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Salinity, a climate-change factor affecting growth, domoic acid and isodomoic acid C content in the diatom *Pseudo-nitzschia seriata* (Bacillariophyceae)

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ABSTRACT

Freshening of the oceans is a predicted outcome of climate change. Marine phytoplankton organisms are in general affected by salinity changes and, given their key role in oceanic food webs and geochemical cycles, it is important to investigate the response of phytoplankton species to salinity changes. Diatom species of the genus *Pseudo-nitzschia* can form massive and, at times, toxic blooms, because several *Pseudo-nitzschia* species produce the neurotoxin domoic acid. Domoic acid can cause amnesic shellfish poisoning in humans and harm animals in the marine food web. The species *Pseudo-nitzschia seriata* can produce domoic acid in cold-water areas, like the Arctic. Hence, it is relevant to investigate the response of *P. seriata* to different salinity levels. Three strains of *P. seriata* were exposed to four different salinity levels (15, 20, 30 and 40). None of the strains grew at salinity 15, and maximum growth rates were found at salinity 30. All three strains contained toxins at salinities 20–40, with the highest cellular content occurring at salinity 20. The peak in toxin content was related to a significantly lower growth rate. However, the higher toxin content overrode the lower growth rate, ultimately resulting in a higher toxin potential at salinity 20. In addition to domoic acid, all strains contained isodomoic acid C in surprisingly high amounts, similar to the domoic acid content.

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INTRODUCTION

Phytoplankton's primary production accounts for approximately 50% of the global carbon fixation, and therefore understanding the effect of climate changes on phytoplankton in the world's oceans is important (Baumert & Petzoldt 2008). Phytoplankton organisms make up the basis of the marine food web and play a critical role in the carbon pump, transporting carbon to the ocean sediment and thereby maintaining the ocean as a carbon sink (Käse & Geuer 2018). With their siliceous frustules, diatoms are furthermore a crucial part of the silicon cycle. Silicon enters the ocean mainly through river run-off and seafloor weathering (Struyf *et al.* 2009). Due to their frustules, diatoms are some of the densest phytoplankton groups and have a higher sinking velocity than other phytoplankton groups (Yool & Tyrrell 2003). Hence, diatoms transport nutrients, carbon and silicon to the ocean sediments (Armbrust 2009).

The effect of climate changes on the phytoplankton community is a concern due to the fundamental role of phytoplankton in the ecosystem (Brander *et al.* 2016). This is particularly relevant in Arctic communities, where massive short-term spring blooms transfer considerable amounts of energy to the upper levels of the food web. In Arctic waters, changes in phytoplankton abundance and timing of blooms may lead to mismatches between phytoplankton and grazers. One outcome could be that smaller, less lipid-rich copepod species will be dominant, and less energy will be transferred up the food web (Søreide *et al.* 2010; Kjellerup *et al.* 2012; Brander *et al.* 2016). The composition of the phytoplankton community is therefore important for the entire Arctic ecosystem. The Intergovernmental Panel on Climate Change predicts that climate changes are going to be particularly extreme in the Arctic regions, a phenomenon called Arctic amplification (Coumou *et al.* 2018; Shu *et al.* 2018; Meredith *et al.* 2019).

Freshening of the Arctic Oceans

The salinity of the Arctic oceans is predicted to decline due to climate changes, with potentially large salinity fluctuations locally and regionally (Li *et al.* 2009; Sejr *et al.* 2017). Increasing temperature in the atmosphere and the ocean is expected to result in melting of land and sea ice and thus directly cause polar salinity fluctuations (Käse & Geuer 2018; Shu *et al.* 2018). As a result of increasing concentrations of atmospheric greenhouse gases, the temperature in the Arctic will increase and the albedo effect of the Arctic decrease as snow and ice cover melts. This will result in a feedback effect with further warming of the Arctic (Winton 2006). Even though there are studies that suggest climate change may lead to increased salinity due to increased evaporation of seawater mainly in subtropical regions (Durack *et al.* 2012), for high precipitation and low evaporation areas such as the polar regions a general pattern of decreasing salinity has been

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suggested (Boyer 2005; Hosoda *et al.* 2009). Climatic changes may also lead to altering of weather patterns and increased precipitation (Nummelin *et al.* 2016) resulting in additional freshwater input to the oceans due to increased runoff from land. Accordingly, freshening of the upper Arctic waters has already been recorded confirming the hypothesized climate-change-induced freshening of the polar oceans (Comeau *et al.* 2011; Straneo *et al.* 2011).

The freshening will inevitably lead to strengthened stratification and less mixing of the nutrient-rich bottom water with the surface water (Li *et al.* 2009), followed by changes in the seasonal cycles and abundance of phytoplankton (Greene & Pershing 2007). The resulting nutrient limitation may affect the magnitude and abundance of algal blooms (Winder & Sommer 2012). On the other hand, an increased runoff from land may cause nutrient enrichment, promoting algal blooms (Zhou *et al.* 2008).

Pseudo-nitzschia seriata – a toxic cold-water diatom

Pseudo-nitzschia species are globally distributed diatoms, particularly abundant in coastal areas where they form harmful blooms (Doucette *et al.* 2008). *Pseudo-nitzschia seriata* (Cleve) H. Peragallo is commonly found in Arctic, Subarctic, and (Northern) temperate waters (Fehling *et al.* 2004; Hansen *et al.* 2011; Tammilehto *et al.* 2012; Harðardóttir *et al.* 2015; Stonik & Orlova 2018; Olesen *et al.* 2020). Reports of *P. seriata* from warmer regions such as Singapore and the Black Sea need to be confirmed (Terenko & Terenko 2012; Tan *et al.* 2016). Presently, 26 *Pseudo-nitzschia* and two *Nitzschia* species are known to produce the neurotoxin domoic acid (DA), with *P. seriata* being one of them (Lundholm & Moestrup 2000; Smida *et al.* 2014; Bates *et al.* 2018). Toxic species may comprise strains that are non-toxic (Bates *et al.* 1998), even strains isolated from the same location as toxic strains (Li *et al.* 2017). However, little is known about strain diversity in toxicity and growth in response to environmental parameters. Grazers, such as zooplankton, mussels, plankton-eating fish, and other filter feeders, ingest toxic diatoms when they are present in the water column, which results in DA accumulation in the body tissues and transfer of the toxin to higher trophic levels. Accumulated DA can lead to neurological disorders and death of marine mammals and seabirds, as well as amnesic shellfish poisoning (ASP) in humans (Trainer *et al.* 2012; Bates *et al.* 2018).

Apart from domoic acid, several isomers of domoic acid (DA-I) are known, mainly from shellfish (DA-IA, -IB, -IC, -ID, -IE, -IF, -IG, and -IH; Lelong *et al.* 2012). However, in *Pseudo-nitzschia* IA and IB have only been found in *P. seriata* (Hansen *et al.* 2011), and IC only in *P. australis* Frenguelli (Holland *et al.* 2005) and *P. subcurvata* (Hasle) G.A. Fryxell (Olesen *et al.* 2021). It has been proposed that isomers of DA are less potent than DA itself (Holland *et al.* 2005; Munday *et al.* 2008).

Pseudo-nitzschia species are frequent in Arctic waters and sea ice (Poulin *et al.* 2011), but knowledge of their diversity, toxicity, and the intensity of blooms in the Arctic is surprisingly restricted (Lewitus *et al.* 2012; Matsuno *et al.* 2014). Wang *et al.* (2018) studied the community structure of the summer bloom in the western Arctic Ocean and found *P. seriata* as one of the dominant species. Presently, two Arctic species, *P. seriata* and *P. obtusa* (Hasle) Hasle & Lundholm, have been shown to be toxic (Hansen *et al.* 2011; Tammilehto *et al.* 2012; Harðardóttir *et al.* 2015). Arctic *P. seriata* strains are potent toxin producers with toxic strains found in Greenland, Iceland, Scotland, and Denmark (Lundholm *et al.* 1994; Fehling *et al.* 2004; Hansen *et al.* 2011; Olesen *et al.* 2020). These are areas where toxic blooms of *P. seriata* have caused DA accumulation in blue mussels (*Mytilus edulis*) up to ten times above the EU regulatory limit (20 mg DA kg^{-1}) (Fehling *et al.* 2004; Lundholm *et al.* 2005; Olesen *et al.* 2020). Under-reporting of toxin syndromes due to toxic phytoplankton is a major problem in Alaska (Lewitus *et al.* 2012) and presumably also in other Arctic areas. Recent surveys of stranded and harvested Arctic mammals in Alaska have revealed DA in 13 different species of Arctic marine mammals, some with toxin levels comparable to dead or severely affected marine mammals from the US West Coast (Lefebvre *et al.* 2016). As pointed out by Levy & Patz (2015), it is expected that toxic algal blooms in the Arctic will occur more frequently in the future due to changing oceanic conditions.

Investigating the potential effects of salinity on growth and toxicity of *Pseudo-nitzschia* is important in order to assess how future climate changes may affect toxic *Pseudo-nitzschia* blooms, with special attention to the Arctic. In this study, we exposed three strains of *P. seriata* to salinity levels varying from 15 to 40, and studied responses in growth, toxin content, and cell morphology. All experiments were performed in triplicate. Morphology was studied because, although temperature is known to affect morphology of *P. seriata* in a way that makes it difficult to differentiate between *P. australis* and *P. obtusa* (Hansen *et al.* 2011), it is not known what effects salinity changes have on the morphology.

MATERIAL AND METHODS

Establishment and maintenance of strains

Three strains of *P. seriata* (I2, I4 and I6) were established by isolation of single cells or chains from Hvalfjörður, Iceland (64°22.18′N, 21°34.08′W) on 1 May 2018. The strains were cultured in L1 medium (Guillard & Hargraves 1993) with a salinity of 30 and a pH of 8.0, at 4°C, and a light intensity of 108 µmol photons m^{-1} s^{-1} in a 16:8 (light:dark) cycle. The strains were identified as *P. seriata* by morphological characteristics and morphometric measurements made on micrographs obtained using a JEM 1010 transmission electron microscope (JEOL Ltd., Tokyo, Japan).

A fixed sample of each strain has been stored in the Natural History Museum of Denmark (reference numbers C-A-99695, C-A-99696 and C-A-99697).

Experimental setup

The experiment was initiated on 26 March 2019 and the cultures were approximately 11 months old when the experiment began. Four different salinities, 15, 20, 30 and 40, of

growth medium L1 (Guillard & Hargraves 1993) were prepared based on filtered seawater with a salinity of 33. The salinities were chosen so that two are lower (15 and 20) than the present oceanic salinity, as well as a slightly higher salinity, exploring the effect of lower as well as more fluctuating salinities in the future Arctic Ocean. The lower salinities, 15, 20 and 30, were prepared by diluting the seawater with distiled water, and salinity 40 was prepared by evaporation. Afterwards pH was adjusted to 7.9–8.0. Acclimatization of the strains was achieved by salinity changes in steps of a maximum of 10 in order to minimize the risk of the strains being exposed to excessive osmotic stress. The strains were acclimatized for a minimum of 14 days, corresponding to 2–5 divisions, after each change.

A batch culture experiment was set up using all three strains in triplicates. The experimental flasks (225-ml flasks) were placed at 4°C and a light intensity of 105–110 µmol photons m^{-1} s^{-1} and moved around to counteract potential differences in light intensity. Based on exponentially growing cultures of all three strains, an initial concentration of approximately 1000 cells ml^{-1} was set for all strains.

Sampling method for cell densities and growth rates during experiment

For measuring cell density, approximately 1.7 ml of sample was transferred to glass vials and Relative Fluorescence Units (RFU; Triology fluorometer, San Jose, CA, USA) measured for all triplicates. One mililitre of one of the triplicates was fixed in 2% acidic Lugol's solution and counted in a Sedgewick-Rafter counting chamber (S52) using an Olympus CKX53 light microscope (Olympus Corp., Tokyo, Japan), counting at least 400 cells or a minimum of 300 squares. Only viable cells were counted. Linear regressions between RFU and cell densities were used to translate RFU to cell densities. The cell density was measured approximately every second day. The growth rate (GR) of each strain at each salinity was calculated following Olesen *et al.* (2020).

Toxin sampling and analysis

Samples for toxin analysis were taken three times during the experiment; on day 0 (the initial phase), in exponential phase and in stationary phase. For each toxin sampling, 45-ml subsamples were centrifuged (Eppendorf Centrifuge 5810 R) for 15 min at 4°C and 1811× *g*, the supernatant removed, and the pellet stored at –20°C until analysis. Strain I4 at salinities 20, 30 and 40, and strain I2 at salinity 40 were sampled when the cultures were entering stationary phase, whereas the others were sampled slightly later, immediately after entering stationary phase. Each replicate was measured once for toxin content.

For analysis of DA, 300 µl of a 1:1 mixture of methanol and 0.03 M acetic acid was added to the pellet that was resuspended with a vortexer for 2 s and transferred to a 2-ml cryotube (Sarstedt micro-tube, Nümbrecht, Germany), and subsequently Lysing Matrix D beads were added until they covered the bottom of the tube. The sample was extracted in a FastPrep (Thermo BIO 101, FastPrep FP120, Illkirch, France) for 45 s at a speed of 6.5 m s^{-1}, centrifuged (Centrifuge 5415 R, Eppendorf, Hamburg, Germany) for 5 min at 16,100× *g*, the supernatant transferred to a centrifugation filter tube (Ultrafree MC HV, Durapore PVDF 0.45 µm, Merck Millipore, Eschborn, Germany) and centrifuged. The filtered sample was transferred to a glass vial and sealed. DA was determined by liquid chromatography (LC 1100 Chromatograph, Agilent, Waldbronn, Germany) coupled to tandem mass spectrometry (LC-MS/MS) on an API 4000 QTrap instrument (Sciex, Darmstadt, Germany) in the selected reaction monitoring (SRM) mode as described in Krock *et al.* (2008). DA was quantified by external calibration against a standard solution of DA (certified reference material programme of the NRC-IMB, Halifax, NS, Canada) and values of isodomoic acid C are expressed as DA equivalents. The limits of detection of DA are given as pg $cell^{-1}$ in Table 1. Collision-induced dissociation (CID) spectra were recorded under identical chromatographic conditions in the enhanced product ion (EPI) mode with a collision energy of 30 V. Toxin content was expressed either per cell or per volume, i.e. toxin cell quota times the cell concentration.

Morphological assessment using TEM

Before the experiment was initiated, 10 ml of each strain were sampled for morphological examination of the frustules. Assessment of the morphological characteristics confirmed the identity of the strains following Hasle & Lundholm (2005; Table S1). After the experiment, 10 ml of all combinations of strain and salinity were sampled. The samples were cleared of organic material following Christensen (1988) and examined using a JEM 1010 transmission electron microscope (JEOL Ltd., Tokyo, Japan). Micrographs were used for measurements and assessment of morphological characteristics. Using length and width of cells, the cell volumes of the three strains at salinities 20, 30 and 40 were calculated according to Ayache *et al.* (2020). At least three valves were used for all morphometric measurements for each strain at each salinity.

Statistical testing

The GR, maximum cell densities, and toxin levels were tested for statistical significance using GraphPad Prism (ver. 9). Comparisons were conducted with One-Way ANOVA and a Tukey's multiple comparisons test. Morphometric data were tested using two-way ANOVA. A significance level of 0.05 was used.

RESULTS

Toxicity

Two out of the three *P. seriata* strains (I2 and I4) produced domoic acid (DA) and another compound with the same mass transition eluting shortly before DA (Fig. 1), whereas strain I6 contained almost only DA. Collision-induced dissociation (CID) spectra of DA and the isobaric compound were almost identical, with only apparent differences between

Table 1. Domoic acid and isodomoic acid C levels (pg cell^{-1}) in exponential and stationary phases of the strains I2, I4 and I6 of *Pseudo-nitzschia seriata*. Mean and *s* are shown for exponential and stationary phases. Values listed as 0.00 ± 0.00 represent samples with toxin content lower than 0.00 pg cell^{-1}, although traces of the toxin were detected.

	Exponential phase			Stationary phase		
	Domoic acid (pg cell^{-1})	Isodomoic acid C (pg cell^{-1})	Level of detection (pg cell^{-1})	Domoic acid (pg cell^{-1})	Isodomoic acid C (pg cell^{-1})	Level of detection (pg cell^{-1})
Salinity 20						
I2	0.33 ± 0.06	0.40 ± 0.09	2.5×10^{-3}	0.38 ± 0.07	0.46 ± 0.09	9.2×10^{-4}
I4	0.35 ± 0.09	0.44 ± 0.13	4.9×10^{-3}	0.69 ± 0.11	0.81 ± 0.14	6.2×10^{-4}
I6	0.03 ± 0.01	nd	1.4×10^{-3}	0.01 ± 0.01	nd	3.5×10^{-4}
Salinity 30						
12	0.01 ± 0.00	0.02 ± 0.01	2.3×10^{-4}	0.02 ± 0.00	0.03 ± 0.00	2.5×10^{-4}
I4	0.01 ± 0.01	0.02 ± 0.01	3.3×10^{-3}	0.06 ± 0.01	0.09 ± 0.02	1.6×10^{-4}
I6	0.02 ± 0.01	nd	6.2×10^{-4}	0.02 ± 0.00	nd	2.4×10^{-4}
Salinity 40						
I2	nd	nd	1.6×10^{-2}	**1.1** ± 0.00	nd	1.2×10^{-4}
I4	0.22 ± 0.04	0.26 ± 0.05	3.1×10^{-3}	0.33 ± 0.14	0.40 ± 0.16	9.0×10^{-4}
I6	nd	nd	9.2×10^{-4}	0.002 ± 0.001	0.002 ± 0.001	4.9×10^{-4}

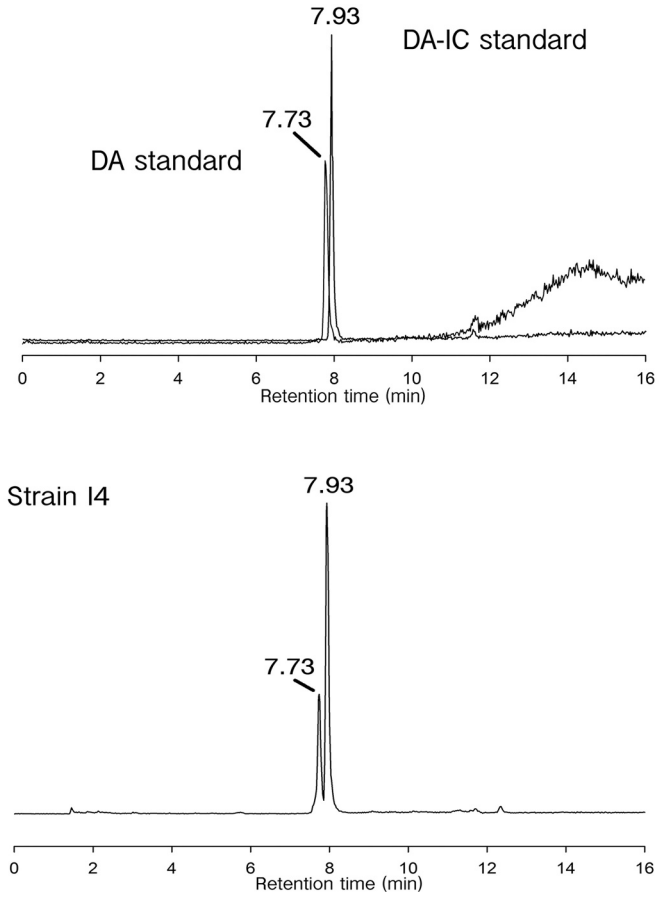

Fig. 1. LC-MS/MS SRM chromatogram of the transition *m/z* 312 > 266 of a standard solution of DA (7.73 min) and DA-IC (7.93 min) (top chart) and *Pseudo-nitzschia seriata* strain I4 (bottom chart).

fragment intensities (Fig. 2). The high similarity of the CID spectra of DA and the unknown compound led to the conclusion that the unidentified compound was an isomer of DA. This hypothesis was verified by an analytical standard of isodomic acid C (DA-IC), showing the same retention time and CID spectrum as the compound of the two *P. seriata* strains (Fig. 2).

Strain I2 showed a higher cellular content of DA at salinity 20 than at salinities 30 and 40 ($p < 0.0001$) in both exponential and stationary phases (Fig. 3). No DA was detected in the exponential phase at salinity 40. Strain I2 showed no significant difference in DA content from exponential to stationary growth phases at salinities 20 and 30 ($p > 0.1$; Fig. S1).

Strain I4 had higher DA contents at salinities 20 and 40 than at salinity 30 ($p < 0.05$), with no significant difference in DA content between salinities 20 and 40 ($p > 0.05$; Fig. 3). Strain I4 had a higher DA content in stationary phase, compared to exponential phase, at salinities 20 and 30 ($p < 0.05$), but with no significant difference between the two growth phases at salinity 40 (Fig. S1).

Strain I6 did not show any difference in DA content at salinities 20 and 30 ($p < 0.05$) (Fig. 3). At salinity 40, no DA was detected in exponential phase in strain I6, and in stationary phase the DA content was lower than at the two other salinities ($p < 0.05$; Fig. S1). There was no significant difference in DA content between the exponential and stationary phases at salinities 20 and 40 ($p > 0.5$).

Comparing toxin contents among strains, strain I6 had significantly lower cell quota ($p < 0.05$) than I2 and I4 in almost all scenarios with only a few exceptions, when DA content was either very low or not detected (Fig. 3; Table 1). Comparisons among strains showed that in the exponential growth-phase strains I2 and I4 had a similar DA content at salinity 20 ($p > 0.5$) and all three strains had a similarly low DA content at salinity 30 ($p > 0.1$; Fig. 3). At salinity 40, strain I4 had a significantly higher DA content than the other two strains, I2 and I6 ($p < 0.0001$). In stationary growth phase, strain I4 had a higher DA content than strains I2 and I6 at all salinities ($p < 0.01$), and strain I2 had a higher DA content than strain I6 at salinity 20 only ($p < 0.005$).

In both strains I2 and I4 the DA and DA-IC cell quotas were similar, but with the DA-IC cell quota being slightly

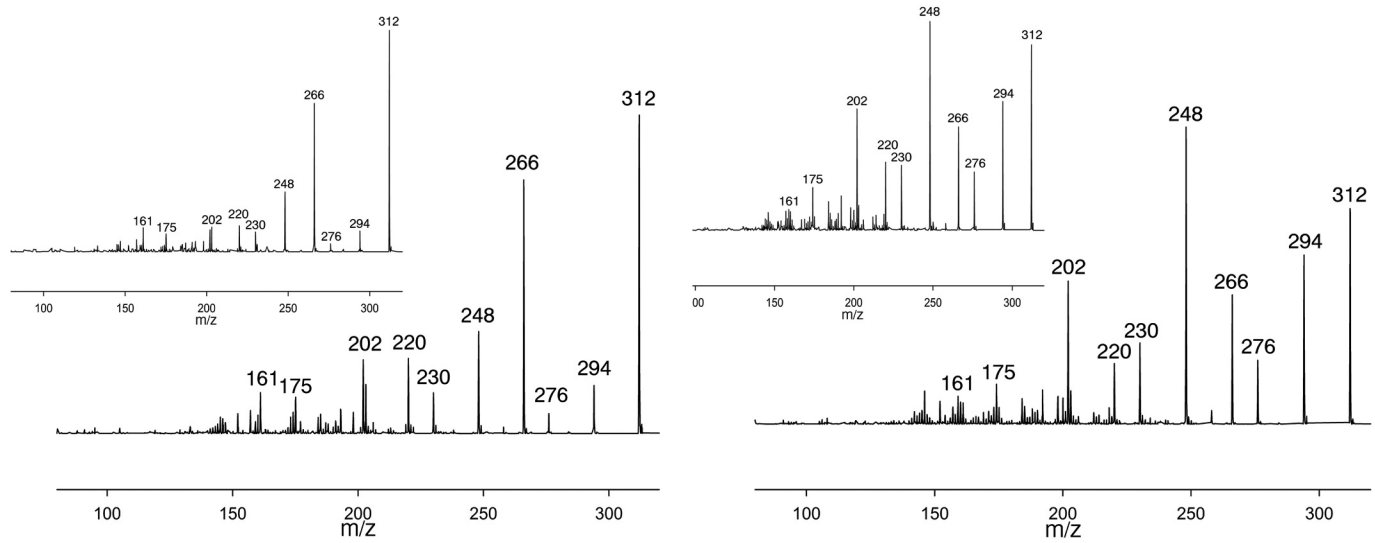

Fig. 2. Collision induced dissociation (CID) spectra of DA (strain I4; left chart) and a DA standard (inset) and CID spectra of IC (strain I4; right chart) and an IC standard (inset).

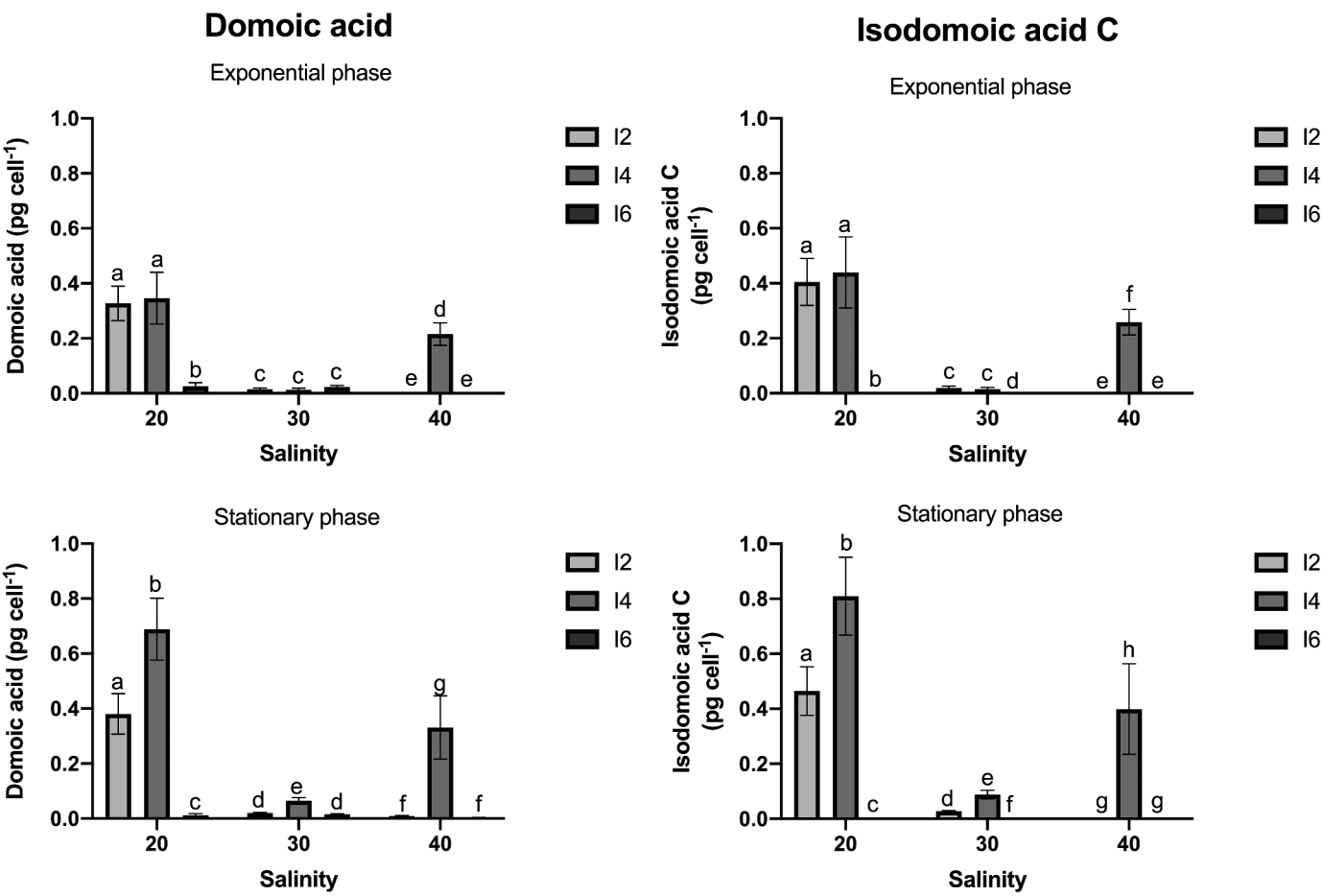

Fig. 3. Domoic acid and isodomoic acid C levels (pg cell^{-1}) in exponential and stationary growth phase of the strains I2, I4 and I6 of *Pseudo-nitzschia seriata* at different salinities (20, 30 and 40). The small letters in each chart indicate significant difference between cell quota of the strains at the same salinity.

higher, in each sample (Table 1). DA-IC was only detected in strain I6 in stationary phase at salinity 40. The total toxin content (DA + DA-IC) per volume of the strains at the different salinities was calculated, taking both cell concentration and toxin cell quotas into consideration. This assessment revealed that the total toxin content was significantly higher in stationary phase, compared to exponential phase ($p < 0.05$; Fig. 4, note different Y-axes) in the salinities where DA and DA-IC were detected in exponential and stationary phase. The salinities at which total DA content was highest among strains during the exponential growth phase were more irregular. In strain I2 there was a significantly higher total toxin content at salinity 20 than at salinity 30 ($p < 0.001$), and no toxins were detected at salinity 40. In strain I4, there was no difference in total toxin content between salinities 20 and 40 ($p > 0.95$) but a lower toxin content was measured at salinity 30 ($p < 0.0001$). In strain I6, similar to strain I2, no toxins were detected at salinity 40, but at salinity 30 strain I6 had a higher total toxin content than at salinity 20 ($p < 0.01$).

In stationary phase, strains I2 and I4 showed similar patterns; significantly higher total toxin content at salinity 20 than at salinities 30 and 40 ($p < 0.0005$), which were not significantly different ($p > 0.08$ and $p > 0.7$, respectively). In contrast, strain I6 showed no difference in total toxin content between salinities 20 and 40 ($p > 0.1$) but a higher content at salinity 30 ($p < 0.01$).

Growth and growth rate

At salinities 20–40, all three strains (I2, I4 and I6) exhibited the typical growth pattern of batch cultures with an exponential growth phase followed by a stationary phase. At salinity 15, none of the strains grew. All cells of strain I4 died at salinity 15, whereas very low cell densities (10–27 cells ml^{-1}) of strains I2 and I6 were seen throughout the 45-day-long experiment (Figs S2–S4).

Overall, the maximum GR of strain I6 was not affected by salinities 20–40 ($p > 0.05$), with intermediate GRs compared to the other two strains (Fig. 5). The maximum GR of strains I2 and I4 were higher at salinity 30 (Fig. 5; Table S2) compared to salinities 20 and 40. For strain I2, the maximum GR at salinity 30 was higher than at the two other salinities ($p < 0.0001$), and the GR at salinity 40 was again higher than at salinity 20 ($p < 0.01$; Fig. 5). For strain I4, the GR at salinity 30 was significantly higher than at 20 and 40 ($p < 0.0001$), but no significant difference was found between

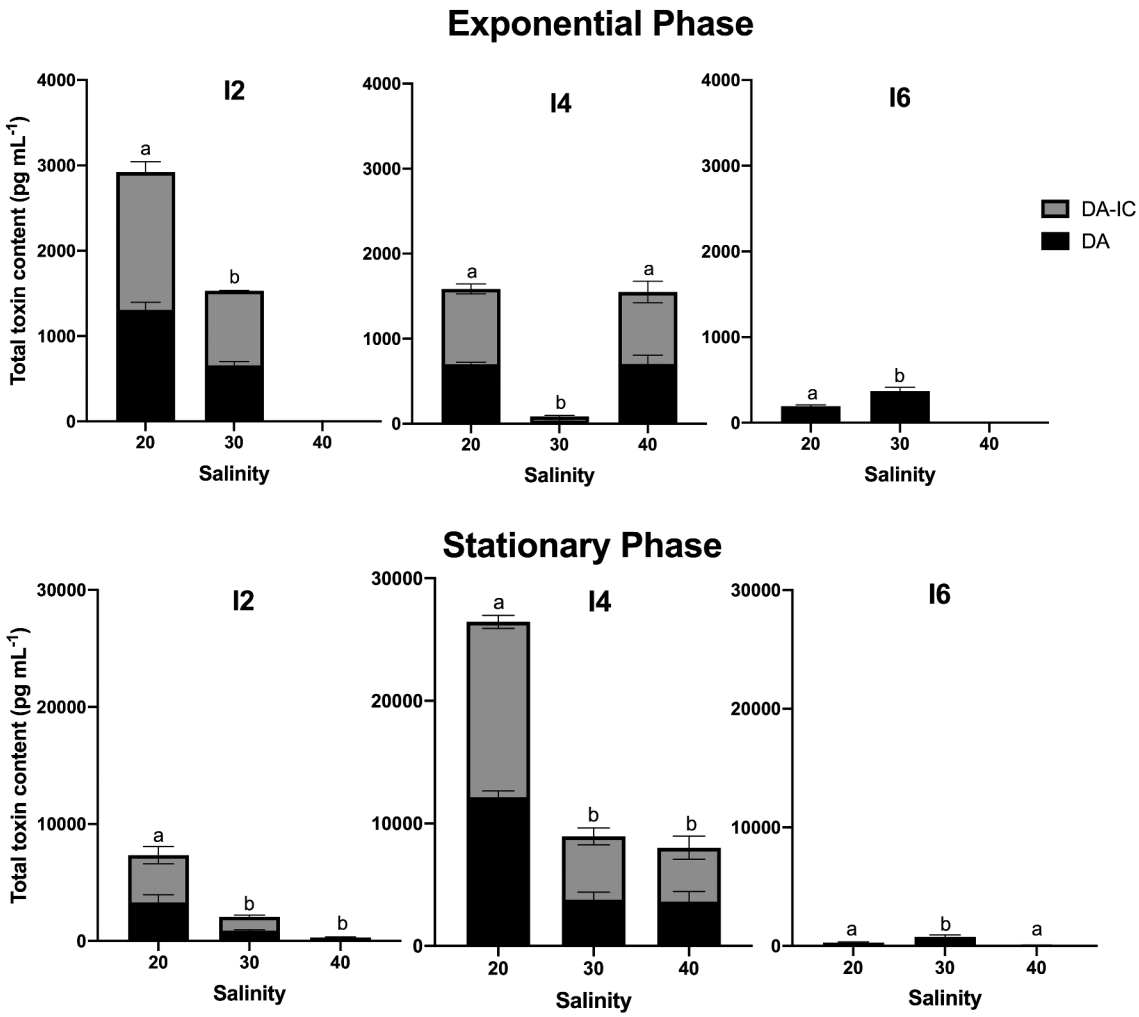

Fig. 4. Total toxin levels (pg ml^{-1}) for strains I2, I4 and I6 of *Pseudo-nitzschia seriata* at different salinities. Top charts show total domoic acid and isodomoic acid C levels in exponential phase; bottom charts show total domoic acid and isodomoic acid C levels in stationary phase. Light grey represents the portion of total toxin made up by DA-IC, while the black represents the portion of DA. Small letters indicate significant differences between total toxin content at the different salinities.

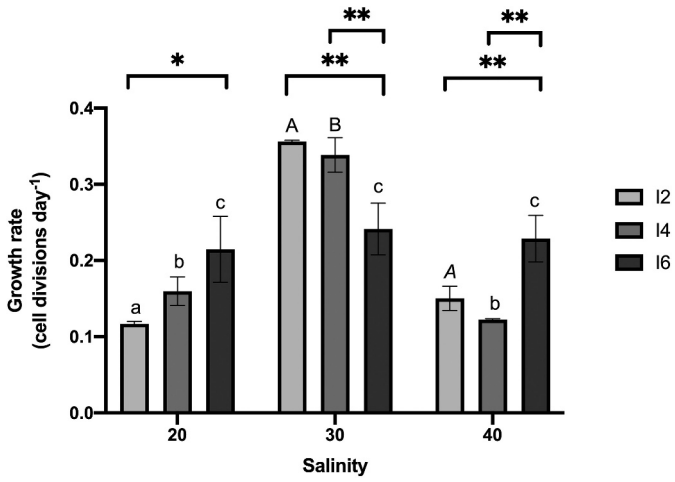

Fig. 5. Growth rate (cell divisions d^{-1}) for the strains I2, I4 and I6 of *Pseudo-nitzschia seriata* at different salinities (20, 30 and 40). The different letters indicate significant differences in growth rate within a strain between the different salinities ($p < 0.05$). Significant differences between strains at the same salinity is indicated by the asterisks; * = significance of 0.5, ** = significance of 0.01.

the GRs at salinities 20 and 40 ($p > 0.05$; Fig. 5). All three strains reached the highest maximum cell density in stationary growth phase at a salinity of 30 ($p < 0.05$; Table S2), although in strain I6 cell densities at salinities 30 and 40 were not different from each other.

Comparison of strains showed a similar growth response at all salinity treatments for I2 and I4, whereas strain I6 had a different response and lacked differences in GR between the salinities. At salinity 20, strain I6 grew significantly faster than I2 ($p < 0.01$) (Fig. 5; Table S2), while neither I2 nor I6 was different from I4 ($p > 0.05$). At salinity 30, strain I6 had a lower GR than both I2 and I4 ($p < 0.005$). In contrast, at salinity 40 the GR of I6 was higher than that of both I2 and I4 ($p < 0.01$). Maximum GRs of I2 and I4 were not significantly different at any salinity ($p > 0.05$).

Morphology

There was no general pattern in morphology in response to the different salinities, and despite single strains varying in single morphometric parameters, morphometries overlapped in all cases. In strain I2 significant changes were seen in valve length at salinities 30 and 40. The valves were longer at salinity 30 than at salinity 40 ($p < 0.001$). In strain I4, a significant change in valve length was observed, with valve length at salinity 30 being longer than at salinities 20 and 40 ($p < 0.0001$). In strain I6, the density of striae was significantly higher in salinity 20 compared to salinity 30 and 40 ($p < 0.05$; Table S1).

DISCUSSION

Toxin content in different growth phases

Pseudo-nitzschia seriata had the same or higher toxin content in stationary vs exponential growth phase, supporting previous studies on *Pseudo-nitzschia* species (Fehling *et al.* 2004; Lelong *et al.* 2012). Our finding of DA in exponential growth phase is in agreement with the high cell quota detected in the exponential phase in *P. seriata* exposed to copepods (Harðardóttir *et al.* 2015). The phylogenetically closely related *P. australis* has on several occasions also shown DA in both exponential and stationary growth phases (Lelong *et al.* 2012; Thorel *et al.* 2014; Martin-Jézéquel *et al.* 2015; Schnetzer *et al.* 2017; Wingert 2017; Ayache *et al.* 2020). In contrast, studies conducted on other species of *Pseudo-nitzschia* have mainly shown DA content only in stationary phase (Lelong *et al.* 2012; Bates *et al.* 2018).

Overall, the strains of *P. seriata* contained higher levels of DA and DA-IC at salinity 20 than at all other salinities, and they were generally most toxic in stationary phase, with DA cell quota in stationary phase of 0.01–0.69 pg DA $cell^{-1}$. These results are similar to measurements by Fehling *et al.* (2004) of DA cell quota in *P. seriata* of 0.16–0.23 pg DA $cell^{-1}$ in stationary phase. Our results also support a study of *P. seriata* that found the highest DA content in (early) stationary phase (Besiktepe *et al.* 2008). Studies on *Pseudo-nitzschia multiseries* (Hasle) Hasle, *Pseudo-nitzschia calliantha* Lundholm, Moestrup & Hasle, and *Pseudo-nitzschia fraudulenta* (Cleve) Hasle found all strains most toxic or only toxic in stationary phase (Thessen *et al.* 2009). Whether and how toxin content varies in relation to growth phases among other species needs to be studied further.

Isodomoic acid C

Our results represent the first finding of DA-IC in *P. seriata*. DA-IC is an isomer of DA, previously found only in *P. australis* (Holland *et al.* 2005). The present study thus expands the diversity of isomers found in *P. seriata*, from previous findings of DA-IA and DA-IB (Hansen *et al.* 2011). In the present study, all three Arctic (Icelandic) strains contained DA and DA-IC, whereas two other Arctic (Greenlandic) strains in Hansen *et al.* (2011) contained DA, DA-IA and DA-IB. DA-IA and DA-IB each made up between 3% and 10% of the total cell quota (Hansen *et al.* 2011), whereas in the present study DA-IC made up 57.9% ± 0.4% of total cell quota (Table S3). Knowledge on isomers of DA in other diatoms is restricted and comprises mainly reports of the isomers A and B. The two isomers made up 5–6% of the total DA in *P. multiseries* and 4–12% in *Pseudo-nitzschia* cf. *delicatissima* (Cleve) Heiden (Kotaki *et al.* 2008) and have also been detected in strains of *Nitzschia bizertensis* Smida, Lundholm, Sakka Hlaili & Hadj Mabrouk (Smida *et al.* 2014). The largest diversity in toxin profiles is known from *Nitzschia navis-varingica* Lundholm & Moestrup, in which five types of toxin profiles have been found: some produce only DA, some produce only DA-IB, some produce both DA and DA-IB, others produce both DA-IA and DA-IB, and some produce all three toxins (Romero *et al.* 2012).

The toxic properties of DA are related to the binding and depolarization of glutamate receptors. The affinity of the isomers of DA to the glutamate receptor is suggested to be lower than DA (Holland *et al.* 2005). Therefore, DA isomers are often not considered a major threat to humans or animals. However, tests on mice have revealed DA-IA to be similarly potent as DA in its capacity to induce seizures, with DA-IC

being approximately 20 times less potent and DA-IB more than 100 times less potent than DA (Sewant *et al.* 2008). Results from previous studies indicate that humans experience toxic effects at much lower doses of DA than rodents and fish (Lefebvre & Robertson 2010). Accordingly, a lesser amount of DA-IC may be sufficient to induce a response in humans compared to rodents. Therefore, it is apparent that different species groups have different DA response thresholds; hence, the effect of the different isomers might also be different among species.

The role or function of DA-IC remains unknown. Hypotheses regarding the role of DA isomers include their being a result of degrading DA or part of the biosynthesis pathway of DA. Studies on *P. multiseries* and on the red alga *Chondria armata* (Kützing) Okamura suggest that DA-IA is involved in the biosynthesis of DA (Brunson *et al.* 2018; Maeno *et al.* 2018), and this may also be the case of DA-IC.

The fact that DA-IC is present in both exponential and stationary phases could imply that it is a part of the biosynthesis of DA. The hypothesis of DA-IC being a degradation product of DA is not supported by our results. Since the percentage of DA-IC of the total toxin content in both exponential and stationary phase is more or less the same (Table S3), degradation of DA to DA-IC is regarded as unlikely.

Changes in toxin content in response to salinity

Strains I2 and I4 had a lower maximum GR at salinity 20 than at salinity 30 (Table S2) but had the highest toxin content at salinity 20 (Table 1). They are thus more toxic at salinity with slower growth.

Studies on the phylogenetically closely related *P. australis* found similarly high levels of DA and low GR at salinity 20, compared to salinities 30, 35 and 40 (Ayache *et al.* 2020), indicating a similar dilution of toxin content. The inverse relation between GR and toxin content could also indicate that metabolic costs are related to DA production, as previously suggested by Lundholm *et al.* (2018). Cells may have to either direct metabolic energy for toxin production or to growth. Here, when cells are restricted to growth due to osmotic challenges, the cells may direct relatively more energy towards DA production.

Volumes of *P. australis* cells were higher at salinity 20 than at higher salinities (Ayache *et al.* 2020). Cell size has been related to toxin content (Mafra *et al.* 2009), and this could be another explanation for the relatively higher cell quota in both *P. seriata* and *P. australis* at salinity 20. The results of the present study on cell volumes indicate no connection between salinity and cell volume in any of the three strains (Table S4).

In contrast to the inverse correlation between toxin content and GR in *P. seriata* and *P. australis, P. multiseries* grew equally fast at salinities 20, 30 and 40, and significantly more slowly at salinity 10. In the same study, *P. multiseries* was significantly less toxic at salinities 10 and 20 compared to 30 and 40 (Doucette *et al.* 2008). The authors suggested that cells allocate energy to maintain homoeostasis and the remaining limited energy to growth rather than DA synthesis at lower salinities. Adaptations to salinity as well as differences in GR may hence affect cell quota in different ways depending on strain and species.

Variation in toxin content among strains

Toxin content varied considerably among strains, both regarding DA content, but also regarding the content of DA-IC. A study on *P. seriata* and *P. australis* (two strains of each species), examining both DA content in the culture and in the medium, also found variation in DA content among strains of the same species (Fehling *et al.* 2004). Additionally, an experiment on five strains of *Pseudo-nitzschia simulans* Yang Li, C. X. Huang & G.S. Xu from Chinese waters revealed variations in toxin content among the strains, with only one of the five strains being found to produce DA (Li *et al.* 2017).

Growth responses to different salinities

We found that none of the *P. seriata* strains could cope with the lowest salinity (15). Only the cells of strain I6 survived at salinity 15 during the whole experimental period (43 days), although at very low cell densities, indicating that *P. seriata* will have difficulties coping with low salinities in e.g. freshwater-rich estuaries or other areas highly affected by freshwater run-off. Similar to our results, two strains of *P. australis* have been shown not to survive at salinities lower than 20 (Ayache *et al.* 2020), and one strain of *Pseudo-nitzschia circumpora* H.C. Lim, Leaw & P.T. Lim did not survive salinities below 25 (Lim *et al.* 2012). In contrast, *Pseudo-nitzschia pungens* (Cleve) Hasle has a wide salinity tolerance range, with detected growth at salinities as low as 4 and 5 (Cho *et al.* 2001; Markina & Aizdaicher 2016; Pednekar *et al.* 2018). Comparing all studies published on salinity effects on *Pseudo-nitzschia* species (Table 2), *P. pungens, P. delicatissima* and *P. multiseries* have all been shown to sustain growth at salinities lower than 10 and seem to have the widest salinity tolerance ranges among examined taxa (Tabl 2). Huge differences in salinity tolerance thus exist among *Pseudo-nitzschia* species.

In the present study, GR and maximum cell densities were significantly lower at salinities 40 and 20 than at salinity 30 in two of the three strains, i.e. with a narrow optimal salinity range, whereas the other strain grew optimally at salinities 20–40, having a wider optimal salinity range. Similarly, narrow salinity ranges have been seen in *P. circumpora, P. pseudodelicatissima* (Hasle) Hasle and *P. pungens* (Lundholm *et al.* 1997; Lim *et al.* 2012). However, most studies have, as for one of our strains of *P. seriata*, shown wider optimum salinity ranges (Table 2), like the closely related *P. australis*, which reached highest cell densities and GR at salinities 30, 35 and 40 (Ayache *et al.* 2020).

This overall variation in optimal salinity ranges illustrates that *Pseudo-nitzschia* species respond differently to changing salinities and that all species comprise intraspecific variation, helping species to survive, thrive and adapt to changing salinities in the ocean.

Salinity changes have previously been shown to alter the morphology of phytoplankton species. For example, strains of the centric diatom *Skeletonema* (Balzano *et al.* 2011) and two species of *Cocconeis* (Leterme *et al.* 2013) changed

Table 2. Overview of studies exploring the effects of salinity on the growth of *Pseudo-nitzschia*. The table shows the salinities at which different species of *Pseudo-nitzschia* had lower limit and upper limit for growth, as well as the salinity range for optimum growth.

Species/strain	Salinity			Reference
	Min.	Max.	Optimal	
Pseudo-nitzschia delicatissima				
LaPn-4	6.25	>45	15–40	Thessen *et al.* (2005)
LaPn-9	6.25	>45	10–30	Thessen *et al.* (2005)
Pseudo-nitszchia pseudodelicatissima				
9D2C1-1-LY	15	>45	25–40	Thessen *et al.* (2005)
9D3C2-1-LY	15	>45	25–40	Thessen *et al.* (2005)
CCMP 1823	15	>45	15–40	Thessen *et al.* (2005)
Løgstør Bredning, Limfjord, Denmark	15	35	25	Lundholm *et al.* (1997)
Pseudo-nitszchia multiseries				
MU 1	10	40	15–40	Thessen *et al.* (2005)
MU 7	7	>45	25–30	Thessen *et al.* (2005)
MU 7	10	40	20–40	Doucette *et al.* (2008)
Chinhae Bay, Korea	10	40	20–40	Cho *et al.* (2001)
Pseudo-nitzschia pungens				
PP-07	4	32	24–32	Markina & Aizdaicher (2016)
PP-08	4	32	32	Markina & Aizdaicher (2016)
PP-081	4	32	16–32	Markina & Aizdaicher (2016)
SP-1	5	35	15–30	Pednekar *et al.* (2018)
Chinhae Bay, Korea	10	50	20–30	Cho *et al.* (2001)
Pseudo-nitzschia seriata				
I2	20	40	30	This study
I4	20	40	30	This study
I6	20	40	20–40	This study
Pseudo-nitzschia circumpora				
	25	35	30	Lim *et al.* (2012)
Pseudo-nitzschia australis				
IFR-PAU-16.1	20	40	30–40	Ayache *et al.* (2020)
IFR-PAU-16.2	20	40	30–40	Ayache *et al.* (2020)

morphologically due to changed salinity. Changes in other abiotic factors, such as temperature, have also led to changes in morphology, like morphological changes in *P. seriata* in response to changes in temperature (Hansen *et al.* 2011). In this study, however, no major or consistent morphological changes were observed in response to changes in salinity.

Intraspecific variation in growth responses

Salinity changes significantly affected the growth of two of the three *P. seriata* strains with the highest maximum GR for both strains found at salinity 30, revealing a GR two to three times higher than at salinities 20 and 40. The growth of the third strain (I6) was not affected by salinities of 20–40. Among the three strains, growth responses to salinity changes hence varied considerably, except that none of the strains grew at salinity 15. In contrast, two strains of the phylogenetically very closely related *P. australis* exposed to abrupt changes in salinity levels as well as salinity-acclimated strains showed only minor differences in growth response between strains (Ayache *et al.* 2019). Similar to our results, an intraspecific variation in growth in response to different salinities (4, 8, 16, 24 and 32) has also been reported for three strains of *P. pungens* (Markina & Aizdaicher 2016), with strains responding differently to the five salinities. Likewise, a study on the effects of salinity on growth of *Pseudo-nitzschia* species showed intraspecific variation in the range of optimal salinity for two strains of *P. delicatissima*, three strains *P. pseudodelicatissima* and two strains *P. multiseries* (Thessen *et al.* 2005). *Pseudo-nitzschia* species are known to comprise genetic variation among strains (Evans *et al.* 2004), but the diversity in physiological responses to environmental parameters among strains is still relatively little studied (Bates *et al.* 2018). This is most likely because including two strains instead of one doubles the effort in physiological experiments. Field populations of diatom species comprise numerous genetically different strains (Casteleyn *et al.* 2010; Tammilehto *et al.* 2017). Hence, when considering the diversity seen in the present study among only three strains, we suggest that future studies exploring physiological responses should include several strains in order to acquire more relevant physiological responses and thus an improved understanding and forecasting of harmful algal blooms.

Ecological implications

All strains of *P. seriata* contained most toxins at salinity 20, indicating that future freshening of the Arctic oceans might result in more toxic cells of *P. seriata*. On the other hand, lower salinity led to a lower GR and a lower maximum cell density. The strains I2 and I4 had 2–3 times lower GR at salinity 20 than at salinity 30, but the cell quota at salinity 20 was 12–33 times higher than at salinity 30. Therefore, it is reasonable to suggest that higher cell quota will override the lower GR at salinity 20. According to the results of the total toxin content (Fig. 4), a decrease in ocean salinity to e.g. 20 would lead to a higher total toxic content compared to the current salinity in two of the strains (I2 and I4). However, for the last strain (I6) a decrease in ocean salinity would have a negative effect on total toxin content. Based on predictions on salinity changes due to climate change, temperate and polar regions are expected to face a decrease in salinity (Boyer 2005; Hosoda *et al.* 2009). An experiment conducted in the Arctic Ocean investigating the interactive effects of temperature, CO_2 levels and salinity on the phytoplankton community found an increase in *Pseudo-nitzschia* species with higher temperatures, increased CO_2 levels and lower salinity (Sugie *et al.* 2020). Hence, even with multiple environmental factors changing and in competition with other species, *Pseudo-nitzschia* may increase in abundance. As *P. seriata* is particularly common in Arctic regions, it is possible that we will see an increase in abundance of *P. seriata*, as well as an increase in cell toxin content with a decrease in ocean salinity.

In regions where evaporation exceeds precipitation, salinity is expected to increase. In our study, only one of the three strains

(I4) – and only in exponential phase – had a significantly higher total cell quota at salinity 40 compared to salinity 30. Since two of the three strains displayed none or very low amounts of toxins at salinity 40, it is suggested that waters of increased salinity may result in less toxic *P. seriata* cells.

The crude calculations based on our experiments indicate that, despite fewer cells at lower salinities, the higher toxin content of the cells will probably result in overall higher toxic potential at salinity 20 than at salinity 30. Whether that increases the risk for toxic blooms and accumulation of DA during blooms will depend on several other factors, like the *P. seriata* fraction of the total bloom, and other toxin-inducing factors, as well as other biological interactions like allelopathic and grazing interactions. Even small changes may have effects on the bloom scale. The high variation among the strains underlines a potentially extensive physiological intraspecific variation and the need for more knowledge on variation among strains in the scale of a bloom.

Because temperature could be the main driver of changes in ocean salinity, it would be interesting to investigate the coupling effect of those two factors: salinity and temperature. Studies on the effect of temperature and salinity on *Pseudo-nitzschia* species is limited to Lundholm *et al.* (1997) coupling salinity and temperature for *P. calliantha* (as *P. pseudodelicatissima*). GR peaked at salinity 25 with a temperature of 25°C (which was also the highest temperature tested). Strains grew at three of the tested salinities (salinity 15, 25 and 35), and GR increased with temperature at all salinities. At all temperatures, GR was highest at salinity 25. This might indicate that optimum temperature and optimum salinity remain the same regardless of the other factor changes, simply amplifying GR when combined. However, additional studies are needed on other species of *Pseudo-nitzschia* to reveal if this is a general pattern. Lundholm *et al.* (1997) found no apparent connection between domoic acid production and salinity and temperature, hence it needs further study as well.

CONCLUSION

Since all three strains of *P. seriata* grow and produce the highest amount of toxin cell quota at a salinity of 20, this suggests an increase in HABs due to *P. seriata* with future freshening of the oceans. The significantly higher toxin content markedly overrides the lower growth rate at salinity 20. This study presents the first account of DA-IC in *P. seriata*. Furthermore, growth and toxicity varied greatly in response to salinity. Future studies are needed to determine the role of DA isomers and to investigate how pronounced the intraspecific variation is in *Pseudo-nitzschia*.

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