{-1}$ in the exponential growth phase and the stationary growth phase, respectively. In contrast to strain L2-D2, strain L4-B1 showed a significant accumulation of GDA at high salinities in the stationary growth phase with a maximum of 27.22 ± 3.45 pg $cell^{-1}$ at salinity 40 ($p \leq 0.01$). Strain L4-B9 also showed a trend to higher GDA cell quotas at high salinities in the stationary growth phase, but was subject of strong variations.

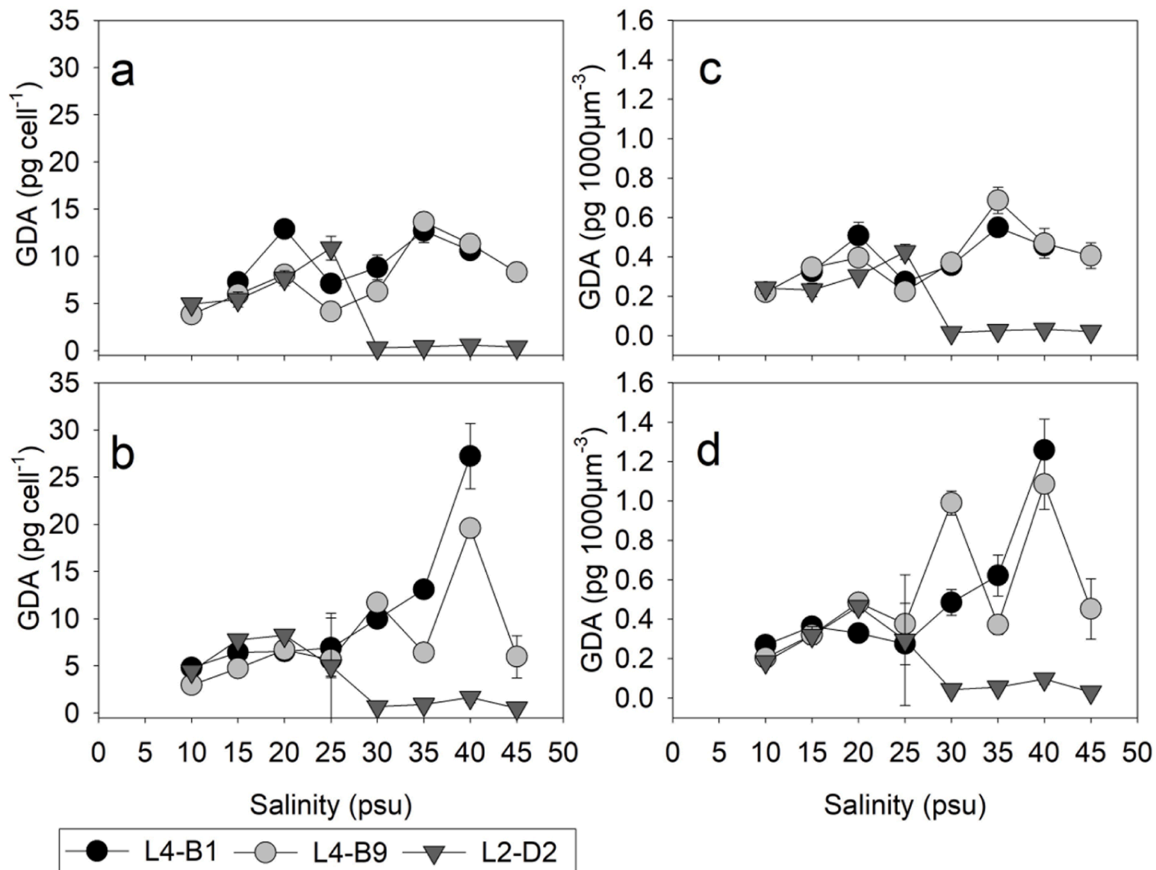

Fig. 4. Goniodomin A cell quotas (a, b) and GDA normalized to cell volume (c, d) of *A. pseudogonyaulax* at different salinities. a, c=exponential growth phase, b, d=stationary growth phase. n=3, ± 1 SD.

A strain comparison revealed a significant difference of the GDA cell quotas at different salinities between L2-D2 and the two other strains for both growth phases ($p<0.001$).

As salinity only had minor effects on cell volume, a normalization of GDA to 1000 μm^3 of cell volume revealed similar patterns (Fig. 4c, d).

3.3. CO_2 experiment

The pH measured in the cultures in the exponential growth phase was 8.21 ± 0.08, 8.14 ± 0.09 and 7.79 ± 0.05 at 250, 400 and 1000 µatm CO_2 respectively, and no significant differences compared to the abiotic controls (8.22 ± 0.02, 8.11 ± 0.02 and 7.85 ± 0.03) were detected. In the stationary phase, the pH slightly increased to 8.44 ± 0.08, 8.34 ± 0.08 and 7.85 ± 0.06 at 250, 400 and 1000 µatm CO_2 respectively and differed significantly from the exponential growth phase and the abiotic controls ($p\leq0.001$).

3.3.1. Growth

Growth rates at different CO_2 conditions ranged from 0.19 ± 0.07 to 0.26 ± 0.003 d^{-1} (Fig. 5), but no significant effects between the treatments or between the strains were detected ($p>0.05$). Maximum cell densities reached 4800 cells mL^{-1} for strain L4-B1, 6200 cells mL^{-1} for strain L2-D2, and 7600 cells mL^{-1} for strain L4-B9 (Table S3, Figure S5). Differences in maximum cell density between strains were tested significant ($p\leq0.05$), but the different CO_2 concentrations had no significant effect.

Cell volumes ranged from 13,000 to 27,000 μm^3 in the exponential growth phase and from 20,000 to nearly 35,000 μm^3 in the stationary phase (Figure S6 a-c). Cells in the stationary phase had significantly higher volumes than cells in the exponential growth phase for each tested strain under each condition ($p\leq0.05$). However, CO_2 concentrations had no effect on cell volumes. In contrast to the temperature and salinity experiments, cells of the strain L2-D2 were significantly smaller than cells of strains L4-B1 and L4-B9 in both growth phases ($p\leq0.01$).

3.3.2. Goniodomin A content

GDA was detected in all strains at all different concentrations of CO_2. Cell quotas in the exponential growth phase ranged from 7.2 ± 0.4 to 8.2 ± 0.5 pg $cell^{-1}$ for strains L4-B1 and L4-B9 (Fig. 6a), but no significant differences between treatments or strains were detected. Strain L2-D2 had significantly lower GDA cell quotas in the exponential growth phase than the other strains ($p\leq0.001$), ranging from 0.09 ± 0.02 to 0.17 ± 0.11 pg $cell^{-1}$. In the stationary growth phase all strains accumulated GDA at different conditions, reaching 13.7 ± 1.3 to 19.3 ± 1.7 pg $cell^{-1}$, 18.4 ± 4.0 to 25.2 ± 1.9 pg $cell^{-1}$ and 0.45 ± 0.23 to1.2 ± 0.5 pg $cell^{-1}$ for strains L4-B1, L4-B9 and L2-D2 respectively (Fig. 6b). The

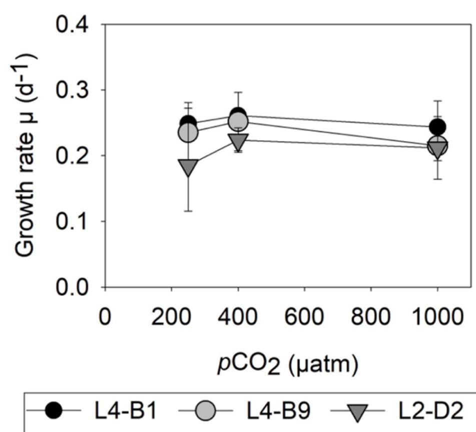

Fig. 5. Growth rates of the three tested strains of *A. pseudogonyaulax* at different CO_2 concentrations. n=3 ± 1 SD.

accumulation of GDA in the stationary growth phase was tested significant for all strains at all conditions ($p\leq0.001$). Furthermore, the accumulation of GDA in the stationary phase was significantly higher in strain L4-B9, than in strain L4-B1 ($p\leq0.05$). Compared with GDA cell quotas from the 20 °C treatment of the temperature experiment, only minor differences in the exponential growth phase were detected but in the stationary growth phase, GDA cell quotas of strains L4-B1 and L4-B9 were up to 3-fold higher. Normalized to 1000 μm^3 of cell volume, the accumulation of GDA in the stationary growth phase was less distinct, but differences were still tested significant ($p\leq0.05$) (Fig. 6c, d).

To test if the low GDA cell quota of strain L2-D2 was due to a generally reduced GDA production over time, or if the low cell quotas were a result of the experimental conditions, a follow- up experiment was performed, where triplicate cultures were grown with and without aeration by compressed air. The results show that small scale turbulences led to a decrease in GDA production by about 90 % (Figure S7).

4. Discussion

4.1. The autecology of A. pseudogonyaulax from the Limfjord

4.1.1. Growth responses

The wide latitudinal distribution of *A. pseudogonyaulax* from the Mediterranean Sea to northern Europe is an indicator for a tolerance to a wide range of temperatures of this species. With positive growth rates between 12 and 30 °C for two of the tested strains and an upper limit of 27 °C for the third strain, the *A. pseudogonyaulax* population of the Limfjord is indeed well adapted to a wide range of temperatures, with significant overall differences between the strains in growth rate, but also in cell volume. While strain L4-B1 had the highest measured growth rate, it also had the narrowest optimum range. Intraspecific differences within a population is an important characteristic, as it increases the total niche width of the population (Bolnick et al., 2003), and also stabilizes it (Łomnicki and Sedziwy, 1989), and our results show variability within the Limfjord population. Similar results have been reported for *Alexandrium ostenfeldii* from Finland and Chile (Salgado et al., 2015), but also for *Protoceratium reticulatum* isolated from arctic, temperate, and subtropical areas (Wang et al., 2019). However, only one study on the effect of temperature on growth of *A. pseudogonyaulax,* isolated from Japan, has been published. In that study, growth was detected at 15, 20 and 25 °C, but no further temperatures were tested (Lim et al., 2007). It would therefore be beneficial to record full growth versus temperature curves using strains from warmer regions like the Mexican Pacific or Mediterranean lagoons. A comparison with other *Alexandrium* species from temperate regions, such as *A. fundyense* (Etheridge and Roesler, 2005) and *A. catenella* (Navarro et al., 2006), show that both cannot tolerate temperatures much warmer than 20 °C, while *A. pseudogonyaulax* from the Limfjord still grew at temperatures that are nowadays rarely reached in northern Europe.

Beside temperature, salinity is another factor affecting growth. In this study, two of the tested strains showed relatively constant growth at all tested salinities, and only minor shifts in cell size, suggesting a high capability of osmoregulation, and no uncontrolled in- or outflow of water. Furthermore, intraspecific variability in cell volumes was detected, as strain L4-B9 had smaller cells compared to the other strains at all conditions. No growth was observed at salinity 5, what has been described as a critical salinity, causing hyperosmotic stress to freshwater phytoplankton and hypoosmotic stress marine phytoplankton species (Flöder et al., 2010). Similar findings have been reported from an *A. pseudogonyaulax* isolate from Japan where growth was detected at salinities between 10 and 30, but no upper limit was tested and no growth was observed at salinity 5 (Lim et al., 2007). Studies on an *A. pseudogonyaulax* strain from a temporarily hypersaline Mediterranean lagoon (Gaaloul et al., 2022) also reported growth over a salinity range from 20 to 37, but neither lower nor upper limits were tested (Zmerli Triki et al., 2015). As no complete growth versus salinity curves of

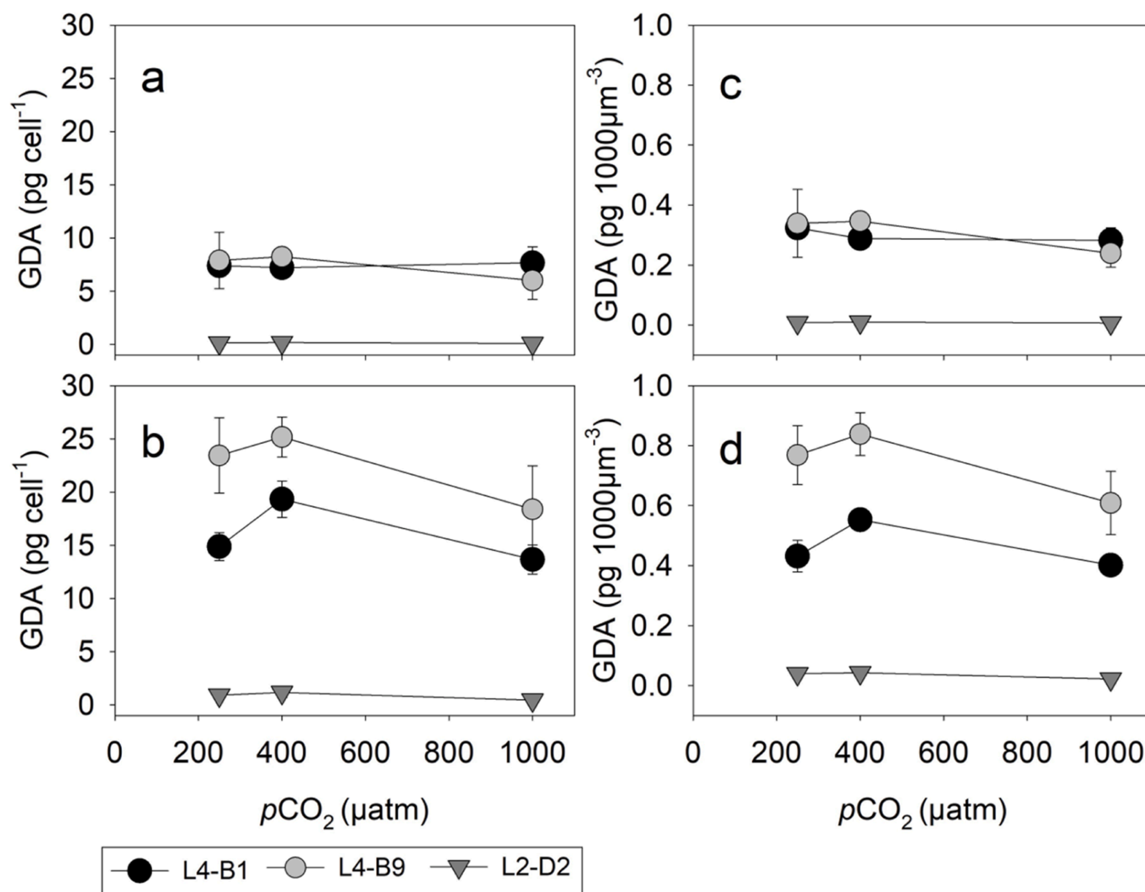

Fig. 6. Goniodomin A cell quotas (a, b) and GDA normalized to cell volume (c, d) of *A. pseudogonyaulax* at different CO_2 concentrations. a, c=exponential growth phase, b, d=stationary growth phase. n=3, ± 1 SD.

A. pseudogonyaulax exist, it is not possible to estimate potential population differences that have been described for other species, in particular for *A. ostenfeldii*, that is adapted to salinities as low as 3 in the northern Baltic Sea, while strains isolated from the North Sea showed no growth at salinities below 20 (Kremp et al., 2009; Suikkanen et al., 2013).

In contrast to temperature and salinity, varying CO_2 concentrations had no effect on the growth rates of all tested strains from the Limfjord and this has also been observed for one strain of *A. catenella* (reported as *A. tamarense*) (Van De Waal et al., 2014). Yet, cell volumes were higher in the stationary phase compared to cultures without aeration. However, while aerated, strain L2-D2 had overall smaller cells compared to the other strains, which is a contrast to the previous experiments at different temperatures and salinities. Generally, cultures that were exposed to higher pCO_2 in the experiment grew denser than cultures in closed culture flasks. Maximum cell densities of *A. pseudogonyaulax* are described as very low in comparison to other *Alexandrium* species e.g. *A. affine* and *A. catenella* (Aguilera-Belmonte et al., 2011; Nguyen-Ngoc, 2004), with densities not always reaching 3000 cells mL^{-1} (Montresor and Marino, 1996; Zmerli Triki et al., 2015), and these findings are in line with the results from the temperature and salinity experiments. Cultures grown under higher pCO_2, however, always reached 4000 cells mL^{-1} and in most cases over 6000 cells mL^{-1}. These findings suggest that cultures without additional CO_2 supply experience carbon limitation and enter the stationary growth phase earlier.

4.1.2. Goniodomin cell quota

Bottom-up factors did not only influence growth dynamics, they also have significant influence on the GDA cell quotas of *A. pseudogonyaulax*. Different studies have reported that temperature and salinity may lead to changing toxin quota up to 5-fold in STX producing *Alexandrium* species (Eckford-Soper et al., 2016; Etheridge and Roesler, 2005; Navarro et al., 2006), and in the present study, GDA cell quotas of strain L2-D2 changed even >400-fold, considering all tested conditions and growth phases. These findings emphasize that bottom-up factors can amplify or weaken the toxicity of HABs.

During the temperature experiment, low temperatures led to high toxin quotas in all tested strains, as it was also seen for the yessotoxin (YTX) producing species *Protoceratium reticulatum*, and it is suggested that toxins accumulate due to shifts in the ratio of growth rate to toxin production (Sala-Pérez et al., 2016). In contrast to temperature, the effects of salinity on the GDA cell quotas of *A. pseudogonyaulax* are less clear. Cell quotas of strains L4-B1 and L4-B9 were subject to small variations at all salinities in the exponential growth phase, with usually lower cell quotas at high growth rates. Similar findings have been published for an *A. ostenfeldii* strain from Canada, and it is suggested that salinity has no effect on toxin production, but differences are assumed to be a result of changing growth rates (Maclean et al., 2003). While in the stationary growth phase, GDA cell quotas of strain L4-B9 were subject to strong variations at different salinities, strain L4-B1 showed a clear accumulation of GDA at high salinities. An accumulation of toxins in the early stationary phase when cell division significantly slows down has also been described for *A. minutum* (Grzebyk et al., 2003; Hwang and Lu, 2000) and *A. catenella* (reported as *A. tamarense*) (Parkhill and Cembella, 1999). In the present study, however, such an accumulation of GDA was only detected at high salinities. Remarkably, the GDA cell quotas of strain L2-D2 decreased 20-fold at salinities of 30 or higher in both growth phases. Beside the GDA cell quota, the cell volumes also decreased at salinities of 30 or higher. If high salinities led to a decreased GDA production, or if the low cell quotas of strain L2-D2 to a certain

extend are a result of osmotic stress leading to a leakage of toxins remains unclear, as no extracellular toxins were measured. In any case, recent studies have shown that the major part of GDA is intracellular (Hintze, 2021), and that extracellular GDA is rapidly transformed to GDA-seco acid in the surrounding medium (Harris et al., 2023). Moreover, more recent studies, applying purified and dissolved GDA and GDA-seco acid suggest that GDA and GDA-seco acid have no harmful effect on fish gills (Möller et al., 2024b) or other phytoplankton species (Gaillard et al., 2024).

Reduced GDA cell quotas of strain L2-D2 compared to the other strains have also been detected when cultures were aerated with different CO_2 concentrations. These results may suggest that for this strain, stress caused by factors such as high salinities, small scale turbulences, and high temperatures lead to a significant decrease of GDA cell quota, which is accompanied with a reduced cell size. Whereas varying CO_2 concentrations had no detectable influence on growth and GDA cell quota of all three strains of *A. pseudogonyaulax*, a study on *A. catenella* isolated in southern California revealed that high CO_2 concentrations can lead to higher cell quotas of saxitoxin and analogues (Tatters et al., 2013), and it is suggested that the surplus carbon is available and utilized for toxin production. In the present study, however, no increasing GDA cell quotas in the exponential growth phase between the CO_2 treatments were detected, but a strong accumulation of GDA in the stationary growth phase was observed leading to GDA cell quotas being significantly higher than in cultures in the temperature and salinity experiment. Altogether, our findings suggest that, in contrast to *A. catenella* (Tatters et al. 2013), available carbon during exponential growth is primarily used to produce biomass, and GDA only accumulates when cell division is reduced in the stationary growth phase. The utilization of available carbon for growth instead of toxin production has also been reported for an *A. ostenfeldii* strain from New Zealand (Beuzenberg et al., 2012).

No clear correlation between temperature, salinity or CO_2 concentration and GDA cell quotas was detected in the present study, suggesting that cell quotas are influenced by multiple parameters. A related study on the same strains of *A. pseudogonyaulax* reported that light intensity and nitrogen source had minor effect on the GDA cell quotas which in turn were mainly influenced by the growth phase (Möller et al., 2024a).

4.2. Ecological implications

4.2.1. Alexandrium pseudogonyaulax in the Limfjord

Whether *A. pseudogonyaulax* has been a background species in the Limfjord for a long time, or whether it is a new species in this area remains unclear, but during the last two decades, a shift from the two formerly dominant species *A. ostenfeldii* and *A. catenella* to *Alexandrium pseudogonyaulax* was described (Kremp et al. 2019). No data on the autecology of other strains of *A. pseudogonyaulax* from northern Europe are available, but a comparison with *A. ostenfeldii* from the Limfjord (Jensen and Moestrup, 1997) show that both species have a similar tolerance to low temperatures, but the growth of *A. ostenfeldii* was observed to already decrease above 23 °C and to stop at 26 °C. A more recent study on *A. ostenfeldii* from the Limfjord showed a significantly decreased growth rate at a temperature of 22 °C compared to 16 °C (Medhioub et al., 2011). Compared to the findings of the present study these results show again that the *A. pseudogonyaulax* population from the Limfjord is well adapted to high temperatures and thus to climate change. It is therefore reasonable to assume that conditions in the Limfjord that regularly reach temperatures above 20 °C (Maar et al., 2010), at some point became unfavourable for *A. ostenfeldii*, which in turn may have opened an ecological niche for more temperature tolerant *A. pseudogonyaulax*.

4.2.2. Alexandrium pseudogonyaulax in the Baltic Sea

The three tested strains of *A. pseudogonyaulax* grow well at low salinities. However, without detailed information about the lower salinity tolerance of *A. pseudogonyaulax* from regions that are characterized by higher salinities, it is impossible to evaluate if the Limfjord population is already adapted to low salinities, or if this is a general species characteristic.

A. pseudogonyaulax is already established in the Limfjord (Kremp et al., 2019), and the spreading into the Baltic Sea is seemingly still ongoing (Wasmund et al., 2017; Zettler et al., 2020). With respect to salinities, the Baltic Sea can be separated into three regions: 1. Salinities of 10 and higher, where growth of *A. pseudogonyaulax* was detected during the experiments, 2. Salinities between 5 and 10 where growth might be possible, 3. Salinities below 5 where none of the Limfjord strains could grow (Fig. 7). Consequently, the very low salinities of 5 and lower (Feistel et al., 2010) of the eastern and north-eastern Baltic Sea may act as a natural barrier for a further spreading of *A. pseudogonyaulax*. While the data in Fig. 7 only show the salinities at a certain time point in September 2022, the overall pictures of salinity distribution based on a 5-year mean (Jaspers rt al., 2021) or the 60- year mean for the month of September (Bäck et al., 2019), are similar. However, as the salinity of the Baltic Sea is suggested to decrease within the next decades due to increased freshwater inflow as a result of climate change (Andersson et al., 2015; Neumann, 2010), the expansion range of *A. pseudogonyaulax* may decrease as well. Furthermore, short term fluctuations of salinity caused by heavy rainfall and river discharge, especially in coastal and estuary regions (Moeller and Hansen, 1994), might also shape the expansion range and velocity of *A. pseudogonyaulax*. For example, it has been described that heavy rainfall terminated an *A. ostenfeldii* bloom in a brackish creek in the Netherlands (Brandenburg et al., 2017). However, such effects of extreme events or rapid salinity shifts on *A. pseudogonyaulax* were not tested in the present study.

The low saline areas of the eastern and northern Baltic Sea host various dinoflagellate species i.e. *Peridiniella catenata* (Gromisz and Witek, 2001), *Dinophysis* spp., *Protoperidinium* spp. (Hällfors et al., 2013), and *Alexandrium ostenfeldii* (Hakanen et al., 2012). These species are clearly adapted to the low salinities of the Baltic Sea and are regularly contributors to blooming events (Hakanen et al., 2012; Spilling et al., 2006) and *A. ostenfeldii*, for example, has been suggested to be separated from all other *A. ostenfeldii* populations since the formation of the Baltic Sea (Tahvanainen et al., 2012). On the other hand, the Baltic Sea is connected to the North Sea, and thus migrations of euryhaline phytoplankton species into the Baltic Sea is probably a normal process. A well-studied example is *Prorocentrum cordatum*, which was first detected in the Skagerrak area in 1979 and reached the northern Baltic Sea and the Gulf of Gdansk with salinities as low as 3 in the 1990s, while an isolate from the Kattegat only had reduced growth at low salinities (Hajdu et al., 2000). Likewise, it is possible that *A. pseudogonyaulax* can spread further into the Baltic Sea. However, it must always be taken into account that spreading is not determined solely by the acclimation and adaptability of *A. pseudogonyaulax*, but to a large extent by competition and interactions with the complex assemblage of other species in the plankton community. In any case, during a field campaign in late summer of 2022, LC-MS/MS based toxin analyses from vertical plankton net tows revealed low GDA levels also in areas with a salinity below 10 (own unpublished results, Fig. 7), and in summer 2019, high densities of 3.3×10^4 cells L^{-1} of *A. pseudogonyaulax* were detected in the western Baltic Sea (Zettler et al., 2020), but unfortunately, no toxin data are available from this study. This cell density is the highest that was described in northern Europe for *A. pseudogonyaulax,* being one order of magnitude higher than cell densities in the Limfjord (Kremp et al., 2019). The salinity of about 8 that was described for this area was not tested during the salinity experiment, but no growth was detected at a salinity of 5 and only low growth rates at 10 indicating that salinities below 10 do not favour *A. pseudogonyaulax* bloom formation. To test if *A. pseudogonyaulax* from this area is already adapted to lower salinities on a population level, further growth experiments with strains from the

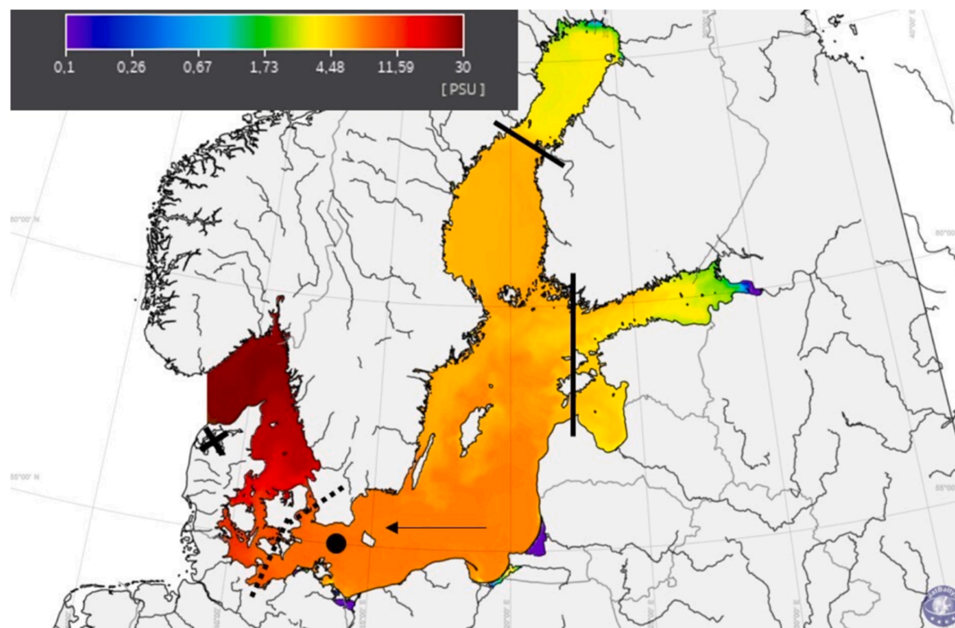

Fig. 7. Sea surface salinity of the Baltic Sea in Sep. 2022. Data from the project Satbaltyk (satbaltyk.pl). Solid lines bound areas in the eastern and northern Baltic Sea with salinities < 5 that has been described as a critical salinity (Flöder et al., 2010) and where no growth was detected during the salinity experiment. Dotted line shows the border at a salinity of 10, where growth was still detected in laboratory experiments. X marks the Limfjord, the arrow points to a sampling station with a salinity of about 7.5, where GDA was detected in Sep. 2022 (unpublished). The dot shows an area where elevated *A. pseudogonyaulax* densities were detected in 2019 (Zettler et al., 2020).

Baltic Sea are necessary. Furthermore, studies on other species of the genus *Alexandrium* have shown that the salinity tolerance might be influenced by the surrounding temperature (Bill et al., 2016; Lim et al., 2007), and multifactorial experiments and also multi-species experiments will help to better understand the ecology of *A. pseudogonyaulax*.

5. Conclusions

The results of this study revealed the high tolerance of the *A. pseudogonyaulax* population of the Limfjord to a wide range of temperatures and salinities. While the high temperature tolerance might have favoured a species shift from *A. catenella* and *A. ostenfeldii* to *A. pseudogonyaulax* in the Limfjord, the ability to grow in a broad range of salinities suggest a potential for a further spreading in northern Europe. While different CO_2 concentrations had no influence on the GDA cell quotas of the three tested strains, temperature and salinity had variable, most intriguing in the contrary responds of strain L4-B9 and L2-D2 at high salinities. These results highlight intraspecific variability within the Limfjord population and point out the importance of multiple strain experiments.

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CRediT authorship contribution statement

Simon Tulatz: Conceptualization, Investigation, Writing – original draft. **Bernd Krock:** Conceptualization, Investigation, Writing – review & editing. **Urban Tillmann:** Conceptualization, Investigation, Writing – review & editing. **Cédric Leo Meunier:** Conceptualization, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

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