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The impact of urea on toxic diatoms – Potential effects of fertilizer silo breakdown on a *Pseudo-nitzschia* bloom

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ABSTRACT

In spring 2016, two silos containing liquid nitrogen-containing fertilizer collapsed on a harbor in Fredericia, Denmark. More than 2,750 tons of fertilizer spilled into inner Danish waters. A bloom of *Pseudo-nitzschia* occurred approximately one month after the incident. The bloom caused a 5-week quarantine of numerous mussel-harvesting areas along the eastern coast of Jutland. The levels of domoic acid measured up to 49 mg kg^{-1} in mussel meat after the bloom. In the months following the event, the species diversity of phytoplankton was low, while the abundance was high comprising few dominant species including *Pseudo-nitzschia.* The main part of the liquid nitrogen-containing compound was urea, chemically produced for agricultural use.

To investigate the potential impact of urea on *Pseudo-nitzschia,* four strains, including one strain of *P. delicatissima,* two of *P. seriata* and one of *P. obtusa*, were exposed each to three concentrations of urea in a batch culture experiment: 10 µM, 20 µM and 100 µM N urea, and for comparison one concentration of nitrate (10 µM). Nitrate, ammonium, and urea were metabolized at different rates. *Pseudo-nitzschia obtusa* produced domoic acid and grew best at low urea concentrations. Both *P. seriata* strains had a positive correlation between urea concentration and growth rate, and the highest growth rate in the nitrate treatment. One strain of *P. seriata* produced domoic acid peaking at low N loads (10 µM N urea and 10 µM N nitrate). In conclusion, the ability to adapt to the available nitrogen source and retain a high growth rate was exceedingly varying and not only species-specific but also strain specific.

1. Introduction

1.1. Nitrogen spill and ecosystem response

On 3 February 2016, two silos containing a total of 4750 tons of liquid nitrogen-containing fertilizer collapsed on the harbor of Fredericia, close to Lillebælt, Denmark (Fig. 1). The amount of nitrogen-containing fertilizer reaching the adjacent sea was at least 2750 tons (Schjødt, 2018). The vast majority of the fertilizer consisted of urea (Markager, 2016), but ammonium and nitrate were also a part of the spill. The composition of the fertilizer included both N16 and N32 liquid nitrogen-containing fertilizer. N16, which consists of urea solely, was in the magnitude of 750 tons. 2000 tons of the spill was N32, which is a combination of N sources: 50% of the total N is from urea, 25% from nitrate, 25% from ammonium (Markager, 2016). No inhibitor was added to either of the fertilizer types. Inhibitors are chemical components working against urease and other enzymes to decrease the rate the fertilizer is utilized. The average yearly supply of nitrogen from land in the Lillebælt area is 5517 tons N, the collapse thus constituted an abrupt supply amounting over 50% of the annual terrestrial input in N (Markager, 2016). The timing of the collapse was about two weeks prior to the usual spring bloom in the area (Markager, 2016). In Danish

Abbreviations: DA, Domoic Acid; PN, Pseudo-nitzschia; ASP, Amnesic Shellfish Poisoning; HAB, Harmful Algal Bloom; CDA, Cellular Domoic Acid; N, Nitrogen; PSP, Paralytic Shellfish Poisoning; RFU, Relative Fluorescence Unit; TIN, Total Inorganic Nitrogen; TS, Total Silicate; TP, Total Phosphate

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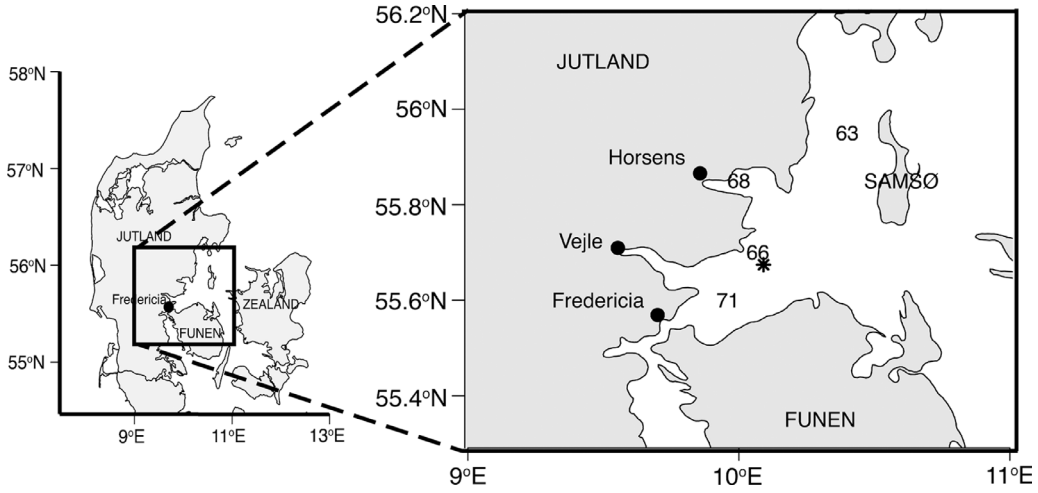

Fig. 1. Map of Denmark showing location of the city Fredericia (●) where the two silos collapsed on the harbor. The location of the 4 mussel farms is also indicted (63, 66, 68 and 71). These were quarantined after the bloom of *P. seriata* in March and April 2016. The Lillebælt Nord (VEJ0006870) monitoring station (*) is located near mussel harvesting area 66.

waters, phytoplankton growth is light limited from November to January. The spring bloom usually initiates in early February and utilizes the majority of the available nitrogen and phosphate in February and March. Hereafter growth is limited, mainly due to nutrient depletion, until October (Markager, 2016). The additional supply of N hence most likely favored phytoplankton growth, and notably, a bloom of domoic acid-producing *Pseudo-nitzschia* (PN) occurred approximately one month after the breakdown of the silos.

In Denmark, all mussel-harvesting areas are closed as soon as domoic acid (DA) or any other phycotoxin is detected. The decision on the management action is based upon the on-going risk assessment carried out by the authorities. The risk assessment takes into account the combined information on algal toxins and the quantitative occurrence of toxic algae. The mussel-harvesting areas are always to be closed if the algal toxin concentrations exceed the international regulatory limit, for DA of 20 mg DA kg^{-1}. Levels of DA up to 49 mg kg^{-1} in mussel meat were measured (Fig. 2). Detection of DA forced closure of numerous mussel-harvesting areas along the eastern coast of Jutland and resulted in an economical deficit of more than 1 million DKK. The added nitrogen was speculated to increase nitrogen availability during the following months and potentially years and lead to other ecosystem responses such as reduced underwater vegetation as a result of light limitation (e.g. eelgrass) (Markager, 2016). The Lillebælt area is characterized by strong currents and rapid water flow, which therefore potentially distributed the added nitrogen, primarily to the north (Kattegat). The area is characterized by estuarine circulation (Nielsen et al., 2017). This phenomenon occurs when surface and bottom currents flow in opposite directions, meaning that even if the nitrogen load was transported northwards with the surface waters, the uptake and sedimentation in the bottom waters may have retained the nitrogen in the area.

1.2. Global urea usage

Since the 1970s, the global use of nitrogen-containing fertilizer has increased drastically (Galloway and Cowling, 2002). In 2013, the global use of industrial N was of a magnitude of >170 megatons N yr^{-1} (Glibert et al., 2014). Since the 1970s, the use of urea has increased more than 100-fold, and at the same time, the composition of the fertilizer has changed, comprising a higher amount of urea. In 2005, urea constituted >50% of the nitrogenous fertilizer used worldwide. As a consequence of this change, urea has become a nitrogen source available to marine and coastal phytoplankton (Glibert et al., 2006).

Apart from organic urea, nitrogen in the ocean mainly occurs as inorganic nitrate and ammonium. The different forms of nitrogen may be utilized with varying efficiency by different phytoplankton species,

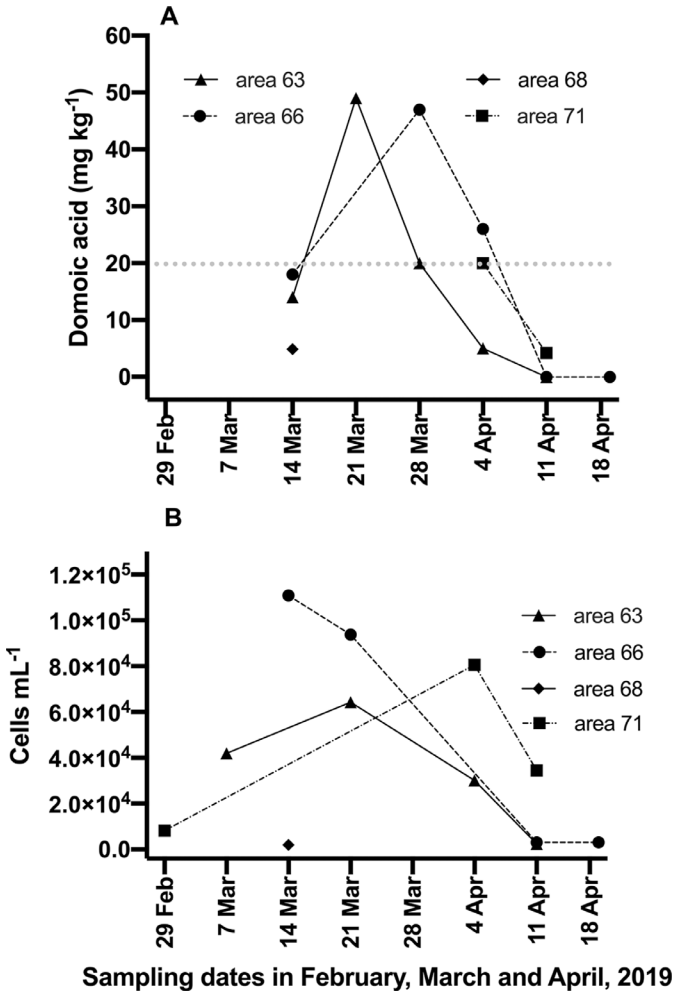

Fig. 2. Contents of domoic acid (**A**) in mussel meat and *Pseudo-nitzschia* cell abundances (**B**) in Lillebælt (catchment sites 63, 66, 68 and 71, see Fig. 1) during March and April 2016. A dotted gray line (20 mg kg^{-1}) on A indicates the international regulatory limit (WHO) of domoic acid in mussel meat.

affecting species competition and thus phytoplankton composition (e.g. Antia et al., 1991). In general, diatoms are considered mainly to utilize nitrate whereas most other phytoplankton is better adapted for using ammonium (Glibert et al. 2016), but the majority of studies regarding nitrogen sources confirm the capability of both freshwater and marine phytoplankton species to utilize urea, either partly or as a sole nitrogen

source (e.g. Cira et al., 2016; Cochlan et al., 2008; Donald et al., 2013, Glibert et al., 2014).

The impact of the changing nitrogen composition in agricultural fertilizer along with the increasing use of fertilizer is gaining attention. There is ample evidence for the detrimental impact of the increasing nitrogen usage in aquatic ecosystems (Howarth et al., 2002). The increasing use of urea in fertilizer has been coupled with an increase in documented incidences of Paralytic Shellfish Poisoning (PSP) (Glibert et al., 2006) caused by several toxic dinoflagellate Harmful Algal Bloom (HAB) species. No investigation has yet linked this with Amnesic Shellfish Poisoning (ASP) incidents in the field.

Growth and toxin content of HAB species have also been investigated in the laboratory in relation to nitrogen sources. The dinoflagellate *Alexandrium catanella* showed e.g. no increase in toxin production related to nitrogen type (Griffin et al., 2019). Contrary the same species (reported as *A. tamarense*) had a higher toxin content when grown on urea than compared to nitrate, but highest on ammonium (Leong et al., 2004). Another dinoflagellate, *Karenia brevis*, also showed much higher toxin content when supplied with urea than without urea supplement (Shimizu et al., 1993).

In general, the urea concentration in aquatic ecosystems is lower than the concentration of the inorganic N-sources (nitrate and ammonium), but in a few instances, concentrations up to 25–50 μM N have been reported (Glibert et al., 2005). The fact that each molecule of urea contains two atoms of nitrogen contributes to some confusion, as some refer to units of urea (e.g. 10 μM urea) and some to the nitrogen content in urea (e.g. 20 μM N urea), both examples are equivalent to the same concentration of urea. In the current study, nitrogen content in urea is used, as this unit is dominant in the literature.

1.3. Pseudo-nitzschia toxicity - impact and induction

The diatom genus PN comprises 52 marine species of which 26 are confirmed toxigenic (Bates et al., 2018). *Pseudo-nitzschia* is a globally distributed pennate diatom genus. Cells are efficient in utilizing nutrients in upwelling systems as well as during spring and autumn mixing of waters (Lelong et al., 2012), and some species of PN can cause major toxic blooms. The toxin is a simple neurotoxic amino acid called domoic acid (DA). The chemical compounds, metabolic precursors, and the penultimate enzymatic steps for the biosynthesis of DA were recently described (Maeno et al., 2018; Brunson et al., 2018; Harðardóttir et al., 2019) but the final isomerization reaction, sources, and compartmentalization are yet to be elucidated.

Toxic blooms may have severe impacts on marine ecosystems as DA can accumulate in primary consumers, e.g. filtrating mussels, zooplankton, and smaller fish. This can cause intoxication and mortality of secondary consumers like marine mammals, seabirds and humans (Teitelbaum et al., 1990; Lefebvre et al., 2016; Nash et al., 2017). Furthermore, HABs have large economic effects on the mussel industry and fisheries, due to closure of the fishery, lost revenue and discard of already harvested mussels (Fehling et al., 2006; McCabe et al., 2016; Bates et al., 2018).

Domoic acid production is affected by various factors, and it has been suggested that dissimilar species are affected differently (Gai et al., 2018). Nutrient composition and levels have a major influence on DA production. Changes in nitrogen have been found to affect DA production, but notably, nitrogen is a component in DA synthesis, and deficiency may, therefore, limit DA production (Trainer et al., 2012 and references therein). Depletion of silicate and phosphate generally induces high cellular DA (Ryan et al., 2017), and species and strain variation in cellular DA content in response to several environmental changes have been observed for iron concentration, salinity, temperature, pH, light, and presence of bacteria and grazers (Bates et al., 2018).

In addition to the variety of inducing environmental factors, the impact of different growth stages on DA production, is still unclear. Some studies have revealed how PN only produces DA in the stationary growth phase, while other studies have shown that DA production is active in the exponential growth phase (Lelong et al., 2012 and references therein).

1.4. Nitrogen and pseudo-nitzschia

From field studies, it is well established that PN benefits from environmental nitrogen loading, both with regard to growth and toxicity (e.g. Downes-Tettmar et al., 2013; Husson et al., 2016; Thorel et al., 2017). Studies assessing the impact of inorganic nitrogen on PN are increasing in number (Auro and Cochlan, 2013; Bates et al., 1993; Bates and Trainer, 2006; Thessen et al., 2009), as are those focusing on organic nitrogen sources such as urea and amino-acids (Auro and Cochlan, 2013; Hillebrand and Sommer, 1996; Howard et al., 2007; Kudela et al., 2008; Martin-Jézéquel et al., 2015.; Radan and Cochlan, 2018). Different nitrogen sources seem to affect PN differently and presently no general pattern in growth, biomass and DA production has been established.

The effect of urea on DA production is, like other DA-inducing factors, unclear in the few studies performed. Urea has been shown to result in higher or lower cellular DA content when compared with other N-sources. When exposing *P. cuspidata* to different N-sources, an increase in DA content was found when grown with nitrate or ammonium as N-source compared to urea (Auro and Cochlan, 2013). On the contrary, higher DA content was found when *P. australis* was grown on urea rather than on inorganic N substrates (Howard et al., 2007). Similarly, *P. multiseries* contained more DA when growing on urea compared to inorganic N-sources (Radan and Cochlan, 2018).

Among PN species, there also seems to be a considerable variation in growth rates in response to N-sources (Radan and Cochlan, 2018). In some, although not all, strains of *P. multiseries, P. calliantha* and *P. fraudulenta,* a significant decrease in growth rate was found when growing on urea as the sole nitrogen source compared to ammonium or nitrate (Thessen et al., 2009). On the contrary in another study, *P. multiseries* and *P. australis* had similar growth rates when growing on nitrate, ammonium, and urea (Martin-Jézéquel et al., 2015)

The effects of different nitrogen sources have been compared, whereas the effects of different *concentrations* of urea have presently not been assessed in PN. As urea contains nitrogen, crucial for cell growth and DA production, variations in urea concentration are, however, expected to have an impact on both cell growth and DA content.

1.5. North Atlantic Pseudo-nitzschia species for testing effects of urea

A wide variety of PN species are confirmed present in Danish waters and other parts of the North Atlantic area (Lundholm et al., 2010; recently reviewed in Bates et al., 2018), and several have been confirmed as toxigenic: *P. seriata* (Lundholm et al., 1994), *P. pungens* (Rhodes et al., 1996), *P. fraudulenta* (Rhodes et al., 1998), *P. australis* (Fehling et al., 2004) and *P. granii* (Trick et al., 2010). The presence of toxigenic PN-species in Danish and neighboring waters poses a risk for toxic events. In 2005, a bloom of *P. seriata* caused a closure of mussel harvesting areas in Denmark, close to the areas affected in 2016 (Lundholm et al., 2005), and in other North Atlantic areas, several bivalve fishery areas have previously been quarantined e.g. in western Scotland (Fehling et al., 2006, and references therein).

Data were gathered on the phytoplankton community, DA in mussels and the environmental conditions in the months before and following the collapse of the fertilizer silo. The aim was to explore whether urea can induce growth and affect DA content in PN species and hence explain the development of the observed toxic bloom. Did the fertilizer spill have any quantitative or qualitative impact on the phytoplankton community? Can urea induce growth and hence initiate a *P. seriata* bloom? Can urea induce toxin production in *P. seriata* and other PN species? To answer these questions, batch culture experiments with strains of *P. seriata, P. delicatissima,* and *P. obtusa* were performed.

Pseudo-nitzschia seriata (Fig. S2) have previously been shown toxic in Danish, Scottish and Greenlandic waters (Lundholm et al., 1994; Fehling et al., 2004; Hansen et al., 2011) and recently strains from Iceland were tested positive for DA (Lundholm et al., 2018). *Pseudo-nitzschia delicatissima* has not been found toxic in Danish-, and adjacent waters, but isolates from other geographical regions have been recorded toxic (Bates et al., 2018). *Pseudo-nitzschia obtusa* strains from Greenland are known to produce DA in the presence of copepods (Harðardóttir et al., 2015), but no other inducing factor is confirmed for this species.

2. Materials and methods

2.1. Spring 2016 monitoring data

Toxin in mussel meat: Monitoring data on DA content in mussel meat (*Mytilus edulis*) from four mussel harvesting areas (areas 63, 66, 68 and 71) (Fig. 1) were gathered. All areas are connected to the water at the location of the silo collapse. By including data from all four areas, we ensure a good temporal and areal resolution for improved understanding of DA accumulation in the environment. Toxin data for each harvesting area is restricted as, in Denmark, toxin analyses on fresh mussel meat are performed only when a harvesting area is designated for industrial harvesting. Therefor data is not obtained regularly, independent of the mussel industry. Toxins levels in mussels are measured every week during harvesting periods. Toxin data (from Union of Danish Mussel Fishery) and relating phytoplankton data (from Orbicon/NIRAS) on *P. seriata* type cells were monitored with a frequency of 7–35 days from February to April 2016.

Phytoplankton diversity and abundance, as well as chemical (nutrient levels) and physical (temperature and salinity) parameters: Data was gathered monthly from the monitoring station "Lillebælt Nord Vej0006870" (located close to mussel harvesting area 66, see Fig. 1). The Danish Ministry of Environment and the consulting companies Orbicon and NIRAS provided data. The phytoplankton monitoring station "Lillebælt Nord VEJ0006870", being the monitoring station geographically closest to the silos (on the harbor of Frederica), was chosen as the focus area for the data analyses (Fig. 1). The mussel harvesting area 71 was located even closer to the silos (Fig. 1).

Data on salinity, temperature and nutrients were acquired for 1 m depth from January to April in the period 2010–2016 from the national monitoring program. Monitoring data on the phytoplankton community was gathered from January to April 2013–2016 and was afterward edited by pooling all observations including size fractions of different "species" e.g. cryptophytes <10 μm or >10 μm or by unclear species denotations. Furthermore, the group "unidentified flagellates" was excluded from the analyses as they were not a part of the monitoring data in all years. On occasions with data, they represented a stable high abundance. Phytoplankton samples were studied by electron microscopy to identify PN cells following the procedure of Lundholm et al. (2002) and examined using a transmission electron microscope (TEM) (JEOL, JEM 1010, Tokyo, Japan) to obtain the necessary morphometrics as well as qualitative morphological characters for species identification (see Fig. S1 for detailed micrograph).

2.2. Culturing and species identification

To establish cultures of PN for the experiments, live phytoplankton samples were collected from three different locations in Greenland, Iceland and Denmark in spring 2017 and 2018 (Table 1). The phytoplankton samples were collected using a 20-μm plankton net, hauled through the upper 0–5 m of the water column. Single cells or chains of PN spp. were isolated by micro-pipetting using a hand-drawn Pasteur pipette under an inverted light microscope (CKX53, Olympus, Tokyo Japan) and placed in 96 well plates with L1 medium. The cultures were kept at 4 °C at a light:dark cycle 16:8 h and 100 μmol photons m^{-2} s^{-1} using cool white light bulbs (550 nm wavelength). These conditions were the same as for the experiment.

Identification of PN cultures was performed as mentioned above for phytoplankton samples (see Table S1 and Fig. S2 for detailed micrographs and data). The strains were identified as *P. delicatissima* (strain S5 from 2018, Denmark), two *P. seriata* strains (I1 and I9 from 2018, Iceland) and *P. obtusa* (CA2 from 2017, Greenland) (Fig. S2). See Table 1 for specific locations. Molecular identification (ITS rDNA region) was established for the strain S5 of *P. delicatissima* strain and confirmed the morphological identification (data not shown) as the ITS regions clustered with other *P. delicatissima*a. Light micrographs were taken with a Zeiss Axio Imager.M2 microscope (Zeis, Oberkochen, Germany) equipped with a 63X oil immersion lens and a Zeiss AxioCam HRc digital camera.

2.3. Experimental setup

Growth and toxicity of four PN strains (*P. delicatissima* (S5), two *P. seriata* strains (I1 and I9) and *P. obtusa* (CA2) was studied in batch culture experiments using three concentrations of urea (10 μM, 20 μM, and 100 μM N urea) and one concentration of nitrate (10 μM N). The nitrogen levels were chosen to depict concentrations, that can be found in coastal waters (10 μM N and 20 μM N) and a high concentration (100 μM N) similar to that of extreme situations such as following the breakdown of the fertilizer silos.

The medium used in the experiment: L1 medium was prepared based on seawater with a salinity of 30, and nitrogen in the medium was added to the desired concentrations of urea or nitrate in addition to the N content present in the natural seawater. pH in the medium was measured using a VWR pHenomenal pH meter and adjusted to pH 7 by adding minute amounts of HCl (10 M).

Initiation of the experiment: All experiments (for each strain and nitrogen treatment) were performed in triplicates in 655 mL flasks with 280 mL medium and either approximately 2000 cells mL^{-1} of *P. seriata* (setup A and B) or *P. obtusa* (setup D), whereas 3500 cells mL^{-1} of *P. delicatissima* were used (setup C), as this is a smaller species. Ten days before the experiments, the cultures were diluted to be in the exponential growth phase at the initiation of the experiment.

To monitor temperature stability throughout the experiments, a data logger (Omega OM-SQ204, Manchester, UK) was set up to measure temperature every 5 min for 48 h. Four sensors were placed near the experimental setup in a temperature-controlled room. The average temperature was stable at 4 $^{\circ}C \pm 0.5$ °C.

2.4. Sampling during experiments

Cell density was monitored every second or third day, and before every sampling, the flasks were carefully rotated to ensure equal mixing of the culture.

Sampling frequency: Due to fast growth, *P. delicatissima* (C) was sampled every other day for relative fluorescence units (RFU) and cell counts. RFU and cell counts were sampled every third day for *P. seriata* (A and B) and *P. obtusa* (D). For all species, sampling for nutrient analysis was obtained once at the initiation of the experiment (D0), once during the exponential growth phase [*P. seriata* (**A**): Day 13 for all treatments, *P. seriata* (**B**): Day 14 for 10 μM N nitrate and 100 μM urea, and day 19 for 10 and 20 μM N urea, *P. delicatissima* (**C**): Day 12 and *P. obtusa* (**D**): Day 13] and once in stationary growth phase [*P. seriata* (**A**): Day 19, *P. seriata* (**B**): Day 19 for 10 μM N nitrate and 100 μM urea, and day 21 for 10 and 20 μM N urea, *P. delicatissima* (**C**): Day 15, and *P. obtusa* (**D**): Day 19]. Sampling for DA was obtained in the stationary growth phase, on the same days as sampling for nutrients. To assess if pH was the limiting factor for cell growth in the assays, pH was measured at the end of the experiments.

Table 1
Date, origin and position of strains of *Pseudo-nitzschia* spp. used in this study. The salinity of the water body from which the strain was isolated is also indicated.

Date	Species (strains)	Origin	Latitude	Longitude	Salinity
11 April 2018	*P. delicatissima* (strain S5)	Denmark Helsingør	56° 02′ 35.7″ N	12° 37′ 07.2″ E	24
1 May 2018	*P. seriata* (strain I9)	Iceland Hvalfjörður	64° 22′ 10.8″ N	21° 34′ 4.6″ W	32
23 June 2017	*P. seriata* (strain I1)	Greenland Disko Bay	69° 11′ 1″ N	53° 31′ 1″ W	30
	P. obtusa (strain CA2)				

2.5. Methods for cell density and growth rates

Cell densities: 1.5 mL of each replicate was fixed in 2–3% acidic Lugol's solution. One of the triplicates was counted using a Sedgewick-Rafter counting chamber (S52). A minimum of 400 cells was counted.

RFU: 1.5 mL sample was transferred to a clear glass flask and measured using a Triology fluorometer (San Jose, CA, USA). Triplicates of all treatments of all strains were measured by RFU, and based on standard curves, RFU measurements were translated into cell densities.

Maximum growth rate calculation: These were based on data points when batch cultures were in the exponential growth phase. See Table S4 for specific days.

The calculation of growth rates was based on the formula:

$$N_b = N_a \times e^{\mu \times t_b}$$

Where N_b and N_a are cell concentrations at time b and an (in hours). μ is a specific growth rate hour^{-1}. Subsequently, growth rates (GR_d) (cell divisions day^{-1}) was calculated as follows:

$$GR_d = \mu^{*}24\ h$$

2.6. Nutrient analysis

60 mL subsamples were centrifuged (1811 G for 15 min at 4 °C) separating cells and supernatant and 40 mL supernatant was used. Analyses were performed at the Terrestrial Ecology Section, Dept of Biology, University of Copenhagen. Urea measurements followed the method by Alam et al. (2017) using a photometer (V-1200VWR, Avantor, Radnor, Pennsylvania, US). Ammonium, nitrate and total inorganic nitrogen (a total of nitrate, nitrite, and ammonium) were analyzed using the methods by Foss Tecator 5201 and 5220 2001, using FOSS FIAstar 5000 and Shimadzu TOC-L.

2.7. Toxin analyses

For cellular DA (CDA), 60 mL subsamples were centrifuged (1811 G for 15 min at 4 °C) separating cells and supernatant and the pellet collected in a 1.5 mL centrifugation tube (Eppendorf, Hamburg, Germany). The pellets were frozen and kept at −20 °C until further analysis for CDA.

Each sample was vortexed for 30 s after 10 min of sonication (Sonorex Digitec, Bandelin, Berlin, Germany). The water phase was subsequently separated from the residual cellular particulates by centrifuging the samples for 10 min at 16,100 G. 500 μL supernatant were transferred to centrifugal filters (Merck Millipore, Ultrafree MC HV, Durapore PVDF 0.45 μm) and centrifuged for 1 min at 16,100 G. At last, the filtrates were transferred to 2-mL crimp cap vials and sealed. Analyses of the extracts were performed using a method coupling liquid chromatography to tandem mass spectrometry (LC–MS/MS) as described in Krock et al. (2008). The limit of detection of CDA acid depends on analyzed biomass and is given as pg cell^{-1} in Table S2.

2.8. Statistics

Statistical analyses were conducted using Graphpad Prism (ver. 8). Comparisons were conducted with one-way ANOVA. A significance level (α) was accepted at $p < 0.05$ for all statistical analyses.

3. Results

3.1. Domoic acid in mussels during Pseudo-nitzschia bloom after fertilizer spill

Domoic acid was measured in mussel meat at four locations (area 63, 66, 68 and 71; Fig. 1) approximately five weeks (on 13 March 2016) after the fertilizer spill (Fig. 2). The highest level of DA were measured on 21 March in area 63, on 29 March in area 66, on 14 March in area 68, and on 4 April in area 71 reaching 49, 45, 4.9 and 20 mg DA kg^{-1}, respectively. Domoic acid contents of mussels were well above the World Health Organization regulatory limit of 20 mg DA kg^{-1}. In area 66, cell abundance peaked approximately 14 days before the corresponding DA peak in mussel meat, but conclusions are somewhat hampered by a few data points. At station 63, DA in mussel meat and cell abundances peaked simultaneously. Cell abundances reached the highest level in area 66 (~110,000 cells L^{-1}), and in all areas, a decline in cell abundance was accompanied by a decrease of DA levels in the mussels (Fig. 2). In the national monitoring program sampling frequencies are limited. Thus, we acknowledge that cell abundances could have fluctuated between sampling, unrecorded.

3.2. Nutrient concentrations at Lillebælt Nord

The concentrations of inorganic nutrients for the first four months of 2010–2016 at Lillebælt Nord (VEJ0006870, see Fig. 1), is shown in Fig. 3. Total Inorganic Nitrogen (TIN): In January and February 2010–2015, TIN was on average high (~250 μg L^{-1}), thereafter TIN decreased in March and April. The year 2016 was markedly different

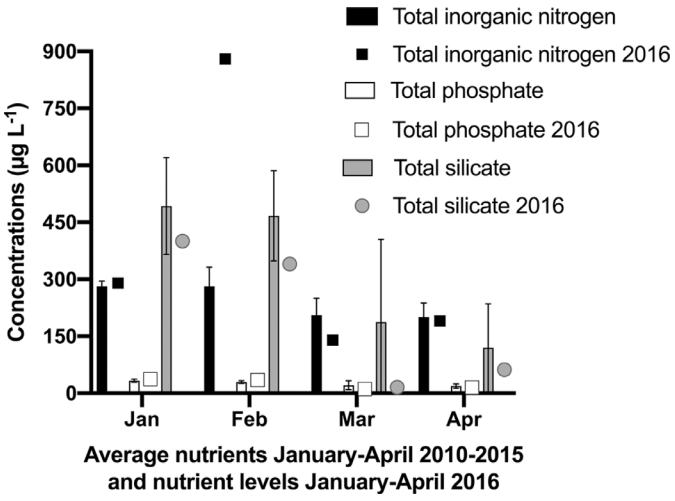

Fig 3. Average levels of total phosphate, total silicate, and total inorganic nitrogen given as μg L^{-1} from January to April 2010–2016 from the monitoring station VEJ0006870. Data are from 1-meter depth. Measurements of the 2016 nutrient levels are indicated separately.

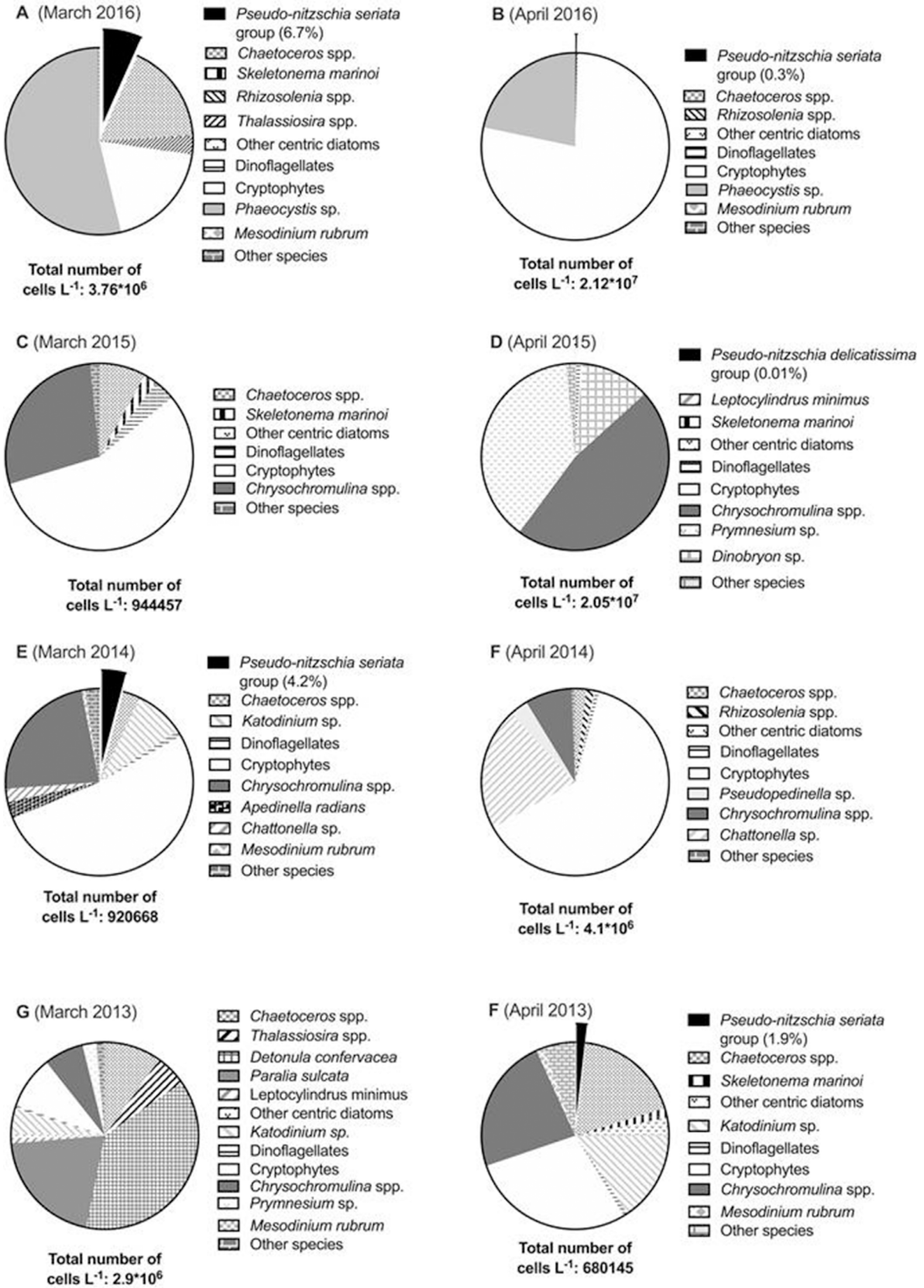

Fig. 4. Abundance of dominant taxonomic groups of phytoplankton in March and April 2013–2016, by monitoring station VEJ0006870. Phytoplankton species and groups representing less than 10,000 cells L^{-1} are clustered in the groups entitled "other species". Total number of cells L^{-1} are given below each diagram.

compared to previous years: In January, the concentration of TIN was similar to 2010–2015, but in February TIN increased to a high level (880 μg L^{-1}). Notably, TIN afterward decreased rapidly and already in March 2016, it was similar to the level measured in March 2010–2015 (Fig. 3).

Total silicate (TS) content decreased from January to February. In spring 2016, silicate levels were similar to previous years (Fig. 3). For all years, total phosphate (TP) concentration was in the range of 1–30 μg L^{-1}; highest in January followed by a slight decrease in the following three months (Fig. 3). The patterns were somewhat similar for all years and nothing exceptional was observed for 2016. Salinity and temperature did not reveal any irregularity in 2016 to explain the development of the phytoplankton community in 2016 (Table S3).

3.3. Phytoplankton community at Lillebælt Nord

From January to March 2013–2015, the phytoplankton community at the Lillebælt Nord station generally developed with a 1 - 4.4-fold increase in cell abundance (Fig. 4). The year 2016 differed significantly from the previous years, as cell densities escalated, increasing 22-fold, from 1.71 × 10^5 cells L^{-1} in January to 3.8 × 10^6 cells L^{-1} in March (Fig. 4). The number of taxonomic groups was lower in 2016 than the previous years. In March 2016, five major phytoplankton groups (major defined as >10,000 cells L^{-1}) accounted for the high cell density. *Phaeocystis* sp. was dominant in March 2016 (Fig. 4A) but contributed less than 10,000 cells L^{-1} at the same time in 2013–15. Similarly, and in contrast to the previous years, PN was one of the major phytoplankton groups in March 2016 (Fig. 4A). The TEM analysis revealed that the PN community solely consisted of *P. seriata* (Fig. S1).

April was for all years characterized by a phytoplankton community with high cell densities, but April 2016 was distinct because the extremely high cell density was combined with low species diversity (only five groups exceeding 10,000 cells L^{-1}) (Fig. 4B). In April 2015, the cell densities were similar to those of April 2016 (app. 21,000.000 cells L^{-1}), but in 2015 this accounted for 11 major phytoplankton groups (with more than 10,000 cells L^{-1}) (Fig 4D).

Hence overall, the phytoplankton community in 2016 was highly unusual compared to the previous years at the monitoring station with a particularly low diversity for March, and April (Fig. 4). The approximate timing of the increasing density of *P. seriata* was at the beginning of March; one month after the breakdown of the silos. This is expected for winter-early spring in Denmark, as most phytoplankton species grow slower at low temperatures and few hours of daylight.

3.4. Pseudo-nitzschia experiments using different concentrations of urea

3.4.1. Maximum growth rates

For ***P. seriata*** strain I9, a positive correlation was found between the maximum growth rate and urea concentration (R^2 = 0.83) (Fig. 5A). The treatments with 10 μM N nitrate and 100 μM N urea had the highest growth rate. Overall, the other *P. seriata* strain (strain I1) had a similar response to *P. seriata* (strain I9), although with lower cell densities and growth rate (Fig. 5B). For strain *P. seriata* (I1), no actual exponential phase was observed in treatment 10 and 20 μM N urea and therefore the growth rate is based on the two days where an increase in cell density was observed. (Fig. 5B and Table S4). There was no difference in growth rate between the 100 μM N urea and 10 μM N nitrate treatment, but both of them were significantly higher than the growth rates at 10 and 20 μM N urea ($p < 0.001$).

For ***P. delicatissima*** (strain S5) (Fig. 5C), the growth rate at 20 μM N urea was higher than at 100 μM N urea and 10 μM N nitrate ($p < 0.05$), and the difference between 10 and 20 μM N urea was not significant. The cell densities reached high levels in all four treatments (Fig. S3).

For ***P. obtusa*** (strain CA2) (Fig. 5D), the growth rates showed a pattern similar to *P. delicatissima*, with the two highest growth rates found in the 10 and 20 μM N urea treatments, but with 20 μM N urea being significantly higher than 10 μM N urea ($p < 0.05$) (Fig. 5D). Growth rates at 20 μM N urea was significantly higher than both 100 μM N urea ($p < 0.0001$) and 10 μM N nitrate ($p < 0.001$). No significant difference was found between the growth rates in 100 μM N urea and 10 μM N nitrate and the 10 μM N urea and 10 μM N nitrate. The highest cell densities were reached in 100 μM N urea, and the lowest in 10 μM N nitrate (See Fig. S3).

When comparing growth rate responses of the different taxa with similar amounts of different sources of nitrogen, 10 μM N nitrate and 10 μM N urea, *P. seriata* had significantly higher growth rate when grown on nitrate, whereas *P. delicatissima* and *P. obtusa* had similar growth rates grown on the two types of nitrogen, although there was a trend of a higher growth rates when grown on urea (Fig. 5).

3.4.2. Domoic acid content

One of the *P. seriata* strains (strain I1) and the *P. obtusa* strain contained DA. *Pseudo-nitzschia seriata* (strain I1) cells contained DA in three of the four treatments (Fig. 6A). The highest DA content was found in the 10 μM N nitrate treatment (0.22 pg $cell^{-1}$), but a slightly lower content, although not significantly different, was found in 10 and 20 μM N urea. The cells grown at 10 μM N urea contained 0.04 pg DA $cell^{-1}$. No significant difference was found between the three treatments, due to the very high variability among triplicates, especially in 20 μM N urea. *P. seriata* did not contain DA above detection limit when grown in 100 μM N urea.

Pseudo-nitzschia obtusa (strain CA2) contained DA in all four treatments (Fig. 6B). The highest DA content was found in the treatments 10 and 20 μM N urea and 10 μM N nitrate ($p > 0.05$). The cells in 100 μM N urea had the lowest DA content which was significantly less than in all other treatments ($p < 0.05$). Hence in both strains, the 100 μM treatment resulted in the lowest (or no) DA content, and cells grown in 10 μM N nitrate had in the highest DA content though this was not significant (Fig. 6), probably because of the high variability of among triplicates.

3.4.3. pH changes during the experiment

The initial pH was 7.8 in all treatments for all strains, whereas pH at the end of the experiments varied (see details in Table 2). For all strains, 100 μM N urea treatments resulted in the highest pH at the end of the experiments. pH in *P. obtusa* was significantly higher than all other strains in the 100 μM N urea treatments ($p > 0.05$).

3.4.4. Nutrients

In all treatments and for most of the strains, the urea concentrations were depleted the latest on day 12 or 13, except for *P. seriata* strain I1 (Fig. 7). Strain I1 utilized the available urea slowly in the 10 and 100 μM N urea treatments, in agreement with the slower growth rates, depleting it on day 19 (100 μM N urea) or not at all.

Total inorganic nitrogen (TIN) was more or less stable throughout the experiment (Fig. 8), except for the 100 μM N urea treatment which for all strains showed a decrease in TIN level during the first 12–14 days. The detailed evolution in TIN in all strains and all treatments varied and the result are hampered by the long period between nutrient assessments. In *P. seriata* (strain I1), TIN levels increased towards the end of the experiment.

Ammonium (Fig. S4) and nitrate (Fig. S5) was utilized at different speeds. Nitrate was rapidly depleted in all strains except *P. seriata* (strain I1), before or latest by day 12–14, although the starting level was higher than that of ammonium. Ammonium was approaching depletion by the end of the experiment in all, but one strain (*P. seriata* strain I1).

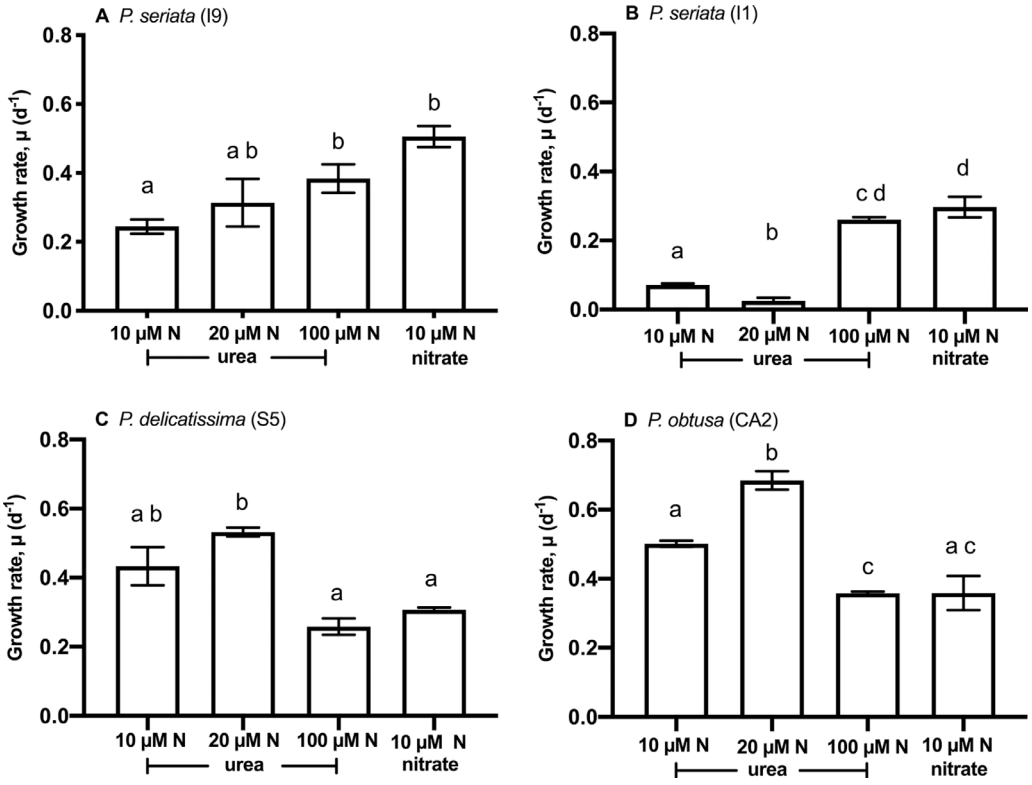

Fig. 5. Maximum growth rates of *P. seriata* (strain I9) (**A**), *P. seriata* (strain I1) (**B**), *P. delicatissima* (strain S5) (**C**) and *P. obtusa* (strain CA2) (**D**) as a function of N-sources (four treatments). Statistically significant differences from One-Way ANOVA analyses between treatments are marked by different letters, whereas statistically non-significant differences are marked by identical letters.

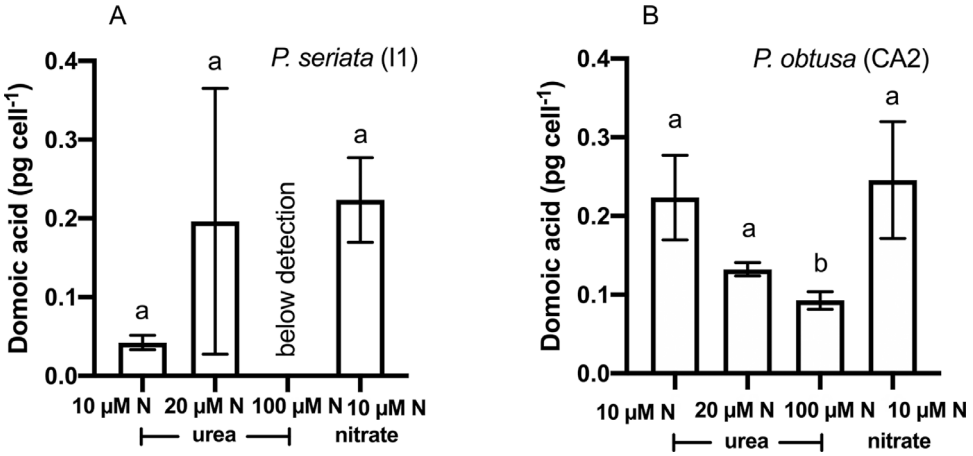

Fig. 6. Domoic acid content of *P. seriata* (strain I1) (**A**) and *P. obtusa* (strain CA2) (**B**) as a function of N-sources. Different letters mark statistically significant differences (One-Way ANOVA analyses) between treatments, whereas identical letters mark statistically non-significant differences.

4. Discussion

4.1. The collapse of the silos and the phytoplankton community

Compared to previous years, the total inorganic nitrogen concentrations measured in mid-February 2016, revealed the impact of the preceding spill (Fig. 3). Nitrogen and light levels are usually the limiting factors for spring phytoplankton blooms (Granéli et al., 1990), also in Danish waters. Hence the supplement of nitrogen and specifically urea, to the seawater adjacent to the harbor of Frederica, could

Table 2
Final pH level in experiment. Initial pH was 7.8. Significant differences between treatments within a strain is marked with different letters. Similar pH levels between treatments of a strain is marked with similar letters. pH for *P. obtusa* in treatment 100 μM N urea was significantly higher than all other strains in all treatments.

	P. seriata I9 Av ± Sd	*P. seriata* I1 Av ± Sd	*P. delicatissima* S5 Av ± Sd	*P. obtusa* CA2 Av ± Sd
10 μM N urea	8.40 ± 0.00^{a}	8.00 ± 0.00^{a}	8.17 ± 0.05^{a}	8.23 ± 0.10^{a}
20 μM N urea	8.47 ± 0.05^{a}	7.83 ± 0.05^{a}	8.52 ± 0.00^{b}	8.61 ± 0.00^{b}
100 μM N urea	8.72 ± 0.00^{b}	8.61 ± 0.00^{b}	8.67 ± 0.05^{b}	9.17 ± 0.05^{c}
10 μM N nitrate	8.27 ± 0.12^{a}	7.93 ± 0.05^{a}	8.12 ± 0.00^{a}	8.23 ± 0.05^{a}

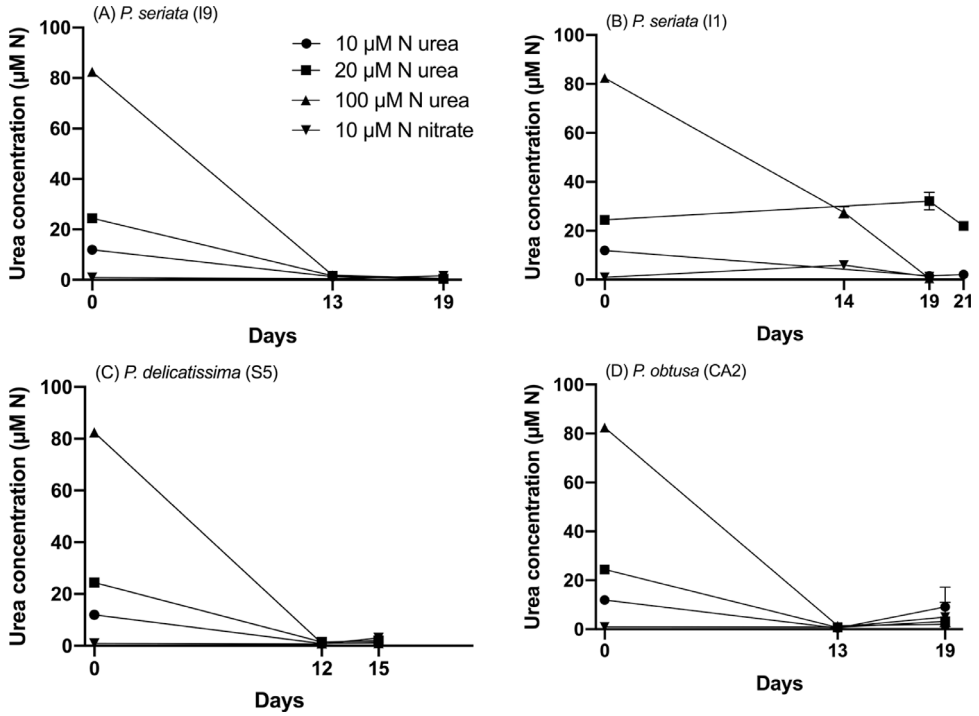

Fig. 7. Concentrations of urea (µM N urea) sampled during the experiments with *P. seriata* (I9) (**A**), *P. delicatissima* (S5) (**C**) and *P. obtusa* (CA2) (**D**) *P. seriata* (strain I1) (**B**) as a function of time (days). The experimental period was between 15 and 21 days.

have caused the increased abundance and the lower diversity of the spring bloom in 2016, compared to the years before.

It has been suggested that growth rates vary greatly depending on the N-source, advocating that a change in the composition of N has a significant impact on a diatom community composition (reviewed in Radan and Cochlan, 2018). An An increasing availability of nitrate can favor the growth of *P. pungens* over *P. multiseries,* and similarly increasing abundance of *P. americana* has been linked to increased ammonium concentrations (Lundholm et al., 2010). It is clear from all investigations conducted that phytoplankton species take up and process nitrogen differently and with varying efficiency (Hillebrand and Sommer, 1996; Martin-Jézéquel et al., 2015).

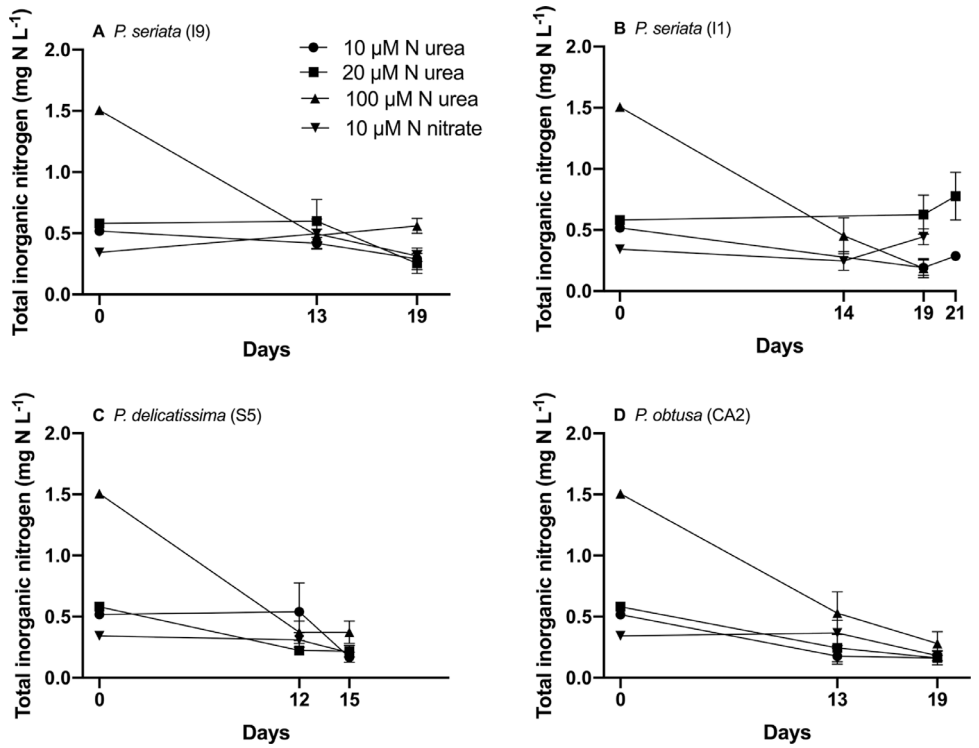

Fig. 8. Concentrations of total inorganic nitrogen (mg N L^{-1}) sampled during the experiment with *P. seriata* (strain I9) (**A**), *P. seriata* strain I1) (**B**), *P. delicatissima* (strain S5) (**C**) and *P. obtusa* (strain CA2) (**D**) as a function of time (days). The experimental period was between 15 and 21 days.

A report published right after the collapse of the silos by Markager (2016) predicted a vast increase in density of the spring phytoplankton bloom depending on the mixing of the heavy fertilizer liquid in the water column in the area adjacent to the harbor. Compared to the magnitudes of the spring blooms of 2013, 2014 and 2015, the year 2016 was undoubtedly a contrasting year, with a phytoplankton density escalating 22-fold faster than previous years and a particular low species diversity (Fig. 4). The Lillebælt area, adjacent to the harbor, is characterized by a frequent and pronounced halocline separating the brackish water entering from the Baltic and the saline water from Skagerrak and the North Sea. The liquid fertilizer had a density of 1320 kg m^{-3}, much heavier than both the upper (~1010 kg m^{-3}) and the lower (~1025 kg m^{-3}) water layers (Markager, 2016). The velocity of the infusion of fertilizer into the water layers would influence the area quite differently. Slow mixing into the water column would have allowed the majority of the fertilizer to settle in the bottom waters, below the photic zone. If this was the case, immediate effects seen in phytoplankton densities would have been limited. Contrary, if the spilled fertilizer was mixed into the water column a massive phytoplankton bloom would theoretically follow it after the collapse, as we could observe from the phytoplankton community analysis (Fig. 4).

The finding of DA in blue-mussel meat in March 2016 and an extensive bloom of *P. seriata* after exposure to a high load of urea-based fertilizer led to the questions assessed with the experiments; how does urea affect growth and toxicity of *P. seriata* and are other species of PN similarly affected?

4.2. Growth related to nitrogen source

The two tested *P. seriata* strains showed a somewhat similar pattern in growth rates in response to the different N treatments; increasing growth rates responding to the increased amount of urea (10–100 μM N urea), with only the exception of strain I1 growth rate was higher in 10 μM N urea than in 20 μM N. The highest growth rate of both strains was found at 10 μM N nitrate, proposing a preference for nitrate over both urea and ammonium (as ammonium concentration increased with urea content) (Figs. S4, S5, and 7). Following the collapse of the fertilizer silo, high TIN concentrations were measured in the area adjacent to the harbor of Frederica. This possibly leading to favorable conditions for *P. seriata,* corresponding well to the results obtained here that *P. seriata* can utilize any N-form and grow fast at high N-levels.

The *P. obtusa* strain (CA2), had higher growth rates in the 20 μM N urea treatment compared to 100 μM N urea and 10 μM N nitrate, suggesting that at least this strain of *P. obtusa* is good at utilizing urea. According to a review by Radan and Cochlan (2018), no studies have found an increase in the growth rate of PN species when growing on urea compared to similar concentrations of other N-sources. Several other studies have, however, found similar growth rates among nitrate, ammonium, and urea (Hillebrand and Sommer, 1996; Martin-Jézéquel et al., 2015; Radan and Cochlan, 2018), as was observed in the current study for *P. delicatissima* and *P. obtusa* (Fig. 5). Thessen et al. (2009) found a significant decrease in growth rate in some, but not all, strains of *P. multiseries, P. calliantha* and *P. fraudulenta* when grown on urea as the sole nitrogen source compared to ammonium or nitrate. This is similar to both *P. seriata* strains in the present study (Fig. 5). Overall the result imply that urea is equally or less efficient as a nitrogen source for PN species compared to nitrate. Even though it was shown here that N-type and load can be a determining factor for growth and toxin production in PN strains additional studies are needed to fully explore the inter- and intra-specific variation.

4.3. Toxin content related to nitrogen source in Pseudo-nitzschia spp

Notably, *P. obtusa* contained DA in all the N treatments (Fig. 6). This is the first report of *P. obtusa* containing DA without it being induced by the presence of copepods. In Hasle and Lundholm (2005) *P. obtusa* did not contain DA as a response to changes in temperature, pH and nutrient levels. In Harðardóttir et al., 2015, *P. obtusa* contained DA as a response to the presence of copepod grazers. In the present study, pH increased to 8.23–9.17 at the end of the experiment, which is not higher than pH levels tested on *P. obtusa* (Hasle and Lundholm, 2005). The difference in DA content in response to environmental factors of *P. obtusa* could be related to strain variation of the same species, as previously shown for other PN species (e.g. Bates et al., 2018). Or as the toxin content is low compared to e.g. *P. seriata* it might be due to the detection levels.

In the present study of *P. obtusa,* a reverse correlation between DA content and urea concentration (Fig. 6). This is surprising, as N is needed for biosynthesis of DA and thus a positive correlation between N availability and DA content would be expected. We find the treatment with the lowest concentration of urea (10 μM N urea) resulting in the highest cellular content of DA, but it is impossible to determine whether the cells were nitrogen depleted because of the remains of inorganic nitrogen. No other study has compared the effect of different concentrations of urea on DA content, but a recent study revealed that nitrogen and urea affect both phytoplankton community dominance and DA content in three PN species (Tatters et al., 2018). The community composition changed depending on CO_2 levels, temperature, and nitrogen source. Tatters et al., (2018) showed nitrogen source to be the most determining factor for the dominance and composition of the phytoplankton community and confirmed that CDA content varies depending on the nitrogen source, in agreement with; Cochlan et al. (2008) and Radan and Cochlan (2018). Previous studies testing the impact of urea on DA content have shown urea resulting in higher DA content than similar N-concentrations of nitrate in *P. multiseries* and *P. australis* (Howard et al., 2007; Radan and Cochlan, 2018), but in lower DA content in *P. cuspidata* (Auro and Cochlan, 2013). In the present study, similar concentrations (10 μM N urea and nitrate) of different N sources resulted in similar DA content (Fig. 6B). This illustrates that the responses to nitrogen sources are completely species-dependent and that caution should be taken when extending conclusions on responses to environmental factors based on one species to explain correlations in other species.

One of the two *P. seriata* strains contained DA.. I In this strain the cellular DA content was similar in all treatments but undetectable in 100 μM urea. As demonstrated, the N concentration has a great impact on both growth and toxicity (Tatters et al., 2018), but as indicated here, the correlation between nitrogen load, DA content, and growth rate is variable both between species and among strains. It is therefore not possible to draw concrete conclusions and we emphasize a need for further studies including several strains of the same species. In *P. seriata,* a high growth rate at the highest urea concentration was related to the lowest DA content (Fig. 6). On the contrary, nitrate resulted in both the highest growth rate of all and the highest DA content (Fig. 5 and Fig. 6). One may speculate, if the result are different responses to the two N-sources, with DA being “diluted” in cells with high growth rates when utilizing urea, while nitrate supports high growth rates and accumulation of DA. The growth response in *P. obtusa* to increasing urea concentrations was, on the contrary different (see above) with no such correlation deduced. Compared with studies including several N sources, the results are in discrepancy; Martin-Jézéquel et al. (2015) and Radan and Cochlan (2018) both included one strain of *P. multiseries* and both found high growth rates and high DA content when growing with urea, i.e. no dilution of DA when utilizing urea for growth. Martin-Jézéquel et al. (2015) also tested one strain of *P. australis* using several N tsources. Here high mean biomass in the stationary phase, linked to low DA content when growing on urea. However, no other N-source sustained high biomass and high DA. We see no support for the speculation on N- source being related to CDA dilution or accumulation.

Domoic acid content has been related to different higher N concentrations in *P. multiseries* (55–880 μM N of nitrate and ammonium) (Bates et al., 1993) demonstrating a limited effect of N-concentrations

until a maximum concentration was reached (at app. 880 μM N). This agrees with the current results in *P. seriata* where N-concentration had almost no effect on the DA content, while in *P. obtusa* DA content was highest in 10 μM N (correlating to 10 μM N urea and 10 μM nitrate) and DA content decreased with increasing urea N concentration (20 and 100 μM N urea). These results support the indications of interspecificity related to responses to nitrogen type and nitrogen concentrations.

4.4. Nitrogen source preferences and Pseudo-nitzschia

Previous studies have demonstrated the ability of diatoms to take up urea, but most studies report inhibitory effects on urea uptake in the presence of nitrate (Thessen et al., 2009; Solomon et al., 2010; Radan and Cochlan, 2018). It has been suggested that nitrate suppress urea uptake in *Pseudo-nitzschia* cf. *seriata* and *P. calliantha* (Garali et al., 2016), and in *P. multiseries* urea uptake was completely inhibited until the nitrate concentrations were below approximately 2 μM N nitrate (Radan and Cochlan, 2018). The diatom *Cylindrotheca fusiformis* demonstrated a complete arrest in growth when urea was the sole N-source and it was suggested that transporters of nitrate were upregulated to a similar level as seen in N-starved cultures, proposing that the strain did not even register urea as a possible N-source (Hildebrand and Dahlin, 2000). In the current study, urea was depleted in most cultures (except in *P. seriata* I1). Depletion of urea could be due to ammonification by bacteria, possibly transforming urea to ammonium, as was probably the case in the adjacent area of the spill leading to high TIN, or simply direct urea uptake by the PN cells. As bacterial driven ammonification of urea could have changed the available N source in this experiment, we suspect the same process could have happened after the spill in 2016.

4.5. Interaction between nitrogen source and other factors

An interesting correlation between growth rates, N-sources and irradiance levels was shown by Radan and Cochlan (2018). *P. cuspidata* grew significantly faster using ammonium than using both urea and nitrate when irradiance was saturated (120 μmol photons m^{-2} s^{-1}). But when growing with unsaturated light (40 μmol photons m^{-2} s^{-1}) the same strain grew significantly faster with urea as the N-source in comparison to both ammonium and nitrate. This relationship potentially explains *P. seriata* growing well after the fertilizer spill in spring 2016, a period characterized by low irradiance and high urea levels (due to the spill). Low irradiance may, if affecting *P. seriata* similar to *P. cuspidata*, have induced fast growth of *P. seriata,* resulting in a bloom. This suggests additional studies of *Pseudo-nitzschia* growth rates at varying irradiance levels and different urea concentrations would be of interest.

5. Conclusion

The phytoplankton community composition exhibited traces of the anthropogenic interference caused by the fertilizer spill and revealed fragility to disturbances in the nutrient balance in Lillebælt in spring 2016. The magnitude of the following spring bloom escalated faster than previous years and species diversity was especially low in 2016 compared to other years. We cannot prove that the PN species were affected by the available nitrogen composition and hence that the reported DA in mussel meat was related to the fertilizer spill. The experiments revealed a variation in growth rate and toxicity between species and among strains dependent on nitrogen source and load. Although all strains were able to utilize urea and some increased in maximum growth rate with higher urea concentration. The results stress both species and strain variability in growth and DA content in the HAB genus PN in response to environmental conditions and call for further investigations increasing the number of strains and/or species.

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

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.hal.2020.101817.

References

Alam, M.S., Casareto, B.E., Suzuki, Y., Sultana, R., Suzuki, T., 2017. Optimization of dissolved urea measurements in coastal waters with the combination of a single reagent and high temperature. J. Oceanogr. 73, 249–258. https://doi.org/10.1007/s10872-016-0400-2.

Antia, N.J., Harrison, P.J., Oliveira, L., 1991. The role of dissolved organic nitrogen in phytoplankton nutrition, cell biology and ecology. Phycologia 30 (1), 1–89.

Auro, M.E., Cochlan, W., 2013. Nitrogen utilization and toxin production by two diatoms of the *Pseudo-nitzschia pseudodelicatissima* complex: *P. cuspidata* and *P. fryxelliana*. J. Phycol. 49, 156–169.

Bates, S.S., Hubbard, K.A., Lundholm, N., Montresore, M., Leaw, C.P., 2018. *Pseudo-nitzschia, nitzschia*, and domoic acid: new research since 2011. Harmful Algae 79, 3–43. https://doi.org/10.1016/j.hal.2018.06.001.

Bates, S.S., Trainer, V.L., 2006. The ecology of harmful diatoms. In: Granéli, E., Turner, J.T. (Eds.), Ecology of Harmful Algae. Springer-Verlag, Berlin, pp. 81–88.

Bates, S.S., Worms, J., Smith, J.C., 1993. Effects of ammonium and nitrate on growth and domoic acid production by *Nitzschia pungens* in batch culture. Can. J. Fish. Aquat. Sci. 50, 1248–1254.

Brunson, J.K., McKinnie, S.M., Chekan, J.R., McCrow, J.P., Miles, Z.D., Bertrand, E.M., Bielinski, V.A., Luhavaya, H., Obornik, M., Smith, G.J., Hutchins, D.A., Allen, A.E., Moore, B.S., 2018. Biosynthesis of the neurotoxin domoic acid in a bloom-forming diatom. Science 361 (6409), 1356–1358. https://doi.org/10.1126/science.aau0382.

Cira, E.K., Paerla, H.W., Wetza, M.S., 2016. Effects of nitrogen availability and form on phytoplankton growth in a eutrophied estuary (Neuse River Estuary, NC, USA). PLoS One 11, 1–15. https://doi.org/10.1371/journal.pone.0160663.

Cochlan, W.P., Herndon, J., Kudela, R.M., 2008a. Inorganic and organic nitrogen uptake by the toxigenic diatom *pseudo-nitzschia Australis* (Bacillariophyceae). Harmful Algae 8, 111–118. https://doi.org/10.1016/j.hal.2008.08.008.

Donald, D.B., Bogard, M.J., Finlay, K., Bunting, L., Leavitt, P.R., 2013. Phytoplankton-specific response to enrichment of phosphorus-rich surface waters with ammonium, nitrate, and urea. PLoS One 8. https://doi.org/10.1371/journal.pone.0053277.

Downes-Tettmar, N., Rowland, S., Widdicombe, C., Woodward, M., Llewellyn, C., 2013. Seasonal variation in *Pseudo-nitzschia* spp. and domoic acid in the Western English Channel. Cont. Shelf Res. 53, 40–49.

Fehling, J., Davidson, K., Bolch, C., Tett, P., 2006. Seasonally of *pseudo-nitzschia* spp. (Bacillariophyceae) in western Scottish waters. Mar. Ecol. Prog. Ser. 323, 91–105. https://doi.org/10.3354/meps323091.

Fehling, J., Green, D.H., Davidson, K., Botch, C.J., Bates, S.S., 2004. Domoic acid production by *pseudo-nitzschia seriata* (bacillariophyceae) in Scottish waters. J. Phycol. 40, 622–630. https://doi.org/10.1111/j.1529-8817.2004.03200.x.

Gai, F.F., Hedemand, C.K., Louw, D.C., Grobler, K., Krock, B., Moestrup, Ø., Lundholm, N., 2018. Morphological, molecular and toxigenic characteristics of Namibian *pseudo-nitzschia* species – including *Pseudo-nitzschia bucculenta* sp. nov. Harmful Algae 76,

80–95. https://doi.org/10.1016/j.hal.2018.05.003.
Galloway, J.N., Cowling, E.B., 2002. Reactive nitrogen and the world: 200 years of change. AMBIO A J. Hum. Environ. 31, 64–71. https://doi.org/10.1579/0044-7447-31.2.64.
Garali, S.M.Ben, Sahraoui, I., de la Iglesia, P., Chalghaf, M., Diogène, J., Ksouri, J., Sakka Hlaili, A., 2016. Effects of nitrogen supply on *pseudo-nitzschia calliantha* and *pseudo-nitzschia* cf. *seriata*: field and laboratory experiments. Ecotoxicology 25, 1211–1225. https://doi.org/10.1007/s10646-016-1675-1.
Glibert, P.M., 2016. Margalef revisited a new phytoplankton mandala incorporating twelve dimensions, including nutritional physiology. Harmful Algae 55, 25–30.
Glibert, P.M., Harrison, J., Heil, C., Seitzinger, S., 2006. Escalating worldwide use of urea – a global change contributing to coastal eutrophication. Biogeochemistry 77, 441–463. https://doi.org/10.1007/s10533-005-3070-5.
Glibert, P.M., Maranger, R., Sobota, D.J., Bouwman, L., 2014. The haber bosch-harmful algal bloom (HB-HAB) link. Environ. Res. Lett. 9, 105001. https://doi.org/10.1088/1748-9326/9/10/105001.
Glibert, P.M., Trice, T.M., Michael, B., Lane, L., 2005. Urea in the tributaries of the chesapeake and Coastal Bays of Maryland. Water. Air. Soil Pollut. 160, 229–243.
Graneli, E., Wallstrom, K., Larsson, U., Graneli, W., Elmgren, R., 1990. Nutrient limitation of primary production in the Baltic Sea area. Ambio 19, 142–151. https://doi.org/10.2307/4313680.
Griffin, J.E., Park, G., Dam, H.G., 2019. Relative importance of nitrogen sources, algal alarm cues and grazer exposure to toxin production of the marine dinoflagellate *alexandrium catenella*. Harmful Algae 84, 181–187. https://doi.org/10.1016/j.hal.2019.04.006.
Hansen, L.R., Soylu, S.í., Kotaki, Y, Moestrup, Ø., Lundholm, N., 2011. Toxin production and temperature-induced morphological variation of the diatom *pseudo-nitzschia seriata* from the Arctic. Harmful Algae 10, 689–696. https://doi.org/10.1016/j.hal.2011.05.004.
Harðardóttir, S., Pančić, M., Tammilehto, A., Krock, B., Møller, E.F., Nielsen, T.G., Lundholm, N., 2015. Dangerous relations in the Arctic marine food web: interactions between toxin producing Pseudo-nitzschia diatoms and Calanus copepodites. Marine drugs 13 (6), 3809–3835. https://doi.org/10.3390/md13063809.
Harðardóttir, S., Wohlrab, S., Hjort, D.M., Krock, B., Nielsen, T.G., John, U., Lundholm, N., et al., 2019. Transcriptomic responses to grazing reveal the metabolic pathway leading to the biosynthesis of domoic acid and highlight different defense strategies in diatoms. BMC molecular biology 20 (1), 7.
Hasle, G.R., Lundholm, N., 2005. *Pseudo-nitzschia seriata f. obtusa* (Bacillariophyceae) raised in rank based on morphological, phylogenetic and distributional data. Phycologia 44, 608–619.
Hildebrand, M., Dahlin, K., 2000. Nitrate transporter genes from the diatom *cylindrotheca fusiformis* (Bacillariophyceae): mRNA levels controlled by nitrogen source and by the cell cycle. J. Phycol. 36, 702–713.
Hillebrand, H., Sommer, U., 1996. Nitrogenous nutrition of the potentially toxic diatom *pseudo-nitzschia pungens f. multiseries* (Hasle). J. Plankt. Res. 18, 295–301.
Howarth, R.W., Sharpley, A., Walker, D., 2002. Sources of nutrient pollution to coastal waters in the United States: Implications for achieving coastal water quality goals. Estuaries 25 (4), 656–676. https://doi.org/10.1007/BF02804898.
Husson, B., Hernández-Fariñas, T., Le Gendre, R., Schapira, M., Chapelle, A., 2016. Two decades of *pseudo-nitzschia* spp. blooms and king scallop (Pecten maximus) contamination by domoic acid along the French Atlantic and English Channel coasts: seasonal dynamics, spatial heterogeneity and interannual variability. Harmful Algae 51, 26–39.
Nielsen, M.H., Vang, T., Lund-Hansen, L.C., 2017. Internal hydraulic control in the little belt, Denmark – observations of flow configurations and water mass formation. Ocean Sci. 13, 1061–1075. https://doi.org/10.5194/os-13-1061-2017.
Howard, M.D.A., Cochlan, W.P., Ladizinsky, N., Kudela, R.M., 2007. Nitrogenous preference of toxigenic *pseudo-nitzschia australis* (Bacillariophyceae) from field and laboratory experiments. Harmful Algae 6, 206–217. https://doi.org/10.1016/j.hal.2006.06.003.
Krock, B., Tillmann, U., John, U., Cembella, A., 2008. LC-MS-MS aboard ship: tandem mass spectrometry in the search for phycotoxins and novel toxigenic plankton from the North Sea. Anal. Bioanal. Chem. 392, 797–803. https://doi.org/10.1007/s00216-008-2221-7.
Kudela, R.M., Lane, J.Q., Cochlan, W.P., 2008. The potential role of anthropogenically derived nitrogen in the growth of harmful algae in California, USA. Harmful Algae 8, 103–110. https://doi.org/10.1016/j.hal.2008.08.019.
Lefebvre, K.A., Quakenbush, L., Frame, E., Huntington, K.B., Sheffield, G., Stimmelmayr, R., Bryan, A., Kendrick, P., Ziel, H., Goldstein, T., Snyder, J.A., Gelatt, T., Gulland, F., Dickerson, B., Gill, V., 2016. Prevalence of algal toxins in Alaskan marine mammals foraging in a changing arctic and subarctic environment. Harmful Algae 55, 13–24. https://doi.org/10.1016/j.hal.2016.01.007.
Lelong, A., Hegaret, H., Soudant, P., Bates, S.S., 2012. *Pseudo-nitzschia* (Bacillariophyceae) species, domoic acid and amnesic shellfish poisoning: revisiting previous paradigms. Phycologia 51, 168–216. https://doi.org/10.2216/11-37.
Leong, S.C.Y., Murata, A., Nagashima, Y., Taguchi, S., 2004. Variability in toxicity of the dinoflagellate *alexandrium tamarense* in response to different nitrogen sources and concentration. Toxicon 43, 407–415.
Lundholm, N., Andersen, P., Jørgensen, K., Thorbjørnsen, B., Cembella, A., Krock, B., 2005. Domoic acid in Danish blue mussels due to a bloom of *pseudo-nitzschia seriata*. Harmful Algae News 29, 8–10.
Lundholm, N., Clarke, A., Ellegaard, M., 2010. A 100-year record of changing *pseudo-nitzschia species* in a sill-fjord in Denmark related to nitrogen loading and temperature. Harmful Algae 9, 449–457. https://doi.org/10.1016/j.hal.2010.03.001.
Lundholm, N., Daugbjerg, N., Moestrup, Ø, 2002. Phylogeny of the bacillariaceae with emphasis on the genus *pseudo-nitzschia* (Bacillariophyceae) based on partial lsu rDNA. Eur. J. Phycol. 37, 115–134. https://doi.org/10.1017/S096702620100347X.
Lundholm, N., Krock, B., John, U., Skov, J., Cheng, J., Pančić, M., Wohlrab, S., Rigby, K., Nielsen, T.G., Selander, E., Harðardóttir, S., 2018. Induction of domoic acid production in diatoms—Types of grazers and diatoms are important. Harmful Algae 79, 64–73. https://doi.org/10.1016/j.hal.2018.06.005.
Lundholm, N., Skov, J., Pocklington, R., Moestrup, Ø, 1994. Domoic acid, the toxic amino acid responsible for amnesic shellfish poisoning, now in *pseudo-nitzschia seriata* (Bacillariophyceae) in Europe. Phycologia 33, 475–478.
Maeno, Y., Kotaki, Y., Terada, R., Cho, Y., Konoki, K., Yotsu-Yamashita, M., 2018. Six domoic acid related compounds from the red alga, chondria armata, and domoic acid biosynthesis by the diatom, *pseudo-nitzschia multiseries*. Sci. Rep. 8 (1), 356. https://doi.org/10.1038/s41598-017-18651-w.
Markager, S., 2016. Notat Om Mulige Miljøeffekter i Lillebælt Og Tilstødende Havområder. Aarhus University and Fredericia Municipality Technical Report.
Martin-Jézéquel, V., Calu, G., Candela, L., Amzil, Z., Jauffrais, T., Séchet, V., Weigel, P., 2015. Effects of organic and inorganic nitrogen on the growth and production of domoic acid by *P. multiseries* and *P. australis* (Bacillariophyceae) in culture. Mar. Drugs 13, 7067–7086. https://doi.org/10.3390/md13127055<.
McCabe, R.M., Hickey, B.M., Kudela, R.M., Lefebvre, K.A., Adams, N.G., Bill, B.D., Gulland, F.M.D., Thomson, R.E., Cochlan, W.P., Trainer, V.L., 2016. An unprecedented coastwide toxic algal bloom linked to anomalous ocean conditions. Geophys. Res. Lett. 43https://doi.org/10.1002/2016GL070023. 366-10.
Nash, S.M.B., Baddock, M.C., Takahashi, E., Dawson, A., Cropp, R., 2017. Domoic acid poisoning as a possible cause of seasonal cetacean mass stranding events in Tasmania, Australia. Bull. Environ. Contam. Toxicol. 98, 8–13. https://doi.org/10.1007/s00128-016-1906-4.
Radan, R.L., Cochlan, W.P., 2018. Differential toxin response of *pseudo-nitzschia multiseries* as a function of nitrogen speciation in batch and continuous cultures, and during a natural assemblage experiment. Harmful Algae 73, 12–29. https://doi.org/10.1016/j.hal.2018.01.002.
Rhodes, L., Scholin, C., Garthwaite, I., 1998. Pseudo-nitzschia in New Zealand and the role of DNA probes and immunoassays in refining marine biotoxin monitoring programmes. Nat. Toxins 6, 105–111.
Rhodes, L., White, D., Syhre, M., Atkinson, M., 1996. *Pseudo-nitzschia* species isolated from New Zealand coastal waters: domoic acid production in vitro and links with shellfish toxicity. In: Yasumoto, T., Oshima, Y., Fukuyo, Y. (Eds.), Toxic phytoplankton; Harmful and toxic algal blooms. UNESCO, pp. 155–158.
Ryan, J.P., Kudela, R.M., Birch, J.M., Blum, M., Bowers, H.A., Chavez, F.P., Doucette, G.J., Hayashi, K., Marin, R., Mikulski, C.M., Pennington, J.T., Scholin, C.A., Smith, G.J., Woods, A., Zhang, Y., 2017. Causality of an extreme harmful algal bloom in Monterey Bay, California, during the 2014–2016 northeast Pacific warm anomaly. Geophys. Res. Lett. 44, 5571–5579. https://doi.org/10.1002/2017GL072637.
Shimizu, Y., Watanabe, N., Wrensford, G., 1993. In: Lassus, P., Arzul, G., Erard-Le-Denn, E., Gentien, P., Marcaillou, C. (Eds.), Lavoisier Publishing, Paris, pp. 351–357.
Schjødt, M., 2018. Afgørelse Om Miljøskade Eller Overhængende Fare for Miljøskade. Fredericia Kommune.
Solomon, C.M., Collier, J.L., Berg, G.M., Glibert, P.M., 2010. Role of urea in microbial metabolism in aquatic systems: a biochemical and molecular review. Aquat. Microb. Ecol. 59, 67–88. https://doi.org/10.3354/ame01390.
Tatters, A.O., Schnetzer, A., Xu, K., Walworth, N.G., Fu, F., Spackeen, J.L., Sipler, R.E., Bertrand, E.M., McQuaid, J.B., Allen, A.E., Bronk, D.A., Gao, K., Sun, J., Caron, D.A., Hutchins, D.A., 2018. Interactive effects of temperature, CO_2 and nitrogen source on a coastal California diatom assemblage. J. Plankton Res. 40, 151–164. https://doi.org/10.1093/plankt/fbx074.
Teitelbaum, J., Zatorre, R., Carpenter, S., Gendron, D., Evans, A., Gjedde, A., NR, C., 1990. Neurologic sequelae of domoic acid intoxication due to the ingestion of contaminated mussels. N. Engl. J. Med. 25, 1781–1787.
Thessen, A., Bowers, H., Stoecker, D., 2009. Intra- and inter- specific differences in *pseudo-nitzschia* growth and toxicity while utilizing different nitrogen sources. Harmful Algae 8, 792–810.
Thorel, M., Claquin, P., Schapira, M., Le Gendre, R., Riou, P., Goux, D., Le Roy, B., Raimbault, V., Deton-Cabanillas, A.-.F., Bazin, P., 2017. Nutrient ratios influence variability in *pseudo-nitzschia* species diversity and particulate domoic acid production in the Bay of Seine (France). Harmful Algae 68, 192–205.
Trainer, V.L., Bates, S.S., Lundholm, N., Thessen, A.E., Cochlan, W.P., Adams, N.G., Trick, C.G., 2012. *Pseudo-nitzschia* physiological ecology, phylogeny, toxicity, monitoring and impacts on ecosystem health. Harmful Algae 14, 271–300. https://doi.org/10.1016/j.hal.2011.10.025.
Trick, C.G., Bill, B.D., Cochlan, W.P., Wells, M.L., Trainer, V.L., Pickell, L.D., 2010. Iron enrichment stimulates toxic diatom production in high-nitrate, low-chlorophyll areas. Proc. Natl. Acad. Sci. USA 107, 5887–5892. https://doi.org/10.1073/pnas.0910579107.