Exploring the factors behind the expansion of the harmful dinoflagellate *Alexandrium pseudogonyaulax* in Northern European waters

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Kristof Sönke Möller
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Examination committee

1. Examiner: Prof. Marko Rohlfs
University of Bremen
Bremen, Germany

2. Examiner: Prof. Björn Rost
University of Bremen
Bremen, Germany

1. Reviewer: Prof. Dedmer van de Waal
University of Amsterdam
Amsterdam, Netherlands

2. Reviewer: PD. Cédric L. Meunier
University of Bremen
Bremen, Germany

3. Reviewer: Prof. Kalle Olli
Estonian University of Life Sciences
Tartu, Estonia

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Summary

Harmful Algal Blooms (HABs) represent a global concern and severely affect coastal nations worldwide. Phycotoxins produced by HAB species may accumulate in marine organisms, such as shellfish or fish, ultimately affecting higher trophic level consumers, such as marine mammals and humans. Other HAB species are ichthyotoxic and can directly cause mortality in natural or commercial fish populations leading to large economic losses in the aquaculture industry. One of the primary causative agents of HABs are dinoflagellates, particularly those belonging to the *Alexandrium* genus. This genus is widely known for causing paralytic shellfish poisoning due to the production of the neurotoxin saxitoxin and its derivatives. Other *Alexandrium* species are producing goniodomins (GDs), which have been associated with the mortality of fish and marine invertebrates. Apart from phycotoxins, many *Alexandrium* species are producing bioactive extracellular compounds (BECs) that are lytic towards a wide range of protists and may play a role in mixotrophy and ichthyotoxicity, yet their chemical structure remains to be discovered. One member, *Alexandrium pseudogonyaulax*, is apparently appearing in increasing frequency and abundance across Northern European waters and, due to its production of GDs and BECs, may pose a risk to local ecosystems and especially fisheries. The aim of this thesis was to investigate this ongoing expansion with the ultimate goal of providing data required for a risk assessment of the potential impact of *A. pseudogonyaulax* on ecosystems and commercial activities. For this purpose, various laboratory experiments were conducted to assess the interplay of bottom-up and top-down factors with *A. pseudogonyaulax*, along with a time series analysis of long-term monitoring stations across Northern Europe.

Publication I was investigating how nitrogen and light, both common drivers of HAB development, influence primarily the growth and toxin content of *A. pseudogonyaulax*. The findings suggest that urea is not an important driving factor of its expansion despite high anthropogenic inputs of urea across Northern Europe due to intensive agricultural farming. Toxin content was primarily driven by the growth phase and higher in the stationary phase, indicating that toxins may accumulate towards later bloom stages. Additionally, high intraspecific variability and resilience to light were observed, suggesting that *A. pseudogonyaulax* is well-equipped for future changes in light availability caused by e.g. climate change or coastal darkening.

Publication II set out to investigate the effects of *A. pseudogonyaulax* on relevant representatives of the pelagic food web, including microalgae (cryptophyte *Rhodomonas salina*), microzooplankton (heterotrophic protist *Polykrikos kofoidii*), mesozooplankton (calanoid copepod *Acartia tonsa*), and fish gill cells (RTgill-W1 cell line of the salmonid *Oncorhynchus mykiss*). One of the primary focuses of these experiments was to untangle the relative contributions of GDs and BECs to the overall toxicity of *A. pseudogonyaulax* by utilizing culture supernatants containing both and comparing them to purified GDs. Although grazers generally ingested *A. pseudogonyaulax*, they suffered adverse fitness effects ultimately resulting in lysis and mortality. Publication II also demonstrated the ichthyotoxic capabilities of *A. pseudogonyaulax*, which are likely driven by BECs and not by GDs.

The third study was designed on the basis of the observation that *A. pseudogonyaulax* suddenly appeared in the Danish Limfjord coinciding with a decrease of other *Alexandrium* species. To investigate whether similar trends occur in the study area, a time series analysis of long-term monitoring programs across Northern Europe, including Danish, Swedish, Norwegian and German time series was conducted. The study confirmed the expansion of *A. pseudogonyaulax* across Northern Europe, which has become a regular component of the summer and autumn microalgal community in the Kattegat, Skagerrak, and southern parts of the Baltic Sea. Surprisingly, this expansion coincided with the arrival of a notorious invading species, the jellyfish *Mnemiopsis leidyi*, which exerts substantial grazing pressure on the zooplankton community, especially during late summer. This enables the proliferation of microalgae, and consequently *A. pseudogonyaulax* might have benefited as well. Additionally, warming temperatures and high concentrations of dissolved organic matter may favour the proliferation of this dinoflagellate. Modelling the yearly calculated probabilities of observing *A. pseudogonyaulax* suggests that the frequency of its occurrences are likely to increase across Northern Europe, even though they may stagnate or even decrease in certain regions, such as the Danish Limfjord.

Altogether, the findings of this thesis demonstrate that *A. pseudogonyaulax* has permanently established itself across Northern European waters and might infiltrate further east into the Baltic Sea. Adverse effects to other protists and grazers, as well as ichthyotoxicity to fish, suggest that *A. pseudogonyaulax* has the potential to disrupt local ecosystems, altering the energy flow between trophic levels. Even though cell densities required for ichthyotoxic effects have rarely been reached across Northern Europe, the potential for economic damages to the shellfish and aquaculture industry remains. The ecological functions of phycotoxins and BECs, as well as the chemical structure of BECs, remain largely elusive warranting further investigations.

Zusammenfassung

Schädliche Algenblüten (HABs) stellen ein ernsthaftes globales Problem dar und betreffen Küstennationen weltweit. Algentoxine, die von HAB-Arten produziert werden, können in Meeresorganismen, wie Muscheln oder Fische, angereichert werden und letztlich höhere trophische Konsumenten, wie Meeressäuger und Menschen, negativ beeinflussen. Andere HAB-Arten sind fischgiftig und können direkt das Absterben natürlicher oder kommerzieller Fischpopulationen verursachen, was zu großen wirtschaftlichen Verlusten in der Aquakulturindustrie führen kann. Einer der Hauptverursacher von HABs sind Dinoflagellaten, insbesondere diejenigen, die zur Gattung *Alexandrium* gehören. Diese Gattung ist als Verursacher von ‚paralytic shellfish poisoning' bekannt, da einige Mitglieder das Neurotoxin Saxitoxin und seine Derivate produzieren. Andere *Alexandrium* Arten produzieren Goniodomine (GDs), die mit dem Tod von Fischen und wirbellosen Tieren in Verbindung gebracht wurden. Abgesehen von Algentoxinen produzieren viele *Alexandrium* Arten zusätzliche bioaktive extrazelluläre Verbindungen (BECs), die gegenüber einer Vielzahl von Protisten lytisch wirken und potentiell in Mixotrophie und Ichthytoxizität involviert sind. Trotzdem wurde ihre chemische Struktur noch nicht aufgeklärt. Eine *Alexandrium* Art, *Alexandrium pseudogonyaulax*, tritt anscheinend in zunehmender Häufigkeit und Abundanz in den nordeuropäischen Gewässern auf und könnte aufgrund seiner Produktion von GDs und BECs ein Risiko für lokale Ökosysteme und insbesondere für die Fischerei darstellen. Ziel dieser Dissertation war es, diese laufende Expansion zu untersuchen, um die für eine Risikobewertung der potenziellen Auswirkungen von *A. pseudogonyaulax* auf Ökosysteme und kommerzielle Aktivitäten erforderlichen Daten bereitzustellen. Zu diesem Zweck wurden verschiedene Laborversuche durchgeführt, um das Zusammenspiel von ‚Bottom-up' und ‚Top-down' Faktoren mit *A. pseudogonyaulax* zu bewerten, sowie eine Zeitserienanalyse von Langzeitüberwachungsstationen in Nordeuropa.

Publikation I untersuchte wie Stickstoff und Licht, beide häufige Treiber von schädlichen Algenblüten, hauptsächlich das Wachstum und den Toxingehalt von *A. pseudogonyaulax* beeinflussen. Die Ergebnisse legen nahe, dass Harnstoff, trotz hoher anthropogener Einträge in Nordeuropa aufgrund intensiver landwirtschaftlicher Bewirtschaftung, kein wichtiger Expansionsfaktor von *A. pseudogonyaulax* war. Der Toxingehalt wurde hauptsächlich von der Wachstumsphase bestimmt und war in der stationären Phase höher, was darauf hindeutet, dass sich Toxine zum Ende einer Algenblüte anreichern können. Darüber hinaus wurde eine hohe intraspezifische Variabilität und Lichtresistenz beobachtet, was darauf hindeutet, dass *A. pseudogonyaulax* gut auf zukünftige Veränderungen der Lichtverfügbarkeit durch den Klimawandel oder Küstenverdunklung vorbereitet ist.

Publikation II untersuchte die Auswirkungen von *A. pseudogonyaulax* auf relevante Vertreter des pelagischen Nahrungsnetzes, darunter Mikroalgen (Kryptophyt *Rhodomonas salina*), Mikrozooplankton (heterotropher Protist *Polykrikos kofoidii*), Mesozooplankton (calanoider Ruderfußkrebs *Acartia tonsa*) und Fischkiemenzellen (RTgill-W1-Zelllinie des Salmoniden *Oncorhynchus mykiss*). Einer der Hauptschwerpunkte dieser Experimente war es die relativen Beiträge von GDs und BECs zur Gesamttoxizität von *A. pseudogonyaulax* zu bestimmen, indem Kulturüberstände, die GDs und BECs enthalten, mit aufgereinigten GDs verglichen wurden. Obwohl Weidetiere generell *A. pseudogonyaulax* gefressen haben, litten sie unter negativen Fitnessauswirkungen, die letztlich zu Lyse und Tod führten. Publikation II zeigte auch die ichthyotoxischen Fähigkeiten von *A. pseudogonyaulax* und stellte fest, dass diese wahrscheinlich durch BECs und nicht durch GDs verursacht wurden.

Die dritte Studie basiert auf der Beobachtung, dass *A. pseudogonyaulax* plötzlich im dänischen Limfjord auftrat, was mit einem Rückgang anderer *Alexandrium*-Arten einherging. Um zu untersuchen, ob ähnliche Trends im Untersuchungsgebiet auftreten, wurde eine Zeitserienanalyse von Langzeitüberwachungsprogrammen in Nordeuropa, einschließlich dänischer, schwedischer, norwegischer und deutscher Zeitreihen, durchgeführt. Die Studie bestätigte die Ausbreitung von *A. pseudogonyaulax* in Nordeuropa, wo es zu einem regelmäßigen Bestandteil der Sommer- und Herbst-Mikroalgen-Gemeinschaft im Kattegat, Skagerrak und den südlichen Teilen der Ostsee geworden ist. Überraschenderweise fiel diese Ausbreitung mit dem Auftreten einer berüchtigten invasiven Art, der Qualle *Mnemiopsis leidyi*, zusammen, die insbesondere im Spätsommer erheblichen Weidedruck auf die Zooplankton-Gemeinschaft ausübt. Dies ermöglicht die Vermehrung von Mikroalgen, und folglich könnte *A. pseudogonyaulax* davon profitiert haben. Darüber hinaus könnten steigende Temperaturen und hohe Konzentrationen an gelöster organischer Substanz das Wachstum dieses Dinoflagellaten begünstigen. Die Abundanz dieser Art wird Modellierungen zufolge wahrscheinlich zunehmen, auch wenn sie in bestimmten Regionen, wie dem dänischen Limfjord, sogar abnehmen könnte.

Insgesamt zeigen die Ergebnisse dieser Dissertation, dass sich *A. pseudogonyaulax* dauerhaft in den nordeuropäischen Gewässern etabliert hat und weiter in den östlichen Teil der Ostsee eindringen könnte. Schädliche Auswirkungen auf andere Protisten und Weidetiere sowie Ichthyotoxizität gegenüber Fischen deuten darauf hin, dass *A. pseudogonyaulax* das Potenzial hat, lokale Ökosysteme zu stören und den Energiefluss zwischen trophischen Ebenen zu verändern. Obwohl die Zelldichten, die für ichthyotoxische Effekte erforderlich sind, in Nordeuropa selten erreicht wurden, bleibt das Potenzial für wirtschaftliche Schäden in der Muschel- und Aquakulturindustrie bestehen. Allerdings bleiben die ökologischen Funktionen von Algentoxinen und BECs sowie die chemische Struktur von BECs weitgehend unklar und erfordern weitere Untersuchungen.

List of Abbreviations

ANOVA	analysis of variance
GD	goniodomin
GDA	goniodomin A
GDB	goniodomin B
GDC	goniodomin C
GDA-sa	goniodomin A seco-acid
BEC	bioactive extracellular compound
C	carbon
Chl *a*	chlorophyll *a*
CTD	conductivity temperature depth
Da	dalton
DIC	dissolved inorganic carbon
DOC	dissolved organic carbon
DOM	dissolved organic matter
DON	dissolved organic nitrogen
DOY	day of the year
EC_{50}	effective concentration of the half-maximal response
ETR	electron transport rate
GAM	general additive model
GLM	generalized linear model
HAB	harmful algal bloom
HPLC	high performance liquid chromatography
IFCB	imaging flowcytobot
MS	mass spectometry
N	nitrogen
P	phosphorus
PSII	photosystem II
PFD	photon flux density
POC	particulate organic carbon
PON	particulate organic nitrogen
PSP	paralytic shellfish poisoning
PST	paralytic shellfish toxin
qPCR	quantitative real-time polymerase chain reaction
RCII	the concentration of functional PSII reaction centers
ROS	reactive oxygen species
UV	ultraviolet

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Chapter 1

Introduction

1.1 Marine microalgae

Microalgae, though constituting less than 1 % of the Earth's photosynthetic biomass, are responsible for approximately 50 % of global net primary production (Falkowski et al. 1998, Field et al. 1998). Photosynthetic microalgae produce complex organic compounds, such as fatty acids and carbohydrates, using dissolved inorganic carbon (DIC) and light energy (W. W. Fischer et al. 2016). These compounds can be utilized for cell growth, energy production, or exuded as dissolved organic carbon (DOC). The ability of microalgae to proliferate via asexual mitotic cell division enables rapid exponential growth, and accordingly microalgae form the base of the pelagic food web constituting around 70 % of marine biomass (Bar-On et al. 2018). However, microalgal biomass is readily regulated by grazing and bacterial or viral lysis resulting in short turnover times of mere days (Falkowski et al. 1998). Grazing by zooplankton provides an entry point for directing nutrients to higher trophic levels, such as planktivorous fish (Lazzaro 1987). Alternatively, DOC can enter the microbial loop, wherein heterotrophic bacteria or archaea recycle DOC into biomass, which, after primary grazing by microzooplankton becomes available again to higher trophic levels of the pelagic food web (Azam and Malfatti 2007). This process increases the overall efficiency of the pelagic food web, as eukaryotic marine organisms can usually not utilize DOC (Hansell and Carlson 2014). Finally, the sinking of microalgae can lead to carbon sequestration in the deep ocean, a process known as the marine biological carbon pump (Sarmiento 2006). Altogether, the interplay between primary production and the microbial food web controls the overall productivity of marine systems, making microalgae a crucial component of aquatic ecosystems, and the global cycles of carbon, nitrogen (N) and phosphorus (P).

There are many ways of assimilating carbon, for instance, mixotrophs can obtain organic carbon through osmotrophy, the uptake of organic substrates, or phagotrophy, the engulfment of prey, while autotrophic organisms can only absorb dissolved inorganic carbon (Flynn et al. 2019). However, many organisms, traditionally labelled as photoautotrophic, can also exploit dissolved organic compounds, suggesting that all planktonic primary producers are rather 'photo-osmo-mixotrophic' (Mitra et al. 2014, Flynn et al. 2019). Especially in low-nutrient environments, osmotrophy may be crucial to mitigate metabolic leakage (Flynn and L. S. Berry 1999). It is important to distinguish between phagotrophic and/or osmotrophic mixotrophs considering the profound implications of mixotrophy on food web structuring and trophic dynamics (Mitra et al. 2014, Ward and Follows 2016). Finally, heterotrophic organisms can only assimilate organic carbon and are therefore not primary producers. The existence of mixotrophy has changed the view on the basic trophic structure of euphotic marine ecosystems as most microalgae can no longer be strictly regarded as producers or consumers (Flynn et al. 2013).

Under certain environmental conditions, cell growth of microalgae can surpass the combined loss processes, leading to the development of an algal bloom, usually characterised by high algal biomass. Algal blooms are driven by a favourable interplay of physical (e.g. turbulence or light), chemical (e.g. nutrient availability) and biological (e.g. grazing) factors, however, the extent to which each factor contributes to bloom development remains controversial (Behrenfeld and Boss 2014). Algal blooms can span large areas, covering more than a million km^2 (Chow et al. 2019), and can persist over many years, such as blooms of *Aureoumbra lagunensis* lasting for over eight years in the Gulf of Mexico (Buskey et al. 2001). In temperate regions, microalgal biomass generally follows a seasonal growth cycle, including a spring and late summer or autumn bloom. The diatom-dominated spring bloom is primarily driven by increasing insolation and temperatures, yet also benefits from high dissolved nutrient concentrations (Sommer and Lengfellner 2008, Almén and Tamelander 2020). Nutrient depletion and increased grazing diminish the spring bloom and maintain low microalgal biomass over summer. At the end of summer or in early autumn, wind-driven mixing can renew the nutrient supply and cause a second bloom, which is usually dominated by dinoflagellates (Winder and Cloern 2010). Altogether, algal blooms may benefit ecosystems, yet sometimes microalgae can inflict harm on other marine organisms and humans.

1.2 Harmful algal blooms

Proliferation of algae that adversely affect ecosystems or humans, termed Harmful Algal Blooms (HABs), represent a growing global concern and severely affect coastal nations worldwide (D. M. Anderson et al. 2012b, Gobler 2020). Hallegraeff et al. (2021) showed that the perceived global increase in frequency and abundance of HABs (D. M. Anderson et al. 2002, D. M. Anderson et al. 2012b) can be primarily attributed to intensified monitoring, mainly due to increased aquaculture production and scientific interest. However, even after normalization to sampling effort, time series data suggests that HAB occurrences are increasing in Central and South America, as well as Europe (G. M. Hallegraeff et al. 2021, Dai et al. 2023). HABs are mainly caused by microalgae, including certain cyanobacteria, such as *Microcystis aeruginosa*. Although HABs of macroalgae, such as the seaweed *Ulva lactuca*, are also encompassed by the term, they will not be further discussed in this thesis.

The common feature of all HABs is that they cause harm. Depending on the underlying cause, HABs can be further grouped into two different categories including toxic and non-toxic HABs, causing harm in other ways. Adverse effects of non-toxic HABs are primarily a side-effect of the rapid development of high biomass leading to oxygen depletion, production of foams or scums, noxious odours and consequently destruction of marine habitats (Granéli, Turner, et al. 2006). In addition, some diatom species possess spines or setae, thin hair-like structures extending from the cell, which may physically damage other marine species, such as the sensitive gills of finfish (Rodger et al. 2011).

In contrast, toxic HABs produce marine toxins, known as phycotoxins, and other structurally undefined bioactive extracellular compounds (BECs). The latter have been referred to as allelochemicals, lytic substances, or ichthyotoxins in the past, but will be described using the more generic term BECs in this thesis. Phycotoxins can accumulate in marine organisms and propagate through pelagic food webs (Fig. 1.1), ultimately affecting higher trophic level consumers such as marine mammals (Doucette et al. 2006, Broadwater et al. 2018), seabirds (Gibble and Hoover 2018), and humans (Berdalet et al. 2016). The main pathway of human intoxication is the consumption of contaminated fish and shellfish. Shellfish poisoning syndromes are named either after inflicted symptoms or after the causative organism and include azaspiracid, amnesic, diarrhetic, neurotoxic and paralytic shellfish poisoning (PSP). Additionally, fish poisonings, such as ciguatera poisoning associated with the benthic dinoflagellate genus *Gambierdiscus*, cause hazardous food-borne illnesses (Loeffler et al. 2021).

In addition to indirect toxic effects, direct exposure to phycotoxins via skin contact (Moreira-González et al. 2021) or aerosols (C. C. Lim et al. 2023), can also provoke adverse human health effects (Fig. 1.1). Moreover, phycotoxins and BECs can deter competing protists (Tillmann et al. 2008) and grazers (Turner 2014) or immobilize prey,

in the case of mixotrophs (Granéli, Turner, et al. 2006, Blossom et al. 2012). Furthermore, HABs may exhibit ichthyotoxic properties, inflicting mortality in natural or commercial fish populations (Dorantes-Aranda et al. 2015, G. Hallegraeff et al. 2017). Apart from harmful health effects, HABs cause significant socioeconomic impacts on the tourism and recreation sector, provoking monitoring and management costs.

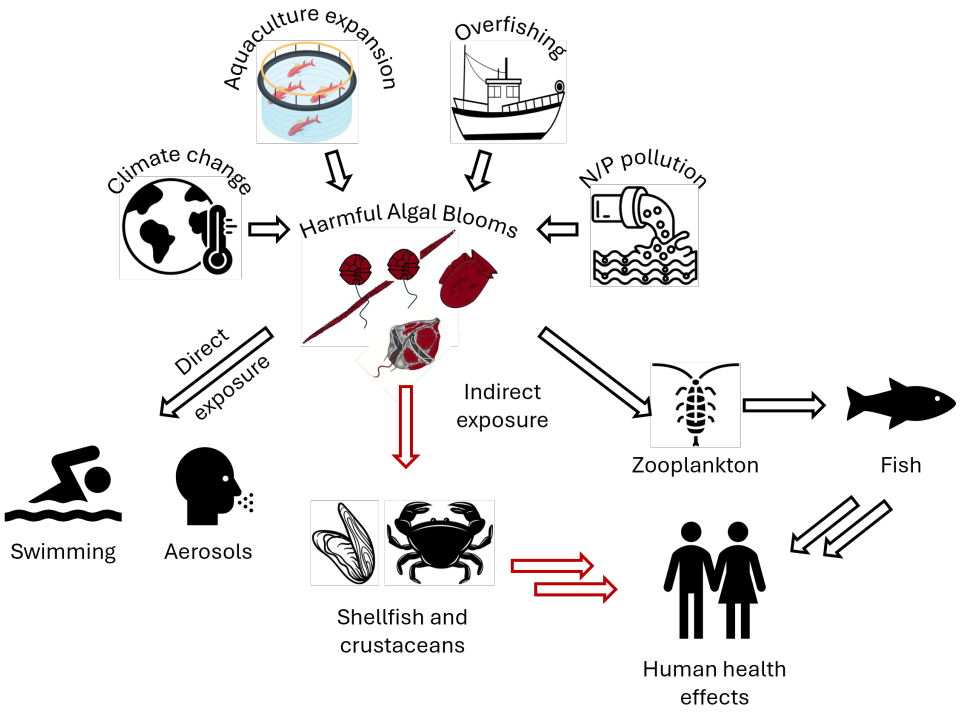

Fig. 1.1: *Main anthropogenic drivers of HABs, including climate change, aquaculture expansion, overfishing and N/P pollution which increase the likelihood of HAB formation; adverse effects in humans can arise from direct exposure (e.g. through swimming or exposure to aerosols) or indirect exposure after consumption of contaminated seafood, which represents the main intoxication pathway.*

The single most devastating HAB event was caused by blooms of the dictyochophyte *Pseudochattonella verruculosa*, leading to losses for the Chilean salmon industry of 800 million US dollars in the austral summer of 2016 (J. I. Mardones et al. 2021). Additionally, blooms of the dinoflagellates *Karenia brevis* (Hoagland et al. 2009), *Alexandrium catenella* (Condie et al. 2019), and *Noctiluca scintillans* (Xiaodong et al. 2023) regularly cause economic losses in the millions of US dollars. Estimations of the economic costs of HABs further include 16 million Canadian dollars for the salmon aquaculture industry in British Columbia from 2009-2012 (Haigh and Esenkulova 2014). However, this phenomenon also affects Europe. In summer 2022, blooms of the brackish haptophyte *Prymnesium parvum* killed approximately 1,000 metric tons of fish, as well as mussels and snails, along 500 km of the Oder River, mainly in Poland and Germany (Köhler et al. 2024). Even though estimating the economic and especially ecological costs due to HABs is complex and likely contains a high degree of uncertainty (Sanseverino et al. 2016), these estimates underscore

the necessity to study the ecology of HAB species and to establish effective monitoring and mitigation strategies.

Members of various microalgal groups can form HABs, including dinoflagellates, diatoms, raphidophytes, haptophytes, and chrysophytes amongst others. Most HAB species are planktonic, although many taxa also have a benthic cyst life stage capable of enduring longer periods of unfavourable environmental conditions (Granéli, Turner, et al. 2006). The coupling between benthic and pelagic life stages has been suggested to initiate the development of HABs, but also their termination (Brosnahan et al. 2020, Brosnahan et al. 2017). Germination of resting cysts has been connected to changing environmental conditions, especially temperature and light, but nutrient concentrations may also play a role (Itakura and Yamaguchi 2001, Genovesi et al. 2009). Notably, some HAB species only have benthic life stages, such as *Gambierdiscus*. While the diversity of HAB species is fascinating, this thesis will focus on one of their primary agents, which are dinoflagellates (Smayda and Reynolds 2003).

Referring to harmful dinoflagellate blooms as HABs may be misleading as the name suggests the occurrence of blooms generally associated with high biomass. Some non-toxic dinoflagellates are known to form high-density blooms with prominent examples including blooms of *Tripos muelleri*, such as in New York Bight in 1976 (Mahoney and Steimle Jr 1979), or blooms of *N. scintillans* capable of reaching cell densities over 10^7 cells L^{-1} (Sulochanan et al. 2014), both associated with bottom-water anoxia provoking the mortality of marine organisms. High-density blooms over 10^6 cells L^{-1} of toxic dinoflagellates include fish-killing *Karenia mikimotoi* (Bresnan et al. 2021) and some *Alexandrium* species, such as *A. catenella* or *A. minutum* (Vila et al. 2005, Bravo et al. 2008). Other dinoflagellates form outbreaks of relatively low abundance in comparison to other planktonic groups, yet this does not prevent them from provoking harm. For instance, cell densities of *Dinophysis* spp. below 10^3 cells L^{-1} (Reguera et al. 2012) or of *K. brevis* exceeding 5×10^3 cells L^{-1} (Gannon et al. 2009) have been associated with shellfish toxicity levels above regulatory limits sufficient to close shellfish beds. Altogether, the multifaceted negative effects of HABs on ecosystems and humans are evident, but how are these phenomena initiated?

1.3 Drivers of HAB proliferation

In pelagic food webs, microalgal biomass is regulated by a combination of physical-chemical bottom-up and biological top-down effects (Granéli, Turner, et al. 2006). These concepts have been widely used to describe ecological mechanisms and substantial progress has been made in integrating them into ecological models (Fauchald et al. 2011, Sunda and Shertzer 2014, Lynam et al. 2017). The complex interactions between bottom-up and top-down factors are disrupted by anthropogenic impacts, primarily climate change (Griffith and Gobler 2020), nutrient pollution (Wurtsbaugh et al. 2019), and overfishing (Jackson et al. 2001, Vasas et al. 2007).

1.3.1 Bottom-up factors

Historically, the study of bottom-up factors focused on the availability of light and macronutrients (e.g. carbon, N, P and silicate) as major drivers of microalgal growth. Today, the importance of micronutrients (e.g. vitamins and trace metals) and other physical or chemical factors (e.g. temperature, salinity, pH) are also recognized (Fig. 1.2). This chapter will focus on macronutrients and light, both important drivers of HABs, as well as temperature and salinity, which are also relevant to the findings in this thesis.

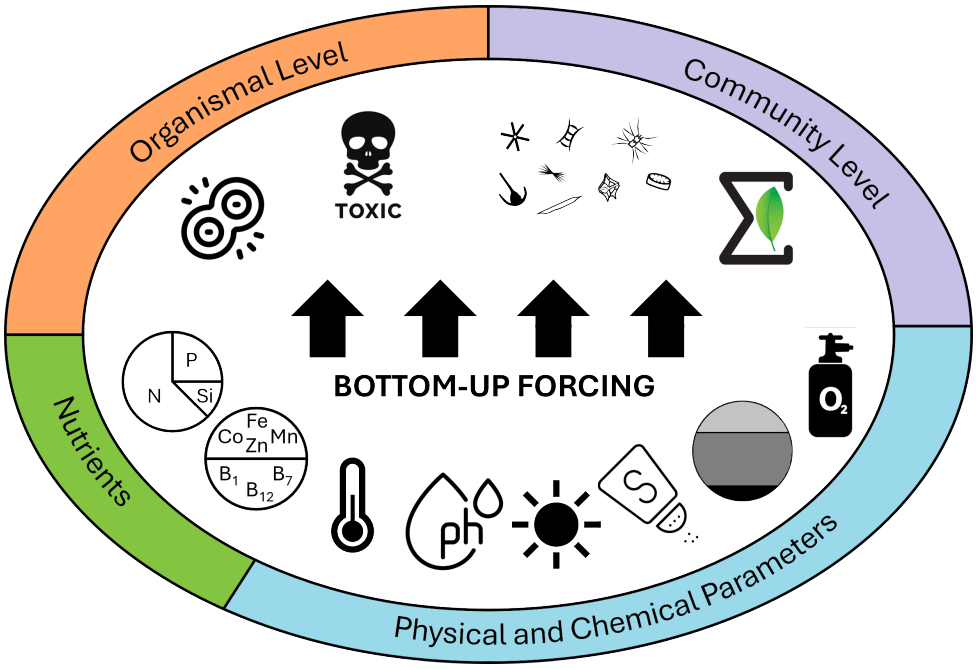

Fig. 1.2: *Schematic overview of the influence of bottom-up factors on microalgal communities on the organismal (growth rate and toxicity) and on the community level (community composition and total biomass); bottom-up factors include macronutrients and micronutrients, as well as physical and chemical parameters (from left: temperature, pH, light, salinity, stratification, dissolved oxygen).*

Light and macronutrients

Light is a major bottom-up factor for microalgae due to its role in photosynthesis. Fish-killing raphidophytes (Zhang et al. 2006) and many *Alexandrium* species, including e.g. *A. pseudogonyaulax*, *A. catenella* and *A. minutum*, are tolerant to high light (> 200 μmol photons m^{-2} s^{-1}) (Etheridge and Roesler 2005, Hwang and Lu 2000, Möller et al. 2024). Cells are protected from photoinhibition by ultraviolet-absorbing compounds (Jeffrey et al. 1999, Carreto and Carignan 2011) or through dissipating light in the xanthophyll cycle (Demers et al. 1991; Laurion and Roy 2009). This adaptation to high light might provide a competitive advantage in shallow stratified waters, especially estuaries, which regularly reach high light intensities. Other dinoflagellates, such as *K. brevis*, *A. ostenfeldii* or brown-tide forming *Aureococcus anophagefferens* appear to be low-light adapted (Maclean et al. 2003, Gobler and Sunda 2012, Magaña and Villareal 2006). Consequently, *A. anophagefferens* shows signs of genetic adaptation to low light, which is of advantage during self-shaded dense blooms (Gobler et al. 2011, Gobler and Sunda 2012). Light has also been demonstrated to influence the toxin content of HAB species, such as *Alexandrium* spp., however no general trend between light intensity and toxin content emerged (Maclean et al. 2003, Laabir et al. 2013, Möller et al. 2024). Additionally, increases in riverine loads of organic material and terrestrial run-off are hypothesized to be connected to 'coastal darkening', which reduces water clarity and thus light availability (A. Garnier et al. 2023, Frigstad et al. 2023). Coastal darkening may reduce primary production, especially in benthic habitats reliant on high light availability (Ask et al. 2009, C. T. Solomon et al. 2015), and thereby shift the proportion of benthic vs. pelagic overall productivity (Ask et al. 2009). Reduced insolation due to coastal darkening may also provoke delays in the microalgae spring bloom counteracting earlier bloom onsets due to global warming (Opdal et al. 2019).

N and P are crucial macronutrients for microalgae as they are required for the synthesis of essential cellular components such as proteins, nucleic acids and lipids. The specific composition and the type of nutrients (e.g. inorganic vs. organic) influences algal community composition (Heisler et al. 2008, R. Gibson et al. 2012, Davidson et al. 2012, Davidson et al. 2014). For instance, dinoflagellates can have an advantage over other microalgal groups in low nitrate and/or high dissolved organic nitrogen (DON) environments since many dinoflagellates are osmotrophic and phagotrophic mixotrophs but are poor competitors for nitrate (Glibert 2016, D. M. Anderson et al. 2002). Under silicate-replete conditions, nitrate generally favours diatom blooms, whereas reduced N-compounds like ammonium or urea are more likely to promote flagellate blooms (Glibert 2016, D. Bronk et al. 2007). Preference for ammonium or DON over nitrate is thought to be driven by the energetic costs associated with the cellular metabolism of the different N-sources (W. P. Cochlan and P. J. Harrison 1991, Levasseur et al. 1993). However, microalgae grown on

either ammonium or nitrate often have similar growth rates (Leong et al. 2004, T.-S. Li et al. 2011, Armstrong et al. 2018, Möller et al. 2024).

Apart from growth, the availability and form of N may significantly impact the toxin content of HAB species (Davidson et al. 2012). For instance, Van de Waal et al. (2014) demonstrated that imbalanced N:P ratios can significantly influence the toxin content of microalgal species producing N-rich toxins such as PSP-toxins, while carbon-rich toxins accumulate under both N and P limitations. Varying toxin content in response to different N-sources has also been demonstrated for *A. catenella* (Leong et al. 2004, Hattenrath et al. 2010, T.-S. Li et al. 2011, J. Xu et al. 2012). The current hypothesis is that toxin synthesis increases as cell growth decreases, suggesting cells become more toxic towards the end of a bloom. However, this relationship is genus- and/or species-specific (Granéli, Turner, et al. 2006) and in natural environments multiple stressors may influence HAB toxicity (Griffith and Gobler 2020). Consequently, accurate predictions of HAB toxicity remain challenging. This is particularly relevant since intense nutrient loading of coastal zones has long been considered a key factor in the proliferation of microalgae. This excessive enrichment of nutrients, particularly N and P, that favours the development of algal biomass is termed eutrophication. Eutrophication is often driven by anthropogenic activities, such as agricultural runoff, wastewater discharge and industrial pollution (Davidson et al. 2014, Wurtsbaugh et al. 2019). This anthropogenic influence provokes multiple problems, including hypoxic dead zones (Breitburg et al. 2018), promotion of HABs (Heisler et al. 2008, Davidson et al. 2014, Wurtsbaugh et al. 2019), stimulation of greenhouse gas release (J. J. Beaulieu et al. 2011) and degradation of sociocultural values of affected water bodies (Wurtsbaugh et al. 2019). Fortunately, long-term reduction of nutrient loading (de-eutrophication) can decrease HAB occurrences as observed in the Sea of Japan and the Black Sea (D. M. Anderson et al. 2002).

While the specific environmental processes selecting for toxic species remain unclear, some models have suggested positive feedbacks between eutrophication-driven bottom-up and top-down controls in the formation of HABs (Mitra and Flynn 2006, Sunda et al. 2006, Sunda and Shertzer 2014, Zhou et al. 2017). These studies indicate that HABs were regularly preceded by one or multiple pre-blooms of fast-growing diatoms that reduced the dissolved nutrient concentrations and stimulated grazer populations, such as zooplankton. This created favourable conditions for HAB species, considering their competitive advantage in low nutrient scenarios, which may also lead to increased toxin production further deterring grazers. Similar bloom dynamics have been observed in the field for *A. anophagefferens* (Gobler and Sañudo-Wilhelmy 2001) and *A. lagunensis* (Buskey 2008).

Other bottom-up factors

Apart from macronutrient and light availability, bottom-up factors can be extended by other abiotic factors, such as temperature or salinity (Fig. 1.2). Temperature is one of the

main environmental factors controlling the metabolic rate, i.e. the rate at which organisms transform energy and materials, and consequently influences microalgal growth (Gillooly et al. 2001, W. F. Cross et al. 2015). While nutrient limitation in oligotrophic systems might counteract temperature-stimulated growth, primary productivity in coastal and upwelling systems is expected to increase (Marañón et al. 2018). Furthermore, temperature influences resource allocation of microalgae and thereby changes the cellular stoichiometry with implications for biogeochemical cycling and food web dynamics (Toseland et al. 2013). Temperature can also alter other physiological processes that shape bloom dynamics, such as motility (Kamykowski and McCollum 1986) or encystment and germination (Montresor and Lewis 2006, A. D. Fischer et al. 2018). Increasing temperatures also stimulate heterotrophic feeding of phagotrophic mixotrophs (Wilken et al. 2013) through enhanced ingestion (Princiotta et al. 2016, You et al. 2020) and/or digestion rates (A. Li et al. 2001). Consequently, increasing temperatures have been demonstrated to accelerate the growth of HAB species, such as *Alexandrium* spp. (Etheridge and Roesler 2005, A. S. Lim et al. 2019b, P. Li et al. 2021), until a certain species-specific thermal affinity is surpassed, after which growth declines. However, temperature-stimulated growth does not verify that HAB species would have a competitive advantage over non-HAB species in an ecosystem setting (Wells et al. 2015). Finally, rising ocean temperatures also influence the geographical expansion of microalgae, which are assumed to expand polewards (Gobler et al. 2017, Kléparski et al. 2024) and to prolong their blooming period (Hjerne et al. 2019, Viitasalo and Bonsdorff 2022) according to their thermal affinities (Thomas et al. 2012). For instance, PSP-producing *A. catenella* is forming blooms in the Bering Strait region (Fachon et al. 2024), which causes accumulation of large cyst beds (D. M. Anderson et al. 2021). Both developments have been primarily associated with global warming (Fachon et al. 2024, D. M. Anderson et al. 2021).

Salinity is another important bottom-up factor that, together with temperature, partially influences stratification, which plays a role in the development of HABs (Carstensen and Jakobsen 2023, Smayda 2002, Berdalet et al. 2014). In fact, rising ocean temperatures cause increased vertical stratification due to stronger surface heating, which likely decreases nutrient supply but prolongs the growing season (Coma et al. 2009, Sarmiento et al. 2004). The motility of dinoflagellates enables the organisms to vertically migrate to surface waters during the day to photosynthesize and downward at night to access deep nutrients (Erga et al. 2015, B. Zheng et al. 2023). This represents a crucial competitive advantage over non-motile microalgae that remain constrained within one water layer, especially during stratified conditions. Moreover, relationships between favourable meteorological conditions, including wind speed and direction, and *Alexandrium* blooms have been reported (Fauchot et al. 2008, Carstensen and Jakobsen 2023). Strong winds may facilitate resuspension of cysts buried in the sediment, while weak winds induce a higher probability of stratification (Carstensen and Jakobsen 2023). Other physical processes,

such as advection, turbulence and turbidity are also influencing stratification and may be considered bottom-up factors as well.

1.3.2 Top-down Factors

Top-down effects primarily describe the predation pressure of higher towards lower trophic levels (Granéli 2008). For microalgae, grazing is the most important top-down effect, but other loss processes such as bacterial infections (Kodama et al. 2006) or viral lysis (Brussaard 2004) are also important, particularly during bloom termination. The main consumers of microalgae are microzooplankton (20–200 μm) and mesozooplankton (> 200 μm), which play a key role in pelagic food webs by linking primary production with higher trophic levels (Calbet and Landry 2004, Tillmann 2004). Notably, microzooplankton account for approximately two-thirds of microalgal mortality in the oceans (Calbet et al. 2003, Calbet and Landry 2004). Consequently, it has been hypothesized that resistance to microzooplankton grazing drives bloom development (Irigoien et al. 2005). Mesozooplankton also represents an important loss factor of larger HAB species, especially dinoflagellates (Calbet 2001). However, HAB species are not passive victims to the top-down grazing pressure, and for instance the production of phycotoxins may act as an efficient defence mechanism.

Grazing by zooplankton on microalgae exerts intense selective pressure for defensive traits. These defences include physiological (e.g. toxicity or bioluminescence (Lindström et al. 2017)), morphological, like chain formation (Bergkvist et al. 2012, Ryderheim et al. 2024), silica shells (Pančić and Kiørboe 2018), spines/keels/helmets (J. J. Gilbert 1966, Harvell 1986)) or behavioural defences, such as adjusted swimming behaviour (Selander et al. 2011). This thesis, however, will focus on HAB species that utilize chemical compounds (phycotoxins and BECs) for the deterrence, immobilization and killing of predators. Toxin production in HAB species, or more precisely the expression of genes associated with toxin production, can be constitutive, i.e. always present, or inducible and only activated upon external stimuli. These mechanisms were first mentioned in host-parasite interactions (Kern 1956), and it was not clearly defined whether inducible mechanisms are completely absent without stimuli or only significantly reduced. Plenty of evidence, including in this thesis (publication I/II), demonstrates that HAB species contain toxins and BECs, when grown in monoculture, suggesting constitutive toxin expression. However, in the last decade, several studies have evidenced that HAB species can increase their toxin content upon exposure to grazers up to 20-fold (Selander et al. 2015, Ryderheim et al. 2021, Park et al. 2023). Notably, Selander and co-workers (Selander et al. 2015, Selander et al. 2016, Grebner et al. 2019) have isolated and identified various copepodamides, taurine conjugated lipids, from multiple copepod species as causative agents of the toxin increase in HAB species and these results reinforce the notion of an inducible mechanism of toxin production.

Ecological models usually presume that the production of secondary metabolites, functioning as chemical defences (e.g. toxins and BECs), comes at a cost, such as a reduced growth rate. Recently, studies by Park and Dam (Park and Dam 2021, Park et al. 2023) have demonstrated direct fitness costs of grazer-induced changes in the toxin production of *A. catenella* by measuring reduced expression of a gene involved in cell division, indicative of cell growth. Additionally, Blossom et al. (2019) found a trade-off between lytic toxicity and the growth rate of multiple *Alexandrium* species, while Brandenburg et al. (2018) demonstrated trade-offs between defensive traits and N assimilation. In contrast, several other studies have found no defence-induced costs in growth rates of *Alexandrium* (Ryderheim et al. 2021, Selander et al. 2008), however, energetic costs associated with the expression of defensive traits may be elusive in short-term laboratory experiments.

The detrimental effects of HAB species on larger mesozooplankton include reduced mobility and ingestion rates (Ryderheim et al. 2021), as well as impaired reproductive mechanisms, including fecundity (Ianora et al. 2003, Turner 2014) , fertilization (Ianora et al. 2003), and egg hatching (Ianora et al. 2003, Turner 2014), all provoking mortality. Microzooplankton experience similar impacts, such as reduced mobility and ingestion rates leading to depressed growth rates and mortality (Tillmann 2004). These effects depend on the specific predator-prey combination, with some predators being more resistant than others (Tillmann 2004, Turner 2014). In fact, geographically separate copepod populations are affected differently by exposure to HAB species suggesting that copepod populations adapt defensive mechanisms (Colin and Dam 2002, Jiang et al. 2011). For instance, recent studies indicate that detoxification in copepods is likely aided by gut microbes (Gorokhova et al. 2021, J. Yang et al. 2024) offering an elegant explanation for the observed local adaptation of copepod populations. Microzooplankton and mesozooplankton are increasingly investigated as biotic top-down control of HABs due to their high grazing rates and the resistance of some species towards toxic prey (Gallardo-Rodríguez et al. 2019).

1.3.3 Trophic cascades

Smayda (2008) noted that:

> "HABs and red tides generally should be viewed as blooms that are regulated by coupled nutrient-grazer processes – nutrient stimulation alone is inadequate, even when exogenous nutrients are not a factor."

Today, field (Frank et al. 2011, Riisgård et al. 2012a) and modelling studies (Fauchald et al. 2011, Lynam et al. 2017) show links between HABs, jellyfish blooms and overfishing, confirming the observation that multiple stressors are interacting during HAB development. The existence of such trophic cascades (Fig. 1.3) implies that fisheries actively modify the ecosystem, rather than being merely beneficiaries of marine resources. Briefly,

overfishing of commercial fish (e.g. tuna, herring, salmon, snapper) reduces the grazing pressure on small, mainly planktivorous fish and jellyfish allowing them to propagate. Subsequently, they decrease herbivore populations, i.e. microzooplankton and mesozooplankton, which then reduces the grazing pressure on HAB species potentially favouring bloom development.

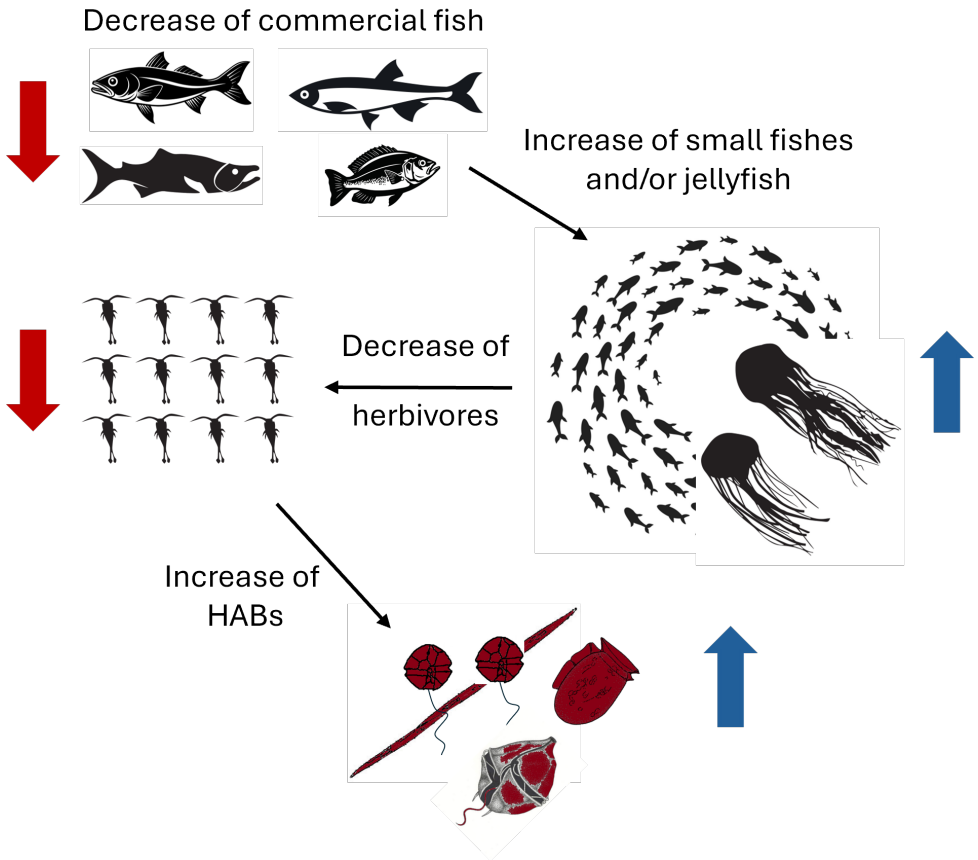

Fig. 1.3: *Schematic representation of a trophic cascade: overfishing reduces the predation pressure on small fishes and/or jellyfish, which decrease herbivore populations allowing microalgae and HAB species to propagate; redrawn from "Eutrophication and Harmful Algal Blooms" by E. Granéli (chapter 7, Olli and Wassmann 2005).*

Unsurprisingly, the complexity of pelagic food webs and the wide range of biological, chemical-physical, meteorological and hydrographic conditions that influence HAB development need to be considered simultaneously. These parameters, together with microalgal abundances, need to be assessed in a high spatiotemporal resolution in order to develop models capable of forecasting HABs. Moreover, models require information about the growth and toxin response of HAB species towards bottom-up and top-down factors, which are still lacking for many HAB species. The high spatiotemporal resolution is of critical importance, considering examples of the field, in which bottom-up and top-down factors are interacting (Rothenberger and Calomeni 2016, Fernández-Alías et al. 2022, Rodríguez-Gálvez et al. 2023).

1.4 The genus *Alexandrium*

One of the primary causative agents of HABs are dinoflagellates, particularly those belonging to the *Alexandrium* genus, which have been extensively studied over the last few decades (D. M. Anderson et al. 2012a, Long et al. 2021). This genus, which belongs to the Gonyaulacales (Saldarriaga et al. 2004), currently contains 34 species (Mertens et al. 2020) known for producing a variety of marine toxins. These include PSP-neurotoxins (D. M. Anderson et al. 2012a), spiroimines (spirolides and gymnodimines, Zurhelle et al. 2018), goniodomins (GDs, Hsia et al. 2006) and BECs (Tillmann and John 2002, Long et al. 2021). Within *Alexandrium*, fourteen species produce PSPs, six species produce GDs, and only *A. ostenfeldii* is known to produce spiroimines (Mertens et al. 2020). The production of lytic BECs has also been demonstrated in some *Alexandrium* species (Mertens et al. 2020). Moreover, several *Alexandrium* species can form long chains, such as *A. catenella* or *A. monilatum*, and many, if not all, can form resting or temporary cysts to endure unfavourable environmental conditions (D. M. Anderson et al. 2012a, Bravo and Figueroa 2014). These adaptive features of *Alexandrium* underline their resilience and ability to colonize various habitats.

The genus *Alexandrium* was formally established by Halim through the description of *Alexandrium minutum* (Halim 1960). Morphological identification requires dissection of thecal plates or imaging by scanning electron microscopy, but the morphological details are out of the scope of this thesis. In molecular phylogeny *Alexandrium* is divided into different clades which partly reflect the subgenera *Alexandrium* and *Gessnerium* as defined by Balech (1990). However, studies also show that the molecularly defined *Gessnerium* group currently contains seven defined species, which are *A. pseudogonyaulax*, *A. monilatum*, *A. hiranoi*, *A. satoanum*, *A. ogatae*, *A. taylorii* and *A. limii* (Abdullah et al. 2023). For these species, the first apical plate is not connected to the apical pore. Some other *Alexandrium* species, namely *A. balechii*, *A. globosum*, *A. foedum*, *A. concavum* and *A. camurascutulum*, also fit this morphological characterization but lack sequence data (U. Tillmann, personal communication). However, some other species of Balech's subgenus *Gessnerium*, such as *A. margalefii*, *A. pohagense*, and *A. insuetum* also lack contact of the first apical plate and the apical core but are not related to the *Gessnerium* clade. Importantly, the species molecularly defined as belonging to *Gessnerium* produce GDs (Hsia et al. 2006, Krock et al. 2018, Tillmann et al. 2020, Abdullah et al. 2023), although the toxin profile of *A. satoanum* has not been analysed yet. Given that GDs are absent in all other analysed *Alexandrium* species (including Balechs *Gessnerium* species *A. margalefii* and *A. insuetum*; personal communication, U. Tillmann), the production of GDs can be considered a chemotaxonomic trait of the *Gessnerium* group, suggesting that GD production evolved in their most recent common ancestor. Other *Alexandrium* species are responsible for PSP-toxin outbreaks along many coastlines worldwide, leading

to substantial economic damage due to the closing of aquacultures and shellfish farms, as well as causing human intoxications (D. M. Anderson et al. 2012a). Human symptoms range from numbness, headache and nausea to fatal cases due to respiratory paralysis (Etheridge 2010).

1.5 *Alexandrium pseudogonyaulax*: An emerging threat across Northern Europe?

1.5.1 Species description

Alexandrium pseudogonyaulax (Fig. 1.4) is a thecate dinoflagellate with a planktonic and a resting cysts benthic life stage (H. Triki et al. 2015). The resting cyst of *A. pseudogonyaulax* is unusual as it has a paratabulation which is not seen in other species of *Alexandrium* (Montresor 1995), and the formation of temporary cysts has also been observed (Kita et al. 1985). Originally identified as *Goniodoma pseudogonyaulax* by Biecheler in the Thau lagoon near Montpellier in France (Biecheler 1952), *A. pseudogonyaulax* has since been reported globally, including in the Mediterranean Sea, the Black Sea, Japan, New Zealand, China, the Gulf of California, the Norwegian Sea, the North Sea and the Baltic Sea (Kita et al. 1985, Montresor 1995, Yahia-Kefi et al. 2001, Morquecho and Lechuga-Deveze 2004, Bravo et al. 2006, Gu et al. 2013, H. Triki et al. 2015, B. Karlson et al. 2021, Abdullah et al. 2023, Klein et al. 2010, Terenko 2005). The widespread distribution of *A. pseudogonyaulax*, primarily in brackish and coastal waters, suggests high adaptive capabilities to varying abiotic parameters, such as temperature and salinity.

This dinoflagellate produces macrocyclic polyketides known as GDs (H. Z. Triki et al. 2016), as well as other BECs (Blossom et al. 2012). Initially considered exclusively photosynthetic, Blossom et al. unravelled the phagotrophic capabilities of *A. pseudogonyaulax* (Blossom et al. 2012). Mixotrophy appears common within the *Alexandrium* genus, considering that they are often observed to contain food vacuoles in the field (Jeong et al. 2010). In the laboratory, however, phagotrophy has only been demonstrated in some members (Blossom et al. 2017), but not in others (A. S. Lim et al. 2019a). Additionally, phagotrophy has been reported for a single strain of *A. ostenfeldii* (Gribble et al. 2005), but not for others (Blossom et al. 2017), indicating potential prey-selectivity or strain-specific variability in phagotrophy. Phagotrophic feeding of *A. pseudogonyaulax* is aided by the production of potentially toxic mucus traps (Fig. 1.4) and leads to enhanced growth rates (Blossom et al. 2012). This unique and effective prey-capture strategy involves the release of sticky mucus, which entraps other protists upon contact and has not been observed in any other *Alexandrium* species yet (Blossom et al. 2017). GDs and/or BECs may be concentrated and stabilized within the mucus, however this hypothesis re-

quires further investigation. Notably, *A. pseudogonyaulax* adversely affects a wide range of microalgae, including other toxic and lytic *Alexandrium* species (Blossom et al. 2012, Blossom et al. 2017).

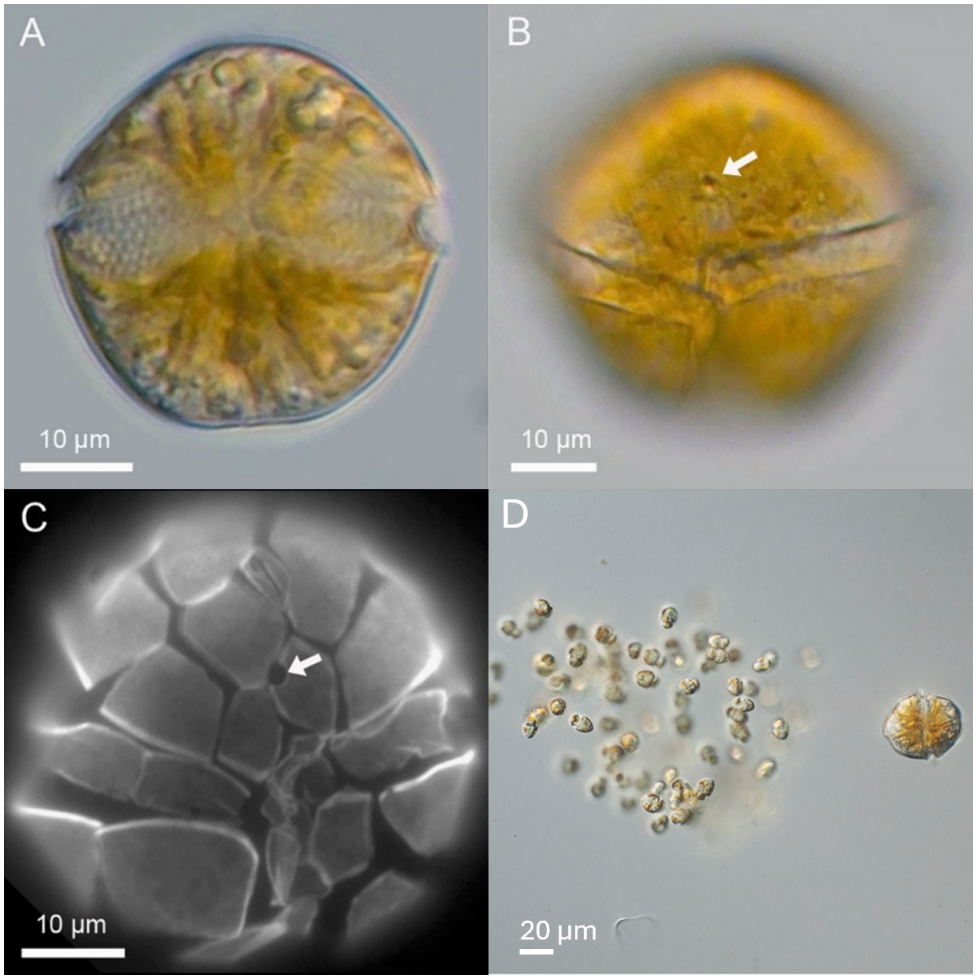

Fig. 1.4: *A-C)* Alexandrium pseudogonyaulax *cells; arrow denotes the position of the ventral pore (microscopy by Urban Tillmann); D)* A. pseudogonyaulax *cell is dragging behind a mucus trap which has caught many* H. rotundata *cells (reprinted from "A search for mixotrophy and mucus trap production in* Alexandrium *spp. and the dynamics of mucus trap formation in* Alexandrium pseudogonyaulax*", 64, Blossom et al. (2017), Harmful Algae, Copyright (2024), with permission from Elsevier.*

Prolonged culturing without prey addition led to the loss of phagotrophic feeding capabilities in one strain of *A. pseudogonyaulax* (Blossom and P. J. Hansen 2021). Thus, discrepancies in or a supposed lack of phagotrophic abilities of *Alexandrium* between studies may reflect the age and culture conditions of strains. Consequently, studies should either utilize recently isolated strains or occasionally provide prey to mixotrophs in laboratory cultures. Interestingly, despite an absence of prey, *A. pseudogonyaulax* retained its ability to produce mucus traps and BECs (Blossom and P. J. Hansen 2021), suggesting that BECs may play additional roles beyond prey assimilation. BECs induced lysis of target cells, thereby increasing the pool of organic carbon and other nutrients, provid-

ing another competitive advantage and potentially offsetting the energetic cost of BEC production and release. This could create a positive feedback loop capable of sustaining HABs (Weissbach et al. 2012, Kang and Gobler 2023). Having established the scientific significance of *Alexandrium*, and especially *A. pseudogonyaulax*, the next chapter will introduce GDs and BECs from a chemical and toxicological perspective.

1.5.2 Goniodomins (GDs) and bioactive extracellular compounds (BECs)

The pioneering work on the isolation and partial structural elucidation of the antifungal toxin goniodomin, isolated from an unidentified dinoflagellate in Puerto Rico, was conducted by Sharma et al. (1968). Twenty years later, Murakami et al. (1988) isolated and purified a compound from a blooming dinoflagellate in Japan with similar properties. The organism was initially identified as *Goniodoma pseudogoniaulax*, nowadays known as *Alexandrium pseudogonyaulax*. It was later confirmed that the organism was in fact *Alexandrium monilatum*, a morphologically similar species, which also produces GDs. Despite this taxonomic mismatch, Murakami and coworkers (1988) successfully elucidated the structure of the isolated compound and named it goniodomin A (GDA) as it was still unclear whether this was the same compound as isolated by Sharma et al. Two decades later Takeda et al. (2008) established the absolute configuration of GDA (Fig. 1.5).

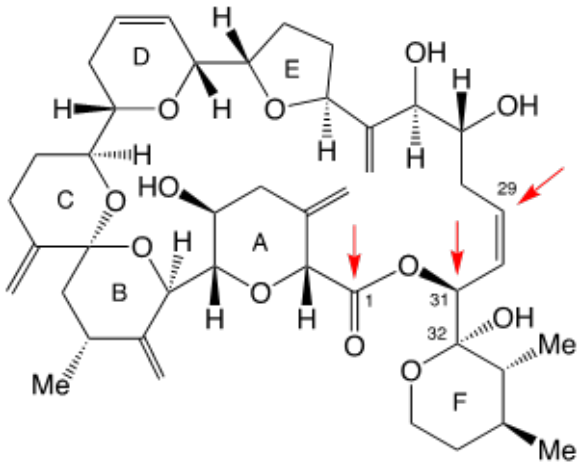

Fig. 1.5: *Absolute configuration of goniodomin A (GDA), red arrows denote possible hydrolysis sites (Takeda et al. 2008, C. M. Harris et al. 2020).*

Goniodomin A is a polyketide macrolide containing six oxygen heterocycles and a lactone group (i.e. a cyclic ester, Fig. 1.5). Under mild conditions, GDA undergoes cleavage of this ester linkage to yield mixtures of goniodomin A seco acids (GDA-sa, Fig. 1.6). Increasing the pH accelerates the cleavage, although ring-opening also occurs at neutral pH (C. M. Harris et al. 2023). Consequently, GDA-sa predominates as the main

extracellular congener, while GDA is primarily found intracellularly (Hintze 2021).

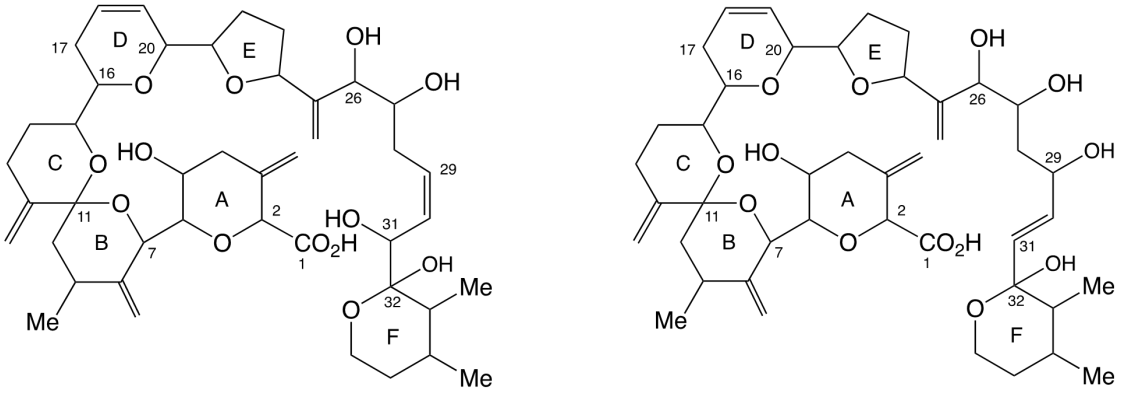

Fig. 1.6: *Two seco acids of goniodomin A (GDA-sa), left: formation of GDA-sa after hydrolysis of the ester linkage at C1 or C31; right: formation of GDA-sa after hydrolysis and allylic rearrangement at C29 (C. M. Harris et al. 2023).*

Furthermore, formation of the isomer goniodomin B (Fig. 1.7, GDB) and the α,β-unsaturated ketone goniodomin C (Fig. 1.7, GDC) primarily under acidic conditions have been reported (C. M. Harris et al. 2023). Additionally, GDC can undergo further hydrolysis, similar to GDA, resulting in the formation of seco acid GDC-sa. Currently it remains uncertain whether GDB, GDC and GDC-sa are artefacts formed during the extraction and analysis of GDs or if they naturally occur in GD-producing microalgae. This isomerization occurs rapidly under acidic conditions, while under alkaline conditions GDA-sa is the primary hydrolysis product. In order to suppress the formation of GDB and GDC, LC-MS/MS analysis of GDs is now conducted under alkaline conditions. The chemical complexity of GDs gets further exacerbated by the discovery of additional congeners, such as goniodomic acid (T. Harris, *under review*).

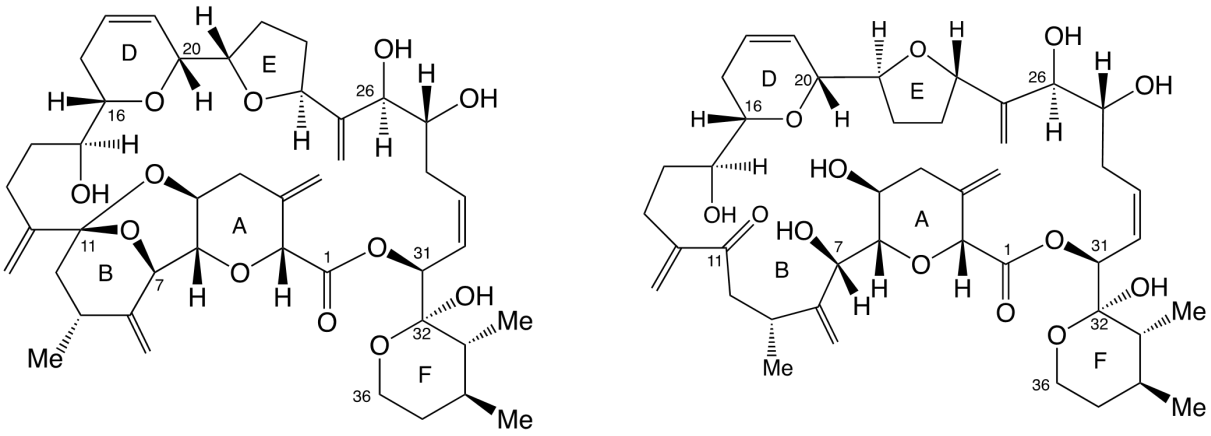

Fig. 1.7: *In acidic environments goniodomin A (GDA) isomerizes to goniodomin B (left, GDB) and an α,β-unsaturated ketone goniodomin C (right, GDC).*

Goniodomin-producing species have long been associated with the mortality of marine organisms. For instance, mass occurrences of *A. monilatum* in the Gulf of Mexico (Connell

and J. B. Cross 1950, Wardle et al. 1975) and eastern Florida (Howell 1953) have been associated with fish kills, as well as the mortality of marine gastropods in the York River in Virginia (Harding et al. 2009). Moreover, *A. monilatum* exhibits toxic effects towards various marine organisms in laboratory experiments, including fish (Gates and Wilson 1960, Sievers 1969), annelids (Sievers 1969), crustaceans (Sievers 1969, Silva et al. 2013), ciliates (Silva et al. 2013) and molluscs (Sievers 1969, May et al. 2010). These studies suggest the involvement of GDs in the toxicity of GD-producing organisms, although a direct causal relationship has yet to be established.

Investigations on the toxic effects of purified GDs have revealed a common feature across all investigated cell assays: interactions with actin (Murakami et al. 1988, Furukawa et al. 1993, Yasuda et al. 1998, Matsunaga et al. 1999, Abe et al. 2002, Espiña et al. 2016). Potentially as a consequence, GDs have been shown to exhibit cytotoxic effects towards mice leading to inflammation and cell damage within liver and thymus (Erker et al. 1985, Terao et al. 1989). In addition, GDA has demonstrated cytotoxicity to rat hepatocytes and human neuroblastoma cells (Espiña et al. 2016). Overall, mammalian cell line bioassays have uncovered adverse effects of GDs, but their toxicity towards marine organisms remains poorly resolved. Given the prevalence of GDA-sa as the primary extracellular GD congener, it is also imperative to specifically investigate its impact on marine organisms.

Alexandrium species are further known for producing BECs, whose molecular structures remain largely uncharacterised. These substances have been previously referred to as allelochemicals, lytic substances, or ichthyotoxins (Long et al. 2021), but will be referred to as BECs in this thesis. Due to the absence of effective isolation and purification protocols (Ma et al. 2009, Ma et al. 2011b), BECs have been primarily characterised by their adverse effects on a wide range of organisms, including protists, metazooplankton, shellfish and fish (reviewed in: (Long et al. 2021). Apart from deterring predators and eliminating competitors, BECs may also directly assist in prey capture, as shown for *A. pseudogonyaulax* (Blossom et al. 2012), which utilizes a potentially toxic mucus to trap and immobilize prey prior to ingestion. Notably, high inter- (Arzul et al. 1999, Tillmann and John 2002, Long et al. 2018) and intra-strain (Tillmann et al. 2009, Alpermann et al. 2010, Van de Waal et al. 2015) variability in the potency of BECs have been reported. As a rare exception, strains completely lacking BEC production have also been described (Tillmann et al. 2009), which are an ideal control in BEC-related comparative studies (publication II, John et al. 2015).

The production of BECs might cause fitness costs, such as a reduced growth rate (Blossom et al. 2019), suggesting a trade-off between carbon utilization for the production of BECs and cell division. In contrast, the lytic activity of *Alexandrium* showed no clear trend to N or P starvation (I. Yang et al. 2011, Zhu and Tillmann 2012). It is unclear which factors are modulating the production of lytic BECs and whether variabilities between

different studies are caused by the specificity of the utilized bioassays or are representing variability in the production of BECs and/or their potency. It can generally be assumed that the adverse effects of lytic BECs on other marine organisms provide *Alexandrium* with a competitive advantage. However, whether this advantage only benefits the producing organism, i.e. a 'private' good, or also other microalgae, i.e. a 'public' good, is still an ongoing debate (Driscoll et al. 2016, Ehrlich et al. 2022). For instance, it has been demonstrated that BECs are protecting non-BEC-producing cells from the same species against grazing (John et al. 2015). On the other hand, rapid dilution of excreted BECs and high cell densities required for lytic activity cast doubt on the theory of public goods, at least in supporting bloom development. However, microalgae have been reported to form dense patches characterised by higher cell densities than their surroundings (Durham et al. 2013, Breier et al. 2018), and thus BEC concentrations may be sufficiently high to cause adverse effects, while benefits might be simultaneously restrained to conspecific microalgae within proximity.

Currently, it remains unclear whether BECs produced by different species of *Alexandrium* are identical, and initial attempts at structural characterisations have yielded contradictory results. The only BEC of *Alexandrium* that has been structurally elucidated so far is alexandrolide, a diatom growth inhibitor, isolated from cell-free culture supernatant of *A. catenella* (Satake et al. 2019). However, alexandrolide is unlikely the only BEC responsible for the adverse effects of *Alexandrium*, as it is a small molecule (528 Da) and most other BECs were found to be much larger (Long et al. 2021). Emura et al. (2004) conducted the first structural study on BECs, revealing potent toxic effects of *A. taylorii* on the brine shrimp *Artemia salina*, as well as haemolytic activity against rabbit and guinea pig erythrocytes. Dependency between cell density and lytic activity suggests continuous excretion of BECs as opposed to leakage from ruptured or dead cells. Furthermore, Emura and coworkers (2004) associated lytic activity with large compounds (> 10 kDa), hypothesizing partial proteinaceous composition due to heat-dependent activity and reduced activity upon treatment with the protease trypsin. Subsequent research by Flores et al. (2012) demonstrated a similar reduction in lytic activity of *A. tamarense* against the ciliate *Tiarina fusus* and the heterotroph protist *P. kofoidii* upon trypsin addition. However, other studies reported no heat sensitivity or loss of lytic activity upon trypsin treatment of *A. tamarense* (likely *A. catenella* due to production of PSP-toxins (Yamasaki et al. 2008, Chen et al. 2015) and *A. catenella* (Ma et al. 2011a, Ma et al. 2011b). It is thus still unclear whether these findings reflect strain and/or assay variability or species-specific differences. Moreover, the potential involvement of reactive oxygen species (ROS) in the lytic activity of some *Alexandrium* spp. (Flores et al. 2012) complicates the characterization of the mode of action of BECs, warranting further research. While some studies suggest BECs to be proteinaceous, others found significant portions of carbohydrates, adding to their chemical complexity (Chen et al. 2015, Galasso et al. 2018).

Moreover, evidence indicates that BECs of *A. catenella* target membrane sterols (Ma et al. 2011a) altering membrane permeability and provoking pore formation akin to various polyketides, such as karlotoxins and karmitoxins (Place et al. 2012, Deeds et al. 2015, Rasmussen et al. 2017), and amphidinols (Wellkamp et al. 2020, Paul et al. 1997). These polyketides are amphipathic sharing a hairpin structure consisting of a polar head and a hydrophobic tail. In the case of karlotoxins, the hydrophobic tail increases membrane permeability (Deeds et al. 2015), while the polar head interacts with membrane sterols outside of the membrane (Waters et al. 2015). Overall, the chemical nature of BECs produced by *Alexandrium* spp. remains largely unknown. Considering their diverse adverse effects on marine organisms and the lack of correlation between lytic activity and phycotoxin concentrations (Tillmann and John 2002, Flores et al. 2012, Shang et al. 2021, Wolf 2021), isolating and characterizing BECs is imperative for assessing their proportion in the overall toxicity of BEC-producing *Alexandrium* species.

1.5.3 Expansion of *A. pseudogonyaulax* across Northern Europe

Alexandrium pseudogonyaulax is not only of scientific interest regarding its production of toxins and BECs, but also considering that this dinoflagellate currently appears in increasing abundance and frequency across Northern Europe. Firstly identified by Kremp et al. (2019), *A. pseudogonyaulax* suddenly appeared in the Danish Limfjord in 2007 and another two years later was the only contributor to the *Alexandrium* community, apparently displacing previously dominating *A. ostenfeldii* and *A. tamarense*. While this study was constrained to the Limfjord, a similar situation emerged in the Skagerrak, Kattegat and the southern and western areas of the Baltic Sea (Fig. 1.8, Möller et al. 2024 *forthcoming*), which feature increasing occurrences of *A. pseudogonyaulax*.

Alexandrium minutum (data not shown) and *A. tamarense* (Fig. 1.8) were seldom part of the *Alexandrium* community in this region, in which it is likely that the majority of records of *A. tamarense* in fact are *A. catenella* due to the production of PSP-toxins. Additionally, occurrences of *Alexandrium* spp. (i.e. no species-level analysis, Fig. 1.8) have not remarkably changed and thus it is unlikely that taxonomic misinterpretations are responsible for the observed changes in the *Alexandrium* community. Notably, *A. ostenfeldii* reached cell densities of more than 10^6 and 10^7 cells L^{-1} in the Limfjord, and Mariager Fjord, respectively. Most of these high-density blooms were in the late 90's, however, in the Limfjord in 2020, *A. ostenfeldii* reached cell densities of 5.4×10^6 cells L^{-1}. In contrast, the highest reported cell density of *A. pseudogonyaulax* was 1.25×10^5 in the Skagerrak next to Arendal, with another nine occurrences featuring cell densities above more than 10^4 cells L^{-1} in the Limfjord.

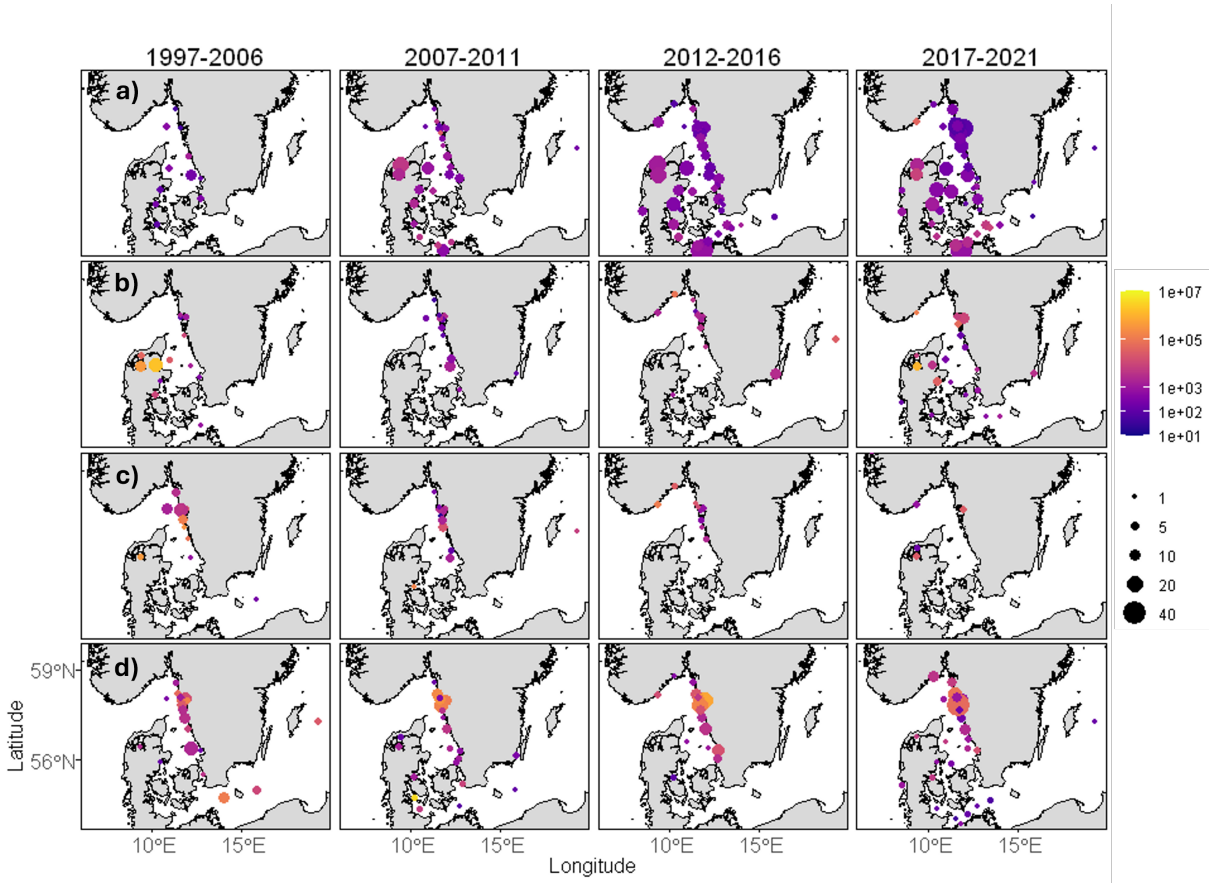

Fig. 1.8: *Mean cell densities of a)* A. pseudogonyaulax*, b)* A. ostenfeldii*, c)* A. tamarense *and d) other* Alexandrium *spp. during the last three decades; colour scheme corresponds to the cell densities (cells L^{-1}) and size to the number of present observations during the respective time-frame (Möller et al., in prep.).*

However, cell densities may imply inaccurate information about the contribution of large dinoflagellates, like *A. pseudogonyaulax*, to the total biomass of microalgal communities. For instance, in the Arkona Basin in 2019, *A. pseudogonyaulax* constituted over half of the total biomass, while only having a cell density of 3.3×10^4 cells L^{-1} (Zettler et al. 2020). Since this thesis focuses on *A. pseudogonyaulax* in the context of the ongoing expansion of Northern Europe, the next chapter will briefly introduce the main regional water bodies.

1.6 Northern European water bodies

1.6.1 General characteristics

The study area in Northern Europe mainly covers the North Sea, including the Skagerrak and Kattegat, as well as the southern part of the Baltic Sea (Fig. 1.9). However, *A. pseudogonyaulax* has occasionally been observed along the Norwegian coastline from Oslofjorden (Dittami et al. 2013) to Finnmark in the Barents Sea (Throndsen et al. 2007). In the south, the Norwegian Sea transitions into the temperate North Sea with depths generally not exceeding 100 m (Rees et al. 2007). The North Sea experiences strong tidal currents and water mixing (Wilde et al. 1992). Consequently, the North Sea is rarely stratified, except for the northern and central areas (Leeuwen et al. 2015). These conditions make the North Sea one of the world's most productive fishing grounds (Conti and Scardi 2010). The Skagerrak-Kattegat area, located between Norway, Sweden and Denmark, acts as a transitional zone between the Baltic Sea and the North Sea. Notably, the Skagerrak is much deeper ($\approx$ 700 m) than the North Sea and primarily stratified (Leeuwen et al. 2015).

The Baltic Sea is a brackish, shallow sea with limited water exchange, which can be divided into several sub-basins based on distinct salinities and nutrient loads. During winter, the water column is mixed, while in summer extensive areas of the Baltic Sea are permanently stratified (Liblik and U. Lips 2019). Unlike in open oceans, this stratification is not only driven by temperature but also by salinity differences. The drainage basin of the Baltic Sea is inhabited by 85 million people, which exert strong anthropogenic pressures, such as nutrient pollution and overfishing, on the Baltic Sea (Korpinen et al. 2012). Due to its narrow connection to the North Sea and large riverine freshwater input, the Baltic Sea features a strong salinity gradient from about 12 in the southwest to 3 in the northeast (Olofsson et al. 2020). Salinity fluctuations can influence the osmoregulation and growth rates of microalgae and consequently, a high salinity tolerance of HAB species may be favouring their expansion into the Baltic Sea.

The Baltic Sea is dominated by dinoflagellates, even during the spring bloom, which is unique among large water bodies (Hjerne et al. 2019, Klais et al. 2011). In contrast, dinoflagellate abundances in the North Sea have generally declined along with an increase in diatom biomass (Alvarez-Fernandez et al. 2012, Beaugrand et al. 2014, Bedford et al. 2020), potentially as a consequence of decreased riverine nutrient inputs and increased sea surface temperatures (Desmit et al. 2020, Di Pane et al. 2022). The general assumption of a competitive advantage of dinoflagellates over diatoms in permanently stratified conditions matches the community composition in the Baltic Sea. However, in the North Sea, even permanently stratified regions are dominated by diatoms (Leeuwen et al. 2015). Hence, other factors than stratification, such as nutrient levels, might be more important

in shaping community composition in the North Sea.

Water bodies across Northern Europe are heavily utilized for fishing (Fig. 1.9), tourism and recreation activities. Thus, the increasing likelihood of *A. pseudogonyaulax* blooms could pose significant risks to both ecosystems and economies. Besides anthropogenic pressures, climate change is likely the most influential factor affecting water bodies in Northern Europe and therefore, expected changes will be introduced in the next chapter.

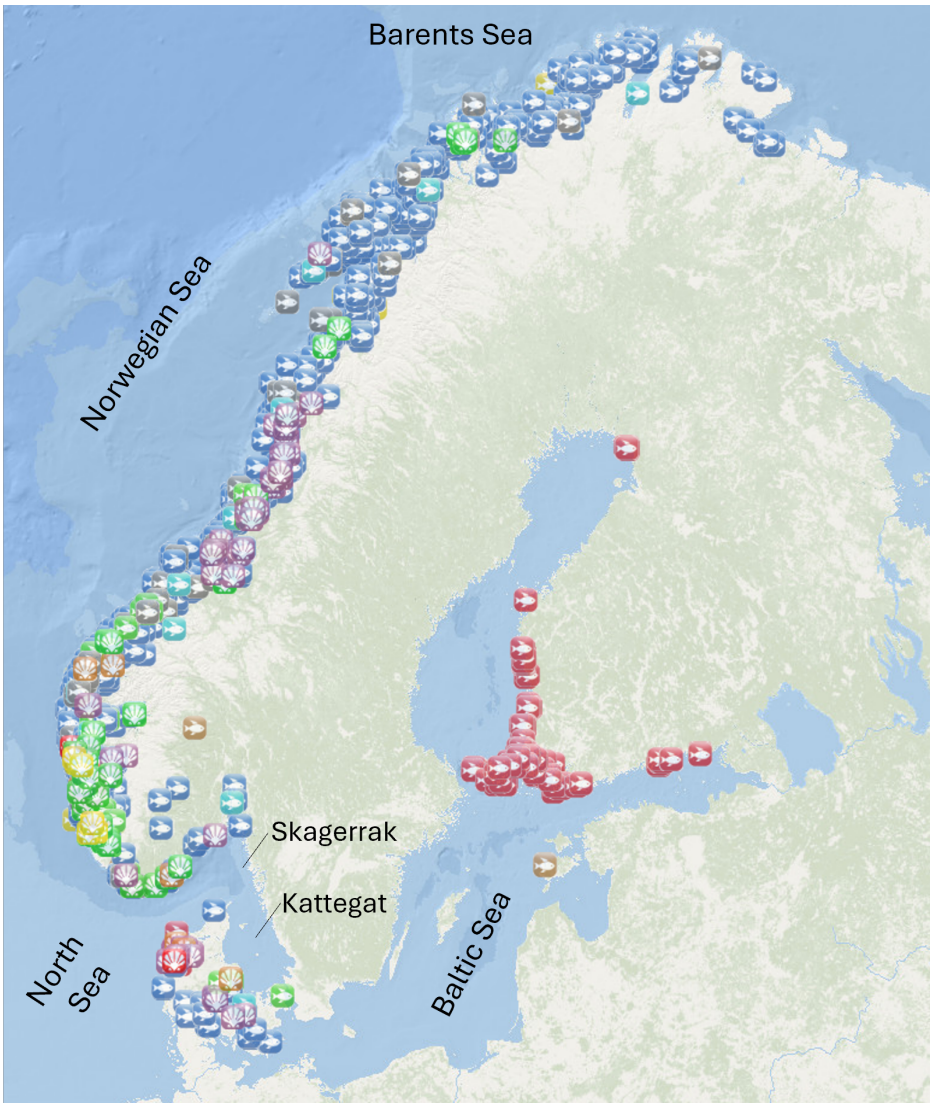

Fig. 1.9: *Map of northeastern Europe depicting the major sea areas discussed in this thesis and including shellfish farms and aquacultures sites according to the "European Atlas of the Seas" by the European Commission; colours correspond to different cultured shellfish or fish species.*

1.6.2 Future changes

The oceans have absorbed approximately 40 % of anthropogenic CO_2 (Sabine et al. 2004, Gruber et al. 2019) and 89 % of excess heat (Von Schuckmann et al. 2023), mitigating climate change effects but resulting in ocean acidification and warming. This trend will likely continue as climate models predict accelerating atmospheric warming towards the end of the 21st century (H. Lee et al. 2023). The Baltic Sea and the North Sea were the fastest-warming water bodies worldwide until 2006 (Belkin 2009). Consequently, the microalgal growing season has been prolonged in the Baltic Sea (Kahru et al. 2016, Wasmund et al. 2019) primarily due to enduring warm temperatures in autumn. Interestingly, warmer winters may even delay the spring bloom as shown for the German Bight (Wiltshire and Manly 2004) potentially due to longer persistence of zooplankton grazers in winter and early spring depressing algal biomass-build up. Warming-related shifts in microalgal succession have the potential to lead to predator/prey resource mismatches and induce regime shifts (Richardson 2008, Boersma and Meunier 2020). Indeed, zooplankton abundances and diversity in the North Sea have sharply declined in the mid-2000s and copepods have generally shifted from larger herbivores to smaller carnivores, which may alter the energy transfer to higher trophic levels (Durant et al. 2019, Deschamps et al. 2024, Di Pane et al. 2024). Additionally, warming-related sea-ice reduction in the northernmost basins of the Baltic Sea has been associated with increased dinoflagellate abundance during the spring bloom (Klais et al. 2011). Increasing temperatures are also predicted to shift food web structures towards greater heterotroph biomass (O'Connor et al. 2009), and stimulate feeding heterotrophic feeding of phagotrophic mixotrophs (Wilken et al. 2013). Similarly, increased temperatures are also associated with higher zooplankton grazing rates (Ratnarajah et al. 2023). Considering the wide range of prey that *A. pseudogonyaulax* can feed on and its lytic activity towards prey (Blossom et al. 2012) and predators (publication II), it could thus be a beneficiary of increasing temperatures.

1.7 Aims and scopes of this thesis

This thesis aimed to investigate the apparent expansion of the toxic dinoflagellate *A. pseudogonyaulax* in Northern European waters and to provide data that are required for a risk assessment regarding its potential impact on ecosystems and commercial activities, such as aquacultures. For this purpose, various laboratory experiments were conducted, along with a time series analysis of long-term monitoring stations in Northern Europe.

1st study: How do nitrogen and light, both common drivers of HAB development, influence growth and toxin content of *A. pseudogonyaulax*?

The objectives of the first study were to investigate the influence of bottom-up factors (N and light) on the ecophysiology, especially growth and toxin content, of three *A. pseudogonyaulax* strains with the perspective of guiding the subsequent time series analysis in the 3rd study. The objectives also included the measurement of photosynthesis versus irradiance curves to empirically assess the relationship between light and photosynthesis of *A. pseudogonyaulax*. This relationship revealed substantial intraspecific differences that were matching similar differences in the growth response towards low light. Consequently, S. Thoms extended the study by modelling the photophysiological response, particularly the photosynthetic electron transport. The investigated N-sources included the common inorganic ammonium and nitrate, with the expectation that *A. pseudogonyaulax* would grow better on ammonium, as dinoflagellates generally prefer more reduced N-sources. Additionally, urea was included due to extensive anthropogenic inputs of urea in Danish waters, primarily from extensive pig farming, suggesting a link to the expansion of *A. pseudogonyaulax*. Additionally, urea is considered a driver of coastal HAB formation, as many (or all) HAB dinoflagellates can use organic N-sources through osmotrophy.

2nd study: Does *A. pseudogonyaulax* adversely affect relevant representatives of the pelagic food web and are these effects primarily driven by GDs or BECs?

The second study aimed to evaluate the effects of *A. pseudogonyaulax* on multiple marine trophic levels, including microalgae (cryptophyte *R. salina*), microzooplankton (heterotrophic protist *P. kofoidii*), mesozooplankton (calanoid copepod *A. tonsa*), and fish gill cells (RTgill-W1 cell line of the salmonid *O. mykiss*). One of the primary focuses of these experiments was to untangle the relative contributions of GDs and BECs to the overall toxicity of *A. pseudogonyaulax* by utilizing culture supernatants containing both and comparing them to purified GDs. Although *A. pseudogonyaulax* has been demonstrated to exhibit lytic effects on other microalgae, quantitative data was lacking. Thus, this study also conducted bioassays with *R. salina* and GDs to establish dose-response curves, which are crucial in interpreting adverse effects of *A. pseudogonyaulax* on microalgae commu-

nity structures. Furthermore, this study investigated effects of *A. pseudogonyaulax*, when offered as prey, to different predators. *Polykrikos kofoidii* was chosen as representative of microzooplankton, being a ubiquitous grazer of toxic planktonic algae, cohabiting with *A. pseudogonyaulax* across Northern Europe. Similarly, the calanoid copepod *A. tonsa* was selected to represent mesozooplankton. Last but not least, the adverse effects of *A. pseudogonyaulax* and GDs on fish, or rather fish gill cells, were assessed. Given that *A. monilatum*, another GD-producer, is ichthyotoxic and that blooms have been associated with fish mortality, this study also sought to explore the potential fish toxicity of *A. pseudogonyaulax*.

3rd study: Is *A. pseudogonyaulax* appearing in higher frequency and abundance across Northern Europe and if so, which abiotic factors are driving this change?

This thesis was primarily designed upon the observation of Kremp et al. (2019) showing a sudden appearance of *A. pseudogonyaulax* in the Danish Limfjord coinciding with a decrease of other *Alexandrium* species. In order to investigate whether similar trends occur in the study area, a time series analysis of long-term monitoring programs across Northern Europe, including Danish, Swedish, Norwegian and German time series was conducted. The first objective was to confirm whether *A. pseudogonyaulax* is appearing in increasing frequency and abundance and to model the future trend of *A. pseudogonyaulax* across Northern Europe. Furthermore, this study aimed to identify potential abiotic driving factors of this expansion, such as nutrient concentrations, stratification or wind conditions.

In the last chapter, the major findings of the thesis are summarised and contextualized. Furthermore, the results are discussed in the context of environmental changes across Northern Europe and an overview of future research directions is provided.

List of publications and declaration of own contribution

Publication I

Effects of bottom-up factors on growth and toxin content of a harmful algae bloom dinoflagellate

by Möller, K., Thoms, S., Tillmann, U., Krock, B., Koch, F., Peeken, I., and Meunier, C. L.

Published in *Limnology & Oceanography* 2024

Contribution: The study was designed by U. Tillmann, B. Krock, F. Koch, C. L. Meunier and myself. S. Thoms constructed and employed the photophysiological model. *A. pseudogonyaulax* strains were isolated by U. Tillmann. I. Peeken and B. Krock provided facilities for pigment and toxin analysis, respectively. All laboratory experiments and analyses, including statistical analysis and plotting of data, were conducted by myself. The manuscript was written by myself and revised with the help of all co-authors.

Publication II

Toxic effects of the emerging *Alexandrium pseudogonyaulax* (Dinophyceae) on multiple trophic levels of the pelagic food web

by Möller, K., Tillmann, U., Pöchhacker, M., Porreca, F., Varga, E., Krock, B., Koch, F., Harris, T. M. and Meunier, C. L.

Accepted (pending minor revision) in *Harmful Algae* 2024

Contribution: The multiple studies in this manuscript were designed by U. Tillmann, B. Krock, E. Varga, F. Koch, C. L. Meunier and myself. *A. pseudogonyaulax* strains were isolated by U. Tillmann. U. Tillmann conducted the *R. salina* bioassays with purified GDs and *A. monilatum*. M. Pöchhacker conducted some additional replications of the RTgill-W1 bioassays. F. Porreca conducted the *R. salina* bioassays with *A. pseudogonyaulax*. E. Varga provided the RTgill-W1 cell line and facilities for the bioassays. B. Krock and C. L. Meunier provided facilities for toxin analysis and copepod experiments, respectively. T. M. Harris provided purified GDs for analytical standards and various bioassays. All other laboratory experiments and all analyses, including statistical analysis and plotting of data, were conducted by myself. The manuscript was written by myself and revised with the help of all co-authors.

Publication III

Time series analysis of the expansion of *Alexandrium pseudogonyaulax* across Northern Europe

by Möller, K., Jakobsen, H., Engesmo, A., Karlson, B. and Carstensen, J.

To be submitted - *Draft*

Contribution: The study was designed by H. Jakobsen, J. Carstensen and myself. H. Jakobsen prepared the Danish monitoring dataset. A. Engesmo prepared the Norwegian monitoring dataset. J. Carstensen designed methodological approaches for the time series analysis. B. Karlson provided help with the Swedish dataset. All data analysis and coding were done by myself. The draft of the manuscript was written by myself.

The declaration of own contribution to multi-author articles and manuscripts can be found at the end of the thesis.

Chapter 2

Publication I

Effects of bottom-up factors on growth and toxin content of a harmful algae bloom dinoflagellate

LIMNOLOGY and OCEANOGRAPHY

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Effects of bottom-up factors on growth and toxin content of a harmful algae bloom dinoflagellate

Kristof Möller [1*] **Silke Thoms** [1] **Urban Tillmann** [1] **Bernd Krock** [1] **Florian Koch** [1] **Ilka Peeken** [1] **Cédric L. Meunier** [2]

[1]Alfred Wegener Institut-Helmholtz Zentrum für Polar- und Meeresforschung, Bremerhaven, Germany
[2]Alfred Wegener Institut-Helmholtz Zentrum für Polar- und Meeresforschung, Helgoland, Germany

Abstract

The toxin-producing dinoflagellate *Alexandrium pseudogonyaulax* has become increasingly abundant in northern European waters, replacing other *Alexandrium* species. *A. pseudogonyaulax* produces goniodomins and lytic substances, which can be cytotoxic toward other organisms, including fish, but we still know little about the environmental conditions influencing its growth and toxicity. Here, we investigated the impacts of different nitrogen sources and light intensities, common bottom-up drivers of bloom formation, on the growth and toxin content of three *A. pseudogonyaulax* strains isolated from the Danish Limfjord. While the growth rates were significantly influenced by nitrogen source and light intensity, the intracellular toxin contents only showed strong differences between the exponential and stationary growth phases. Moreover, the photophysiological response of *A. pseudogonyaulax* showed little variation across varying light intensities, while light-harvesting pigments were significantly more abundant under low light conditions. This study additionally highlights considerable physiological variability between strains, emphasizing the importance of conducting laboratory experiments with several algal strains. A high physiological plasticity toward changing abiotic parameters points to a long-term establishment of *A. pseudogonyaulax* in northern European waters.

The concepts of bottom-up and top-down population controls have been widely used to describe ecological mechanisms in which resource availability or grazing by predators, respectively, determine the abundance of populations (Frederiksen et al. 2006; Jacox et al. 2016). Bottom-up factors include the availability of light and macro- (e.g., C, N, P) and micronutrients (e.g., vitamins, trace metals), which are essential for microalgal growth. While intense nutrient loading of coastal zones has long been implicated as a key factor in the proliferation of algal and/or harmful algal blooms (HABs), the availability, ratio, and/or type of nutrients (e.g., inorganic vs. organic) can also influence algal community composition (Heisler et al. 2008; Anderson 2009). For instance, increases in nitrogen due to eutrophication can result in low Si : N ratios favoring non-silicious species or cause phosphorus limitation (Burson et al. 2016). Furthermore, dinoflagellates have been shown to have an advantage over other plankton groups in low nitrate and/or high dissolved organic nitrogen (DON) environments since many species are phagotrophic mixotrophs and/or are able to utilize DON but are poor competitors for nitrate (Anderson et al. 2002; Glibert 2016). Nitrate is generally suggested to favor diatom blooms, whereas other more reduced nitrogen compounds, such as ammonium or urea, are more likely to promote flagellate blooms (Bronk et al. 2007; Glibert 2016). A preference toward ammonium or urea over nitrate is driven by the energetic costs associated with the cellular metabolism of the different nitrogen sources (Cochlan and Harrison 1991; Levasseur et al. 1993). After transportation into the cell, urea is broken down enzymatically with ammonium as the final N product, and hence the reductant requirements are similar (Levasseur et al. 1993). Nitrate, on the other hand, has to be reduced to nitrite, followed by reduction to ammonium, before it can be utilized by the cell, both at the expense of energy (Thompson et al. 1989). However, in many instances, plankton grown on either ammonium or nitrate were found to have similar or

*Correspondence: kristof.moeller@awi.de

Additional Supporting Information may be found in the online version of this article.

Author Contribution Statement: K.M., U.T., B.K, F.K., and C.L.M. conceived of and designed the study. K.M. wrote the original draft while all other authors participated in reviewing and editing the manuscript. K.M, S.T., U.T., and I.P. conducted the experiments and analyzed the data.

even faster growth rates when subjected to nitrate (Leong et al. 2004; Li et al. 2011; Armstrong et al. 2018).

In addition to nutrients, light is another crucial bottom-up factor influencing the growth of microalgae, particularly for those HAB species blooming in eutrophic and often turbid environments, such as *Aureococcus anophagefferens* (Gobler et al. 2011) or *Karlodinium veneficum* (Coyne et al. 2021).

While the frequency and intensity of HABs have been increasing globally, factors responsible for bloom initiation and factors governing their toxicity are still poorly understood (Heisler et al. 2008; Anderson 2009). HABs are primarily caused by dinoflagellates, including many species of the genus *Alexandrium*, which produce various phycotoxins (Long et al. 2021). Some of these toxins can directly affect other protistan species, but others can be transferred through the food web and subsequently accumulate in higher trophic levels (e.g., in bivalves) (James et al. 2010; Long et al. 2021).

Alexandrium pseudogonyaulax, originally described from the Thau Lagoon in France (Biecheler 1952), has been increasingly prevalent in northern European waters, replacing other previously dominating *Alexandrium* species such as *Alexandrium ostenfeldii* (Kremp et al. 2019; Karlson et al. 2021). However, environmental factors that promoted this proliferation remain unresolved. This proliferation may be especially problematic as *A. pseudogonyaulax* is known to produce the phycotoxins goniodomins (GDs) (Zmerli Triki et al. 2016), which belong to a class of macrocyclic secondary polyketides including a lactone group sensitive to hydrolysis (Sharma et al. 1968; Harris et al. 2020). GDs are cytotoxic, likely due to their disturbance of the actomyosin ATPase activity and the F-actin meshwork (Terao et al. 1989; Furukawa et al. 1993; Espiña et al. 2016). *Alexandrium monilatum*, another GD producer, is also cytotoxic toward a variety of organisms, suggesting that *A. pseudogonyaulax* has a similar toxicity (Hsia et al. 2006; May et al. 2010). In addition, *A. pseudogonyaulax* also produces lytic substances that aid in mixotrophic feeding (Blossom et al. 2012, 2017), as many species of *Alexandrium* (Long et al. 2021). However, these substances have not been structurally elucidated yet and the extent to which GDs or lytic substances are responsible for the observed toxicity is unknown (Ma et al. 2011; Long et al. 2021). Several studies have investigated the effects of bottom-up factors on the toxin content of other *Alexandrium* species (Ogata et al. 1987; Béchemin et al. 1999; Parkhill 1999; Leong et al. 2004; Griffin et al. 2019), highlighting a diverse response to different bottom-up factors and underlining significant inter- and intraspecific variability of HAB species. In contrast, there is limited information available about the effects of different bottom-up factors on the physiology of *A. pseudogonyaulax* and their subsequent impact on toxin production (Zmerli Triki et al. 2015, 2016).

This study aimed to examine the impacts of light availability and different nitrogen sources on primarily the growth and toxin content of three northern European *A. pseudogonyaulax* strains isolated from the western Danish Limfjord through conducting bottle incubation experiments. In addition, it aimed to assess whether varying light intensities affect the photophysiological and pigment response of *A. pseudogonyaulax*.

Material and methods

Cell isolations

All three *A. pseudogonyaulax* strains (L2-D2 (A), L4-B1 (B), L4-B9 (C)) used in this study were established by micropipette isolation from live net tow samples under a M5A stereomicroscope (Wild) during an expedition with the R/V *Uthörn* in August 2020 in the western Danish Limfjord close to Thyborøn. The isolated single cells were transferred into 96-well tissue culture plates (TPP), each containing 250 μL of K medium (Keller et al. 2007) prepared from 0.2 μm sterile-filtered seawater from the sampling location. All strains were identified as *A. pseudogonyaulax* by cellulose staining with solophenyl flavine and observation under an inverted fluorescence microscope (Chomérat et al. 2017).

Culture maintenance

The non-axenic stock cultures were maintained under semi-batch conditions, at 20 °C, 25 PSU salinity, and 80 μmol photons m^{-2} s^{-1} (photon flux densities [PFDs]) of cool fluorescent light on a 16 : 8 h light : dark cycle. Salinity was measured on a benchtop conductivity meter (Symphony SB80PC; VWR) and converted to practical salinity units according to the standard electrical conductivity method 2520. PFDs were measured inside of the culture bottles with a light sensor (ULM-500; Walz GmbH). Stock cultures were diluted every other week to keep cells in the exponential growth phase (GP). All K/2 media (K media with half of the original nutrient concentrations, pH 8.1, 441 μmol L^{-1} NO_3^-, 25 μmol L^{-1} NH_4^+) employed in the present study was prepared from the same batch of aged seawater collected from the German North Sea near Helgoland. The original K-medium receipt was modified by replacing the organic phosphorus source with 3.62 μmol L^{-1} Na_2HPO_4. Ambient concentrations of NO_3^-, NH_4^+, and PO_4^{3-} in this seawater were 5.6, 4.6, and 0.178 μmol L^{-1}, respectively.

Experimental design

Nitrogen experiment

The impact of four different nitrogen treatments (N-deplete, NH_4^+, NO_3^-, and urea) on the growth and toxin content of two *A. pseudogonyaulax* strains (A and B) were investigated by means of bottle incubation experiments. The N-deplete treatment consisted of an aged seawater K/2 medium without any nitrogen addition. Cells were preconditioned for 2 weeks in the K/2 medium only containing the corresponding experimental nitrogen source (50 μmol L^{-1}, NH_4^+, NO_3^-, or urea), while cells in the N-deplete treatment were preconditioned in K/2 medium.

Then, exponential phase cells were collected by gently filtering over a 20 μm mesh to reduce nutrient carryover and transferred to the incubation treatment bottles. The targeted initial cell density for all treatments was 100 cells mL^{-1}. Triplicate bottles were prepared for each treatment in 500 mL sterilized screw-top polycarbonate bottles (TPP) and incubated under the same temperature and light conditions as the stock cultures. Samples for toxin and particulate organic carbon/particulate organic nitrogen (POC/PON) (Supporting Information Section S2.1) analysis were collected at the late exponential phase and in the stationary phase as identified by reaching a plateau in the sigmoidal growth curve. The urea treatment was not sampled within the exponential GP as stagnation of growth was reached unexpectedly and upon repetition, stagnation of growth appeared at even lower cell densities insufficient for the nutrient and toxin analysis. Cell densities at the start and during the experiment were determined in duplicates by microscopic counts of cells of 0.2–2 mL Lugol's iodine-fixed subsamples taken every 2nd day. The cell size was determined at both harvest points by measuring the radius of 35–90 cells and the cell volume was calculated presuming a spherical shape ($V_{cell} = 4/3 \times \pi \times r^3_{cell}$). The NH_4^+, NO_3^-, and urea treatment received an additional recovery spike of either 50 μmol L^{-1} NH_4^+ or NO_3^- once growth stagnated or declined. All treatments of strain C died during the onset of this experiment with no identifiable cause, and hence, the nitrogen experiment was only conducted with the other two strains (A and B).

Light experiment

The influence of three different PFDs (20, 100, and 200 μmol photons m^{-2} s^{-1}) on the growth and toxin content of three *A. pseudogonyaulax* strains (A, B, and C) was investigated. Cultures were grown in K/2 medium and were preconditioned to the experimental PFDs for 2 weeks. Cultures of the 20 μmol photons $m^{-2}s^{-1}$ treatment were acclimated at 60 μmol photons $m^{-2}s^{-1}$ due to slow growth. The targeted initial cell density of all treatments was 100 cells mL^{-1}. Triplicate bottles of each treatment were prepared in 250 mL sterilized screw-top polycarbonate bottles (TPP) and incubated for a period of 20–25 d under constant temperature (20 °C). Samples for toxin, POC/PON (Supporting Information Section S2.1) and pigment (Supporting Information Section S2.2) analysis were collected in the exponential phase and toxin samples a second time in the stationary phase. In addition, chlorophyll *a* (Chl *a*) fluorescence measurements were performed on a Fast Repetition Rate fluorometer (FRRf) coupled to a FastAct laboratory system (FastOcean PTX, both from Chelsea Technologies Group) detailed in the Supporting Information Section S2.3.1. Cells of the 20 μmol photons $m^{-2}s^{-1}$ treatment were only harvested in the late exponential phase. As growth declined, low light (20 μmol photons $m^{-2}s^{-1}$) treatments of strain B and C were transferred to 60 μmol photons $m^{-2}s^{-1}$. Cell densities and cell sizes were determined as described in the Nitrogen experiment.

Toxin analysis

Toxin extraction

At both cell harvests, 25 mL of each incubation bottle were harvested by centrifugation at 3220 × *g* for 10 min (Eppendorf 5810 R). Cell pellets were re-suspended in methanol (500 μL) and transferred to FastPrep tubes containing 0.9 g lysing matrix D (Thermo-Savant). Samples were homogenized by reciprocal shaking for 45 s at maximal speed (6.5 m s^{-1}) in a FastPrep instrument (Thermo-Savant) and subsequently centrifuged for 15 min at 16,100 × *g* and 10 °C (Eppendorf 5415 R). Supernatants were transferred into spin filters (Ultra-free; Millipore) and filtered by centrifugation for 30 s at 10 °C and 5000 × *g*. The resulting filtrates were transferred to high-performance liquid chromatography (HPLC) vials and stored at −20 °C until mass spectrometric analysis.

Toxin analysis

Water was deionized and purified (Millipore Milli-Q) to 18 MΩ cm^{-1} or better quality. Formic acid (90%, p.a.), acetic acid (p.a.) and ammonium formate (p.a.) were purchased from Merck. The solvents, methanol and acetonitrile, were HPLC grade (Merck). LC-MS/MS samples were analyzed by ultrahigh-performance liquid chromatography coupled with tandem quadrupole mass spectrometry (LC-MS/MS). An alkaline elution system was used with eluent A consisting of 6.7 mmol L^{-1} aqueous NH_3 and eluent B consisting of 9 : 1 (vol : vol) acetonitrile and 6.7 mmol L^{-1} aqueous NH_3. All other chromatographic and instrumental parameters were set according to Harris et al. (2023). Quantification of GDA was performed by calculating the absolute peak areas of m/z 786.5 → 733.5 with a four-point (36.67, 366.67, 825, and 1100 pg μL^{-1} GDA) external calibration curve ($R^2 = 0.99$). Finally, toxin quotas were normalized to the cellular molar carbon content (mol GDA mol^{-1} C^{-1}) or to the cell volume (pg GDA μm^{-3}).

Data analysis and statistics

All statistical analyses and plotting of data were performed using the R 4.1.2 software (R Core Team 2021). All statistical comparisons were performed by conducting a non-parametric Kruskal–Wallis test (Kassambara 2023a) followed by a Conover–Iman post hoc test after the rejection of the null hypothesis, including a p value adjustment according to Benjamini and Hochberg ($\alpha = 0.05$) (Benjamini and Hochberg 1995). Two-factor designs, involving one factor repeatedly measured over time, were analyzed by a repeated measures ANOVA with time as a dependent and the second factor as an independent variable (Kassambara 2023*a*). In case of deviations from sphericity, a Greenhouse–Geisser correction was performed to correct the degrees of freedom and hence the *F*-value of the ANOVA test results. In case of a significant

interaction between treatment and time, the effect of the treatment was further analyzed at each time point by a one-way ANOVA and in case of a rejection of the null hypothesis, further pairwise *t*-tests (Kassambara 2023*a*) were performed. Effect sizes, only shown between treatments and control, were calculated as Cohen's *d* and corrected according to hedges h (Kassambara 2023*a*) and interpreted as small (< 0.2), medium (0.2–0.8), and large (> 0.8). Specific growth rates (μ d^{-1}) over the exponential phase of growth were determined by fitting the cell count data with an exponential growth model featuring a heuristic linear method (Petzoldt 2022) containing five data points (only three points for strain B in the light experiment) similar to the method of Hall et al. (2014). Briefly, this approach considers a window of five-time points (points 1–5), calculates the slope, and moves then one-time point (points 2–6) further until the maximum slope is determined. All plots were generated with ggplot2 (Wickham et al. 2019) with the help of extrafont (Chang 2023), ggthemes (Arnold 2021), ggtext (Wilke and Wiernik 2022), ggprism (Dawson 2022), ggpubr (Kassambara 2023b), and patchwork (Pedersen 2024). General data transformations were performed within the tidyverse (Wickham et al. 2019). Packages were managed with pacman (Rinker and Kurkiewicz 2018) and package citations were generated with the grateful package (Rodrigues-Sanchez and Jackson 2023).

The PFDs needed for the saturating growth rate (y_{max}) in the light experiment were calculated from the fitted growth rates using a regular asymptotic regression (Eq. 4) through a Nelder–Mead method (Soetaert and Petzoldt 2010). Then, the minimum light saturation irradiance (I_K) was calculated as the intersection of a line, with the initial slope of the regression, with the horizontal line of y_{max}.

$$y = a - (a - b)e^{-cx}. \quad (4)$$

Results

Nitrogen experiment

Growth responses

Both strains (A and B) of *A. pseudogonyaulax* grew on all three nitrogen sources (NH_4^+, NO_3^-, urea), yielding significantly higher growth rates over the N-deplete treatment (NH_4^+, NO_3^-: $p < 0.01$; urea: $p_A < 0.1$ and $p_B < 0.05$; Fig. 1), also reflected in large effect sizes ($d = 4.1$–6.3). Furthermore, a significant interaction between nitrogen source and time indicated a time-dependency of the nitrogen source effect (strain A: $F_{16,48} = 42.41$, $p < 0.001$, strain B: $F_{16,48} = 12.33$, $p < 0.001$). Each sampling point was separately analyzed, revealing a consistent significant growth effect of the nitrogen source (strain A: $p < 0.001$, strain B: $p < 0.05$) except on the first 2 d, which can be regarded as the lag phase. Pairwise comparisons showed that after the lag phase, mean cell counts of strain A in the NH_4^+ and NO_3^- treatments were significantly

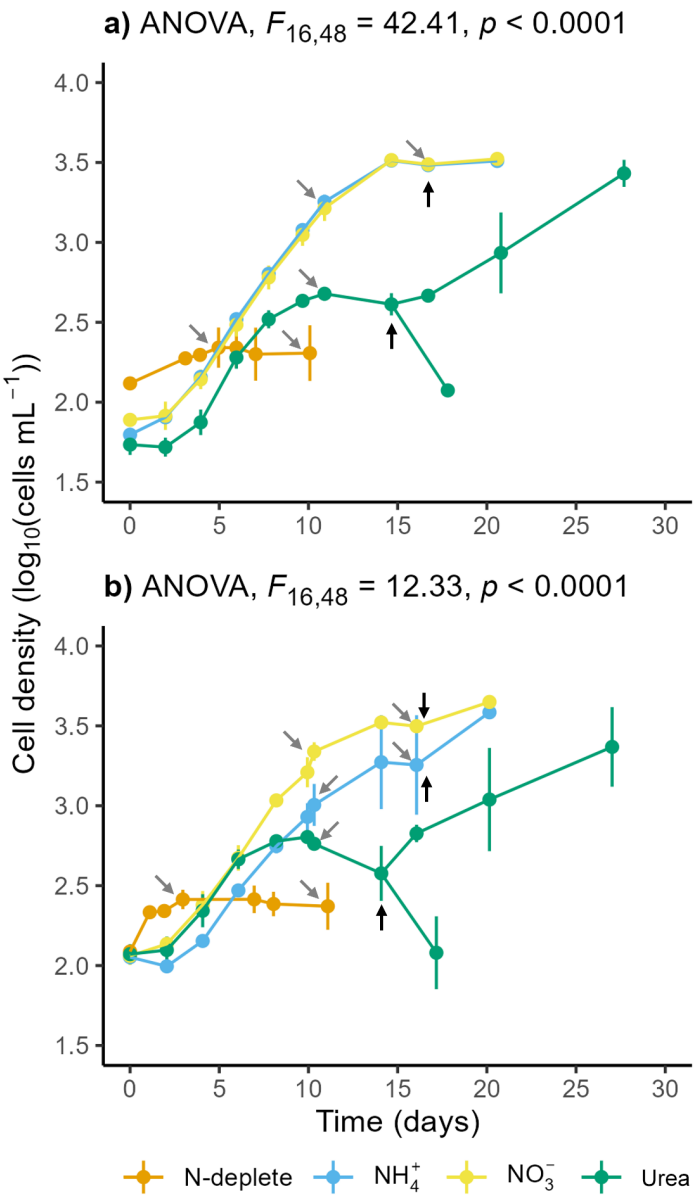

Fig. 1. Growth curves of *Alexandrium pseudogonyaulax* strains A (a: L2-D2) and B (b: L4-B1) subjected to different nitrogen sources (N-deplete, NO_3^-, NH_4^+, urea); points represent mean including standard deviations of $n = 3$ biological replicates; after a decline in cell densities (urea) or after a few days in the stationary phase (NH_4^+ and NO_3^-), aliquots from all nitrogen addition treatment flasks were spiked with additional NH_4^+ or NO_3^- at times indicated by the black arrows; particular nutrients, and toxins were sampled at times indicated by the gray arrows; test statistics correspond to a two-way repeated measures ANOVA examining the effects of time and nitrogen source on the cell counts.

($p < 0.01$) higher than in the urea treatment. Similarly, the mean cell counts of strain B in the NO_3^- treatment were significantly higher ($p < 0.01$) than the urea treatment, however only after day 8, while the mean cell counts of the NH_4^+ treatment were significantly lower than the urea treatment on days 4 and 6 ($p < 0.05$) and higher after day 10 ($p < 0.05$). No

significant differences between the NH_4^+ and the NO_3^- treatment were found at any sampling day for strain A, while for strain B the cell counts of the NO_3^- treatments were significantly higher than the NH_4^+ treatment between days 2 and 10 ($p < 0.05$).

In addition, mean growth rates of both strains in the NH_4^+ and NO_3^- treatments (Fig. 2) did not differ significantly and maximum cell densities were in the same range (NH_4^+: 3100–3800; NO_3^-: 3100–3850). In contrast, urea addition for both strains yielded significantly lower growth rates ($\leq 0.3\ d^{-1}$, $p < 0.05$) and maximum cell densities (< 1 k cells mL^{-1}, $p < 0.01$) than the inorganic nitrogen treatments. Furthermore, urea cell densities declined rapidly after reaching their maximum (Fig. 1). Growth in the urea treatment was swiftly recovered after the addition of a recovery spike of NO_3^- or NH_4^+. In contrast, further addition of nitrogen to the NH_4^+ or NO_3^- treatments resulted in no or only marginal continuative growth (Fig. 1).

Finally, the growth rates in all nitrogen treatments were the same between strains A and B ($H_1 = 0.05$–2.3, $p > 0.05$), however, the growth rate of the urea treatment was significantly higher in strain A ($H_1 = 3.86$, $p < 0.05$) compared to strain B.

Toxin quotas

Mean goniodomin A (GDA) quotas ranged between 0.74 and 6.73 pg GDA $cell^{-1}$ or 2.64×10^{-6}–3.51×10^{-5} mol GDA mol^{-1} C^{-1}. Toxin quotas of strain A (pg GDA $cell^{-1}$ and mol GDA mol^{-1} C^{-1}) were significantly higher in the stationary than in the exponential GP for all treatments ($p < 0.001$; Fig. 3). Even though the Kruskal–Wallis test for strain B yielded a p value of 0.066 (Fig. 3), subsequent post hoc tests were conducted motivated by the exploratory nature of this study. This revealed the same pattern for strain B in the NH_4^+ treatment ($p < 0.1$; Fig. 3). In the exponential GP, toxin quotas (pg GDA $cell^{-1}$ and mol GDA mol^{-1} C^{-1}) of strain A were significantly higher in the inorganic nitrogen treatments than in the N-deplete treatment (NH_4^+: $p < 0.1$, $d = 1.8$; NO_3^-: $p < 0.001$, $d = 6.1$), while they did not differ significantly for strain B. Within the stationary GP, the toxin contents (mol GDA mol C^{-1}; Supporting Information Table S3) of both strains in the inorganic nitrogen treatments were also significantly higher than in the N-deplete treatment ($p < 0.01$). However, the same pairwise comparisons did not yield significant results when analyzed as GDA per cell. In addition, within the stationary GP, toxin quotas of both strains were significantly lower in the urea (strain A: $p < 0.001$, strain B: NH_4^+: $p < 0.05$, NO_3^-: $p < 0.1$) than in the two inorganic nitrogen treatments, as well as significantly lower than in the N-deplete treatment (strain A: $p < 0.01$, $d = 2.1$; strain B: $p < 0.1$, $d = 0.3$). Finally, toxin quotas of strain B were significantly higher than those of strain A in the exponential phase of the N-deplete and the NH_4^+ treatment ($H_1 = 3.86$, $p < 0.05$).

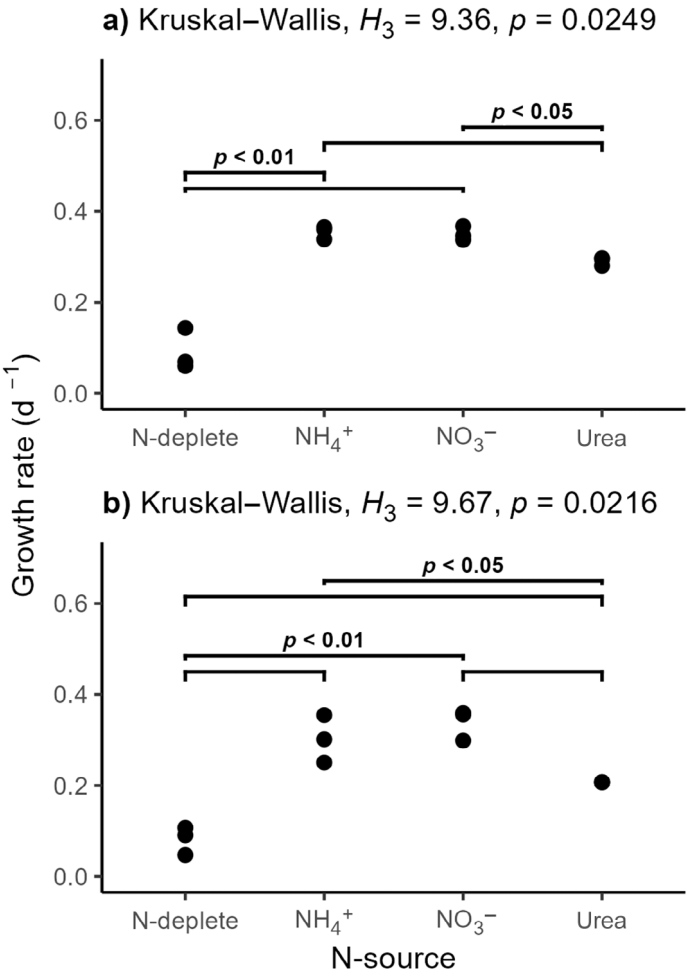

Fig. 2. Growth rates (d^{-1}) of *Alexandrium pseudogonyaulax* strains A (a: L2-D2) and B (b: L4-B1) subjected to different nitrogen sources corresponding to the growth curves of Fig. 1; points correspond to single data points of biological replicates ($n = 3$); growth rates were calculated with an exponential growth model containing five data points (2); test statistics and pairwise comparisons correspond to the Kruskal–Wallis rank sum test and to selected Conover–Iman post hoc test results, respectively.

Light experiment

Growth responses

All three strains grew under medium and high light intensities (medium light "ML": 100 μmol photons $m^{-2}s^{-1}$, high light "HL": 200 μmol photons $m^{-2}s^{-1}$), while only strain A grew continuously under low light (low light "LL": 20 μmol photons $m^{-2}s^{-1}$). A significant interaction between PFDs and time indicated a time-dependency of the light effect (strain A: $F_{18,54} = 452.21$, $p < 0.001$, strain B: $F_{14,42} = 259.64$, $p < 0.001$, strain C: $F_{18,54} = 143.15$, $p < 0.001$). Each sampling point was separately analyzed, revealing a consistent significant growth effect of the PFDs ($p < 0.001$, at day 2: $p < 0.05$), except for the initial day. Pairwise comparisons revealed that from the second day on, the mean cell counts of the ML and HL treatment were significantly higher ($p < 0.001$) than the LL treatment

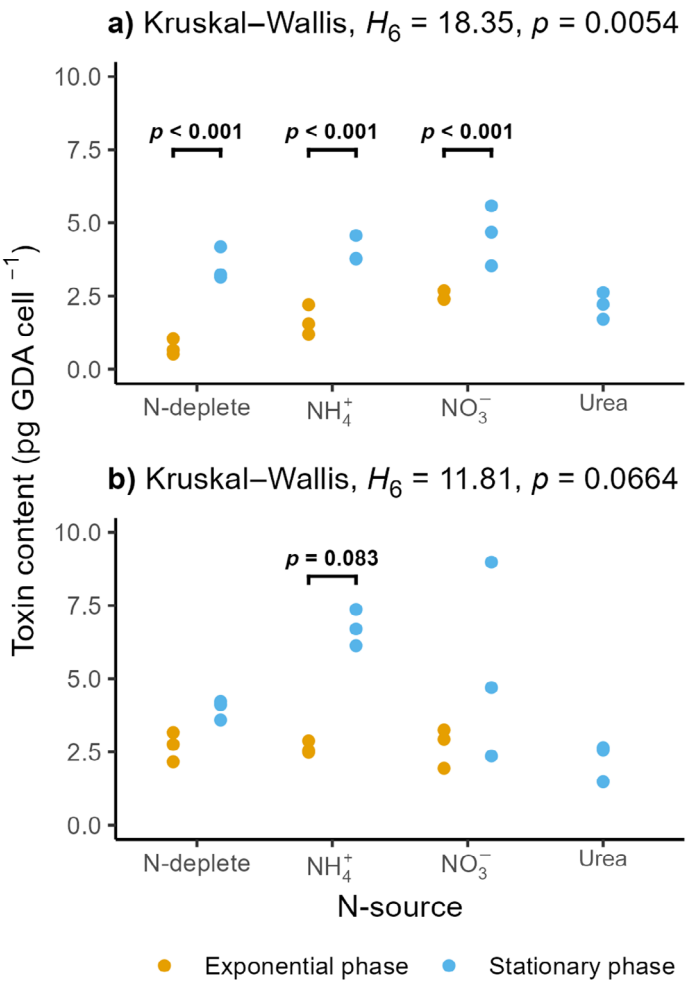

Fig. 3. Intracellular GDA toxin quotas (pg GDA cell^{-1}) of *Alexandrium pseudogonyaulax* strains A (a: L2-D2) and B (b: L4-B1) subjected to different nitrogen sources in the exponential (brown) and stationary (blue) growth phase; points correspond to single data points of biological replicates ($n = 3$); test statistics and pairwise comparisons correspond to the Kruskal–Wallis rank sum test and to selected Conover–Iman post hoc test results, respectively.

(Fig. 4). In addition, each strain grew significantly faster (strain A: $p < 0.05$, strain B/C: $p < 0.01$) in the HL than in the ML treatment, although the effect sizes were not substantially different (strain A: 59.7/40.4; strain B: 11.2/13.1; strain C: 72.0/67.5 ML/HL, respectively). Contrary, the maximum cell densities of strain A and strain B were significantly higher ($p < 0.05$) in the ML (strain A: 8800; strain B: 10,000 cells mL^{-1}) than in the HL (strain A: 6800; strain B: 9000 cells mL^{-1}) treatment, while they were the same for strain C (≈ 5600 cells mL^{-1}). In addition, all strains in ML and HL grew significantly faster (strain A/C: $p < 0.05$, strain B: $p < 0.001$ $d = 11$–72) compared to the LL treatment. The LL treatment of strain A exhibited strongly impeded growth and significantly lower cell densities at the end of the experiment (< 1.000 cells mL^{-1}, $p < 0.001$), while strains B and C did not grow continuously under LL. However, the growth of strains B and C could be stimulated by increasing the light intensity to 60 μmol photons m^{-2}s^{-1} (Fig. 4). Finally, intraspecific variabilities in the growth rates (Fig. 5) were found for the LL and ML treatment ($H_2 = 6.5$–7.2, $p < 0.05$). A subsequent post hoc analysis revealed that the growth rate of strain A was significantly higher ($p < 0.05$) than of strain B and strain C in both the LL and ML treatment, while they were the same for strain B and strain C.

A strong positive correlation (Spearmans $\rho = 0.94$, $p < 0.001$) between light intensities and growth rates was observed, which can be adequately represented by an asymptotic regression fit (Fig. 6). This analysis revealed that the minimum light saturation irradiance (I_K) was 57 μmol photons m^{-2}s^{-1} and that the maximum growth rate (γ_{max}) of *A. pseudogonyaulax* was 0.37 d^{-1}. Furthermore, the light compensation point, at which net growth is zero, was 9 μmol photons m^{-2} s^{-1}.

Toxin quotas

GDA quotas ranged between 1.0–11.8 pg GDA cell^{-1} or 7.7×10^{-6}–1.3×10^{-5} mol GDA mol C^{-1} across all treatments and strains in the light experiment (Fig. 7; Supporting Information Table S4). Toxin quotas of each strain in the stationary GP were significantly higher than in the exponential GP within the same treatment (strain A/B: $p < 0.01$, strain C: $p < 0.05$; Fig. 7). In addition, toxin quotas of strain B in the exponential GP were significantly higher in the ML and HL treatment than in the LL treatment ($p < 0.05$; Fig. 7). Significant intraspecific variabilities ($H_2 = 7.2$, $p < 0.05$) were found for all three light intensities during the exponential and stationary GP. A subsequent post hoc analysis revealed that toxin contents within each treatment of strain C were always significantly higher ($p < 0.05$) than those of strain A, which in turn were significantly higher ($p < 0.05$) compared to strain B.

Pigment composition and abundances

The following pigments were identified in *A. pseudogonyaulax* cells: Chl *a*, peridinin, *β*-carotene, Chl c_2, violaxanthin (only in strain A and strain B), diadinoxanthin, dinoxanthin, diatoxanthin, and zeaxanthin. While pigment ratios were similar across treatments, significant differences emerged in the molar ratios ($H_2 = 7.2$, $p < 0.05$) of light-harvesting (LH) to light-protecting (LP) pigments. The LH : LP in the LL treatment for each strain was significantly higher ($p < 0.05$) than ML and HL treatments, with approximately threefold differences (Supporting Information Table S5). More detailed results about pigment abundances can be found in the Supporting Information Section S3.2.1.

Photophysiological responses

In strain A, the ML treatment exhibited a significantly higher ($H_2 = 7.2$, $p < 0.05$) dark-adapted maximum quantum yield (F_v F_m^{-1}) compared to LL and HL treatment (Supporting Information Table S4). In addition, the concentration of functional PSII reaction centers (RCII) was significantly higher in

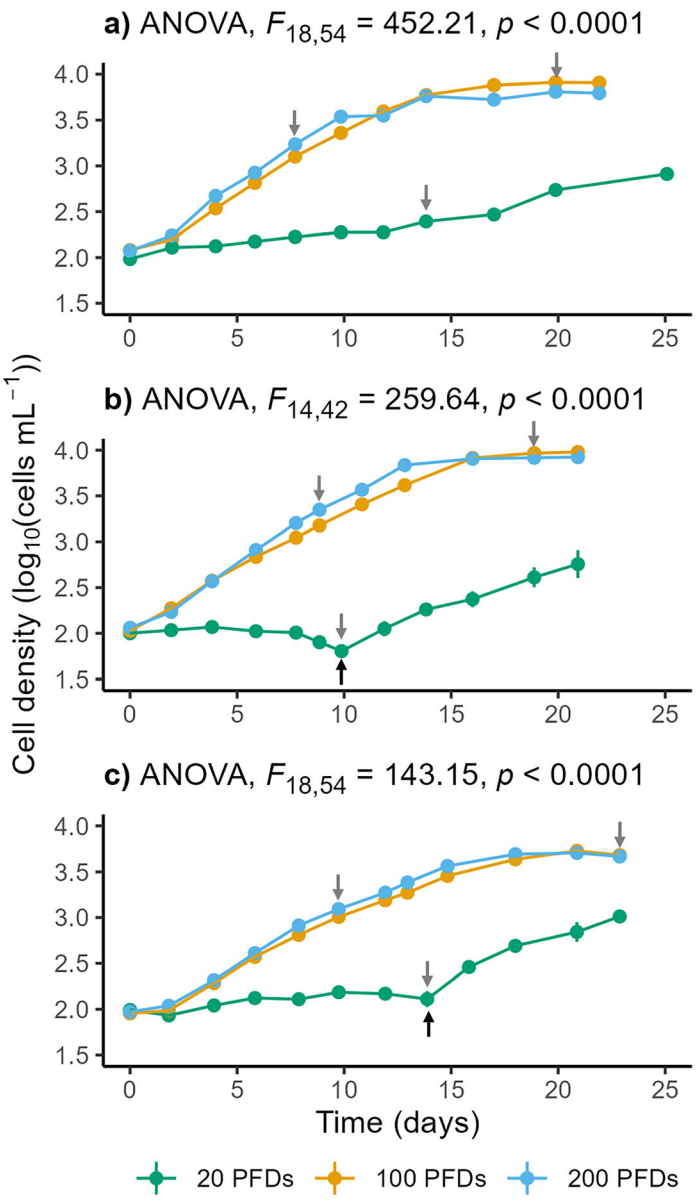

Fig. 4. Growth curves of *Alexandrium pseudogonyaulax* strains A (a: L2-D2), B (b: L4-B1), and C (c: L4-B9) subjected to different photon flux densities (20, 100, and 200 μmol photons m^{-2} s^{-1}, PFDs); points represent mean including standard deviations of $n = 3$ biological replicates; low light (20 μmol photons m^{-2}s^{-1}) treatments of strain B and C were transferred to 60 μmol photons m^{-2}s^{-1} after the onset of a decline in cell densities as indicated by the black arrow; particular nutrients, pigments and toxins were sampled at times indicated by the gray arrows; test statistics correspond to a two-way repeated measures ANOVA examining the effects of time and light intensity on the cell counts.

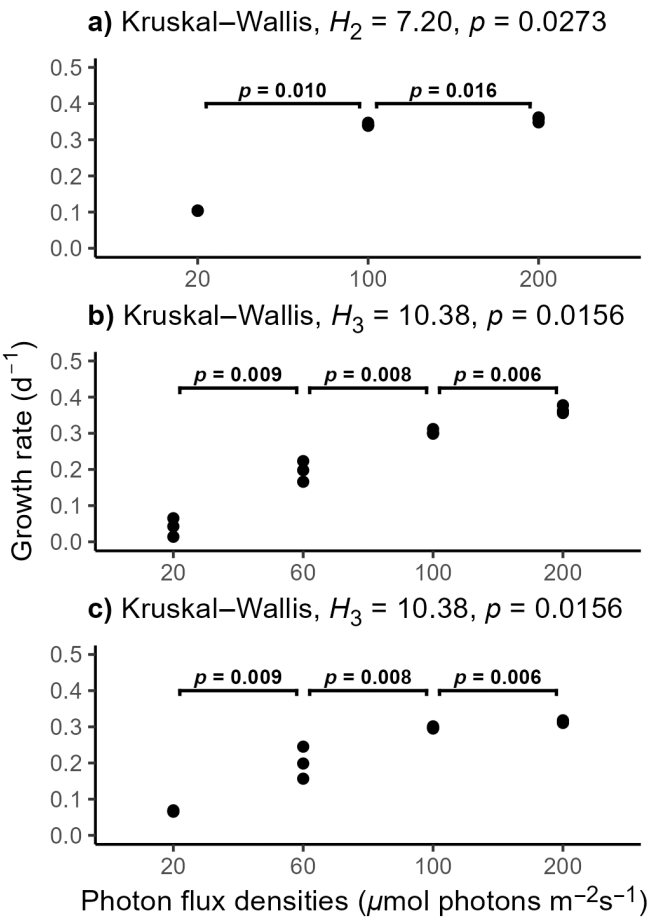

Fig. 5. Growth rates (d^{-1}) of *Alexandrium pseudogonyaulax* strains A (L2-D2), B (L4-B1), and C (L4-B9) subjected to different photon flux densities (μmol photons m^{-2} s^{-1}) corresponding to the growth curves of Fig. 4; points correspond to single data points of biological replicates ($n = 3$); growth rates were calculated with an exponential growth model containing five data points; test statistics and pairwise comparisons correspond to the Kruskal–Wallis rank sum test and to selected Conover–Iman post hoc test results, respectively.

the ML than in the HL treatment ($p < 0.001$), but RCII in the HL treatment remained significantly higher than in the LL treatment ($p < 0.05$). RCII remained the same across all treatments for strain B and C. Finally, for strain A, the maximum electron transport rate (ETR) ETR_{max} and the minimum saturating light irradiance (I_K) in the ML treatment were significantly lower ($p < 0.05$) than in the LL and HL treatment (Fig. 8; Supporting Information Table S4). In accordance, multiplying the ETR with RCII to yield the ETR_{cell} showed that ETR_{cell} increased concomitantly with the PFDs (only calculated for strain A; Fig. 8). In general, ETRs per PSII of all treatments and strains were high compared to other algal species such as green algae. Strain A was chosen to be considered in more detail within the photophysiological model due to its significant differences in RCII concentrations and ETR_{max}, consequently affecting ETR_{cell}, unlike strains B and C, which displayed similar ETR_{cell} values across all treatments (Supporting Information Fig. S1; Supporting Information Table S4). Increasing the rate constants for the mobile electron carriers plastoquinone, plastocyanin, and ferredoxin within the photophysiological model (Supporting Information Section S2.3.2) enabled to account for the potential

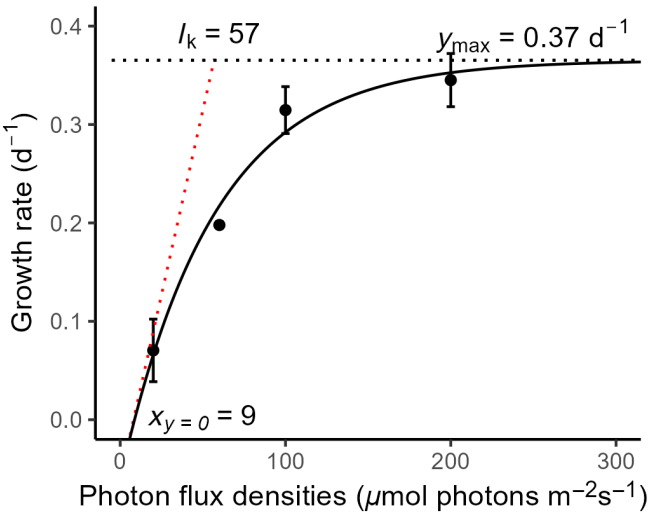

Fig. 6. Asymptotic regression analysis of the growth rates (d^{-1}) of three *Alexandrium pseudogonyaulax* strains corresponding to Fig. 5, including the minimum saturating light irradiance (I_K) and the maximum growth rate (y_{max}, d^{-1}); points correspond to the mean ($n = 3$) of the mean of each strain ($n = 3$) including standard deviations.

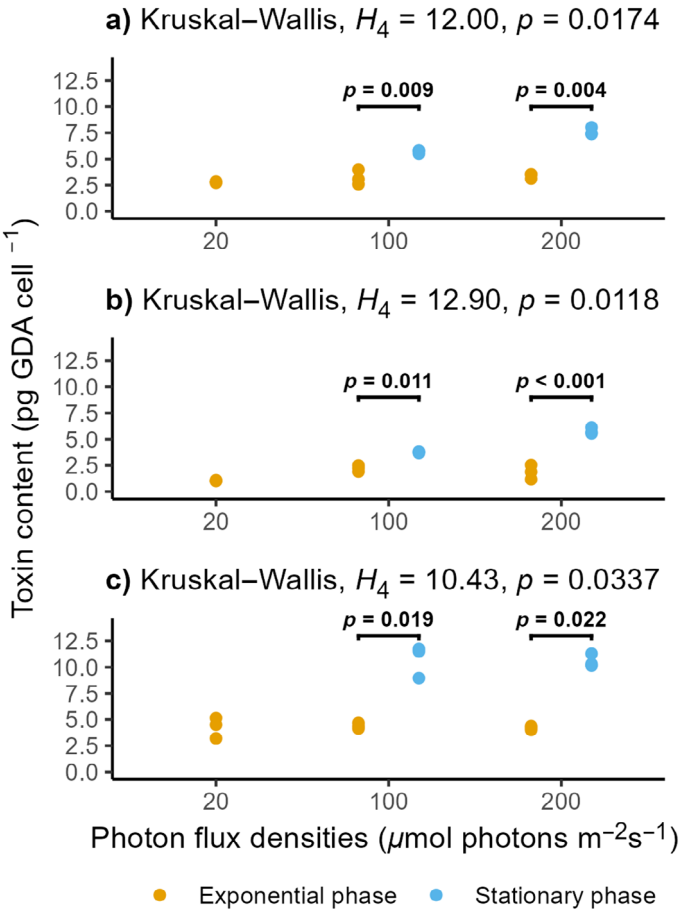

Fig. 7. Intracellular GDA toxin quotas (pg GDA cell^{-1}) of *Alexandrium pseudogonyaulax* strains A (a: L2-D2), B (b: L4-B1), and C (c: L4-B9) subjected to different photon flux densities in the exponential (brown) and stationary (blue) growth phase; points correspond to single data points of biological replicates ($n = 3$); test statistics and pairwise comparisons correspond to the Kruskal–Wallis rank sum test and to selected Conover–Iman post hoc test results, respectively.

impact of different RCII concentrations on the diffusion of the mobile electron carriers. The used parameters of this model adapted to *A. pseudogonyaulax* are shown in the Supporting Information Tables S1, S2 and more detailed information about the photophysiological response can be found in Supporting Information Section 3.2.2.

Discussion

A. pseudogonyaulax and the role of nitrogen

Nitrogen plays a crucial role in the formation of HABs and is typically the major limiting nutrient in coastal marine systems (Heisler et al. 2008; Gobler 2020), hence investigating N-preference is essential for characterizing the ecophysiology of *A. pseudogonyaulax*.

Inorganic nitrogen sources, but not urea, sustained growth

The growth response of two *A. pseudogonyaulax* strains observed in this study emphasize the complex nature of nitrogen utilization by microalgae. The nitrogen concentrations (50 μmol L^{-1}) employed in this experiment reflect maximum total nitrogen levels observed in various regions of the Limfjord such as the western Nissum Bredning, and the central Skive Fjord, Risgårde Bredning, Lovns Bredning, and Løgstør Bredning (Carstensen et al. 2013; Jakobsen and Markager 2016). While the highest growth rates (0.3–0.4 d^{-1}) were obtained when this dinoflagellate was exposed to inorganic nitrogen sources (NH_4^+ or NO_3^-), no significant differences in growth between the two nitrogen sources were detected (Figs. 1, 2). This finding suggests that *A. pseudogonyaulax* growing on nitrate may compensate for higher reductant requirements through alternative means other than a reduction in growth or that a decrease in growth was marginal and hence, undetectable (Levasseur et al. 1993). Another possibility may be that the growth rate of *A. pseudogonyaulax* is restricted by other unknown intrinsic factors and the conserved reduction potential cannot be converted to enhanced growth. Notably, while lower reduction requirements generally favor NH_4^+ over NO_3^- (Levasseur et al. 1993) no conclusions toward a nitrogen preference in the presence of both NH_4^+ and NO_3^- can be drawn.

Moreover, the growth rate of *A. pseudogonyaulax* was significantly lower when nitrogen was only available as urea (Figs. 1, 2), which is important as the Limfjord features higher organic than inorganic nitrogen concentrations (Carstensen et al. 2013). Despite initial growth, all urea treatments failed to reach high cell densities and the population started to decline before the experiment concluded. This was probably

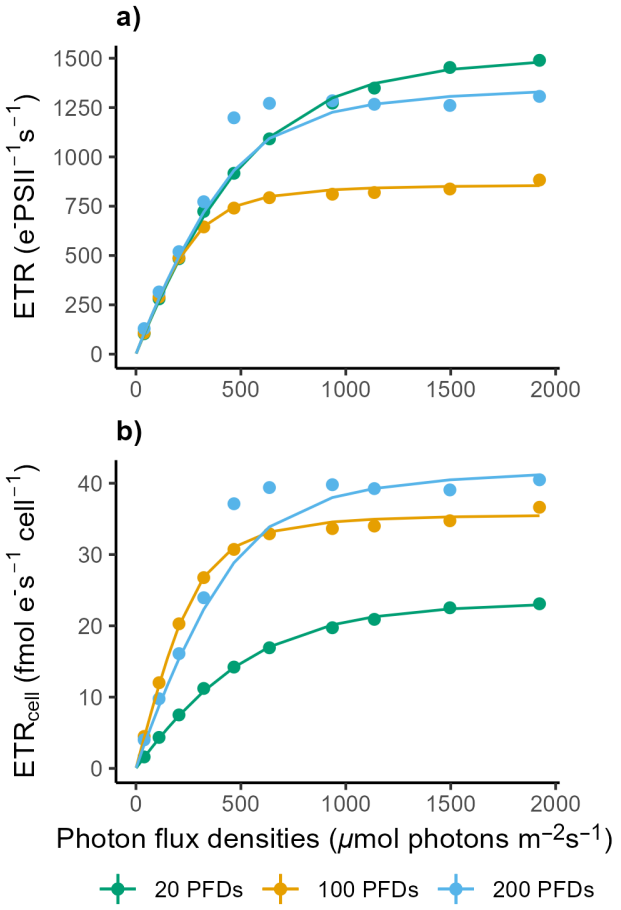

Fig. 8. Measured and calculated ETR of *Alexandrium pseudogonyaulax* strain A (L2-D2) harvested in the exponential growth phase after exposure to three different photon flux densities (PFDs), normalized to either PSII (a: ETR) or to the entire cell (b: ETR_{cell}); points refer to the measured (mean of $n = 3$) and lines to the modeled ETR, respectively.

caused by nitrogen limitation due to the inability to utilize urea as the only nitrogen source. Given that the reductant requirements of ammonium and urea are similar (Levasseur et al. 1993), it is plausible that lower growth rates in the urea treatment were caused by a reduced efficiency in cellular processes such as urea uptake and/or enzymatic conversion into NH_4^+ (Dyhrman and Anderson 2003). Dyhrman and Anderson (2003) demonstrated that *A. catenella* is able to utilize urea as the sole nitrogen source, but only in the presence of nickel hinting toward nickel-dependent urease activity. Other studies reporting the growth of *Alexandrium* species on diverse nitrogen sources, including urea, without the addition of nickel to the growth media, may therefore be attributed to varying background concentrations of nickel in the employed seawater (Leong et al. 2004; Li et al. 2011; Xu et al. 2012). Hence, it cannot be excluded that the observed growth decline of urea-grown *A. pseudogonyaulax* cells in this study may be partly attributed to insufficient nickel availability, as the employed K/2 media did not contain any additional nickel.

Nevertheless, the findings of this study suggest that urea is suboptimal for the growth of *A. pseudogonyaulax*, which is reinforced by the fact that urea-grown cultures could be easily recovered through the addition of nitrate or ammonium. Hence, it may be hypothesized that urea is not the primary driver of the observed expansion and proliferation of *A. pseudogonyaulax* in the eutrophic Danish Limfjord, despite the high levels of anthropogenic urea inputs induced by intensive agricultural farming (Glibert et al. 2005; Carlsson et al. 2009). However, the inability to utilize urea as a nitrogen source does not seem to be consistent with the well-known mixotrophic capabilities of *A. pseudogonyaulax* and the fact that maximum growth rates were only obtained in the presence of prey (Blossom et al. 2012, 2017). Nevertheless, the utilization of prey-based organic nitrogen has not been explicably shown for this dinoflagellate, which could be clarified by conducting isotope labeling studies such as for *A. catenella* (Kang and Gobler 2023).

Toxin normalization may have significant impacts on the interpretation of results

Studies generally normalize toxin quotas per cell, which can result in a loss of information since it excludes the effect of possible changes in the cell volume and intracellular carbon content, especially between the exponential and stationary GP (Supporting Information Table S3). Consequently, natural differences between both GPs are more likely resolved if toxin contents are normalized to another cell-specific feature such as the cell volume or carbon content. For instance, GDA (mol GDA mol^{-1} C^{-1}) in the exponential GP of the inorganic nitrogen treatments of strains A and B was still higher than in the stationary GP of the N-deplete and urea treatment, while lower if normalized per cell or volume (Supporting Information Fig. S2). Hence, additionally including normalization per carbon and volume may further facilitate the comparability of toxin abundances between studies and/or species and thereby improve the capacity to predict the toxic impact of HABs on ecosystems.

GP rather than nitrogen source drives toxin content of A. pseudogonyaulax

The influence of nitrogen availability on intracellular toxin abundances of PST-producing *Alexandrium* species exhibited a similar diverse pattern as the growth response toward nitrogen. For instance, Xu et al. (2012) reported either strong or only minor effects of differing nitrogen sources on the toxin content of different strains of *A. catenella* (note that Xu et al. reported one of the strains as *A. tamarense*). Modest effects of nitrogen sources on the toxin content of *A. catenella* were also reported by Leong et al. (2004) (reported as *A. tamarense*) and Li et al. (2011). In all three studies, no explicit correlation between GP (i.e., exponential or stationary GP) and toxin content was observed (Leong et al. 2004; Li et al. 2011; Xu et al. 2012). For *A. pseudogonyaulax*, toxin abundances of each treatment were higher in the stationary than in the

exponential GP, implying an accumulation of toxins toward the later stages of algae blooms and hence, potentially increased toxin exposure of other marine organisms and/or reduced grazing pressure toward *A. pseudogonyaulax*. However, during each GP, the nitrogen source did not substantially influence toxin levels, which is likely due to GDs not containing nitrogen. Another explanation may be that the activity of enzymes involved in GD production is independent of nitrogen. Consistently, previous research on PST-producing *A. catenella* generally found a stronger impact of the nitrogen source on the toxin content in agreement with PSP-toxins containing a substantial amount of nitrogen (Béchemin et al. 1999; Leong et al. 2004; Li et al. 2011; Xu et al. 2012). To unravel the relationship between GPs and toxin content, future studies should include multiple toxin sampling days within the same GP. This approach would enable the calculation of toxin production rates, offering additional insights alongside cellular toxin contents (Park et al. 2023).

Furthermore, *A. pseudogonyaulax* cultivated on inorganic nitrogen sources exhibited higher toxin contents compared to those grown on urea in accordance with previous studies observing similar trends with other PST-producing *Alexandrium* species (Leong et al. 2004; Li et al. 2011). This finding further emphasizes the limited significance of urea in assessing the future risk potential of *A. pseudogonyaulax* in northern European waters.

Intraspecific variabilities between two strains isolated at the same location

Even though strain A and strain B were isolated from the same location, intraspecific differences in growth rates, toxin and nutrient contents were found. These findings highlight the necessity to conduct laboratory experiments with multiple strains, especially if the results are subsequently processed in modeling studies featuring long-term predictions.

A. pseudogonyaulax *and the role of light*

Light intensity strongly affected growth and pigment composition

The growth rates of all three *A. pseudogonyaulax* strains increased concomitantly with increasing PFDs (Fig. 5), reflecting the interdependence of phototrophic growth rates and light intensity. More importantly, the relationship between growth rate and light (Fig. 6) suggests that *A. pseudogonyaulax* can grow in a wide range of light conditions. Similarly, studies have shown that *A. catenella* (Parkhill 1999; Etheridge and Roesler 2005; Laabir et al. 2011) (reported as *A. tamarense* and *A. fundyense* by Parkhill et al. and Etheridge et al., respectively), *A. minutum* (Hwang and Lu 2000) and *Alexandrium pohangense* (Lim et al. 2019) can also grow over broad light ranges and are tolerant toward high light (≥ 200 μmol photons $m^{-2}s^{-1}$) scenarios. In contrast, the growth of *A. ostenfeldii* exhibited a sharp maximum at 100 μmol photons $m^{-2}s^{-1}$ followed by a rapid decline (Maclean et al. 2003). Hence, differences in light tolerance may have contributed to the replacement of *A. ostenfeldii* by *A. pseudogonyaulax* in northern European waters. This is especially important in shallow and regularly stratified areas of the Danish Limfjord (Carstensen et al. 2007), in which algae are subjected to high light conditions (≥ 200 μmol photons $m^{-2}s^{-1}$) in surface waters during the summer months (Stæhr et al. 2020).

Furthermore, stagnating growth of cells exposed to LL conditions could be rapidly recovered by increasing the light intensity indicating that *A. pseudogonyaulax* cells can overcome longer periods of reduced light without decaying or forming resting stages. The LL conditions are representative of light conditions at 5 m depth (which reflects well the average water depth for the Limfjord) during summer month and surface waters during winter month in the Limfjord (Stæhr et al. 2020). Nonetheless, carbon acquisition via photosynthesis at LL was insufficient (except for strain A) to meet the cellular carbon demands for growth or, at LL, *A. pseudogonyaulax* may allocate carbon to other cellular processes apart from cell division. Notably, *A. pseudogonyaulax* cells in the LL treatment were acclimated at 60 μmol photons $m^{-2}s^{-1}$, instead of 20 μmol photons $m^{-2}s^{-1}$, and improved acclimatization protocols may enhance the capability of *A. pseudogonyaulax* to thrive in LL conditions. It was further observed that the ML and HL treatment reached two to three times higher cell densities in comparison to the nitrogen experiment. Factors governing the cell densities of dinoflagellates in the stationary phase are, however, still poorly understood. Since macronutrient concentrations were high, differences in cell densities may be explained by an increase in pH. Next to potentially affecting cellular processes directly, elevated pH reduces bicarbonate availability in the carbonate system leading to decreased carbon availability and growth (Hansen 2002; Hansen et al. 2007). Similarly, NO_3^- and NH_4^+ grown cultures in the nitrogen experiment may have also been carbon limited as intracellular carbon contents in the exponential and stationary GP were the same (Supporting Information Table S3). However, pH in the experiments was not measured and thus a relation between light and pH remains elusive and direct effects of light on maximum cell densities through unknown cellular mechanisms may have to be considered.

In addition to growth, light intensity strongly influenced the pigment composition of all three *A. pseudogonyaulax* strains. Pigment ratios in this study are in agreement with previously published results obtained for the same species by Zapata et al. (2012). Under LL conditions, cells upregulated the production of light-harvesting pigments (peridinin, Chl *a*, Chl c_2) and featured up to three times higher LH : LP ratios than ML or HL acclimatized cells (Supporting Information Table S5), a commonly observed acclimatization strategy of microalgae in response to higher light levels (Borowitzka et al. 2016).

Photophysiological response is largely independent of light intensities

The photophysiological response of *A. pseudogonyaulax* showed little variation in response to varying PFDs. Surprisingly, the maximum quantum yield of PSII ($F_v F_m^{-1}$) and high PSII normalized ETR were equal for all three treatments of strains B and C, and hence, it is unlikely that the photosynthetic apparatus was damaged at LL or HL conditions. High PSII normalized ETR that is not translated into cell growth for the LL treatment of strains B and C, may be explained by varying contributions of alternative electron pathways to overall ETR. Alternative electron pathways, such as the Mehler reaction, regulate the ATP/NADPH ratio in photosynthetic light reactions, but do not necessarily contribute to carbon fixation (Roberty et al. 2014). Unintuitively, LL acclimatized cells of strains B and C featured comparable ETR cell^{-1} as ML and HL acclimatized cells, but still did not sustain growth over the timeframe of the experiment. Conversely, the LL acclimatized strain A featured a lower ETR cell^{-1}, however, this strain did sustain growth over the timeframe of the experiment. High intraspecific variability in natural phytoplankton populations has already been observed, implicating the difficulty of abstracting results from monoclonal laboratory experiments onto natural populations and communities (Kremp et al. 2012; Wolf et al. 2018). Similarly, it may be hypothesized that the observed intraspecific variability regarding light increases the resilience of natural *A. pseudogonyaulax* communities toward environmental changes and hence strain A may be specifically adapted to LL scenarios or even to the winter season.

Thylakoid structure may influence diffusion of electron carriers

In plastids of green algae, the two photosystems (PSs) are segregated into different thylakoid subdomains. PSII is mostly located in the grana stacks, while PSI is mainly found in the stroma lamellae (Flori et al. 2017). No such thylakoid subdomains are visible in plastids of dinoflagellates (comparable to diatoms), where loose stacks of mostly three thylakoids are found. These different thylakoid architectures may differently affect the diffusion of the soluble electron carriers. While PSs confinement in subdomains constrains the electron flow in green algae, it may be hypothesized that the less structured thylakoids of dinoflagellates allow a faster diffusion of the soluble electron carriers and therewith a faster redox equilibration between the two PSs. In this study, increasing the rates for the mobile electron carriers in comparison with the original green algae model (Kroon and Thoms 2006) led to a better agreement of measured and modeled ETRs (Fig. 8), strengthening the aforementioned hypothesis.

Depressed growth at low light may indicate a switch to mixotrophic feeding

Interestingly, Lim et al. (2019) reported a significantly lower light compensation point of *A. pohangense* cultures grown mixotrophic compared with phototrophic growth, indicating that ingested prey can serve as a substitute for cellular carbon demands under LL conditions. Factors governing the balance between phototrophic and phagotrophic nutrition modes in mixoplankton species in response to light are, however, not fully understood (Hansen 2011; Flynn et al. 2019; Jeong et al. 2021). While it is known that many mixotrophic dinoflagellates are dependent on light to feed (Hansen 2011), even highly mixotrophic species such as *Karlodinium* spp. (Berge and Hansen 2016), the underlying causality is unclear. For phagotrophy of *A. pseudogonyaulax*, Blossom et al. (2012) reported an exceptional feeding mechanism that involves the production of extracellular mucus traps. These substances, even though not structurally elucidated yet, are large carbon-based macromolecules and hence it may be speculated that their biosynthesis is competing with cell division (Long et al. 2021) and that, below a certain light intensity, the biosynthesis of substances aiding in feeding may impede phototrophic driven cell division of *A. pseudogonyaulax*.

Growth stage rather than light drives toxin content of A. pseudogonyaulax

The relationship between light availability and toxin quotas in *Alexandrium* populations is as versatile as the relationship between macronutrient concentrations and toxin quotas. Different studies on the influence of bottom-up factors on the toxin content of microalgae have reported direct, inverse, and no systematic relationships between PFDs and toxin quotas, highlighting the complexity of cellular toxicity (Parkhill 1999; Maclean et al. 2003; Wang and Hsieh 2005; Laabir et al. 2013). For *A. minutum* and *A. catenella*, the highest PST quotas have also been found at ML intensities resulting in a Gaussian-like relationship between PFDs and toxin quotas (Parkhill 1999; Hwang and Lu 2000). The present study cannot resolve such uncertainties due to a lack of a systematic relationship between PFDs and toxin contents (Fig. 7). Instead, a trend similar to the nitrogen experiment (Fig. 3) was observed with significantly higher toxin quotas in the stationary compared to the exponential GP. These differences, however, were less apparent if toxin contents were normalized per volume instead of per cell (Supporting Information Fig. S3), underlining how toxin normalization may influence the interpretation of results. In addition, toxin contents decreased from strain C to strain A and then to strain B, revealing substantial intraspecific variability. Consequently, the potential adverse effects of *A. pseudogonyaulax* blooms may vary significantly based on the dominant strain within the blooming community. Altogether, these findings suggest that toxin abundances in *A. pseudogonyaulax* are influenced by multiple factors, including light availability and GP, and that the relationship between light intensity and toxin production is complex.

Conclusions

The findings of this study show that the growth rate of the harmful algae bloom dinoflagellate *A. pseudogonyaulax* was significantly influenced by nitrogen source and light intensity, both important drivers of bloom formation. Meanwhile, the intracellular toxin content was predominantly driven by the growth stage. In addition, considerable physiological intraspecific variability of different *A. pseudogonyaulax* strains have been observed, thus emphasizing the importance of conducting laboratory experiments using multiple monoclonal strains. The study further suggests that the observed expansion and proliferation of *A. pseudogonyaulax* in the Danish Limfjord cannot be attributed to high anthropogenic urea inputs, while a broad light tolerance may have favored it. Altogether, the findings point toward a long-term establishment of *A. pseudogonyaulax* in northern European waters. Considering the ongoing expansion of this toxic dinoflagellate, further research into other bottom-up factors (e.g., salinity, temperature, mixotrophy) and their influence on the toxicity of *A. pseudogonyaulax* are needed.

Data availability statement

All data needed to evaluate the conclusions in the paper are present in the paper and/or supporting information and are freely available from the PANGAEA data repository: https://doi.pangaea.de/10.1594/PANGAEA.965195. All code can be found online on GitHub: https://github.com/KristofM854 (accessed on 18 April 2024).

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Effects of bottom-up factors on growth and toxin content of a harmful algae bloom dinoflagellate

Kristof Möller[1*], Silke Thoms[1], Urban Tillmann[1], Bernd Krock[1], Florian Koch[1], Ilka Peeken[1], Cédric L. Meunier[2]

[a]Alfred Wegener Institut-Helmholtz Zentrum für Polar- und Meeresforschung, Bremerhaven, Germany

[b]Alfred Wegener Institut-Helmholtz Zentrum für Polar- und Meeresforschung, Helgoland, Germany

*Correspondence: kristof-moeller@outlook.de

Supporting information

Document includes:

Additional material & methods and results for particulate nutrients and pigments analysis, as well as the analysis and modelling of the photophysiological response.

Tables S1-S5

Figures S1-S3

Material and methods

Particulate and dissolved nutrient analysis

An amount of 100-200 mL from each incubation bottle was harvested by filtration onto pre-combusted (500 °C, 6 h) glass fiber filters (GF/F) (Whatman, Maidstone, United Kingdom) and stored in pre-combusted (500 °C, 6 h) petri-dishes. The GF/F-filters were dried (12 h, 65 °C) and 200 μL of concentrated hydrochloric acid was added to remove inorganic carbon. The GF/F-filters were dried again (12 h, 65 °C) and packed into pre-combusted cubicle tinfoil for analysis. Samples were analysed on a Euro Elemental Analyser 3000 CHNS-O (HEKAtech GmbH, Wegberg, Germany). POC and PON contents were normalized to filtered culture volume and cell densities to yield the mass of carbon or nitrogen per cell and then converted to the amount of substance per cell. Dissolved macronutrient concentrations (combined nitrate + nitrite and ammonium) were analysed colorimetrically according to Grasshoff et al. (2009) on a Quattro autoanalyser (Seal Analyticals, Norderstedt, Germany).

Pigment analysis

An amount of 20-100 mL from each incubation bottle was harvested by filtration onto glass fiber filters (Whatman GF/F) and immediately flash-frozen in liquid nitrogen. Samples were stored at -80 °C in the dark until extraction and analysis of pigments. Pigments were measured and quantified with an Alliance 2695 HPLC Separation Module (Waters GmbH, Eschborn, Germany) connected to a 2996 photodiode array detector (Waters). The system was calibrated with standards from the DHI group (DHI LAB products, Hørsholm, Denmark). For analytical preparation, an internal standard (50 μL, canthaxanthin), 100% acetone HPLC grade (1.5 mL) and glass beads were added to each filter sample on ice and the samples were homogenized for 2 $\times$ 20 seconds in a cell mill (Precellys® Bertin Technologies, Montigny-le-Bretonneux, France) and thereafter centrifuged (5 min, 0 °C, 13,000 rpm). Then, the supernatant was filtered through a 0.2 μm 'PTFE'-filter (Rotilabo, Carl Roth GmbH + Co. KG, Karlsruhe, Germany) and stored at -80 °C prior to analysis. An aliquot of 120 μL was transferred to the autosampler (4 °C), which uses a step function to draw the liquid from the sample and 1 M ammonium acetate solution (4 $\times$ 25 μL each), just prior to analysis, which sufficiently mixes the two solvents. Pigment analysis was conducted by reverse-phase HPLC using a C8 column (Agilent Microsorb-MV3 4.6 $\times$ 100 mm, Santa Clara, USA), and HPLC-grade solvents using a modified method of Barlow et al. (1997). Eluting pigments were detected by absorbance (440 nm) and identified by comparing their retention times with those of pure standards of the library. Pigment contents were quantified based on peak areas of external standards, which were spectrophotometrically calibrated using extinction coefficients according to Bidigare et al. (2013). Experimental losses and volume changes were corrected by normalizing pigment contents to the internal standard canthaxanthin.

In addition, pigment contents were normalized to filtered volume and cell densities.

Photophysiological responses

FRRf analysis

Chlorophyll a (Chl *a*) fluorescence measurements were performed at the end of the light experiment (2.3.1) on a Fast Repetition Rate fluorometer (FRRf) coupled to a FastAct laboratory system (FastOcean PTX, both from Chelsea Technologies Group, Molesey, United Kingdom). To calculate the maximum quantum yield of photosystem II (PSII) $F_v\ F_m^{-1}$ (dimensionless), the minimum (F_0) and maximum (F_m) Chl *a* fluorescence was determined after 10 min of dark acclimatization (Eq. 1, Oxborough and Baker 1997).

$$F_v F_m^{-1} = (F_m - F_0) F_m^{-1} \qquad (1)$$

The functional absorption cross-section of PSII (σ_{PSII}, nm^2), the re-oxidation time of the primary electron acceptor Qa at PSII (τ_{Qa}, μs), the concentration of functional PSII reaction centres (RCII, amol cell^{-1}), the connectivity factor (P, dimensionless) and the non-photochemical quenching were derived using the FastPro8 Software (Version 1.0.55, Chelsea Technologies Group Ltd) from Oxborough et al. (2012). Electron transport rates (ETR) irradiance curves in the exponential phase were conducted by applying 10 irradiances from 0 up to $\approx$ 1900 μmol photons $\mathrm{m}^{-2}\mathrm{s}^{-1}$ (PFDs) for 5 min each. A light sensor (ULM-500, Walz GmbH) measured each PFD emitted from the FastAct Laboratory system. Electron transport rates ($\mathrm{e}^{-}\mathrm{PSII}^{-1}\mathrm{s}^{-1}$) were calculated according to Suggett et al. (2003, 2004) (Eq. 2), whereby $\mathrm{Fq'Fm'}^{-1}$ is the effective PSII quantum yield at ambient light.

$$ETR = \sigma_{PSII} \frac{F_{q'} F'^{-1}_m}{F_v F_m^{-1}} E \qquad (2)$$

Maximum ETR (ETR_{max}, e^{-1} PSII^{-1} s^{-1}), light utilization efficiency (α) and minimum saturating irradiance of the photosystems (I_K, μmol photons m^{-2} s^{-1}) were calculated from the fitted irradiance-dependent ETR (Eq. 3) using the model of Platt et al. (1980) through a Nelder-Mead algorithm programmed on R studio. Within this model, the starting parameters of α and $\mathrm{P_s}$ were estimated as the slope of a linear regression through the first four PFDs and the maximum obtained ETR, respectively. Furthermore, β was initially assumed to be equal to 0.5.

$$ETR(E) = P_s \times [1 - e^{-\alpha \times \frac{E}{P_s}}] \times e^{-\frac{\beta \times E}{P_s}} \qquad (3)$$

The overall ETR per cell (ETR_{cell}) of each A. pseudogonyaulax strain was then calculated as a multiplication of the PSII normalized ETR given in Eq. 2 with the mean concentration of functional PSII reaction centres ([RSII]) of each strain, respectively.

Modelling

The cell model used in this study was adapted from Kroon and Thoms (2006). This model aims to describe phytoplankton steady-state growth by including the main mechanisms and processes in photosynthetic electron transport, involving all intermediary steps from photon absorption and charge separation at photosystem II up to the reduction of the terminal electron carrier ferredoxin at the end of the electron transport chain. In particular, the model includes the representation of a free plastoquinone pool interacting with a QB binding site at PSII, the cytochrome b6/f complex, the Q-cycle, the binding of ferredoxin to the cytochrome b6/f (which allows for cyclic electron transport) and the electron transfer steps within PSI. This model describes photosynthesis in terms of electron flow, which is assumed to be the most elementary product for describing the consequences of different bottom-up factors on well-known growth characteristics (e.g. ETR curve). As a result of the photochemical nature of the model, ETR normalized to photosystem II and ETR of the entire *A. pseudogonyaulax* cell were calculated. The used parameters of this model adapted to *A. pseudogonyaulax* are shown in Table S1 and Table S2.

Results

Nitrogen experiment

Particulate and dissolved nutrients

Mean intracellular C:N ratios ranged between 4.72 – 6.57 mol mol^{-1} in the exponential GP, and between 7.03 – 9.20 mol mol^{-1} in the stationary GP across all treatments and strains (supporting information Table S3). Significant differences in the C:N ratios between the different nitrogen sources and GPs were found for strain A ($H_6 = 15.9, p < .05$) and for strain B ($H_6 = 17.4, p < .01$). A subsequent post hoc analysis revealed that within the exponential GP, the C:N ratio of the inorganic nitrogen treatments was significantly lower ($p < .05$) than the N-deplete treatment, while it did not differ significantly across all treatments in the stationary GP. Furthermore, within each treatment, the C:N ratio was significantly higher ($p < .01$) in the stationary compared to the exponential GP except for the N-deplete treatment of strain A. Finally, no differences in C:N ratios between strain A and strain B were found. Dissolved inorganic nitrogen concentrations ($NH4^+$, $NO3^-$, NO^-) in the stationary GP were at least 5 μM in both inorganic nitrogen treatments.

Cell sizes

Mean cell diameter ranged between 27.5–30.7 μm in the exponential GP and 27.6–30.9 μm in the stationary GP across all treatments and strains (supporting information Table S3). Cell diameters of strain A exhibited significant differences ($H_6 = 18.5$, $p < .01$), while no significant differences were found for strain B ($H_6 = 11.1$, $p > .05$). A subsequent post hoc analysis revealed that cell diameters in the stationary GP were significantly different to the exponential GP for each treatment of strain A ($p < .05$, supporting information

Table S3). Finally, no differences in cell sizes between strain A and strain B were found.

Light experiment

Pigment composition and abundances

The following pigments were identified in *A. pseudogonyaulax* cells: chlorophyll *a* (Chl *a*), peridinin, *β*-carotene, chlorophyll c_2 (Chl c_2), violaxanthin (only in strain A and strain B), diadinoxanthin, dinoxanthin, diatoxanthin and zeaxanthin. However, diatoxanthin was only identified at 200 PFDs and zeaxanthin solely in strain B at 20 PFDs (supporting information Table S5). The carotenoid peridinin was the most abundant pigment in all treatments, closely followed by chlorophyll *a* with mean peridinin:Chl *a* ratios ranging between 1.22 and 1.40 (supporting information Table S5). Significant differences in the peridinin:Chl *a* ratio were found for strain B ($H_2 = 6.5$, $p < .05$) with the peridinin:Chl *a* ratio being significantly higher ($p < .05$) in the LL treatment. The third most abundant pigment was the xanthophyll diadinoxanthin with mean ratios of diadinonoxanthin:Chl *a* ranging from 0.25 to 0.47 in the ML and HL treatment. In the HL treatment the diadinonoxanthin:Chl *a* ratio was significantly higher ($H_2 = 7.5$–7.7, $p < .05$) than in the ML and LL treatment, whereby no diadinonoxanthin was detected in the LL treatment. Mean dinoxanthin:Chl *a* ratios ranged from 0.10 to 0.14 and exhibited significant differences between all treatments in strain B and C ($H_2 = 6.8$–7.1, $p < .05$) with highest ratios in the HL and lowest ratios in the LL treatment. While pigment ratios were similar across treatments, significant differences emerged in the molar ratios ($H_2 = 7.2$, $p < .05$) of light harvesting (LH) to light protecting (LP) pigments. The LH:LP in the LL treatment for each strain was significantly higher ($p < .05$) than ML and HL treatments with approximately threefold differences (supporting information Table S5).

Photophysiological responses

In strain A, the ML treatment exhibited a significantly higher ($H_2 = 7.2$, $p < .05$) dark-adapted maximum quantum yield ($F_v\ F_m^{-1}$) compared to LL and HL treatment (supporting information Table S4). Connectivity between the adjacent photosystems (P) remained constant across all treatments and strains. Significant differences were found in the functional absorption cross sections of PSII (σ_{PSII}) ($H_2 = 7.2$, $p < .05$), whereby the LL treatment was significantly lower ($p < .05$) than the HL treatment in strain A and B. In strain A, the re-oxidation time of the primary electron acceptor Qa at PSII (τ_{Qa}) was significantly higher in the LL and ML compared to the HL treatment ($H_2 = 7.2$, $p < .05$), while it remained unchanged in strains B and C. Further significant differences ($H_2 = 7.2$, $p < .05$) were observed in the concentrations of functional PSII reaction centers (RCII). In strain A, RCII was significantly higher in the ML than the HL treatment ($p < .001$), but RCII in the HL treatment remained significantly higher than in the LL treatment ($p < .05$). RCII remained the same across all treatments for strain B

and C. Finally, for strain A, ETRmax and the minimum saturating light irradiance (Ik) in the ML treatment were significantly lower ($p < .05$) than in the LL and HL treatment (supporting information Table S4 and Fig. 8). In accordance, multiplying the ETR with RCII to yield the ETR$_cell$ showed that ETRcell increased concomitantly with the PFDs (only calculated for strain A, Fig. 8). In general, ETRs per PSII of all treatments and strains were high compared to other algal species such as green algae. Strain A was chosen to be considered in more detail in the photophysiological model, as only this strain showed significant differences in RCII concentrations. Increasing the rate constants for the mobile electron carriers plastoquinone (PQ), plastocyanin (PC) and ferredoxin (Fd) within the photophysiological model (supporting information 2.3.2) enabled to account for the potential impact of different RCII concentrations on the diffusion of the mobile electron carriers.

Cell sizes

Mean cell diameters of all strains ranged between 27.3–29.4 μm in the exponential GP and 31.4–34.0 μm in the stationary GP (supporting information Table S4). Cell diameters in the stationary GP of the ML and HL treatment were significantly higher compared to the exponential GP for each treatment of each strain ($p < .05$, supporting information Table S4). No intraspecific differences between cell sizes were found, except within the exponential GP of the ML treatment ($H_2 = 6.5$, $p < .05$). Here, the cell size of strain A was significantly higher ($p < .05$) than of strain B and strain C.

Table S1: *Chlorophyll contents: Calculated number of chlorophyll a (Chl a) and chlorophyll c_2 (Chl c_2) molecules in photosystem II (PSII) and photosystem I (PSI) are based on measured total Chl a and total Chl c_2 (Table S4) per PSII assuming a heterogeneous distribution of Chl a and c_2 over PSII and PSI. A fraction of total Chl a and total Chl c_2 is assigned to PSII to account for the measured values of the light utilization efficiency (α).*

Parameter	LL treatment	ML treatment	HL treatment
total Chl *a* / PSII (mol mol^{-1})	3815	1021	791
Chl *a* / PSII (mol mol^{-1})	250	531	300
Chl *a* / PSI (mol mol^{-1})	3565	490	491
total Chl c_2 / PSII (mol mol^{-1})	1251	341	274
Chl c_2 / PSII (mol mol^{-1})	250	325	250
Chl c_2 / PSI (mol mol^{-1})	1001	16	24

Table S2: *Rate constants for the interaction of protein complexes (PSII, cyt b6/f, and PSI) and mobile electron carriers (PQ, PC, Fd): Compared to the rate constants of green algae, the rate constant for the PQ-PSII and Ferredoxin-PSI interactions at LL were increased by a factor of 3 and 10, respectively. The higher PSII content at ML and HL may affect the diffusion of the mobile electron carriers, which is represented by smaller rate constants.*

Process	LL treatment	ML treatment	HL treatment
	Rate constant (ms^{-1})		
Release of PQH_2 from PSII	6.000	2.760	5.000
Oxidation of cyt b6/f by PC^+	6.500	6.500	6.500
Proton release by cyt b6/f	3.861	1.776	3.217
Proton binding by cyt b6/f	3.900	1.794	3.250
Docking of Fd^- to cyt b6/f	0.039	0.039	0.039
Reduction of Fd by PSI	2.010	0.925	1.675
Terminal Fd^- re-oxidation	12.00	12.00	12.00

Table S3: *Cell sizes; intracellular C:N ratios and goniodomin content of* Alexandrium pseudogonyaulax *strains A (L2-D2) and B (L4-B1) subjected to different nitrogen sources in the exponential and stationary growth phase; all values represent the mean ± standard deviation of three biological replicates; ex = exponential, st = stationary, POC/PON = particulate organic carbon/nitrogen, GDA = goniodomin A.*

strain	N-source	cell diameter (μm)	POC (nmol cell^{-1})	PON (nmol cell^{-1})	C:N ratio (mol mol^{-1})	GDA:C (mol mol^{-1})	GDA:volume (pg μm^{-3})	GDA (pg cell^{-1})	growth phase
Strain A	N-deplete	27.5 ± 0.1	0.32 ± 0.07	0.05 ± 0.01	6.57 ± 1.11	2.64e-06 ± 1.06e-06	6.03e-05 ± 1.26e-05	0.74 ± 0.28	ex
		28.3 ± 0.3	0.54 ± 0.15	0.07 ± 0.02	8.20 ± 0.66	7.54e-06 ± 1.66e-06	2.70e-04 ± 9.78e-05	3.52 ± 0.57	st
	NO_3^-	28.7 ± 0.3	0.18 ± 0.02	0.04 ± 0.00	4.91 ± 0.03	1.03e-05 ± 3.70e-06	1.35e-04 ± 4.75e-05	1.64 ± 0.51	ex
		27.6 ± 0.5	0.19 ± 0.02	0.02 ± 0.00	9.20 ± 0.54	2.36e-05 ± 2.76e-06	3.63e-04 ± 1.42e-05	4.04 ± 0.46	st
	NH_4^+	29.3 ± 0.3	0.17 ± 0.01	0.03 ± 0.00	5.30 ± 0.13	1.66e-05 ± 3.90e-08	1.92e-04 ± 6.12e-06	2.49 ± 0.17	ex
		28.5 ± 0.3	0.19 ± 0.02	0.02 ± 0.00	8.68 ± 1.43	2.76e-05 ± 5.67e-06	3.73e-04 ± 1.01e-04	4.60 ± 1.03	st
	urea	30.2 ± 0.4	0.30 ± 0.05	0.04 ± 0.00	8.40 ± 0.61	8.06e-06 ± 1.03e-06	1.52e-04 ± 3.80e-05	2.18 ± 0.46	st
Strain B	N-deplete	28.6 ± 0.3	0.30 ± 0.06	0.05 ± 0.01	6.40 ± 0.47	1.04e-05 ± 3.16e-06	2.09e-04 ± 5.09e-05	2.69 ± 0.50	ex
		30.9 ± 0.8	0.49 ± 0.09	0.06 ± 0.02	9.11 ± 1.30	9.16e-06 ± 1.45e-06	2.81e-04 ± 1.71e-05	3.97 ± 0.34	st
	NO_3^-	28.8 ± 0.4	0.24 ± 0.05	0.05 ± 0.01	4.72 ± 0.12	1.25e-05 ± 2.32e-06	2.07e-04 ± 1.60e-05	2.64 ± 0.21	ex
		29.0 ± 0.4	0.23 ± 0.09	0.03 ± 0.02	7.03 ± 1.16	3.51e-05 ± 9.18e-06	5.39e-04 ± 7.94e-05	6.73 ± 0.62	st
	NH_4^+	30.7 ± 0.3	0.15 ± 0.01	0.03 ± 0.00	5.14 ± 0.32	1.98e-05 ± 4.50e-06	2.06e-04 ± 7.08e-05	2.70 ± 0.68	ex
		29.1 ± 1.8	0.20 ± 0.01	0.02 ± 0.00	9.12 ± 0.95	3.06e-05 ± 2.04e-05	4.01e-04 ± 3.23e-04	5.34 ± 3.36	st
	urea	29.8 ± 0.3	0.29 ± 0.01	0.04 ± 0.00	8.17 ± 0.59	8.48e-06 ± 2.52e-06	1.60e-04 ± 4.50e-05	2.23 ± 0.64	st

Table S4: *Photophysiological responses of three* Alexandrium pseudogonyaulax *strains: The dark-adapted maximum quantum yield (F_v F_m^{-1}), the connectivity between adjacent photosystems (P), the functional absorption cross section of PSII (σ_{PSII}), the time constant for electron transfer at PSII (τ_{Qa}) and the concentration of functional reaction centers for PSII (RCII); the maximum electron transport rate (ETR_{max}), minimum saturating irradiance (I_k) and the light utilization efficiency (α) were determined by fitting the PI curves (Fig. S1) with a model (supporting information 2.3.1) according to Platt et al.(1980); all values represent the mean ± standard deviation of three biological replicates as determined in the exponential growth phase (if not mentioned otherwise) after exposure to different light intensities; ex = exponential; st = stationary; GP = growth phase; PFDs = photon flux densities.*

strain	PFDs (μmol photons m^{-2} s^{-1})	F_v/F_m (dimensionless)	P (dimensionless)	σ_{PSII} (nm^2)	τ_{Qa} (μs)	Chl *a* (fmol $cell^{-1}$)	Chl c_2 (fmol $cell^{-1}$)	RCII (amol $cell^{-1}$)	ETR_{max} (e^- $PSII^{-1}$ s^{-1})	ETR_{cell} (fmol e^- s^{-1} $cell^{-1}$)	I_k (μmol photons m^{-2} s^{-1})	α (dimensionless)	cell diameter (μm) exp. GP	cell diameter (μm) st. GP	C:N (mol:mol)	GDA:C (mol:mol)
Strain A	20	0.40 ± 0.03	0.41 ± 0.03	4.04 ± 0.29	646.30 ± 25.78	57.46 ± 1.26	18.82 ± 0.39	15.51 ± 3.33	1517 ± 432	23.79 ± 8.69	525 ± 99	2.86 ± 0.318	28.1 ± 1.0	/	/	/
	100	0.47 ± 0.02	0.44 ± 0.02	4.25 ± 0.10	634.47 ± 14.02	41.83 ± 3.24	13.96 ± 1.08	41.49 ± 4.82	850 ± 59	35.08 ± 2.10	241 ± 20	3.53 ± 0.065	29.4 ± 0.6	33.5 ± 1.8	5.28 ± 0.05	9.44e-06 ± 1.39e-06
	200	0.40 ± 0.03	0.41 ± 0.03	5.98 ± 1.91	528.44 ± 82.69	24.32 ± 1.13	8.41 ± 0.39	31.26 ± 4.41	1335 ± 544	39.66 ± 9.15	332 ± 79	3.93 ± 0.679	28.5 ± 1.0	34.0 ± 0.6	5.36 ± 0.31	1.07e-05 ± 5.51e-07
Strain B	20	0.43 ± 0.05	0.42 ± 0.05	4.14 ± 0.20	600.74 ± 37.26	51.55 ± 2.42	23.80 ± 0.86	31.95 ± 17.61	1079 ± 62	34.80 ± 20.48	400 ± 31	2.70 ± 0.071	27.7 ± 1.1	/	/	/
	100	0.46 ± 0.03	0.46 ± 0.03	5.06 ± 1.02	582.99 ± 30.52	36.41 ± 5.30	12.47 ± 5.71	29.24 ± 3.28	1175 ± 263	34.90 ± 11.76	298 ± 2	3.94 ± 0.865	27.6 ± 1.0	31.4 ± 0.8	5.33 ± 0.30	7.77e-06 ± 1.47e-06
	200	0.42 ± 0.04	0.42 ± 0.03	4.69 ± 0.13	553.35 ± 12.99	24.35 ± 1.28	9.03 ± 10.68	26.02 ± 3.44	1069 ± 36	27.83 ± 3.81	273 ± 18	3.92 ± 0.215	27.3 ± 0.9	32.2 ± 0.4	5.17 ± 0.29	7.72e-06 ± 2.53e-06
Strain C	20	0.38 ± 0.02	0.39 ± 0.01	4.17 ± 0.48	619.38 ± 18.95	53.08 ± 5.33	19.83 ± 4.80	21.60 ± 4.80	1162 ± 73	24.93 ± 4.74	386 ± 32	3.03 ± 0.39	27.8 ± 0.1	/	/	/
	100	0.49 ± 0.03	0.44 ± 0.01	4.26 ± 0.14	596.60 ± 19.80	26.60 ± 2.69	11.12 ± 6.93	18.55 ± 6.93	984 ± 101	17.98 ± 5.76	307 ± 27	3.21 ± 0.15	28.5 ± 0.4	33.4 ± 1.1	5.49 ± 0.34	1.25e-05 ± 9.64e-07
	200	0.41 ± 0.05	0.41 ± 0.04	4.98 ± 0.90	541.92 ± 49.22	18.84 ± 1.21	7.73 ± 4.94	17.88 ± 4.94	1323 ± 323	24.40 ± 11.65	352 ± 12	3.76 ± 0.89	28.1 ± 0.8	33.5 ± 0.9	5.63 ± 0.20	1.00e-05 ± 5.77e-08

Table S5: *The ratio of light harvesting (LH = peridinin, Chl c2, Chl a) to light protective pigments (LP = diadinoxanthin + dinoxanthin + diatoxanthin + β-carotene + zeaxanthin + violaxanthin) of three* Alexandrium pseudogonyaulax *strains determined in the exponential growth phase after exposure to different photon flux densities; all values represent the mean ± standard deviation of three biological replicates; peri = peridinin, Chl = chlorophyll, diadino = diadinoxanthin, dino = dinoxanthin, diato = diatoxanthin; PFDs = photon flux densities.*

Strain	PFDs (μmol photons m^{-2} s^{-1})	LH:LP	Peri:Chl *a*	Peri:Chl c_2	Chl c_2:Chl *a* (mol:mol)	Diadino:Chl *a*	Dino:Chl *a*	Diato:Chl *a*
Strain A	20	18.1 ± 1.1	1.30 ± 0.01	3.97 ± 0.03	0.33 ± 0.00		0.10 ± 0.00	
	100	6.6 ± 0.6	1.30 ± 0.02	3.87 ± 0.03	0.33 ± 0.01	0.25 ± 0.00	0.10 ± 0.01	
	200	5.4 ± 0.1	1.22 ± 0.05	3.52 ± 0.12	0.35 ± 0.01	0.34 ± 0.01	0.10 ± 0.01	0.03 ± 0.00
Strain B	20	16.9 ± 0.2	1.40 ± 0.05	3.04 ± 0.34	0.46 ± 0.04		0.10 ± 0.01	
	100	5.6 ± 0.2	1.29 ± 0.02	3.77 ± 0.01	0.35 ± 0.01	0.34 ± 0.01	0.13 ± 0.01	
	200	4.5 ± 0.0	1.26 ± 0.02	3.41 ± 0.10	0.37 ± 0.01	0.47 ± 0.01	0.14 ± 0.00	
Strain C	20	18.8 ± 0.4	1.27 ± 0.05	3.41 ± 0.18	0.38 ± 0.01		0.10 ± 0.00	
	100	6.8 ± 0.1	1.32 ± 0.02	3.15 ± 0.07	0.42 ± 0.01	0.30 ± 0.01	0.12 ± 0.00	
	200	4.9 ± 0.1	1.26 ± 0.02	3.07 ± 0.17	0.41 ± 0.03	0.42 ± 0.01	0.13 ± 0.01	0.04 ± 0.01

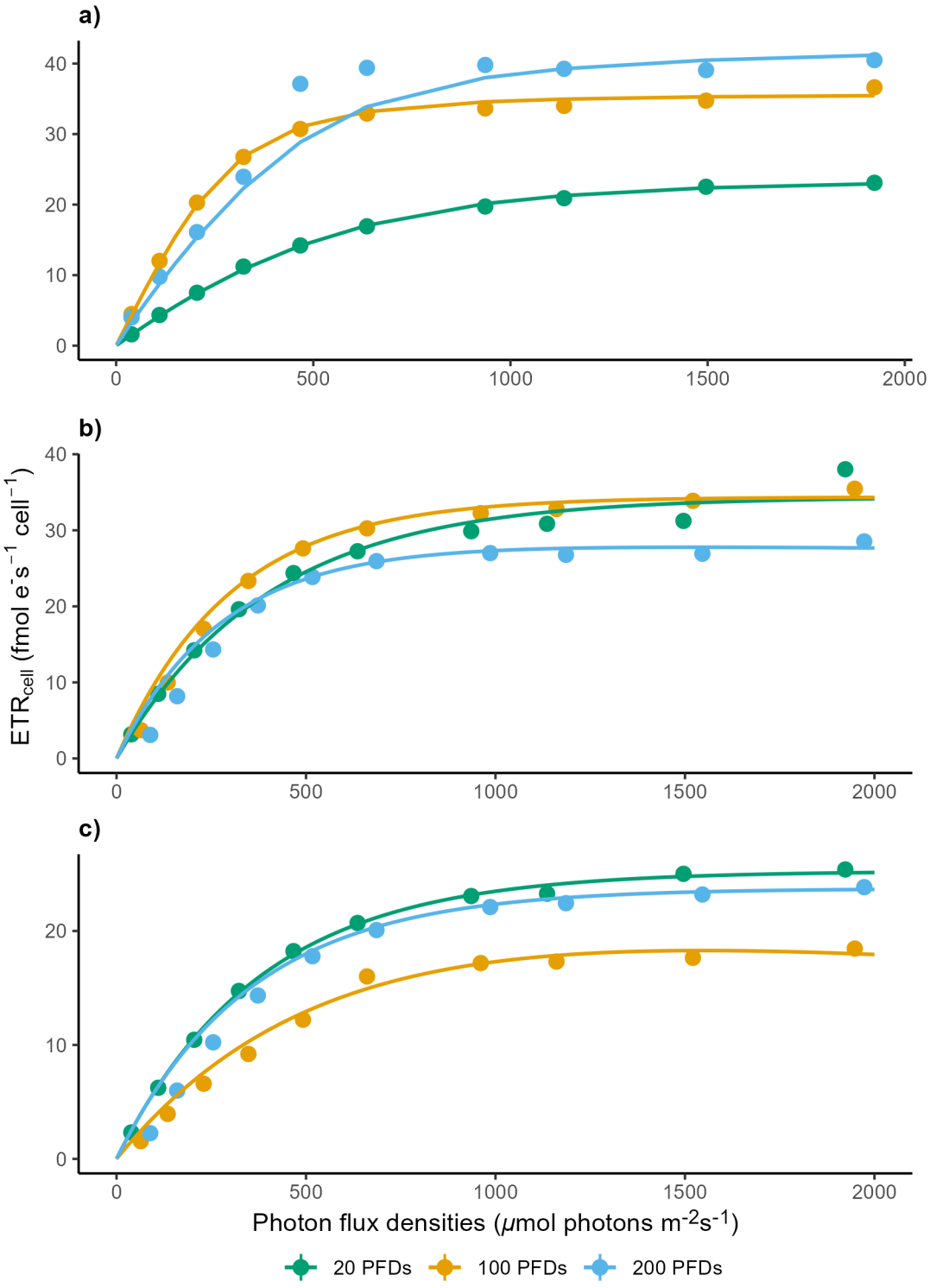

Fig. S1: *Measured, calculated (for strain A (**a**) L2-D2)) and fitted (for strain B (**b**) L4-B1) and C (**c**) L4-B9)) ETRs of three Alexandrium pseudogonyaulax strains harvested in the exponential growth phase, normalized to the entire cell; points refer to the measured (mean of n = 3) and lines to the modelled/fitted ETR, respectively; ETR = electron transport rate.*

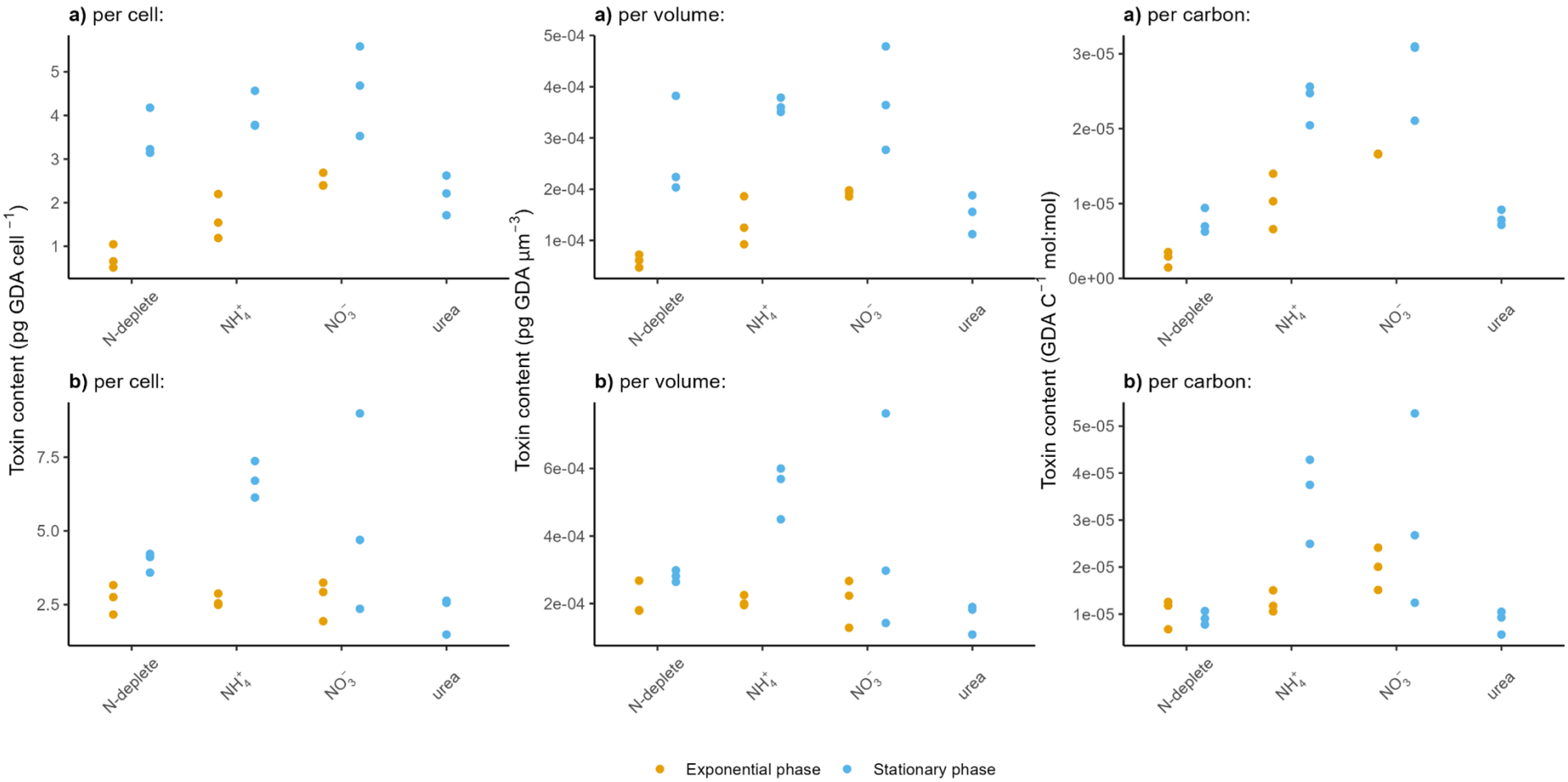

Fig. S2: *Intracellular toxin quotas (pg GDA cell^{-1}, pg GDA µm^{-3}, GDA C^{-1} mol mol^{-1}) of two Alexandrium pseudogonyaulax strains A (**a**) L2-D2) and B (**b**) L4-B1) subjected to different nitrogen sources in the exponential (brown) and stationary (blue) growth phase; points correspond to single data points of biological replicates.*

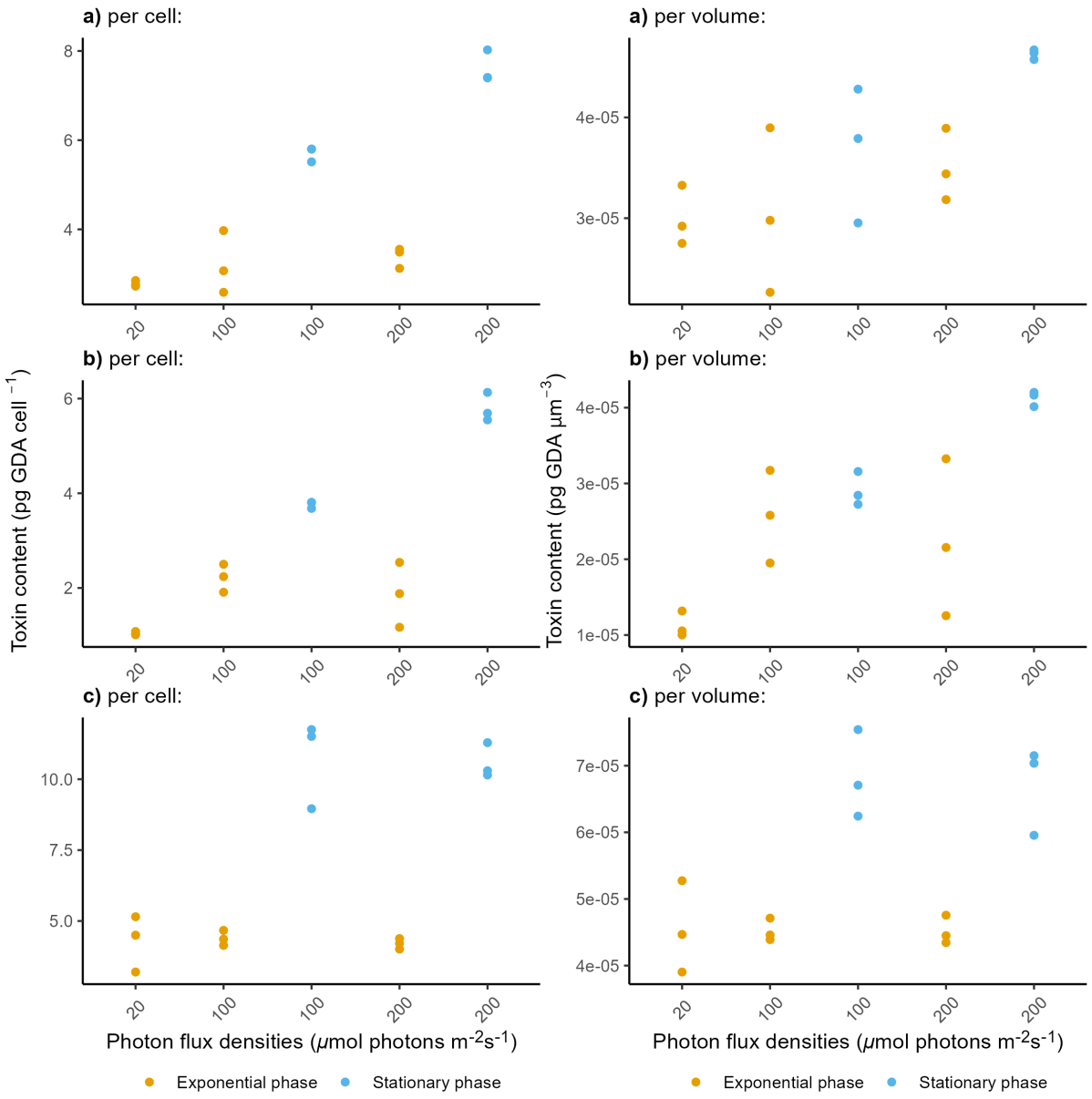

Fig. S3: *Intracellular toxin quotas (pg GDA cell^{-1}, pg GDA µm^{-3}) of three Alexandrium pseudogonyaulax strains A (**a**) L2-D2), B (**b**) L4-B1) and C (**c**) L4-B9) subjected to different photon flux densities in the exponential (brown) and stationary (blue) growth phase; points correspond to single data points of biological replicates.*

Chapter 3

Publication II

Toxic effects of the emerging Alexandrium pseudogonyaulax (Dinophyceae) on multiple trophic levels of the pelagic food web

Toxic effects of the emerging *A. pseudogonyaulax* (Dinophyceae) on multiple trophic levels of the pelagic food web

Kristof Möller[1], Urban Tillmann[2], Magdalena Pöchhacker[2,3,4], Elisabeth Varga[2,3], Bernd Krock[1], Francesco Porreca[1], Florian Koch[1], Thomas M. Harris[5], Cédric L. Meunier[6]

[1] Section Ecological Chemistry, Alfred-Wegener-Institut Helmholtz-Zentrum für Polar- und Meeresforschung, Bremerhaven, Germany
[2] Department of Food Chemistry and Toxicology, University of Vienna, Vienna, Austria
[3] Unit Food Hygiene and Technology, Centre for Food Science and Veterinary Public Health, Clinical Department for Farm Animals and Food System Science, University of Veterinary Medicine Vienna, Vienna, Austria
[4] Vienna Doctoral School in Chemistry (DoSChem), Faculty of Chemistry, University of Vienna, Währinger Str. 42, 1090 Vienna, Austria
[5] Department of Chemistry, Vanderbilt University, Nashville, TN, 37235, USA
[6] Section Shelf Sea Ecology, Alfred-Wegener-Institut Helmholtz-Zentrum für Polar- und Meeresforschung, Helgoland, Germany

*Correspondence: kristof-moeller@outlook.de

Running head: Top-down factors *A. pseudogonyaulax*

Abstract

The dinoflagellate *A. pseudogonyaulax*, a harmful algal bloom species, is currently appearing in increasing frequency and abundance across Northern European waters, displacing other *Alexandrium* species. This mixotrophic alga produces goniodomins (GDs) and bioactive extracellular compounds (BECs) that may pose a threat to coastal ecosystems and other marine resources. This study demonstrated the adverse effects of *A. pseudogonyaulax* on four marine trophic levels, including microalgae (*Rhodomonas salina*), microzooplankton (*Polykrikos kofoidii*) and mesozooplankton (*Acartia tonsa*), as well as fish gill cells (RTgill-W1, *Oncorhynchus mykiss*), ultimately leading to enhanced mortality and cell lysis. Furthermore, cell-free supernatants collected from *A. pseudogonyaulax* cultures caused complete loss of metabolic activity in the RTgill-W1 cell line, indicating ichthyotoxic properties, while all tested GDs were much less toxic. In addition, cell-free supernatants of *A. pseudogonyaulax* led to cell lysis of *R. salina*, while all tested GDs were non-lytic. Finally, reduced egg hatching rates of *A. tonsa* eggs exposed to cell-free supernatants of *A. pseudogonyaulax* and impaired mobility of *P. kofoidii* and *A. tonsa* exposed to *A. pseudogonyaulax* were also observed. Altogether, bioassay results suggest that the toxicity of *A. pseudogonyaulax* is mainly driven by BECs and not by GDs, although further research into factors modulating the lytic activity of *Alexandrium* spp. are needed.

Introduction

Pelagic primary production serves a critical role in marine ecosystems, as phyto- and mixo-plankton constitute the basis of the pelagic food web, generate oxygen through photosynthesis and contribute significantly to the global carbon cycle. However, blooms of certain microalgal species, known as harmful algal blooms (HABs), may pose serious ecological threats causing mortality among shellfish and finfish, as well as impacting tourist and recreational industries (Hallegraeff 1993; James et al. 2010). Furthermore, HABs have the potential to disrupt marine food web structures, for instance by negatively impacting zooplankton (Granéli and Turner 2006), which may favour the proliferation of these harmful organisms (Riebesell et al. 2018). Marine toxins, or phycotoxins, produced by HAB species can accumulate in marine organisms and propagate through pelagic food webs, ultimately affecting higher trophic level consumers such as marine mammals (Broadwater et al. 2018), seabirds (Gibble and Hoover 2018), and ultimately, humans (Berdalet et al. 2016). Moreover, phycotoxins may deter competing protists (Tillmann et al. 2008), prey or zooplankton grazers (Tillmann 2003; Granéli and Turner 2006; Adolf et al. 2007; Turner 2014). Zooplankton, including both microzooplankton (< 200 μm) and mesozooplankton (> 200 μm) play a key role in pelagic food webs by linking primary production with higher trophic levels (Calbet 2001; Calbet and Landry 2004; Tillmann 2004) and it is thus crucial to study the potential regulatory effect of protistan and other zooplankton grazers as a 'top-down control' in regulating bloom development and termination. Microzooplankton may be an especially suitable candidate, considering their short generation times (Tillmann 2004). The detrimental effects of HAB species on larger mesozooplankton include reduced mobility and/or ingestion rates (Ryderheim et al. 2021), impaired reproductive mechanisms, including fecundity (Ianora et al. 2003; Turner 2014), fertilization (Ianora et al. 2003), and egg hatching (Ianora et al. 2003; Turner 2014), which all may result in mortality. In addition, HABs can also exhibit ichthyotoxic properties, harming natural or commercial fish populations (Dorantes-Aranda 2023), which can result in large economic losses in the aquaculture industry.

The predominant group of microalgae causing HABs are dinoflagellates, particularly those belonging to *Alexandrium* (Anderson et al. 2012). Members of this genus produce an array of marine toxins, each possessing distinct toxicological properties (Hallegraeff 1993; Long et al. 2021). *Alexandrium* species are further known for producing BECs, whose molecular structures remain largely uncharacterized. These substances have been referred to as allelochemicals, lytic substances, or ichthyotoxins in the past (Long et al. 2021). Due to the absence of effective isolation and purification protocols for these substances, they have been primarily characterized by their adverse effects on a wide range of organisms, encompassing protists, metazooplankton, shellfish, and fish (reviewed in: Long et al. 2021). The exact mode of action of BECs is poorly understood, however, for BECs

produced by *A. catenella*, Ma et al. (2011) hypothesized that, similarly to karlotoxins (Place et al. 2012; Deeds et al. 2015), BECs may target membrane sterols, resulting in an alteration of membrane permeability and pore formation. Apart from deterring predators and eliminating competitors, BECs may also directly assist in prey capture as shown for *A. pseudogonyaulax* (Blossom et al. 2012), which utilizes a potentially toxic mucus to trap and immobilize prey prior to ingestion. *A. pseudogonyaulax* is further known for producing GDs (Zmerli Triki et al. 2016), phycotoxins belonging to a class of macrocyclic polyketides featuring a lactone group (Sharma et al. 1968; Harris et al. 2020). Under alkaline conditions, goniodomin A (GDA) undergoes hydrolysis to give the goniodomin A seco-acid (GDA-sa) and thus a mixture of GDA and GDA-sa exists in seawater, where pH is usually 8 or slightly above. Goniodomin A was the predominant intracellular GD congener (4–27 pg cell^{-1}, usually $\approx$ 75 %), while GDA-sa was the predominant extracellular GD congener (2–9 pg cell^{-1}, usually $\approx$ 75 %) in *A. pseudogonyaulax*, *Alexandrium hiranoi*, *Alexandrium monilatum* and *Alexandrium taylorii* (Hintze 2021). Normalized per cell, intracellular GDs were always more abundant than extracellular GDs in all analysed strains (Hintze 2021). Additionally, formation of the isomer goniodomin B (GDB) and the α,β-unsaturated ketone goniodomin C (GDC) under acidic conditions have been reported (Harris et al. 2023). Currently, it remains uncertain whether GDB and GDC are artefacts formed during the extraction and analysis of GDs or if they naturally occur in GD-producing microalgae. The chemical complexity of GDs gets further exacerbated by the discovery of additional congeners, such as goniodomic acid (T. Harris, unpublished). Goniodomins have been shown to exhibit cytotoxic effects towards mice (Erker et al. 1985; Terao et al. 1989) leading to inflammation and cell damage within liver and thymus, presumably through disrupting actomyosin ATPase activity and the F-actin meshwork (Furukawa et al. 1993; Espiña et al. 2016). This disruption may subsequently alter the reorganization of the cytoskeleton (Espiña et al. 2016). Additionally, the main goniodomin congener goniodomin A (GDA) is cytotoxic to rat hepatocytes and human neuroblastoma cells (Espiña et al. 2016).

Bioassays have revealed adverse effects of GDs and BECs, but the toxicity of *A. pseudogonyaulax* towards marine organisms remains poorly resolved. This information is crucial since the occurrence of *A. pseudogonyaulax* in Northern European waters has markedly increased in the last 15 years (Kremp et al. 2019, Möller et al. in prep.). In addition, GDs have been linked to fitness reductions and mortality of other marine species (Long et al. 2021). For instance, the GD-producing species *A. monilatum* exhibited toxic effects towards fish (Gates and Wilson 1960; Sievers 1969), annelids (Sievers 1969), crustaceans (Sievers 1969), and molluscs (Sievers 1969; May et al. 2010) making it plausible that *A. pseudogonyaulax* has similar toxic effects. Furthermore, *A. monilatum* has been associated with the mortality of marine gastropods (Harding et al. 2009) and fish (Table 1 in May et al. 2010) in natural settings. There is evidence that GD-producing HAB

species also produce BECs (Long et al. 2021), however, differentiating between their effects has been notoriously challenging. This is mainly due to the unknown chemical nature of BECs and because most studies utilized strains containing both phycotoxins and BECs (Ma et al. 2011; Long et al. 2021). In light of the current expansion of *A. pseudogonyaulax* in Northern European waters, this study aimed to evaluate the effects of *A. pseudogonyaulax* towards multiple marine trophic levels, including microalgae (cryptophyte *R. salina*), microzooplankton (heterotrophic protist *P. kofoidii*), mesozooplankton (calanoid copepod *A. tonsa*), and fish gill cells (RTgill-W1 cell line of the salmonid *O. mykiss*). A main emphasis of these experiments was to untangle the relative influence of GDs and BECs on other organisms by utilizing culture supernatants containing both and comparing them to purified GDs.

Materials and methods

Algae isolation and maintenance

Three *A. pseudogonyaulax* strains (L2-D2 (A), L4-B1 (B), L4-B9 (C)) were used in this study. These were isolated from live net tow samples during an expedition with the R/V Uthörn in August 2020 in the western Danish Limfjord close to Thyborøn (56°37'48.0"N; 8°17'24.0"E) using a M5A stereomicroscope (Wild, Heerbrugg, Switzerland). Other *Alexandrium* species utilized for comparison included *Alexandrium catenella* (strain Alex5), *Alexandrium monilatum* (strain YRK2007, provided by Kimberley S. Reece, Virginia Institute of Marine Science) and *Alexandrium limii* (strain Atay99Shio-02, provided by Satoshi Nagai, Japan Fisheries Research and Education Agency). *Alexandrium catenella* was isolated from the Scottish east coast of the North Sea (Tillmann et al. 2009) and was chosen, because it has not exhibited any lytic effects (Tillmann et al. 2009). However, *A. catenella* produced paralytic shellfish toxins (PSTs), which were dominated by neosaxitoxin, N-sulphocarbamoyl C toxins and saxitoxin being most prominent (Tillmann and Hansen 2009). *Alexandrium monilatum* strain YRK2007, isolated from the York River estuary (Virginia, USA) in 2007 and identified by light microscopy and LSU gene sequencing, was chosen because, similarly to *A. pseudogonyaulax*, it also produces both GDs and BECs. *Alexandrium limii*, isolated in 1999 from Shioya Bay, Japan (Abdullah et al. 2023) was chosen as it produces GDs, but no BECs (Tillmann, unpublished). The cryptophyte *Rhodomonas salina* (strain KAC30 from the Kalmar culture collection) was used, since it has previously served as a model organism for studying protistan cell lysis (Tillmann et al. 2008). The non-axenic microalgae stock cultures were maintained under semi-batch conditions, at 20 °C, salinity of 25 (*A. catenella* and *A. limii* at salinity of 33) and 80 μmol photons $m^{-2}s^{-1}$ of cool fluorescent light on a 16:8 hour light:dark cycle. Salinity was measured using a benchtop conductivity meter (Symphony SB80PC,

VWR). Stock cultures were diluted every other week to maintain cells in exponential growth. Cells were grown in K/2 medium (K-medium with half of the original nutrient concentrations, pH = 8.1, 441 μM NO_3^-, 25 μM NH_4^+). The original K-medium recipe was modified by replacing the organic phosphorus source with 3.62 μM Na_2HPO_4. All media used in this study were prepared from the same batch of aged seawater collected from the North Sea near Helgoland. Salinity adjustments of the seawater were made by dilution with deionized and purified water (Millipore Milli-Q, < 18 MΩ cm^{-1}). Finally, the seawater was autoclaved, nutrients were added and then the seawater was additionally filter sterilized (0.2 μm).

Zooplankton isolation and maintenance

Polykrikos kofoidii was isolated in August 2020 from the Danish Limfjord at Rønbjerg Havn (56°53'47.9"N; 9°9'9.66"E) and subsequently grown as described in Tillmann and Hoppenrath (2013). Briefly, *P. kofoidii* cultures were fed weekly with *A. catenella* cultures at a ratio of 10:1 and inoculated on a slowly rotating (1 rpm) plankton wheel under subdued light conditions at 20 °C. *Polykrikos kofoidii* was chosen as a representative of microzooplankton as it is a common, ubiquitous grazer of toxic planktonic algae cohabiting with *A. pseudogonyaulax* across Northern Europe (Tillmann and Hoppenrath 2013). The calanoid copepod *Acartia tonsa* was selected to represent mesozooplankton and was cultivated according to Meunier et al. (2016). Briefly, *A. tonsa* organisms were cultured in 200-litre cylindrical tanks at 18 °C at a 12:12 light:dark cycle. Copepods were fed ad libitum with *R. salina*, a well-established food source for zooplankton rearing. Eggs were collected daily from the bottom of the tanks and stored in seawater at 4 °C for later use. For the experiments, *A. tonsa* eggs were incubated in fresh seawater, collected from the German Bight in front of Helgoland, at 20 °C and a salinity of 33. Hatched nauplii were collected between 24- and 36-hours post-incubation to minimize age discrepancies among individuals. Beginning 24 hours after hatching, *A. tonsa* was fed daily with *R. salina*. The *R. salina* cultures were maintained in F/2 medium under semi-batch conditions, at 20 °C, salinity of 33 and 150 μmol photons $m^{-2}s^{-1}$ of cool fluorescent light under a 16:8 light:dark cycle and bubbled with ambient, filter sterilized (0.2 μm) air. Cell densities of *R. salina* were monitored using a Coulter Counter (Multisizer 4e, Beckman) and maintained in exponential growth phase (2×10^5 to 5×10^5 cells mL^{-1}).

Rainbow trout gill cell line (RTgill-W1) maintenance

Rainbow trout (*O. mykiss*) gill cells (RTgill-W1, provided by Kristin Schirmer, EAWAG) were cultivated at 19 °C in the dark in L-15 complete medium, composed of 500 mL Leibovitz's L-15 medium (Thermo Fisher Scientific, Waltham, MA, USA), 50 mL fetal calve serum (FCS Eurobio Scientific, Les Ulis, France) and 5 mL penicillin/streptomycin

solution (Thermo Fisher Scientific). Gill cells were passaged weekly after reaching confluency under sterile conditions according to Dayeh et al. (2013). Gill cells were seeded in 96-well plates (Sarsted AG & Co. KG, Nümbrecht, Germany; 20-22 $\times$ 10^3 cells per well) and then incubated for 48 h at 19 °C in the dark prior to the start of an assay.

Analysis of particulate organic nutrients

On the day of the feeding experiments involving *A. tonsa*, the particulate organic carbon, nitrogen and phosphorus (POC, PON and POP, respectively) content of the prey items was analysed. Therefore, triplicate cultures of *R. salina* (3–7 $\times$ 10^6 cells total) or *A. pseudogonyaulax* (2–12 $\times$ 10^4 cells total) in the exponential growth phase were harvested by filtration onto pre-combusted (500 °C, 6 hours) GF/F-filters (Whatman, Maidstone, United Kingdom) and stored in 6 well-plates (TPP, Techno Plastic Products AG). For POC and PON analyses, GF/F-filters were dried (12 hours, 65 °C), packed into pre-combusted cubicle tinfoil and analysed with an elemental analyser (Elementar vario MICRO cube). For POP analysis, GF/F-filters were frozen at -20 °C until spectrometric determination (Thermo Scientific Multiscan Spectrum) of orthophosphate according to Grasshoff et al. (2009). Finally, particulate nutrient contents were normalized per cell.

Goniodomins: extraction, analysis, and standards

Goniodomin extraction of *A. pseudogonyaulax* and *A. monilatum* followed by subsequent LC-MS/MS analysis was conducted as described in Möller et al. (2024). Briefly, cells were harvested by centrifugation at 3,220 $\times$ g for 10 min (Eppendorf 5810 R, Hamburg, Germany), after which cell pellets were re-suspended in methanol (500 μL) and transferred to FastPrep tubes containing 0.9 g lysing matrix D (Thermo-Savant, Illkirch, France). Samples were homogenized by reciprocal shaking for 45 s at maximal speed (6.5 m s^{-1}) in a FastPrep instrument (Thermo-Savant) and subsequently centrifuged for 15 min at 16,100 $\times$ g and 10 °C (Eppendorf 5415 R). Supernatants were transferred into spin filters (Ultra-free, Millipore, Eschborn, Germany) and filtered by centrifugation for 30 s at 10 °C and 5,000 $\times$ g. The resulting filtrates were transferred to HPLC vials and stored at -20 °C until mass spectrometric analysis. Cellular GD contents of all *Alexandrium* spp. utilized in this study were quantified. Moreover, the extracellular GD concentrations of cell-free supernatants of *Alexandrium* strains utilized in the *R. salina* and RTgill-W1 bioassays were quantified. Supernatants were harvested by centrifugation, transferred into spin filters (Ultra-free, Millipore, Eschborn, Germany) and filtered by centrifugation for 30 s at 10 °C and 5,000 $\times$ g. The resulting filtrates were transferred to HPLC vials and stored at -20 °C until mass spectrometric analysis. Quantification of GDA was performed by calculating the absolute peak areas of m/z 786.5 $\rightarrow$ 733.5 with a three to four point (5-1000 pg μL^{-1} GDA) external calibration curve ($R^2 = 0.99$). Finally, toxin quotas (mass

cell^{-1}) were converted to molar toxin quotas (molar mass cell^{-1}) and normalized to the molar carbon content per cell (GDA C^{-1}). Purified GDs (GDA, GDA-sa, GDB and GDC) utilized in this study, for both sample quantification (GDA) and toxicological assays (other GDs) were obtained following extraction and purification procedures as described by Harris et al. (2020).

Quantification of mortality and ingestion rates of *P. kofoidii* feeding on *A. pseudogonyaulax*

This experiment aimed to evaluate the interaction of *A. pseudogonyaulax* with *P. kofoidii* by quantifying the mortality and ingestion rates of *P. kofoidii* when exposed to *A. pseudogonyaulax* compared to *A. catenella*. Mortality (i.e. decrease in cell densities) and ingestion rates of *P. kofoidii*, both essential characteristics of predator/prey interactions, were determined by exposing *P. kofoidii* to either *A. pseudogonyaulax* (strain B, n = 6) or *A. catenella* (n = 3) for 72 hours in 12-well plates (TPP) under dim light conditions and at 20 °C. The targeted initial cell densities were 300 cells mL^{-1} for *A. pseudogonyaulax* and *A. catenella* and 10 cells mL^{-1} for *P. kofoidii* in a total volume of 2.5 mL. Given the small experimental volume, continuous mixing was not necessary during the experiment. Additionally, a 'hunger control' devoid of any prey addition was included to distinguish the effects of starvation from those of ingesting toxic prey. The number of ingested cells within food vacuoles of *P. kofoidii* and the number of *P. kofoidii* cells were determined by direct microscopic counts of formalin-fixed samples on an inverted fluorescence microscope. Quantification of ingested cells was limited to the initial 27 hours of the experiment, as subsequent advanced digestion and/or overlapping of prey cells within the food vacuoles of *P. kofoidii* prevented accurate counting. Finally, ingestion rates were calculated by dividing the number of ingested cells by the total number of *P. kofoidii* and normalized to time.

Investigating selective feeding capabilities of *P. kofoidii* feeding on mixtures of *Alexandrium* spp.

The objective of the following experiment was to estimate the contributions of GDs and BECs to the overall toxicity of *Alexandrium* spp. on *P. kofoidii* by using different combinations of toxin producers as prey. In addition, the study aimed to investigate whether *P. kofoidii* actively selects for non-toxic prey. A minimum of triplicate *P. kofoidii* cultures were thus exposed to prey mixtures consisting of either *A. pseudogonyaulax* (GDs + BECs) and *A. catenella* (no GDs and BECs, but PSTs) or of *A. pseudogonyaulax* and *A. limii* (GDs, no BECs) for 6 hours in 12-well plates. Each mixture included one species labelled with the live stain 7-amino-4-chloromethylcoumarin (CMAC). The combination of

A. pseudogonyaulax and *A. catenella* was assessed in both dye combinations to ensure no interfering effects of the dye. No interfering effects of the fluorescent dye were found and thus ingestion rates of both dye combinations of the mixture of *A. pseudogonyaulax* and *A. catenella* were combined for the statistical analysis. The targeted initial cell densities were 150 cells mL^{-1} for each prey (i.e. *A. pseudogonyaulax*, *A. catenella* and *A. limii*) and 10 cells mL^{-1} for *P. kofoidii* in a total volume of 2.5 mL. Mortality and ingestion rates of *P. kofoidii* were obtained as outlined above.

Effects of *A. pseudogonyaulax* on *A. tonsa*

Ingestion rates of *A. tonsa* and toxin cell content of *A. pseudogonyaulax* Ingestion rates of *A. tonsa* feeding on *A. pseudogonyaulax* were determined through bottle incubation experiments using a minimum of six replicates per treatment. In addition, four replicates of *A. pseudogonyaulax* were included as a control, in order to assess growth rates, essential for subsequent calculation of ingestion rates according to Frost et al. (1972), and toxin contents in the absence of grazers. Predator and prey or only *A. pseudogonyaulax* were incubated in 250 mL culture flasks (TPP) for 24 h at 20 °C on a plankton wheel at dim light. The targeted initial cell densities were 100, 200 and 300 cells mL^{-1} for *A. pseudogonyaulax*, corresponding to advancing life-stages of *A. tonsa* with a total of 40, 25 or 20 N_4-nauplii, C_4-copepodites and adult copepods, respectively. At the end of the experiment, algae and copepods were separated by gently sieving the latter onto a 50 μm mesh. Subsequently, copepods were transferred to a Bogorov's counting chamber, fixed with Lugol's iodine solution, and enumerated under a stereomicroscope. Similarly, cell densities of *A. pseudogonyaulax* were determined at the start and the end of the experiment by direct microscopic counts of 2 mL Lugol's iodine-fixed subsamples. Ingestion rates were further converted to ingested carbon according to the molar carbon content of prey organisms determined at the onset of the experiment. Goniodomin A extraction and analysis was performed from 50 mL subcultures, following the separation of *A. tonsa* at the end of the experiment.

Influence of *A. pseudogonyaulax* on the developmental rate of *A. tonsa*

The effects of *A. pseudogonyaulax* on the development of *A. tonsa* were assessed through bottle incubation experiments conducted using a minimum of six replicates in 250 mL culture flasks (TPP). N_2-nauplii, representing the first feeding stage of *A. tonsa*, and prey (*R. salina* or *A. pseudogonyaulax*) were co-incubated for 4 or 10 days on a plankton wheel in dim light. The targeted initial cell densities of *A. pseudogonyaulax* and *R. salina* were 200 cells mL^{-1} or 4,000 cells mL^{-1}, respectively, with a total of 200 N_2-nauplii per bottle. Prey concentrations were calculated to provide a similar prey biovolume to *A. tonsa*, considering the larger cell size of *A. pseudogonyaulax*. Prey organisms were changed

daily by gently sieving *A. tonsa* onto a 50 μm mesh and immediately placing them back into a flask with fresh seawater and prey organisms. During the 4 days experiment, six replicates were prepared for sampling after 2, 3 and 4 days, respectively, to perform daily photo and video observations of live and fixed nauplii using a stereomicroscope (SCX16, Olympus, Tokyo, Japan). Copepods were enumerated and staged at the end of the 10-day experiment using a stereomicroscope.

Hatching rate of *A. tonsa* eggs subjected to the supernatant of *A. pseudogonyaulax*

The effects of *A. pseudogonyaulax* on the hatching rate of *A. tonsa* eggs were assessed by exposing the eggs to cell-free supernatants, collected from strains A-C of exponentially grown *A. pseudogonyaulax* cultures ($\approx$ 3,000 cells mL^{-1}), for 48 h at 20 °C in the dark. Between 50 and 300 eggs per replicate were incubated in glass petri dishes with 100 mL of cell-free supernatant in six replicates for each *A. pseudogonyaulax* strain. Another six replicates consisting of *A. tonsa* eggs in filtered seawater, adjusted to a salinity of 25, served as a control. The hatching rate was determined by counting both unhatched eggs and N_1 nauplii in each petri dish under a stereomicroscope.

Toxin cell content of *A. pseudogonyaulax* after exposure to copepodamides

A. pseudogonyaulax cultures were exposed to copepodamides from either *Calanus finmarchicus* or *A. tonsa*, and a potential increase in cellular toxin contents of *A. pseudogonyaulax* was assessed. Copepodamides from *C. finmarchicus* were extracted and purified from freeze-dried organisms as detailed in Selander et al. (2015). Copepodamides from *A. tonsa* were obtained by methanol extraction of several hundred adult individuals over 24 hours at -20 °C and the crude extract was utilized without further purification or partitioning. In both experiments, copepodamides (*C. finmarchicus*: 0.1–5 nmol L^{-1}; *A. tonsa*: crude extract), dissolved in methanol, were coated onto the glass floor of 250 mL conical flasks and after evaporation of the methanol, 20 mL of *A. pseudogonyaulax* culture with a targeted initial cell density of 1500 cells mL^{-1} was added. The *C. finmarchicus* copepodamides blend contained copepodamides 22:6, 20:5, 18:4, 16:0, 14:0 and dihydrocopepodamides 22:6, 20:5, 18:4, (Grebner et al. 2019) with a ratio of 86:14 copepodamides:dihydrocopepodamides in a total concentration of 28.8 μmol L^{-1}. After 24 hours, cell densities of *A. pseudogonyaulax* were determined by direct microscopic counts of 200 μL subsamples and the remaining *A. pseudogonyaulax* cells were utilized for extraction of GDs.

Effects of *A. pseudogonyaulax* and goniodomins on RTgill-W1 fish gill cells

RTgill-W1 gill cells from the rainbow trout (*O. mykiss*) were exposed to either cell-free supernatants collected from exponentially grown *A. pseudogonyaulax* cultures or purified GDs dissolved in methanol (stock: 100 ng μL^{-1}) for 24 hours at 19 °C. Different supernatant (300 μL) or goniodomin (100 μL) concentrations were prepared by dilution in K/2- or L-medium, respectively. Goniodomin concentrations were selected to ensure that final methanol concentrations did not exceed 0.5 %, previously demonstrated to have no effect on gill cell viability (Dorantes-Aranda et al. 2011). After 24 hours, 50 μL subsamples were transferred to a new 96-well plate for the assessment of lytic activity using a lactate dehydrogenase (LDH) cytotoxicity assay kit (CyQUANTTM, Thermo Fisher Scientific, C20301) following the standard protocol provided by the manufacturer. Subsequently, residual supernatants or GDs were thoroughly siphoned off and the metabolic activity of the remaining gill cells was assessed using a cell viability assay kit (CellTiter-Blue©, Promega, G8080) following the standard protocol provided by the manufacturer. Both assays were analysed fluorometrically using a cell-imaging multi-mode reader (Cytation 3 Cell Imaging Multi-Mode Reader, BiotekR). All data presented encompass a minimum of four biological gill cell line replicates, and four to five varying concentrations of *A. pseudogonyaulax* supernatants or four concentrations of GDA, GDA-sa or GDA + GDA-sa. Each supernatant or GD concentration comprised of three to six technical replicates during each biological gill cell replicate.

Effects of *Alexandrium* spp. and goniodomins on the cryptophyte *R. salina*

The lytic activity of supernatants, collected by centrifugation of exponentially grown *A. monilatum* or *A. pseudogonyaulax* cultures, diluted with K/2-medium, or purified GDs (GDA, GDB, GDA-sa and GDA + GDA-sa) were estimated using a *R. salina* lysis assay described in detail by Tillmann et al. (2009). Briefly, triplicate *R. salina* cultures were subjected to varying concentrations of supernatants (equivalent to 3– approximately 3000 cells mL^{-1}) or GDs (0.2–180 pg μL^{-1}) for 3, 24 or 48 hours including two sets of controls (*R. salina* in K/2-medium (salinity of 25) including or not 0.1 % of methanol) with a total bioassay volume of 4 mL. After the incubation period at 20 °C in the dark, subsamples were fixed with Lugol's solution (2 % final concentration) and the number of intact *R. salina* were determined by inverted microscopic counts. EC_{50}-concentrations, i.e. the effective concentration at which the half-maximal response is obtained (Lakshmanan et al. 2022), were estimated by fitting the data points, normalized to the control, with a dose response curve (DRC).

Data Analysis

All statistical analyses and plotting of data were performed using the R 4.1.2 software (R Core Team 2021). First, analysed data were checked for equal variances (homoscedasticity) by a Levene's test, package rstatix v 0.7.2 (Kassambara 2023a), and for normal distribution by a Shapiro Wilk test and by visual observation of a histogram. In addition, common data transformations, such as inversion, logarithmic conversion, and square root extraction, were applied if they resulted in improved normality. If sample sizes were bigger than five, outliers were detected and removed through application of a Dixon test (Komsta 2022). For single-factor designs, data was analysed by a one-way ANOVA (Kassambara 2023a) followed by a Tukey honest significance difference (HSD) post hoc test after rejection of the null hypothesis of the ANOVA ($\alpha = 0.05$), if normality and homoscedasticity were fulfilled. If one criterion was not fulfilled, data was analysed by a non-parametric Kruskal-Wallis test (Kassambara 2023a) followed by a Conover Iman post hoc test after rejection of the null hypothesis. Obtained p-values were adjusted according to Benjamini & Hochberg (Benjamini and Hochberg 1995). Two-factor designs, typically involving one factor repeatedly measured over time, were analysed by a repeated measures ANOVA (rmANOVA) with time as a dependent and the second factor as an independent variable (Kassambara 2023a). Dose-response curves (DRCs) were generated with the drc-package (Ritz et al. 2015). All plots were generated with ggplot2 (Wickham et al. 2019) with the help of extrafont (Chang 2023), ggthemes (Arnold 2021), ggtext (Wilke and Wiernik 2022), ggprism (Dawson 2022), ggpubr (Kassambara 2023b) and patchwork (Pedersen 2024). General data transformations were performed within the tidyverse (Wickham et al. 2019). Packages were managed with pacman (Rinker and Kurkiewicz 2018) and package citations were generated with the grateful package (Rodrigues-Sanchez and Jackson 2023).

Results

Effects of *A. pseudogonyaulax* on *Polykrikos kofoidii*

When *P. kofoidii* was offered monoalgal prey consisting of either *A. pseudogonyaulax* or *A. catenella* (Fig. 1), both algae were ingested, however significant differences in ingestion rates ($F_{1,7} = 45.9$, $p < .001$) and cell densities ($F_{2,9} = 225.6$, $p < .001$) of *P. kofoidii* were found (Möller et al. 2024b). After 48 hours, all three treatments showed significant differences in cell densities (pairwise t-tests, $p < .01$) underlining that the *P. kofoidii* population propagated when feeding on *A. catenella* yet declined when feeding on *A. pseudogonyaulax* (Fig. 1). Notably, the *P. kofoidii* population remained stable in the 'hunger control'. In addition, pairwise comparisons of the ingestion rates confirmed, that *A. catenella* was ingested 2–25 times more (pairwise-t-test, $p < .001$) than *A. pseudogonyaulax* throughout the experiment (Fig. 1). In another experiment, *P. kofoidii* was offered a mixture of either *A. catenella* and *A. pseudogonyaulax* or of *A. limii* and *A. pseudogonyaulax*. Over the course of the first six hours, the ratios of ingested prey were 1.5–5.1 for the combination of *A. catenella* and *A. pseudogonyaulax*, while they were 0.8–1.1 for the combination of *A. limii* and *A. pseudogonyaulax* (Table 1). Moreover, for the combination of *A. catenella* and *A. pseudogonyaulax*, the former got ingested significantly more often after one and three hours ($H_1 = 8.3$–8.4, $p < .01$), but not after six hours ($H_1 = 3.2$, $p > .05$). No significant differences for the other prey combination were found ($H_1 = 0.1$–2.7, $p > .05$) at any time points.

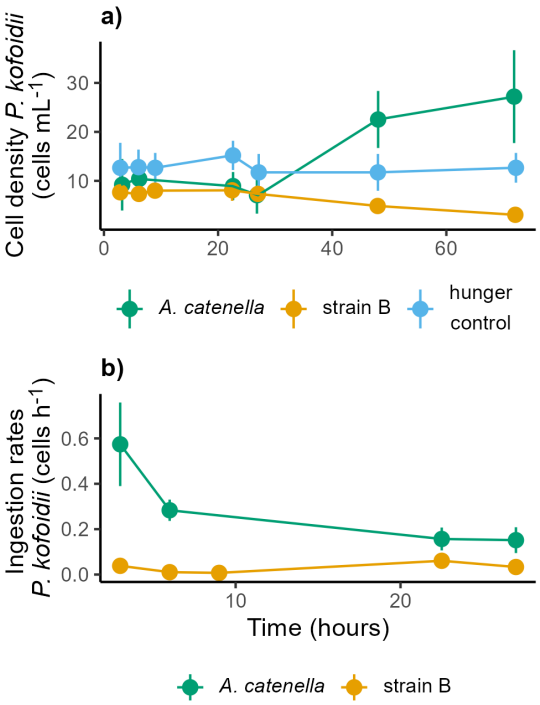

Fig. 1: a) *Cell densities (cells mL^{-1}) over time of P. kofoidii feeding on monoalgal prey (A. catenella or A. pseudogonyaulax strain B) including an additional hunger control; points represent mean with 95 % confidence intervals from 3–6 biological replicates;* ***b)*** *corresponding ingestion rates (cells h^{-1}), only assessed during the first 27 hours.*

Table 1: *Mean ingested prey cells with 95 % confidence intervals of* P. kofoidii *exposed to prey mixtures consisting of* A. pseudogonyaulax *and* A. catenella *or* A. limii.

Prey 1	Prey 2	Time (h)	Mean ingested prey 1 (cells copepod^{-1})	Mean ingested prey 2 (cells copepod^{-1})	Prey 2 : Prey 1 ratio
A. pseudogonyaulax strain B	*A. catenella* strain Alex5	1	0.38 ± 0.09	0.66 ± 0.07	1.74
		3	0.10 ± 0.03	0.52 ± 0.08	5.20
		6	0.10 ± 0.03	0.15 ± 0.04	1.40
	A. limii strain Atay99Shio-02	1	0.23 ± 0.10	0.26 ± 0.12	1.13
		3	0.30 ± 0.05	0.25 ± 0.16	0.83
		6	0.12 ± 0.01	0.14 ± 0.01	1.17

Effects of *A. pseudogonyaulax* on *A. tonsa*

All three *A. pseudogonyaulax* strains (A, B, C) were consumed by all three life-stages of Acartia tonsa examined in this experiment, namely N_4-nauplii, C_4-copepodites and adult copepods (Möller et al. 2024c). Ingestion rates increased with progressing life-stages of *A. tonsa* and showed minimal variation between the three examined strains of *A. pseudogonyaulax* (Fig. 2).

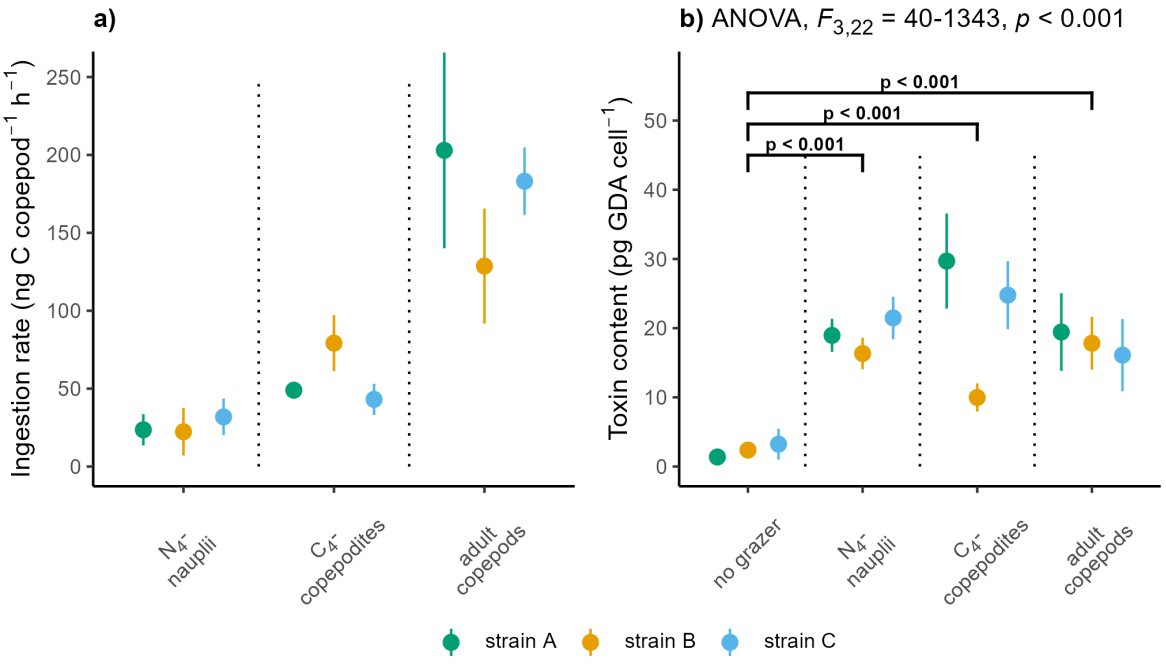

Fig. 2: a) *Ingestion rates (ng C copepod^{-1} h^{-1}) of three developmental stages of* A. tonsa *(N_4-nauplii, C_4-copepodites, adult copepods) feeding on* A. pseudogonyaulax *strains A-C and* ***b)*** *corresponding toxin content (pg GDA cell^{-1}) of* A. pseudogonyaulax *both with 95 % confidence intervals from 3 to 10 replicates; ingestion rates were calculated according to Frost et al. and converted to ingested carbon according to the carbon content of* A. pseudogonyaulax.

The intracellular toxin contents of *A. pseudogonyaulax* in this experiment ranged from 3.6×10^{-6} to 2.1×10^{-4} mol GDA mol C^{-1} or 1.3 to 45.7 GDA pg $cell^{-1}$ (Fig. 2). At the end of the experiments, significant differences within the toxin quotas (GDA $cell^{-1}$) of all three *A. pseudogonyaulax* strains were found (A: $F_{3,22} = 1343.2$, $p < .001$; B: $F_{3,20} = 95.5$, $p < .001$; C: $F_{3,24} = 40.2$, $p < .001$), whereby the toxin contents of each *A. pseudogonyaulax* strain were significantly ($p < .001$) higher in the presence of *A. tonsa* than in the control. The initial experiment, designed to investigate the influence of *A. pseudogonyaulax* on the development of juvenile *A. tonsa*, was conducted for a period of 10 days. However, the experiment yielded a > 98 % mortality rate and only small fragments of the exoskeleton of *A. tonsa* were detected (Table 2). In contrast, approximately 80 % of *A. tonsa* reached stage C_4 after 10 days when fed with *R. salina* (Table 2). Since the experiment was only sampled at the end, the precise time of death of *A. tonsa* exposed to *A. pseudogonyaulax* could not be determined. Consequently, the experiment was repeated under identical conditions with a higher sampling frequency. Qualitative observations clearly depicted a gradual decline in the mobility of nauplii exposed to *A. pseudogonyaulax* and several individuals getting stuck in the mucus produced by *A. pseudogonyaulax*. Finally, nauplii perished after three to four days. Significant differences ($F_{3,20} = 20.8$, $p < .001$) in the hatching success of *A. tonsa* eggs exposed to the supernatant of three strains of *A. pseudogonyaulax* were found compared to the control (Fig. 3). Subsequent post hoc analyses revealed that exposure to the supernatants of *A. pseudogonyaulax* provoked a significant reduction (> 20 %, Tukey HSD, $p < .001$) of egg hatching success of *A. tonsa*. No significant differences in hatching success between the three examined strains of *A. pseudogonyaulax* were identified.

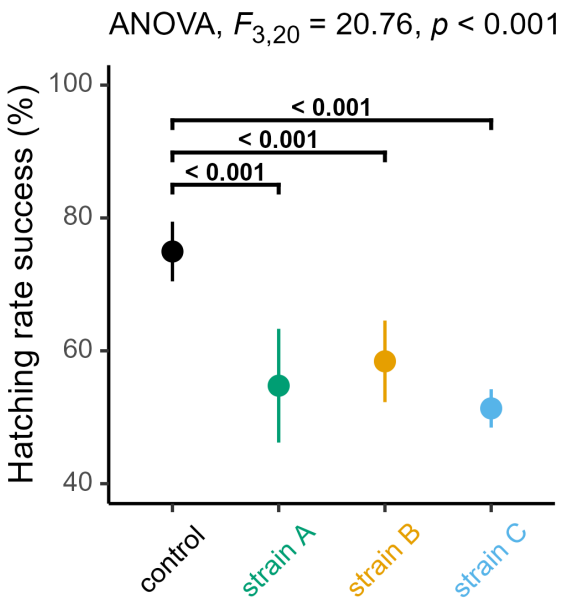

Fig. 3: *Mean hatching rate success with 95 % confidence intervals of* A. tonsa *eggs exposed to the cell-free supernatant of* A. pseudogonyaulax *strains A-C with the control being filtered seawater, test statistics and pairwise comparisons correspond to an ANOVA test and to selected Tukey Honest Significant Differences test results, respectively.*

Table 2: *Developmental stages of* A. tonsa *after exposure to* A. pseudogonyaulax *for 10 days in comparison to* R. salina*; values represent mean with 95 % confidence intervals of six replicates; note that in the A. pseudogonyaulax treatments only single organisms were left in each treatment and thus percentages of the developmental stages and confidence intervals have little relevance; C = copepodite.*

Prey	Developmental stage of *A. tonsa* (%)					Total number *A. tonsa* (#)
	C_1	C_2	C_3	C_4	C_5	
R. salina	/	/	12.2 ± 10.8	77.7 ± 7.0	10.1 ± 6.4	128, 103, 31
A. pseudogonyaulax strain A	33.3 ± 75.0	33.3 ± 75.0	33.3 ± 75.0	/	/	3, 2, 1, 1 0, 0
A. pseudogonyaulax strain B	20.0 ± 55.5	60.0 ± 68.0	/	/	/	1, 1, 1, 1, 1, 0
A. pseudogonyaulax strain C	/	/	/	/	/	/

Toxin cell content of *A. pseudogonyaulax* after exposure to copepodamides

The copepodamide blend of *C. finmarchicus* showed a significant, albeit small, effect ($F_{6,28} = 2.5$, $p < .05$, Fig. 4) on the cellular toxin content of *A. pseudogonyaulax*. However, subsequent post hoc tests did not detect any significant differences between cells exposed to copepodamides and the control. Similarly, the crude copepodamide extract of *A. tonsa* did not result in an increased toxin content of *A. pseudogonyaulax* (Fig. 4).

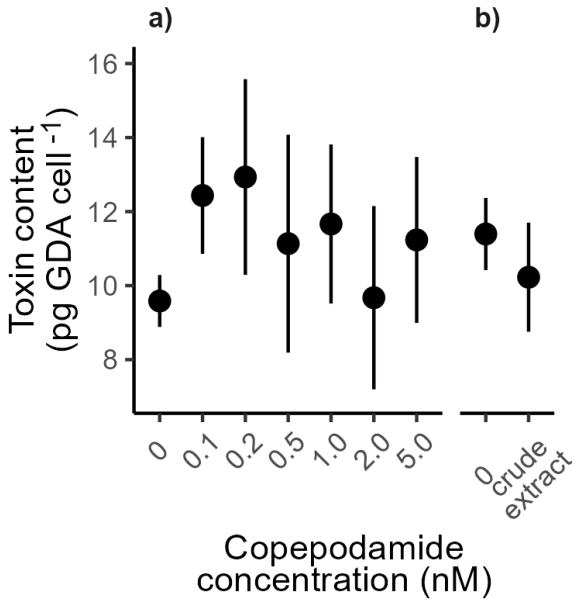

Fig. 4: *Toxin content (pg GDA cell$_{-1}$) of* A. pseudogonyaulax *cells after exposure to copepodamides from* ***a)*** C. finmarchicus *or* ***b)*** A. tonsa*; points represent mean with 95 % confidence intervals from six replicates.*

Effects of *A. pseudogonyaulax* and goniodomins on RTgill-W1 fish gill cells

Supernatants of *A. pseudogonyaulax* and purified GDs both exhibited cytotoxic and lytic effects on the RTgill-W1 cell line varying greatly in their effect as visualized in the dose response curves (DRCs, Fig. 5, Möller et al. 2024d). Cell-free supernatants of *A. pseudogonyaulax*, equivalent to high cell densities (> 4,000 cells mL^{-1}), led to a total loss of gill cell viability and approximately 30 % gill cell lysis (Table 3). In contrast, the highest evaluated GDA concentration (500 pg μL^{-1}) only reduced gill cell viability by 30 %, while GDA-sa did not induce any viability loss in the applied concentration range. The lytic activity of GDA was negligible and GDA-sa exhibited no lytic effect. In addition, no synergistic interaction between GDA and GDA-sa was observed. The cytotoxicity and lytic activity of a mixture of GDA and GDA-sa was depressed in comparison to pure GDA. EC_{50}-values of the cytotoxic and lytic activity ranged from 1,246–1,360 cells mL^{-1} to 2,003–2,178 cells mL^{-1}, respectively, for *A. pseudogonyaulax* and were 225 and 282 pg μL^{-1} for GDA (Table 3) for the cytotoxic and lytic activity. No intraspecific variability in cytotoxic or lytic effects of the three *A. pseudogonyaulax* strains were found.

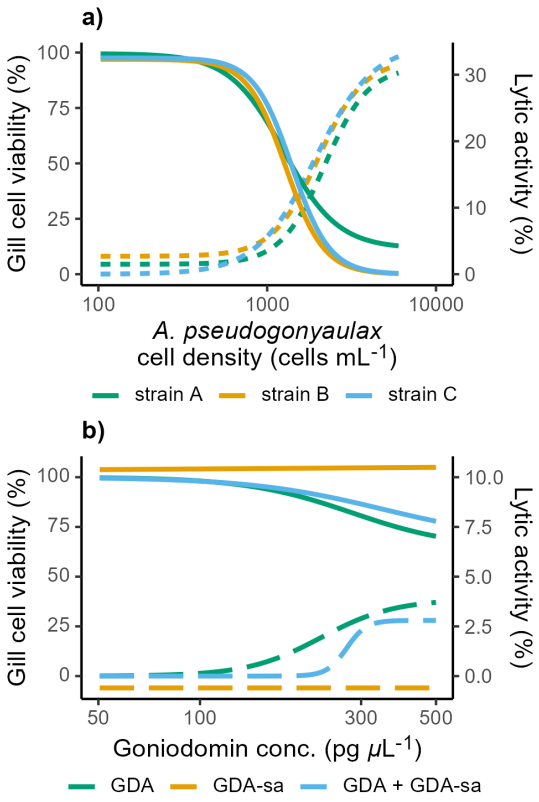

Fig. 5: *Dose response curves of the gill cell viability (%, left y-axis) and lytic activity (%, right y-axis) of RTgill-W1 fish cells conducted with* ***a)*** *cell-free supernatants of A. pseudogonyaulax strains A-C and* ***b)*** *purified GDs; all data presented encompass a minimum of four biological gill cell line replicates, including four to five concentrations of A. pseudogonyaulax supernatants and four concentrations of GDA, GDA-sa or GDA + GDA-sa each comprised of three to six technical replicates.*

Table 3: *Meta-data and EC_{50}-values of the RTgill-W1 and* R. salina *bioassays with supernatants of* A. pseudogonyaulax *and* A. monilatum*, respectively, as well as purified goniodomins.*

Treatment	Strain/substance	EC_{50} viability / lytic activity	Time (h)	Total loss of viability?	Assay
cell-free supernatant of *A. pseudogonyaulax*	strain A	1,254 / 2,190 cells mL^{-1}	24	yes	RTgill-W1
	strain B	1,286 / 2,010 cells mL^{-1}			
	strain C	1,390 / 1,921 cells mL^{-1}			
pure substance dissolved in K/2-medium	GDA	225 / 228 pg μL^{-1}		no	
	GDA-sa	no effect			
	GDA + GDA-sa	294 / 272 pg μL^{-1}			
cell-free supernatant of *A. monilatum*	strain YRK2007	60-454 cells mL^{-1}	3		*R. salina*
	strain YRK2007	40-322 cells mL^{-1}	24		
cell-free supernatant of *A. pseudogonyaulax*	strain A	1,229 cells mL^{-1}	24	yes	
	strain B	1,260 cells mL^{-1}	24		
	strain C	520 cells mL^{-1}	3		
	strain C	240-461 cells mL^{-1}	24		
pure substance dissolved in K/2-medium	GDA GDA-sa GDA + GDA-sa GDB GDC	no effect	3, 24, 48	no	

Effects of *Alexandrium* spp. and goniodomins on the cryptophyte *R. salina*

Rhodomonas salina cells exposed to supernatants of *A. pseudogonyaulax* or *A. monilatum* were completely lysed after 3 or 24 hours (Fig. 6, Table 3, Möller et al. 2024e). In stark contrast, goniodomin (GDA, GDA-sa, GDB, GDC) concentrations up to 180 pg μL^{-1} did not result in any measurable cell lysis after 3, 24, or 48 hours (Fig. 6, Table 3, Möller et al. 2024f). In addition, no synergistic interaction between GDA and GDA-sa was observed. *A. pseudogonyaulax* strain C had a stronger lytic effect (lower EC_{50}, Table 3) than strain A and strain B (Fig. 6). For both strains, for which two independent dose-response curves were recorded (*A. pseudogonyaulax* strain C, and *A. monilatum*), there was substantial intra-strain variability (Fig. 6, Table 3). EC_{50}-values (Table 3) for *A. pseudogonyaulax* and *A. monilatum* ranged from 240–1,260 and 40–454 cells mL^{-1}, respectively.

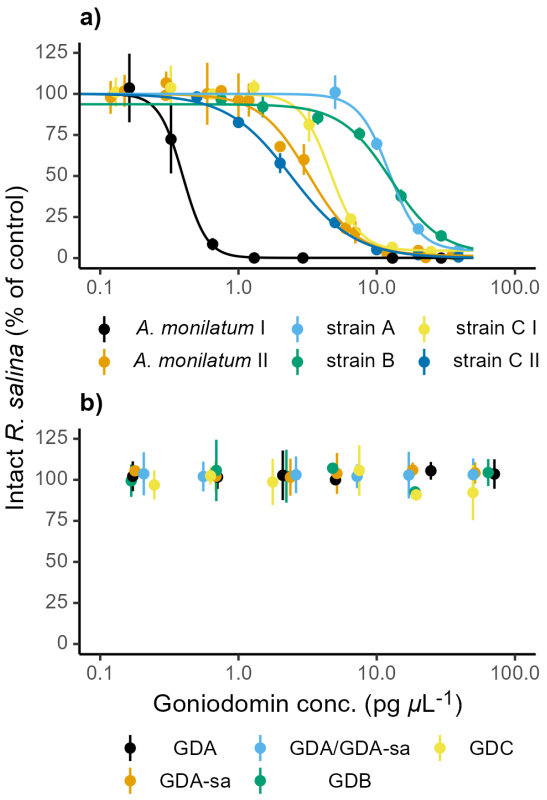

Fig. 6: *Dose response curves of the* R. salina *bioassays over 24 hours with* ***a)*** *cell-free supernatants of* A. monilatum *strain YRK2007 and* A. pseudogonyaulax *strains A-C and* ***b)*** *purified goniodomins; points represent mean with 95 % confidence intervals from three replicates, while lines represent modelled dose-response curves; goniodomin concentrations in cell-free supernatants correspond to the maximum value if all intracellular toxins were excreted, i.e. if all cells died; corresponding EC_{50}-values are listed in table 3.*

Discussion

This study revealed adverse effects of *A. pseudogonyaulax* on four marine trophic levels, including algae, microzooplankton, mesozooplankton, and fish gill cells, ultimately resulting in enhanced mortality and cell lysis. Altogether, the results of this study indicate that *A. pseudogonyaulax* may pose a threat to marine ecosystems and that harmful effects are mainly driven by BECs and not by GDs.

Protistan grazers are unlikely to control *A. pseudogonyaulax* bloom development

This study investigated the effects of *A. pseudogonyaulax* on the heterotroph protist *P. kofoidii*, which coexists with *A. pseudogonyaulax* across Northern European waters. This heterotroph protist is a ubiquitous grazer of mainly dinoflagellates including a number of toxic species (Jeong et al. 2001; Tillmann and Hoppenrath 2013). Hence, *P. kofoidii* can be considered representative of the microzooplankton fraction, which has been shown to be a prominent consumer of primary production (Calbet and Landry 2004). Protistan grazers, such as *P. kofoidii*, possess high ingestion and reproduction rates (Tillmann 2004) making them promising candidates for biotic mitigation of HABs. However, the findings of this study cast doubt on micrograzing as an effective top-down mechanism controlling *A. pseudogonyaulax* blooms. While *A. pseudogonyaulax* was initially ingested by *P. kofoidii*, it could not sustain growth, and cell densities decreased until eventually the population collapsed within three days. Interestingly, starvation of *P. kofoidii* displayed a weaker negative effect on the population density than exposure to *A. pseudogonyaulax* highlighting the detrimental effects of *A. pseudogonyaulax* on this microzooplankton grazer. Cell lysis of *P. kofoidii* has previously been shown when co-incubated with lytic *A. catenella* (Tillmann et al. 2008; Kim et al. 2016) and various other *Alexandrium* species (Kang et al. 2018). Microscopic observations indicated that exposure to *A. pseudogonyaulax* initially caused a decline in the swimming velocity of *P. kofoidii*, which may lead to reduced prey encounter and thus ingestion rates (Kiørboe et al. 1996). *Polykrikos kofoidii* avoided ingestion of *A. pseudogonyaulax* to some extent and exhibited a preference for *A. catenella*, which is supported by previous studies demonstrating selective feeding capabilities of *P. kofoidii* on mixtures of red-tide dinoflagellates (Jeong et al. 2001). Since *A. catenella* strain Alex5 produces PSTs, but not BECs, these findings also support the literature evidence that PSTs are not primarily responsible for adverse effects towards protistan grazers (Flores et al. 2012). However, no selective feeding of *P. kofoidii* was observed when it was offered *A. pseudogonyaulax* and *A. limii*. This might suggest that GDs are involved in prey rejection as both strains produce GDs. However, compounds other than lytic BECs might have cause reduced grazing on *A. limii*, as it has been pre-

viously shown for *A. tonsa*, where feeding behaviour responses were not related to lytic activity or PST content of various *Alexandrium* strains (Xu et al. 2017). Altogether, the findings of the present study suggest that although micrograzers, such as *P. kofoidii*, can ingest HAB species, the subsequent negative impacts on their fitness (swimming and survival) may prevent them from exerting a significant top-down control on *A. pseudogonyaulax*. Instead, *P. kofoidii* may even alter phytoplankton species succession patterns by selectively grazing on faster growing non-toxic microalgae, ultimately favouring the development of harmful blooms.

A. pseudogonyaulax impairs hatching and subsequent development of *A. tonsa*

The intracellular GDA cell quota of all three *A. pseudogonyaulax* strains were greatly enhanced after one day of co-incubation with *A. tonsa*, suggesting that GD production (Van Donk et al. 2011) may be upregulated upon grazer signals. However, exposure of *A. pseudogonyaulax* to varying concentrations of copepodamides from *C. finmarchicus* did not increase cellular toxin content. While copepods may have specific copepodamide profiles (Grebner et al. 2019), the crude copepodamide extract of *A. tonsa*, produced from the same mass cultures that were used for the predator-prey interaction experiments in this study, did not result in an increased toxin content of *A. pseudogonyaulax*. The reasons for the high GD cell quota after the first experiment could thus not be fully clarified. On the one hand, cell quota estimates after grazing may be biased when a large share of toxins may be associated with faecal material and cell debris and thus not only to the intact cells. However, the total toxin content at the start of the experiment, i.e. the intracellular toxin content of the control of *A. pseudogonyaulax* multiplied by the total number of cells, was 2–19 times lower than total toxins in grazing treatments at the end of the experiment supporting the assumption of an upregulated toxin production. On the other hand, toxin production in *A. pseudogonyaulax* may have been stimulated by specific chemical signatures released by conspecific cells upon grazing, and further studies are needed for clarification. Major increases of toxin production by copepodamides has already been shown for other members of the *Alexandrium* genus and for a variety of other toxic phytoplankton species (Selander et al. 2012, 2015; Lundholm et al. 2018; Griffin et al. 2019; Ryderheim et al. 2021), but all inflicting paralytic or amnesic shellfish poisoning toxins are not belonging to the class of polyketide phycotoxins. All examined life-stages of *A. tonsa* (N_4-nauplii, C_4-copepodites, adult copepods) ingested all three *A. pseudogonyaulax* strains tested. Ingestion rates were in a similar range as those previously published for *A. tonsa* feeding on a variety of organisms (Stoecker and Egloff 1987; Kleppel et al. 1998; Thor 2002; Broglio et al. 2003; Colin and Dam 2005; Besiktepe and Dam 2020). Although, this hints towards *A. pseudogonyaulax* being a nutritionally adequate

food organism for *A. tonsa*, here the ingestion of toxic cells resulted in adverse effects on fitness. It is well established that phycotoxins can have adverse effects on zooplankton including enhanced mortality or sublethal effects, such as reductions in food intake or fecundity (Frangópulos et al. 2000; Colin and Dam 2005; Vasconcelos et al. 2010). However, previously studied predator-prey interactions of copepods have often been restricted to copepodites or adult copepods even though juvenile nauplii are the most vulnerable life-stages towards environmental stressors (Hopp and Maier 2005). Exposing juvenile N_2-nauplii to *A. pseudogonyaulax* was usually lethal with almost no nauplii reaching the copepodite life-stage. Similarly, Blanda et al. (2016) demonstrated a three-fold increase of nauplii mortality exposed to just 20 cells mL^{-1} of *A. pseudogonyaulax*, however 100 cells mL^{-1} did not lead to enhanced nauplii mortality. Notably, the partial or total cell lysis of nauplii at the end of the experiment is strong evidence for a dominant role of BECs of *A. pseudogonyaulax* in nauplii impairment. The resulting increase of released organic carbon and other nutrients may compensate the energetic cost of BEC production and release, and potentially create a positive feedback loop capable of sustaining HABs (Kang and Gobler 2023). Early sublethal effects on nauplii included a reduced swimming velocity which may reduce ingestion rates (Kiørboe et al. 1996). In addition, nauplii got caught in the mucus produced by *A. pseudogonyaulax*. Blossom et al. (2012) demonstrated that *A. pseudogonyaulax* produces mucus traps to capture prey. These traps are regularly abandoned (Blossom et al. 2012) and upon formation of larger mucus traps, it is plausible that these can also act as a deterrent towards grazers or hinder prey assimilation. Sublethal effects further included reduced egg hatching rates when eggs of *A. tonsa* were subjected to the supernatant of dense *A. pseudogonyaulax* cultures. Previous studies have shown deleterious effects of toxins on the hatching rate success of copepod eggs when adults were fed toxic or nutritional inadequate algae or exposed to extracts of toxic algae (reviewed in: Turner 2014). However, as already pointed out by Jónasdóttir et al. (1998), utilizing extracts of algae has a low ecological relevance because extracts may contain different substances and/or different concentrations compared to supernatants and the target organism may never be exposed to these substances in situ. Overall, the findings of this study demonstrate that exudates of toxic microalgae possess the potential to impair hatching and subsequent larval development of copepods.

Loss of metabolic activity and lysis of fish gill cells were likely driven by BECs and not by GDs

This study identified a complete loss of metabolic activity of gill cells exposed to cell-free supernatants of *A. pseudogonyaulax* compared to only marginal effects after exposure to purified GDs. Consequently, the findings strongly suggest that ichthyotoxicity of *A. pseudogonyaulax* is primarily driven by the BECs and not by the GDs. Also, the GD

concentrations in the cell-free supernatants (≈ 1 pg μL^{-1}) were two magnitudes lower than the highest tested GD concentration of 180 pg μL^{-1}. Recently, Tainter et al. (2020) showed that GDA forms strong complexes with alkali ions, especially with potassium, suggesting that ionophoric properties may be involved in its toxicity. These properties hint towards a similar mechanism of toxicity as has been described for karlotoxins, which cause fish kills by creating membrane pores leading to leakage of electrolytes or other small molecules (Deeds et al. 2015). However, membrane pores may also enable the influx of molecules and it can thus not be ruled out that BECs and GDs are acting synergistically, whereby the former creates membrane pores enabling the latter to infiltrate. Increasing the GD concentration in cell-free supernatants should lead to enhanced toxic effects, but preliminary results do not support this assumption (data not shown). Nevertheless, ichthyotoxic properties in this study cannot be solely explained through the presence of GDs, which did not lead to total loss of gill cell viability. In addition, the lytic activity of GDs was marginal in comparison to the BEC-containing supernatants and cannot explain the observed lytic effects towards RTgill-W1. However, it needs to be considered that marginal adverse effects of GDs may add up in the long run or make the target organism more susceptible to other stressors. Altogether, these results suggest that the toxicity of *A. pseudogonyaulax* on fish is primarily driven by the BECs or by a synergistic action of BECs and GDs, but not exclusively by GDs.

Lytic effect of *Alexandrium* spp. on microalgae was likely driven by BECs.

In the bioassays, cell-free supernatants of *A. monilatum* and *A. pseudogonyaulax* resulted in total cell lysis of *R. salina* already after 3 hours. In contrast, exposure to various GD congeners with concentrations up to 180 pg μL^{-1} induced no lysis after up to 48 hours. The dissolved GD concentrations of the cell-free supernatants (≈ 1 pg μL^{-1}) were up to two orders of magnitudes lower than the highest tested GD concentrations. Hence, the results of this study suggest that the toxicity of *A. pseudogonyaulax* towards microalgae is primarily driven by the BECs, but not by the GD congeners. *Alexandrium* species are well known to produce extracellular toxins that can have immobilizing effects on other protists (Tillmann and John 2002) and *A. pseudogonyaulax* has been shown before to act lytic towards some prey species (Blossom et al. 2012). This study presents the first dose-response curves of the lytic activity of *A. pseudogonyaulax*. Corresponding EC_{50}-values of 240–1,260 cells mL^{-1} were well within the middle of the broad range of lytic activity reported for other *Alexandrium* species (Tillmann et al. 2009, 2020; Hakanen et al. 2012; Van de Waal et al. 2015; Long et al. 2018; Blossom et al. 2019). However, the intra-strain variability in lytic activity of *A. monilatum* and *A. pseudogonyaulax* as quantified on two separate occasions underline the limited mechanistic understanding of factors modulating

the production of lytic BECs.

Conclusions

This study revealed adverse effects of *A. pseudogonyaulax* across four marine trophic levels, including phytoplankton, microzooplankton and mesozooplankton, and fish gill cells. These effects ultimately resulted in enhanced cell lysis and mortality highlighting the potential harm of this dinoflagellate to marine ecosystems. Furthermore, sublethal effects on health parameters of zooplankton, such as impaired mobility and reproduction, were observed. Notably, supernatants of *A. pseudogonyaulax* exhibited much stronger ichthyotoxic properties than purified GDs, suggesting that BECs are the key driver of the fish toxicity of GD-producing organisms. While increased toxin contents of *A. pseudogonyaulax* in the presence of *A. tonsa* were detected, no toxin induction after exposure to copepodamides of *C. finmarchicus* or *A. tonsa* was observed. Although a definitive conclusion regarding the toxin induction of GDs cannot be drawn, all other toxicity estimations suggest a limited significance of GDs for the overall toxicity of *A. pseudogonyaulax* towards the marine targets tested here. Nonetheless, further research into the toxicity of BECs and the various GD congeners, as well as their potential synergistic interactions, is necessary to comprehensively assess the risks posed by *A. pseudogonyaulax*. The findings of this study may aid in modelling studies and may thus contribute to a better understanding of the establishment and expansion of *A. pseudogonyaulax* in Northern European waters. Finally, the significant contribution of BECs to the overall toxicity highlight the importance of investigating *Alexandrium* species not only for the production of phycotoxins, but also of other BECs.

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Declaration of Generative AI and AI-assisted technologies in the writing process

During the preparation of this work the main author used ChatGPT 3.5 from OpenAI in order to correct the writing with respect to language, choice of words, synonyms and sentence structure. After using this tool, the main author reviewed and edited the content as needed and takes full responsibility for the content of the publication.

Author Contribution Statement

K.M., U.T., E. V. B.K, F.K., and C.L.M. conceived of and designed the study. K.M. wrote the original draft while all other authors participated in reviewing and editing the manuscript. K.M, U.T., and M.P. conducted the experiments and analysed the data. T.M.H. provided purified goniodomins.

Data availability statement

All data needed to evaluate the conclusions in the paper are present in the paper and/or are freely available from the PANGAEA data repository. Datasets on PANGAEA are referenced within the results. All code can be found online on GitHub: https://github.com/KristofM854 (accessed on 10 July 2024).

Conflict of interests

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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Chapter 4

Publication III

Time series analysis of the expansion of Alexandrium pseudogonyaulax across Northern European waters

Time series analysis of the expansion of *Alexandrium pseudogonyaulax* across Northern European waters

Kristof Möller[1], Hans Jakobsen[2], Annette Engesmo[3], Bengt Karlson[4], Jacob Carstensen[2]

[1] Alfred-Wegener-Institut Helmholtz-Zentrum für Polar- und Meeresforschung, Bremerhaven, Germany
[2] Aarhus University, Roskilde, Denmark.
[3] Norwegian Institute for Water Research, Oslo, Norwegen.
[4] Swedish Meteorological and Hydrological Institute, Stockholm, Sweden.

*Correspondence: kristof-moeller@outlook.de

Running head: Expansion of *A. pseudogonyaulax*

Abstract

The harmful dinoflagellate *Alexandrium pseudogonyaulax* is producing goniodomins (GDs) and structurally undefined bioactive extracellular compounds (BECs) that have been associated with the mortality of marine organisms, including fish. In recent decades *A. pseudogonyaulax* has been appearing in increasing frequency and abundance across Northern Europe coinciding with a decrease of the previously dominating *Alexandrium ostenfeldii* and *Alexandrium catenella*. Originally constrained to the Kattegat region, this potentially fish-killing dinoflagellate has expanded eastwards into the Baltic Sea with occurrences at salinities of 7. The seasonality of *A. pseudogonyaulax* is highly conserved across different water bodies, occurring primarily between June and September, but it appears slightly earlier in estuaries than open waters. *A. pseudogonyaulax* likely benefits from high temperatures and high dissolved organic nutrient concentrations. Although cell densities seldom exceed 10^4 cells L^{-1}, damages to fisheries and aquacultures remain conceivable. Altogether, time series data from Germany, Sweden, Denmark and Norway suggest that this dinoflagellate has established itself as regular component of the microalgae community across Northern Europe and likely increases its contribution in the future.

Introduction

Microalgal blooms may be beneficial to marine ecosystems by fixing carbon at the base of the food chain and supporting fisheries. However, Harmful Algal Blooms (HABs) that adversely affect ecosystems or humans represent a growing global concern, affecting coastal nations across the world (D. M. Anderson et al. 2012b, Gobler 2020). While there is no evidence that HAB events are globally increasing in frequency and abundance, HAB events in Europe, and especially in the Baltic Sea, have increased in the last decades (G. M. Hallegraeff et al. 2021, Dai et al. 2023). These blooms, depending on the blooming species, cause a variety of environmental and human health effects. Health effects may be caused directly through exposure to the marine toxins, e.g. while swimming or via aerosols, or indirectly through the consumption of contaminated shellfish or fish (Berdalet et al. 2016). The impacts of HABs further encompass economic losses in fisheries and the tourism and recreation sector. Eutrophication, i.e. the excessive enrichment of nutrients, is widely associated with increases in the frequency and severity of HABs (Heisler et al. 2008, Davidson et al. 2014). Recently, climate driven changes have also been implicated with alterations in the frequency, distribution and intensity of HABs (Gobler 2020, Dai et al. 2023). These alterations are driven by multiple environmental changes such as ocean warming (Gobler et al. 2017), acidification (Riebesell et al. 2018) and deoxygenation (Breitburg et al. 2018). For instance, as ocean temperatures rise, HABs are assumed to expand their range to the poles migrating to new ecosystems (Gobler et al. 2017, Kléparski et al. 2024) and to prolong their blooming period (Hjerne et al. 2019, Viitasalo and Bonsdorff 2022). Consequently, the microalgal growing season has been prolonged in the Baltic Sea (Kahru et al. 2016, Wasmund et al. 2019) and the North Sea (Llope et al. 2009), primarily due to increasing sea surface temperatures (SSTs) and increased insolation. Notably, the Baltic Sea and the North Sea were the fastest-warming water bodies worldwide until 2006 (Belkin 2009).

Warmer winters may even delay the spring bloom as shown for the German Bight (Wiltshire and Manly 2004), potentially due to longer persistence of zooplankton grazers in winter and early spring, depressing biomass-build up. Additionally, warming-related sea-ice reduction in the northernmost basins of the Baltic Sea has been associated with increased dinoflagellate abundance during the spring bloom (Klais et al. 2011). Increasing temperatures are also predicted to shift food web structures towards greater heterotroph biomass (O'Connor et al. 2009), and stimulate heterotrophic feeding of phagotrophic mixotrophs (Wilken et al. 2013). Considering the wide range of prey that *A. pseudogonyaulax* can feed on (Blossom et al. 2012, Möller et al. 2024 *forthcoming*), it could be a beneficiary of increasing temperatures. Overfishing is another anthropogenic pressure that alters marine coastal communities (Steneck 1998, Vasas et al. 2007). Field (Frank et al. 2011, Riisgård et al. 2012a) and modelling studies (Lynam et al. 2017) show links

between overfishing, jellyfish blooms and HABs. This indicates that overfishing can create a trophic cascade, which allows jellyfish and planktivorous fish to propagate, which subsequently decrease herbivore populations, which then reduces the grazing pressure on HAB species. Fortunately, evidence suggests that reductions in nutrient loading, i.e. deeutrophication, can directly correlate with decreases in HAB occurrences as observed in the Sea of Japan and the Black Sea (D. M. Anderson et al. 2002). Nevertheless, the potential for HAB events remains and interdisciplinary efforts to comprehend their complex dynamics are needed to mitigate the consequences of HABs.

HAB events in Northern Europe are composed of a variety of HAB producers. Major species include fish-killing haptophytes, such as *Chrysochromulina leadbeateri* or *Prymnesium polylepsis*, and dictyophytes, such as *Pseudochattonella* (B. Karlson et al. 2021). In addition, dinoflagellates responsible for Diarrhetic (DSP) and Paralytic Shellfish Poisoning (PSP) are a regular component of the microalgae community. Evidence suggests that dinoflagellates have generally increased in abundance in the North Sea and Baltic Sea (Hjerne et al. 2019, Edwards et al. 2006). This may suggest that dinoflagellates are coping better with temperature increases, however the temperature response may also be indirectly if e.g. climate warming enhances stratification (Edwards et al. 2006). For instance, increased precipitation and thus fresh-water input is likely to promote haline stratification, which promotes the formation of HABs (Weise et al. 2002). This competitive advantage is due to the motility of many HAB species in comparison to non-motile diatoms (Smayda 1997). In addition, but constrained to the Baltic Sea, low species richness at low salinities may favour the invasion of non-indigenous microalgae due to low competition and unoccupied ecological niches (Paavola et al. 2005).

A prominent invasive microalgal species in Northern European waters is the dinoflagellate *Prorocentrum cordatum*, which appears in increasing abundancy in the German Bight and in Danish coastal waters and spread north-eastwards into the Baltic Sea (Telesh et al. 2016) after its first detection in the Kattegat in 1981. However, first high-density blooms of *P. cordatum* were first registered at the end of the 90s hinting towards competition with other closely related dinoflagellates (Telesh et al. 2016). Expansion of this dinoflagellate may have been favoured by its wide temperature and salinity tolerance (Tyler and Seliger 1981, Hajdu et al. 2005), low-light adaptation (Harding Jr and Coats 1988), its ability to utilize multiple nitrogen sources (Fan et al. 2003b, Fan et al. 2003a), and its mixotrophic feeding capabilities (Stoecker et al. 1997). The expansion of the non-indigenous *P. cordatum* may be especially harmful as this dinoflagellate has been connected to fish kills (Tango et al. 2005). Other dinoflagellates from the genus *Alexandrium* are among the most prominent HAB species and produce a variety of marine toxins including PSP-toxins, spirolides and/or gymnodimines and goniodomins (e.g. Long et al. 2021). Moreover, *Alexandrium* species are recognized for producing other bioactive extracellular compounds (BECs), whose molecular structures remain largely uncharacterized. These substances have been

primarily characterized by their adverse effects on a wide range of organisms encompassing protists, zooplankton, shellfish and fish (Long et al. 2021). This study investigates the spread of an emerging HAB species, namely the dinoflagellate *A. pseudogonyaulax*, which has recently expanded into Northern European waters (Kremp et al. 2019, B. Karlson et al. 2021). Similarly to *P. cordatum*, *A. pseudogonyaulax* can grow in a wide range of light conditions (Möller et al. 2024) and has a broad temperature and salinity tolerance (Tulatz et al., *in prep.*). This dinoflagellate is a mixotrophic species that utilizes a toxic mucus trap to capture and immobilize prey and only obtains maximum growth rates when feeding (Blossom et al. 2012, Blossom et al. 2017). Its inability to efficiently utilize urea (Möller et al. 2024) suggests that high levels of anthropogenic urea inputs in the Danish Limfjord due to agricultural farming (Glibert et al. 2005, Carlsson et al. 2009) are not the primary driver of the proliferation of *A. pseudogonyaulax*. Exposure to this toxic dinoflagellate has been shown to induce adverse effects on algae, microzooplankton and mesozooplankton, as well as fish gill cells (Möller et al. 2024 *forthcoming*) similarly to the closely related *Alexandrium monilatum*, which is also producing goniodomins and BECs (Sievers 1969, May et al. 2010, Harding et al. 2009). The mucospheres utilized to capture prey may also contribute significantly towards carbon export similarly to *Prorocentrum cf. balticum* (Larsson et al. 2022) or *Phaeocystis antarctica* (Balaguer et al. 2023), although average cell densities of *A. pseudogonyaulax* are much lower.

The aim of this study was to evaluate whether *A. pseudogonyaulax* has established itself across Northern European waters and if the abundance of other *Alexandrium* species simultaneously decreased. Furthermore, this study aimed to evaluate whether calm and stratified water bodies, such as the Danish Limfjord, act as a breeding ground for *A. pseudogonyaulax*, i.e. neighbouring water bodies are injected with *A. pseudogonyaulax* and do not sustain a population. Finally, this study investigates the hypothesis that increases in SST and high concentrations of reduced nitrogen sources (e.g. ammonium or urea) favoured the expansion of *A. pseudogonyaulax*. .

Materials and Methods

Study area

This study analyses the spread of *A. pseudogonyaulax* in the Norwegian Sea, Norwegian part of the Barents Sea, eastern North Sea, Skagerrak, Kattegat and Baltic Sea covering a wide range of hydrographical and meteorological conditions.

The Norwegian Sea is characterized by deep waters with an average depth of 2,000 metres (ICES 2022). Influenced by the warm North Atlantic Current and the cold East Icelandic Current,the Norwegian Sea features strong thermal stratification, high salinities, and nutrient-rich deeper water layers, supporting diverse marine life and crucial spawn-

ing grounds for fish (ICES 2022). In the south, the Norwegian Sea transitions into the temperate, shallow North Sea. The North Sea experiences strong tidal currents and water mixing (Wilde et al. 1992). Consequently, the North Sea is rarely stratified, except for the northern and central areas (Leeuwen et al. 2015). The Skagerrak-Kattegat area, located between Norway, Sweden and Denmark, serves as a transitional zone between the Baltic and North Sea. Notably, the Skagerrak is much deeper ($\approx$ 700 m) than the North Sea and primarily stratified (Leeuwen et al. 2015). The Baltic Sea is a brackish, shallow sea with limited water exchange, which can be divided into several sub-basins, each with distinct salinities and nutrient loads. During winter, the water column is mixed, while in summer extensive areas of the Baltic Sea are permanently stratified (Liblik and U. Lips 2019). In contrast to open oceans, this stratification is driven by temperature and salinity differences. The narrow connection to the North Sea coupled with large freshwater input result in a strong salinity gradient from about 12 in the southwest to 3 in the northeast (Olofsson et al. 2020).

Sample collection

Marine monitoring data were extracted from the Swedish Ocean Archive (SHARK) database provided by the Swedish Meteorological and Hydrological Institute, the ODIN 2 database provided by the Leibnitz Institute for Baltic Sea Research, the national marine monitoring program for the Aquatic and Terrestrial Environments (NOVANA) provided by the National Environmental Research Institute and the mussel harvesting program of the Danish Veterinary and Food Administration in Denmark, as well as from the Norwegian Institute for Water Research (NIVA). Plankton data was generally analysed following standard quantitative phytoplankton techniques as described under Annex C-6 of the HELCOM-COMBINE manual. With respect to *A. pseudogonyaulax* being a large distinct dinoflagellate and high scientific interest in the *Alexandrium* genus, we assume that it was correctly identified if present.

Data analysis

All statistical analyses and plotting of data were performed using the R 4.1.2 software (R Core Team 2021). General data transformations were performed within the tidyverse (Wickham et al. 2019). All plots were generated with ggplot2 (Wickham 2016) with the help of extrafont (Chang 2014), ggthemes (Arnold et al. 2021), ggtext (Wilke and Wiernik 2020), viridisLite (S. Garnier et al. 2018), ggprism (Dawson 2022), ggpubr (Kassambara 2018) and patchwork (Pedersen 2020). Maps were generated with ggOceanMaps (Vihtakari 2022) and ggspatial (Dunnington and B. Thorne 2020). Packages were managed with pacman (Rinker and Kurkiewicz 2018).

Data about the wind conditions where additionally extracted from the national surveillance program for the aquatic environment and nature (NOVANA) of the Danish Meteorological Institute (DMI).

Data pre-processing

Microalgae data was filtered for *Alexandrium* spp. occurring in Northern Europe, specifically *Alexandrium pseudogonyaulax*, *Alexandrium minutum*, *A. ostenfeldii*, *A. tamarense* and unidentified *Alexandrium* spp. Monitoring stations were retained if they conducted sampling every year from 2010 to 2020, allowing for a maximum of three missing years, and if sampled monthly from May throughout September, with at least 10 sampling days each year. Physical and chemical water characteristics were averaged over a water depth of 10 m to correspond to the phytoplankton cell count data. Station data differing by only one day were averaged, which was particularly necessary for the Norwegian dataset due to frequent one-day offsets between phytoplankton counts and physical parameters caused by bad weather conditions. If necessary, latitude and longitude were converted from degrees, minutes, seconds (DMS) to decimal degrees (DD). Finally, stations in proximity, herein defined as stations within 0.025° latitude and longitude ($\approx$ 2.5 km), were combined and averaged on the same dates. Applying the filtering criteria to the dataset resulted in a subset of 54 stations, which are primarily located in the Skagerrak, Kattegat and the south-eastern Baltic Sea (Fig. 1). Additionally, few stations in the Norwegian Sea and the north-eastern Baltic Sea were also retained.

Calculation of the stratification index

Seawater density was calculated using an equation from a UNESCO publication (supporting Information, Eq. 1), with temperature (T) in °C and salinity (S) unitless. Then, a binomial stratification key (stratified yes/no) was introduced by comparing the density difference between the average density of the upper and lower two metres of the water column with a threshold of 1 g cm^{-3}.

Towards understanding the expansion of an emerging HAB species

Assessing temporal changes in the probability of presence of a single species

The objective of this procedure was to evaluate whether the abundance of *A. pseudogonyaulax* in Northern European waters has increased and to determine the seasonality of its presence. In addition, it aimed to assess whether an increase in abundance is restrained to certain water bodies. A binomial probability approach was chosen, wherein the presence of *A. pseudogonyaulax* was categorized as either present or absent, independent of the cell densities. Then a generalized linear model (GLM) was used to analyse the effect

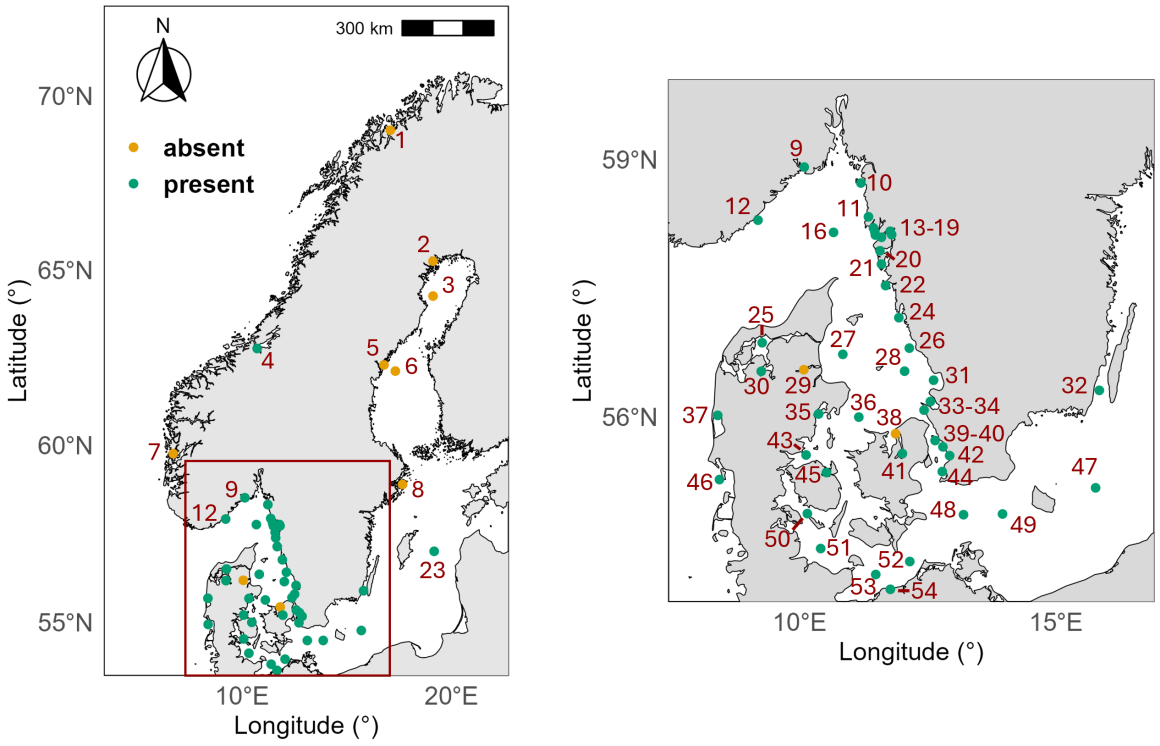

Fig. 1: *Sampling stations that passed all filtering criteria; stations with occurrences of* A. pseudogonyaulax *are shown in green and those without in orange; stations are sequentially numbered from high to low latitudes.*

of time (month or year) on the binomial probability of observing *A. pseudogonyaulax* at a specific station (Eq. 1a), whereby ε denotes the error term. For each station, data was restricted to years after the first appearance of *A. pseudogonyaulax*, respectively. Confidence intervals (95 %) were calculated with the confint function and the coefficients of the GLM were back-calculated to probabilities according to Eq. 1b, yielding the probability of observing *A. pseudogonyaulax* during the respective time-frame. Finally, a logistic regression model was fitted to the yearly probability pattern to assess whether a significant trend or pattern exists in the probability of observing *A. pseudogonyaulax* over the years.

$$\text{logit}(\text{probability}) = \text{factor}(\text{month or year}) + \varepsilon \tag{1a}$$

$$\text{probability} = \frac{e^{\text{coefficient}}}{1 + e^{\text{coefficient}}} \tag{1b}$$

$$\text{logit}(\text{probability}) = f(\text{DOY}) + \varepsilon \tag{1c}$$

The relationship between the probability of observing *A. pseudogonyaulax* and the day of the year (DOY, Eq. 1c) at a specific station was modelled by a generalized additive model (GAM) with a smoothing function featuring a cyclic cubic regression spline with penalized complexity to prevent overfitting, whereby ε denotes the error term. GAMs were only further processed if the smoothing function was statistically significant ($p < .05$).

Knots were manually specified at day 0 and day 365 based on the annual cycle. The predict function was used to estimate the predicted probabilities, which were subsequently back-calculated to probabilities according to Eq. 1b. Key characteristics of the smoothing functions were extracted including the time window during which the probability of observing *A. pseudogonyaulax* exceeds 10 % and the DOY at which the probability was maximal. Next, all stations were categorized into three distinct water bodies, including estuary, coastal and open ocean stations. Then, a one-way ANOVA was conducted to compare the mean key characteristics and to assess whether *A. pseudogonyaulax* blooms occurred earlier, persisted longer, or emerged later in any of the three distinct water bodies. If the one-way ANOVA revealed a significant effect, post hoc tests (Tukey's HSD) were conducted to identify statistically significant different groups.

Analysing which abiotic parameters influence the short-term presence of a single species

The objective of this analysis was to identify environmental conditions influencing the presence of *A. pseudogonyaulax* in the short-term. Seasonal variations in abiotic parameters at each station (temperature, salinity, secchi depth, ammonium, nitrate, phosphate, wind speed, total nitrogen (TN), total phosphorus (TP) and silicate) were modelled as the sum of a sinusoidal and cosinusoidal function according to Eq. 2. In addition to the seasonal variation, this model describes if the presence or absence of *A. pseudogonyaulax* is associated with an anomaly (deviation from the general seasonal variation) in the abiotic parameter indicating a tendency for higher or lower values during the presence or absence of *A. pseudogonyaulax*. All abiotic parameters, except for the temperature, were log-transformed prior to this analysis. Input data was restricted to years after the first year of observation and to May throughout October. This model did not account for potential temporal trends in the specified abiotic parameter. The parameters of this model, amplitude (amp) and phase, were estimated by fitting a nonlinear least squares (NLS) model to the data with initial parameters set at 2 for amplitude and 0 for phase.

$$\text{abiotic parameter} = \text{amp} \cdot \sin\left(\frac{2\pi \cdot \text{doy}}{365} + \text{phase}\right) + \text{amp} \cdot \cos\left(\frac{2\pi \cdot \text{doy}}{365} + \text{phase}\right) \quad (2)$$

Calculating monthly means of abiotic parameters

Monthly means, between May and October, of each abiotic parameter at each station and its potential association with the monthly probability of observing *A. pseudogonyaulax* or *A. ostenfeldii* were modelled in a GLM according to Eq. 3. This model estimates the relationship between the specific abiotic parameter with the month, year and the probability of observing *A. pseudogonyaulax* or *A. ostenfeldii*. Nutrient data was filtered to include observations after 2000, since conditions were mostly stable since then. Accord-

ingly, *Alexandrium* data was restricted to the years after the first year of observation, but only including data after 2000 to match the nutrient data. Finally, monthly means and probabilities were averaged for each station and plotted against each other to assess whether it is more likely to observe *A. pseudogonyaulax* or *A. ostenfeldii* under certain nutrient regimes.

$$\text{abiotic parameter} = \text{factor(month)} + \text{factor(year)} + \text{probability} \tag{3}$$

Modelling binomial probability of presence as a function of temperature

This approach aimed to assess the optimal temperature range for growth of *A. pseudogonyaulax*. The probability of its presence was modelled as a function of temperature with a GAM. The model included a smoothing function featuring a cyclic cubic regression spline with penalized complexity to prevent overfitting. For each station, data was filtered to include observations from May throughout October and stations with fewer than 50 total observations were excluded. For significant models ($p < 0.1$), probabilities and 95 % confidence intervals were predicted within a temperature range of 10–22.5 °C.

Results & Discussion

The toxic dinoflagellate *A. pseudogonyaulax* is appearing in increasing abundance and frequency across Northern Europe (Fig. 2). Firstly identified by Kremp et al. (2019), *A. pseudogonyaulax* suddenly appeared in the Danish Limfjord in 2007, and two years later was the only contributor to the *Alexandrium* community, apparently displacing previously dominating *A. ostenfeldii* and *A. tamarense*. While this study was constrained to the Limfjord, a similar situation emerged in the Skagerrak, Kattegat, and the southern and western areas of the Baltic Sea with increasing occurrences of *A. pseudogonyaulax* (Fig. 2, Fig. S1/S2). *A. pseudogonyaulax* occurred at 43 of the 52 analysed stations but has not expanded past Gotland into the northern Baltic Sea yet (Fig. 1).

Noteworthy occurrences with respect to geographical positions, cell densities, and abiotic parameters are summarized in Table S1. Originally constrained to the Kattegat region, including Aarhus Bay, and the northern parts of the Little and Big Belt (Fig. 2, Fig. S1), *A. pseudogonyaulax* has since expanded into the Baltic Sea. While *A. ostenfeldii* was the most frequent *Alexandrium* species from 1994–2004, there has been an increase in the relative contribution of *A. pseudogonyaulax* to the total *Alexandrium* community (Fig. S1/S2). Kremp et al. (2019) showed similar results for three stations located in the Danish Limfjord with *A. pseudogonyaulax* suddenly appearing in 2007 along with declining cell densities of *Alexandrium* spp. corresponding to the results of station 25 and 30 in this study. Considering that both species differ morphologically (Mertens et al. 2020), it can be assumed that they were correctly identified, especially considering

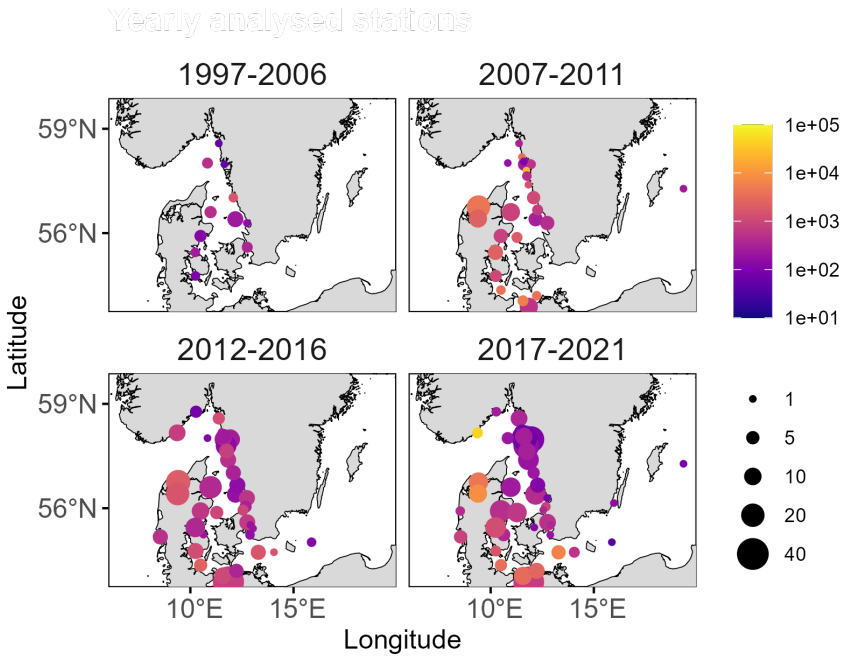

Fig. 2: *Mean cell densities of* A. pseudogonyaulax *during the last three decades; colour scheme corresponds to the cell densities (cells L^{-1}) and the size to the number of present observations during the respective time-frame.*

the scientific interest in *Alexandrium* species. In addition, *Alexandrium* species were generally identified at the species level as *Alexandrium* spp. entries have not markedly increased during the same time-frame (Fig. S1d). *A. tamarense* (Fig. S1c) and *A. minutum* (data not shown) were seldom part of the *Alexandrium* community in this region. Notably, *A. ostenfeldii* reached cell densities $> 10^6$ and $> 10^7$ cells L^{-1} in the Limfjord, and Mariager Fjord, respectively. Most of these high-density blooms were in the late 90's, however *A. ostenfeldii* bloomed in the Limfjord in 2020 reaching cell densities of 5.4×10^6 cells L^{-1}. In contrast, the highest reported cell density of *A. pseudogonyaulax* was 1.25×10^5 in the Skagerrak next to Arendal, with another nine occurrences with cell densities above 10^4 cells L^{-1} in the Limfjord. Considering that *A. pseudogonyaulax* is a large dinoflagellate, cell densities may be misleading, as for instance in the Arkona Basin in 2019 *A. pseudogonyaulax* constituted over half of the total biomass, while only having a cell density of 33,000 cells L^{-1} (Zettler et al. 2020). Cell densities are likely lower than required to cause toxic effects to fish gill cells (Möller et al. 2024 *forthcoming*). However, the RTgill-W1 bioassay is generally less sensitive than whole fish bioassays (Tanneberger et al. 2013) and microalgae have been reported to form dense patches characterized by higher cell densities than their surroundings (Durham et al. 2013, Breier et al. 2018). Hence, natural bloom concentrations of *A. pseudogonyaulax* may still cause ichthyotoxic effects. Occurrences of *A. pseudogonyaulax* will likely increase across Northern Europe in the future (Fig. 3), even though they may stagnate in some regions, such as the Danish Limfjord. Thus, the potential for ecological damages to aquaculture industries remains

and further research into the toxicity of this emerging dinoflagellate is required.

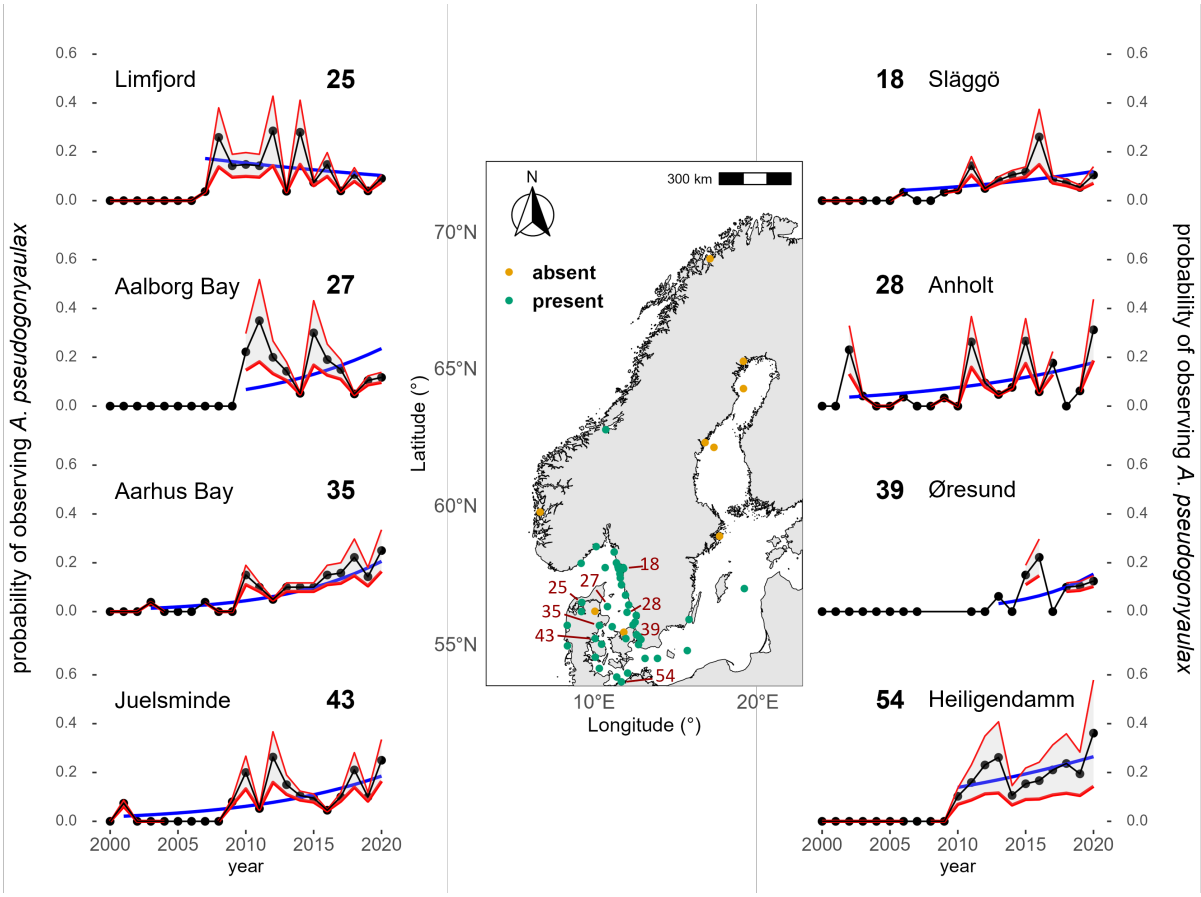

Fig. 3: *Probability of observing* A. pseudogonyaulax *across Northern European waters; black points and lines correspond to yearly calculated probabilities; red lines and grey ribbons to calculated 95 % confidence intervals and the blue line to a logistic regression model fitted to the yearly probability pattern.*

Seasonality of *A. pseudogonyaulax* is conserved, but different to *A. ostenfeldii*

We hypothesized that calm and stratified estuaries, such as the Limfjord, act as a breeding ground for *A. pseudogonyaulax* and inject neighbouring water bodies at the start of the summer season. Therefore, we analysed after which day the probability of observing *A. pseudogonyaulax* exceeded 10 % and expected this day earlier in estuaries. This approach enables to perform conventional parametric statistics with time series data and can be transferred to the analysis of other microalgae. The seasonality of *A. pseudogonyaulax* turned out to be highly conserved across all stations (Fig. 4, Fig. S3), but *A. pseudogonyaulax* occurred slightly earlier in estuaries than in coastal or open ocean stations ($F_{2,24} = 3.2$, $p < 0.1$). No significant differences ($p > 0.1$) in the end or peak of the growing season were found. *A. pseudogonyaulax* most frequently occurred between June and September (Fig. 4), while *A. ostenfeldii* was also part of the spring bloom. These results suggest that *A. pseudogonyaulax* maintains a consistent presence throughout all

water bodies.

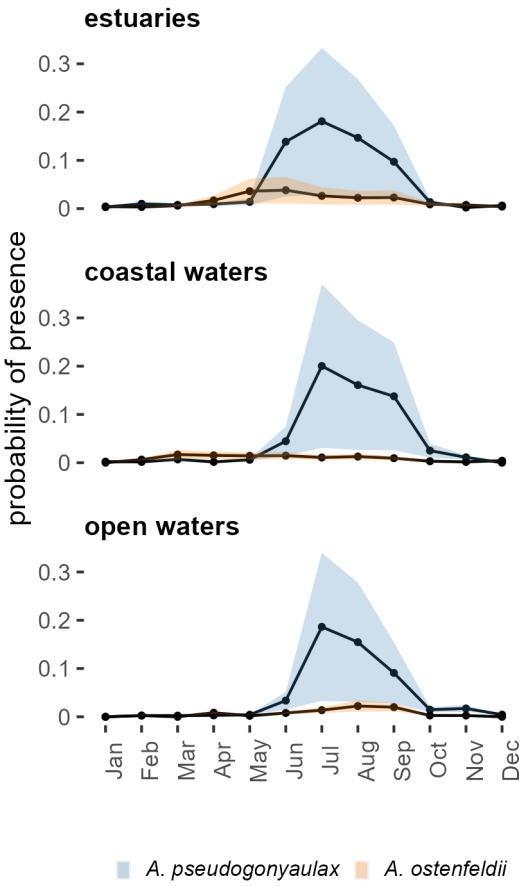

Fig. 4: *Seasonality of* A. pseudogonyaulax *and* A. ostenfeldii *across three water bodies; black points correspond to calculated probabilities and the coloured ribbon to the 95 % confidence interval, respectively.*

Salinity and temperature may play a role in shaping *Alexandrium* community composition

Increasing occurrences of *A. pseudogonyaulax* along the salinity gradient of the Baltic Sea (Fig. 5, Table S1) suggest that *A. pseudogonyaulax* is adapted to a wide range of salinities. Plotting seasonal means of abiotic parameters against the probability of observing *Alexandrium* further indicates that *A. pseudogonyaulax* prefers intermediate salinities and higher temperatures (Fig. 5).

Water temperatures are around 18 °C when the probability of observing *A. pseudogonyaulax* is highest (Fig. S4), associated with the late summer blooming season. Positive growth rates of *A. pseudogonyaulax* up to 25 °C (Tulatz et al., *in prep.*) suggest that this dinoflagellate may benefit from increasing water temperatures. Additionally, *A. pseudogonyaulax* has been observed in the Baltic Sea at salinities below 8 (Table S1)

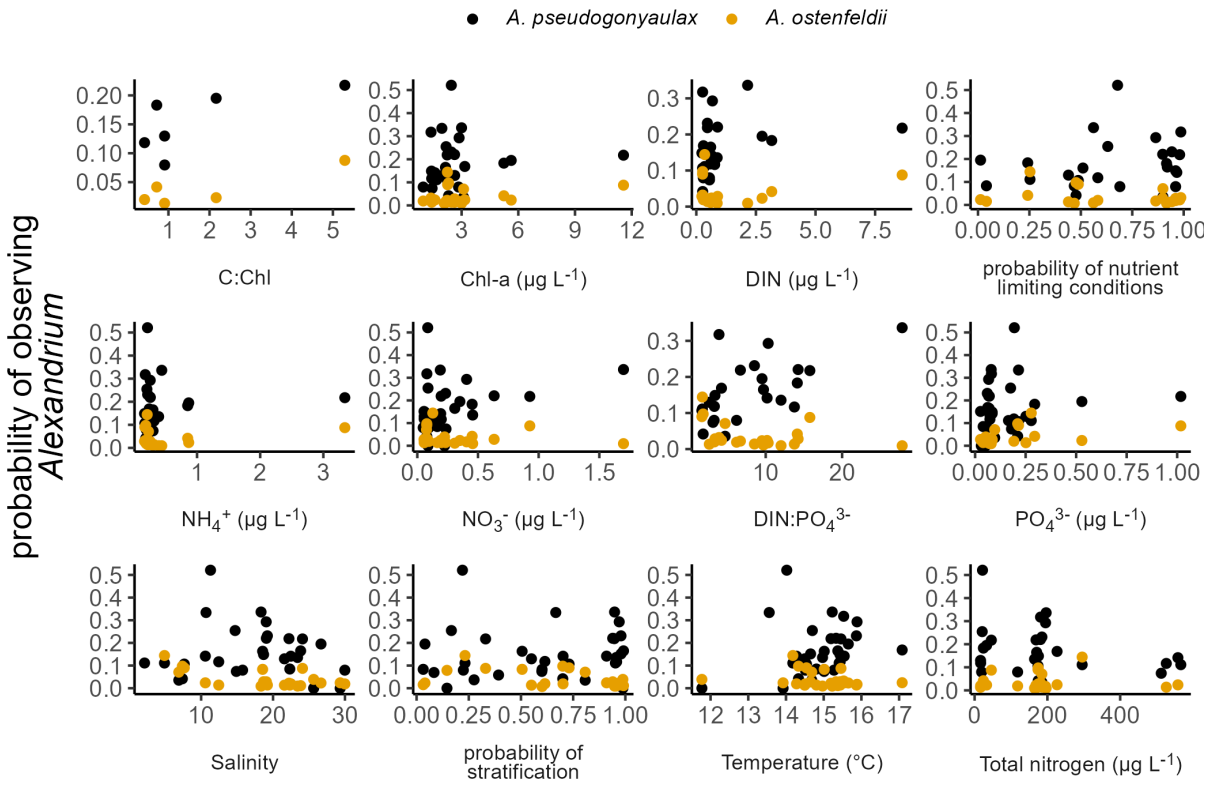

Fig. 5: *Seasonal means (May throughout October) of abiotic parameters vs. the seasonal probability of observing A. pseudogonyaulax (black) or A. ostenfeldii (orange); nutrient limiting conditions correspond to DIN < 2 and PO_4 < 0.2 µmol L^{-1}; each point corresponds to one station.*

and goniodomins, a chemotaxanomic trait of *A. pseudogonyaulax* in Northern Europe, were found during a research cruise in late summer 2022 east of Bornholm at a salinity of 7.5 (data not shown). While *A. ostenfeldii* is currently dominating the *Alexandrium* community in the eastern and northern parts of the Baltic Sea (Kremp et al. 2009), *A. pseudogonyaulax* may become a serious competitor in the future.

A. pseudogonyaulax might benefit from the senescence of algal blooms through mixotrophic feeding

The findings in this study indicate that it is more likely to observe *A. pseudogonyaulax* when the carbon to chlorophyll ratio is high (Fig. 6), which suggests that *A. pseudogonyaulax* can efficiently utilize high dissolved organic matter (DOM) concentrations. This high carbon to chlorophyll ratio could arise from a senescent pre-bloom of other algae, which is a scenario that has been demonstrated in ecological models (Mitra and Flynn 2006, Sunda et al. 2006, Sunda and Shertzer 2014) and in the field for brown-tide forming *Aureococcus anophagefferens* (Gobler and Sañudo-Wilhelmy 2001) and *Aureoumbra lagunensis* (Buskey 2008). This is particularly important, as blooms of both brown-

tide species have been associated with high DOM concentrations (Gobler and Sunda 2012, Gobler et al. 2004) and both species are osmotrophic mixotrophs (Berg et al. 1997, Muhlstein and Villareal 2007). Additionally, but only in the southwest of the Baltic Sea (station 51–53), the occurrence of *A. pseudogonyaulax* was associated with relatively increased phosphate concentrations (Fig. 6). So far, no clear relationships between other abiotic factors and the presence of *A. pseudogonyaulax* have been elucidated warranting further investigations.

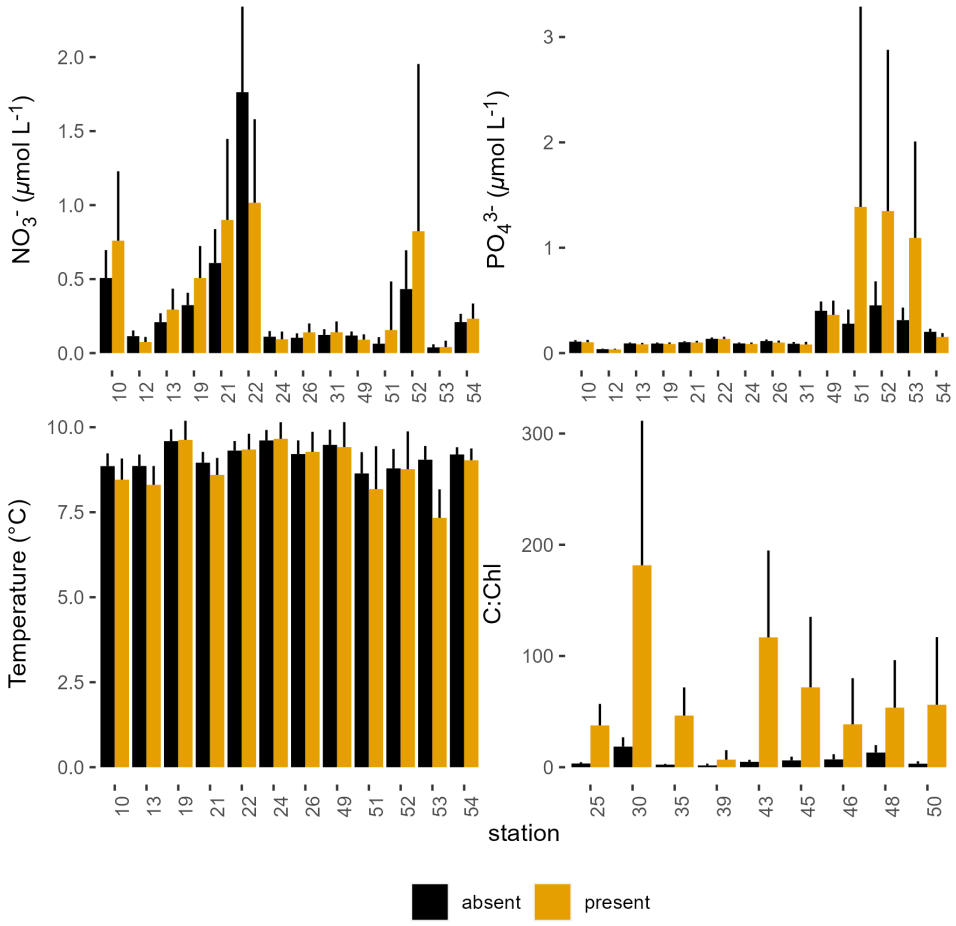

Fig. 6: *Seasonal variations in abiotic parameters at each station were modelled as the sum of a sinusoidal and cosinusoidal function, this model describes if the presence or absence of* A. pseudogonyaulax *is associated with an anomaly (deviation from the general seasonal variation) in the abiotic parameter indicating a tendency for higher or lower values of the respective abiotic parameter during the presence or absence of* A. pseudogonyaulax.

Conclusions

The harmful dinoflagellate *A. pseudogonyaulax* has established itself as a regular component of the *Alexandrium* community across Northern European waters, primarily the

Kattegat, Skagerrak and the southern part of the Baltic Sea. Simultaneously, the likelihood of observing previously dominating *A. catenella* and primarily *A. ostenfeldii* have decreased. Time series analysis suggests that temperature and salinity play a role in shaping the *Alexandrium* community. Furthermore, a high salinity tolerance of *A. pseudogonyaulax* combined with sightings as far east as Gotland suggest that *A. pseudogonyaulax* may become a serious competitor for *A. ostenfeldii* in the Baltic Sea. This ongoing expansion of also underscores the necessity of further research into the toxicity of *A. pseudogonyaulax*, which remains elusive due to lacking analytical quantification of BECs. The abundance of this species is likely to increase across Northern Europe and thus economic and ecological damages remain conceivable.

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Conflict of interests

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.

Data availability statement

The Swedish and German datasets are publicly available on the Sharkweb and ODIN 2 website, respectively. All code can be found online on GitHub: https://github.com/KristofM854 (accessed on 10 July 2024).

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Time series analysis of the expansion of *Alexandrium pseudogonyaulax* across Northern European waters

Kristof Möller[1], Hans Jakobsen[2], Annette Engesmo[3], Bengt Karlson[4], Jacob Carstensen[2]

[1] Alfred-Wegener-Institut Helmholtz-Zentrum für Polar- und Meeresforschung, Bremerhaven, Germany
[2] Aarhus University, Roskilde, Denmark.
[3] Norwegian Institute for Water Research, Oslo, Norwegen.
[4] Swedish Meteorological and Hydrological Institute, Stockholm, Sweden.
*Correspondence: kristof-moeller@outlook.de

Running head: Expansion of *A. pseudogonyaulax*

Supporting information

Document includes:

Equation for the calculation of water density required for the determination of the binomial stratification key.

Tables S1

Figures S1-S4

Equation S1: $$\textit{water density}\ (g\ cm^{-3}) = 999.842594 + 6.793952 \times 10^{-2}\ T - 9.095290 \times 10^{-3}\ T^2 + 1.001685 \times 10^{-4}\ T^3 - 1.120083 \times 10^{-6}\ T^4 + 6.536332 \times 10^{-9}\ T^5 + (8.24493 \times 10^{-1} - 4.0899 \times 10^{-3}\ T + 7.6438 \times 10^{-5}\ T^2 - 8.2467 \times 10^{-7}\ T^3 + 5.3875 \times 10^{-9}\ T^4)\ S + (-5.72466 \times 10^{-3} + 1.0227 \times 10^{-4}\ T - 1.6546 \times 10^{-6}\ T^2)\ S^{3/2} + 4.8314 \times 10^{-4}\ S^2$$

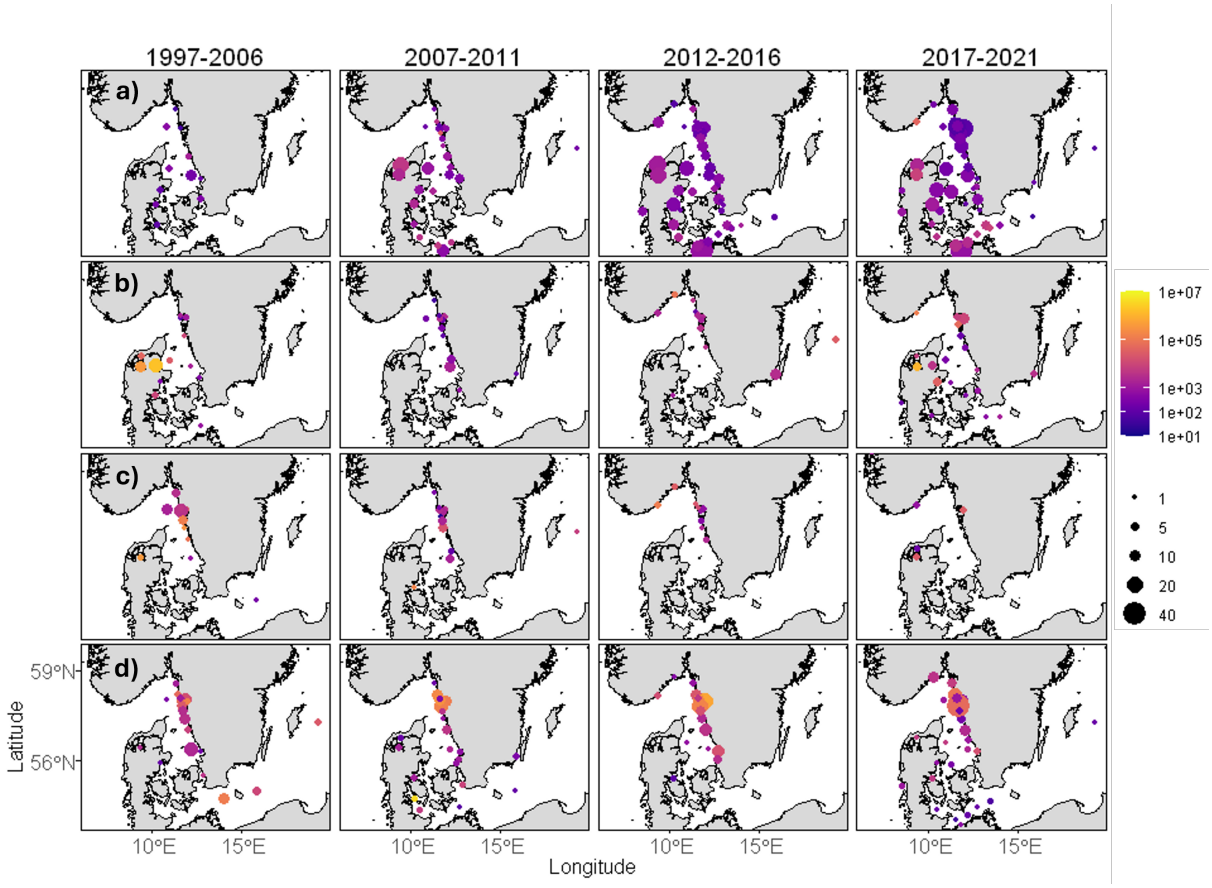

Fig. S1: *Mean cell densities of a)* A. pseudogonyaulax, *b)* A. ostenfeldii, *c)* A. tamarense *and d) other* Alexandrium *spp. during the last three decades; colour scheme corresponds to the cell densities (cells* L^{-1}*) and size to the number of present observations during the respective time-frame.*

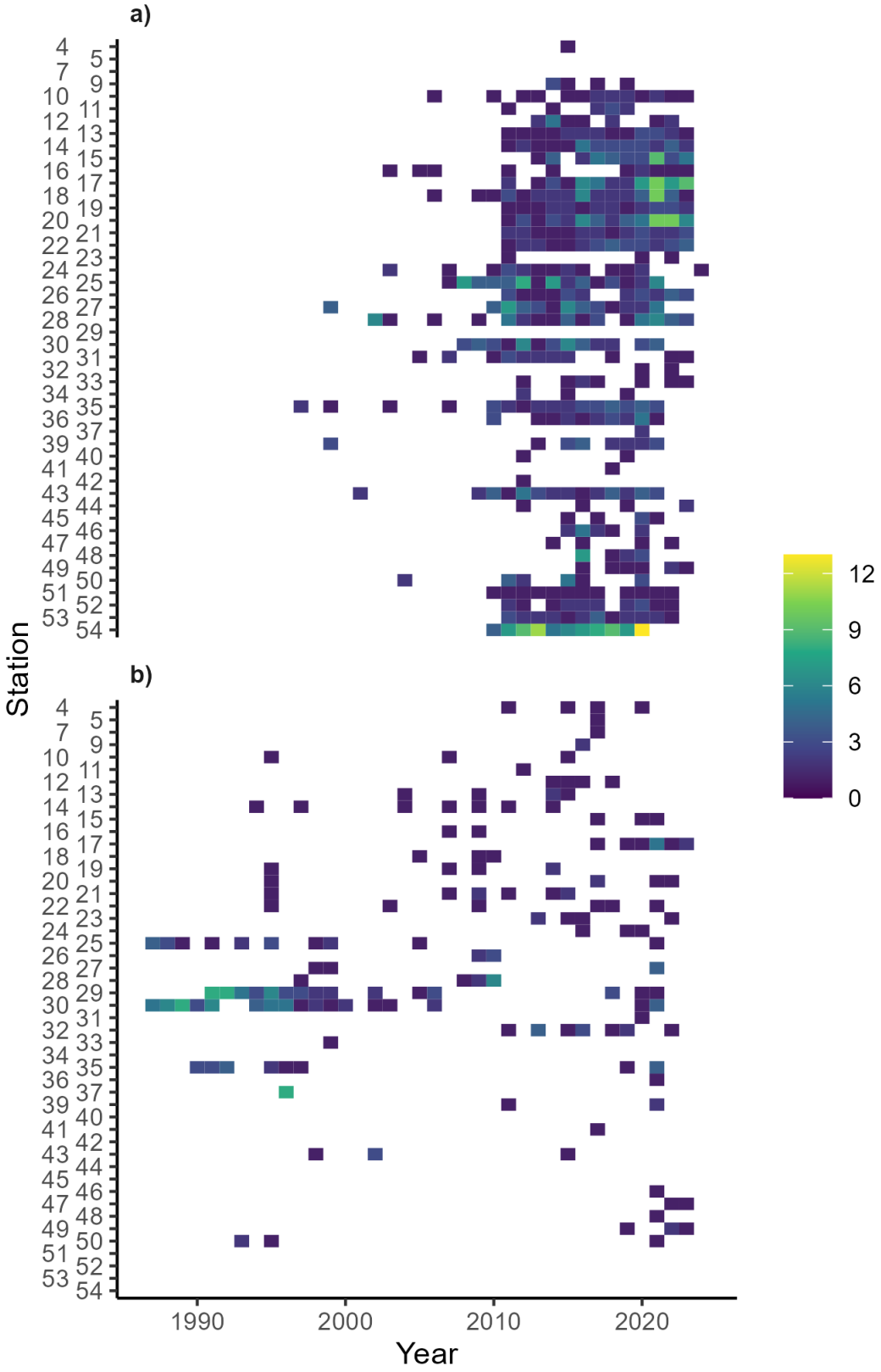

Fig. S2: *Heatmap of the yearly present observations of a)* A. pseudogonyaulax *and b)* A. ostenfeldii, *colour scheme indicates the number of present observations in each year.*

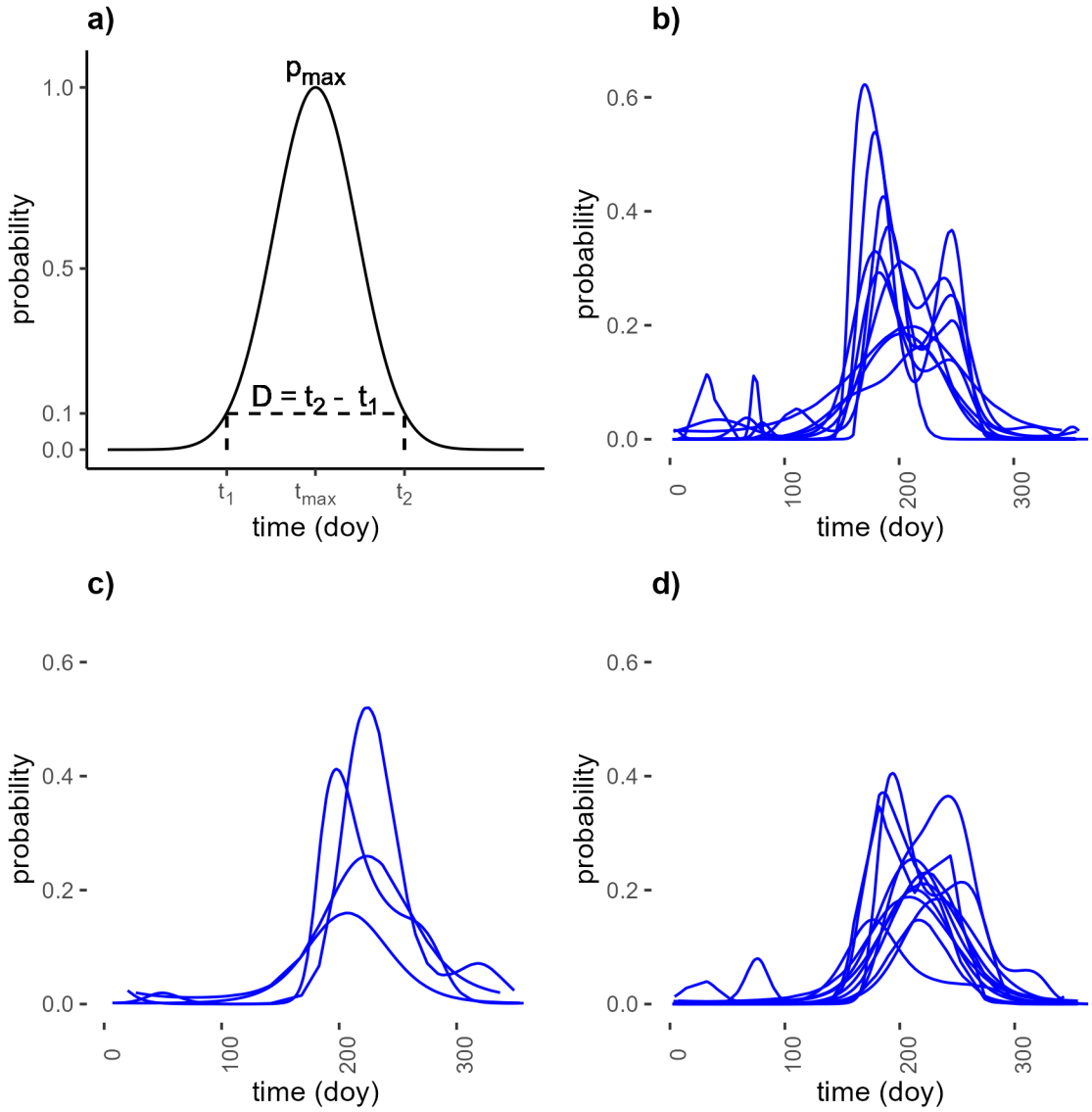

Fig. S3: *Seasonality of* Alexandrium pseudogonyaulax *across three different water bodies, including estuary (B), coastal (C) and open ocean (D) water bodies; methodological approach is shown in (A) and included the determination of the DOYs at which the probability of observing* A. pseudogonyaulax *exceeds or undercuts 10 % and the DOY at which the probability is maximum; DOY = day of the year.*

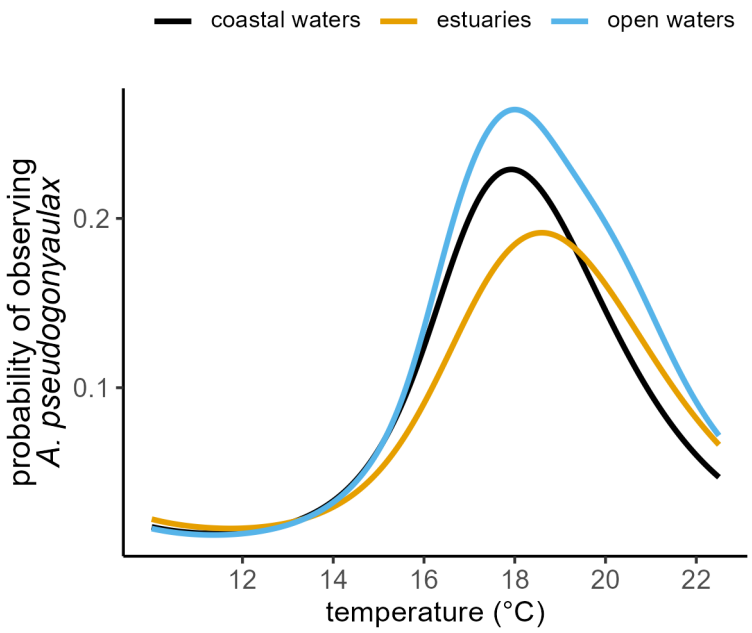

Fig. S4: *Temperature range vs. the probability of observing* A. pseudogonyaulax *across three different water bodies (see table S1).*

Table S1: *Station parameters and characteristics of stations that passed the filtering criteria; 'open' corresponds to open water stations.*

Station characteristics						Characteristics of present observations of *A. pseudogonyaulax*					
Combined stations	Station number	Assigned water body	Latitude (DD)	Longitude (DD)	Sampling period	Number of present observations	Max cell density (cells L^{-1})	Temperature range (°C)	Salinity range	years	months
Kjempebakken	1	estuary	69.79	20.60	2006-2019	0	/	/	/	/	/
RA2, RÅNEÅ-2	2	estuary	65.73	22.45	2004-2023	0	/	/	/	/	/
A13, F9 / A13	3	open	64.71	22.07	1995-2023	0	/	/	/	/	/
Korsfjorden	4	estuary	63.41	10.01	2011-2021	1	3.27e+02	6.4-6.4	31.2-31.2	2015-2015	4-4
GA1, GAVIK-1	5	estuary	62.86	18.26	1997-2023	0	/	/	/	/	/
C3	6	open	62.65	18.95	2000-2023	0	/	/	/	/	/
Bjørnafjorden	7	estuary	60.10	5.47	2013-2022	0	/	/	/	/	/
Fjällsviksviken, Skarpösundet, Svedudden	8	estuary	59.34	18.72	1996-2022	0	/	/	/	/	/
Håøyfjorden	9	coastal	59.03	9.80	2007-2022	6	2.80e+02	4-17	24.3-26.7	2014-2019	3-9
Kosterfjorden NR16	10	coastal	58.87	11.10	1990-2023	17	2.06e+03	4.5-18.7	23.5-27.6	2006-2023	4-9
SLV Bottnefjorden, SLV Sotefjorden	11	estuary	58.47	11.29	2004-2020	9	3.56e+03	/	/	2011-2019	4-7
Arendal	12	coastal	58.38	8.82	2000-2022	14	1.26e+05	9.5-19	19.8-32.6	2013-2022	5-10
Stretudden	13	estuary	58.34	11.40	1995-2023	21	1.84e+03	1.4-21.2	21-27.3	2011-2023	2-9
Havstensfjord	14	estuary	58.31	11.78	1990-2023	31	2.09e+03	2.6-22	7-20.4	2011-2023	1-10
SLV Saltö Fjord	15	estuary	58.30	11.43	2006-2023	39	2.30e+03	/	/	2013-2023	3-9
Å17	16	open	58.28	10.52	1999-2023	11	9.69e+02	10.8-19.5	26.2-32.2	2003-2023	5-9
SLV Havstensfjorden-Ljungskile, Kollhom	17	estuary	58.27	11.81	2004-2023	55	2.07e+03	/	/	2011-2023	3-11
Pricken, Släggö	18	estuary	58.26	11.45	1986-2023	44	1.71e+03	3.8-20.9	19.6-27.9	2006-2023	3-12

Table S1: *Station parameters and characteristics of stations that passed the filtering criteria; 'open' corresponds to open water stations.*

Station characteristics						Characteristics of present observations of *A. pseudogonyaulax*					
Combined stations	Station number	Assigned water body	Latitude (DD)	Longitude (DD)	Sampling period	Number of present observations	Max cell density (cells L^{-1})	Temperature range (°C)	Salinity range	years	months
Koljöfjorden, Borgilefjorden	19	estuary	58.23	11.59	1994-2023	23	3.51e+03	16.4-21.7	20.7-22.6	2011-2023	6-9
Stigfjorden	20	estuary	58.08	11.56	1990-2023	57	2.75e+04	/	/	2011-2023	2-12
Åstol	21	coastal	57.92	11.59	1994-2023	24	2.73e+03	15.8-20.6	17.3-23.4	2011-2023	6-9
Danafjord	22	coastal	57.67	11.69	1990-2023	31	1.35e+03	3.4-20.3	20.2-22.3	2011-2023	1-9
BY15 Gotlandsdj, TF0271, TF0271b	23	open	57.33	20.05	1995-2023	3	2.40e+02	6.9-21.4	7.2-7.2	2011-2022	5-8
N7 Ost Nidingen, Kungsbackafj Mynning	24	coastal	57.29	11.98	1993-2024	19	3.93e+03	13.3-21.1	1.9-23.3	2003-2024	3-10
VIB3708	25	estuary	56.95	9.06	1980-2024	55	3.06e+04	11.7-22.6	23.8-29	2007-2021	5-10
N14 Falkenberg, 5W Glommen	26	coastal	56.94	12.21	1993-2023	25	1.60e+03	3.3-20.8	15.3-23.2	2011-2023	3-10
NOR409, 409 Ålborg Bugt	27	open	56.86	10.79	1995-2023	46	2.00e+03	7-20.2	16.7-27	1999-2021	4-9
Anholt E, Röde Banke S1/S2	28	open	56.67	12.11	1995-2023	45	3.18e+03	13.1-21.5	15-22	2002-2023	2-10
NOR5503	29	estuary	56.66	9.97	1979-2024	0	/	/	/	/	/
VIB3727	30	estuary	56.62	9.08	1980-2024	42	3.35e+04	6.6-22	21.3-27.4	2008-2021	4-9
L9 Laholmsbukten	31	coastal	56.57	12.72	1993-2024	18	4.50e+03	14.8-22.3	1.6-19.4	2005-2023	7-10
REF M1V1	32	coastal	56.37	16.20	1995-2022	2	4.00e+02	9.2-16.1	/	2020-2022	5-7
S-5, S-2, Skälderviken-1	33	coastal	56.32	12.65	1995-2023	8	1.66e+03	17.4-18.1	/	2012-2023	7-9
Övf 1:1 Höganäs	34	coastal	56.22	12.52	1997-2020	4	2.00e+03	14.8-18.2	13.2-13.5	2012-2019	7-9

Table S1: *Station parameters and characteristics of stations that passed the filtering criteria; 'open' corresponds to open water stations.*

Station characteristics						Characteristics of present observations of *A. pseudogonyaulax*					
Combined stations	Station number	Assigned water body	Latitude (DD)	Longitude (DD)	Sampling period	Number of present observations	Max cell density (cells L^{-1})	Temperature range (°C)	Salinity range	years	months
ARH170006	35	coastal	56.15	10.32	1971-2024	37	2.90e+03	12.8-21.4	17.8-27.4	1997-2021	6-10
VSJ20925, 925 Kattegat SW	36	open	56.13	11.16	1998-2023	21	3.30e+03	13.4-22.5	14.7-21.3	2010-2021	6-9
RKB1	37	estuary	56.08	8.22	1980-2024	2	6.20e+02	15.4-22.5	12.2-12.2	2020-2020	6-6
FRB75	38	estuary	55.94	11.92	2000-2023	0	/	/	/	/	/
KBH431, SOOP-SQ019B, W Landskrona	39	coastal	55.86	12.74	1974-2023	20	2.55e+03	10.7-22.6	0.6-24.3	1999-2021	7-11
ÖVF 3:2 Lundåkra, Barsebäck	40	coastal	55.79	12.90	1985-2020	2	3.68e+02	17.3-17.9	9.5-9.5	2012-2019	8-8
ROS60	41	estuary	55.71	12.07	1972-2024	1	1.20e+02	17.5-17.5	14-14	2018-2018	9-9
ÖVF 4:8 Lomma	42	coastal	55.69	13.04	1997-2020	1	2.04e+02	15.1-15.1	9.4-9.4	2012-2012	9-9
VEJ0006870	43	coastal	55.67	10.09	1976-2024	37	9.60e+03	0.1-19.2	15.6-26	2001-2021	3-11
ÖVF 5:1 Höllviken, ÖVF 5:2 Klagshamn	44	coastal	55.50	12.88	1995-2023	5	5.55e+02	15.9-18.7	9.4-9.4	2012-2023	6-9
FYN6900017	45	estuary	55.48	10.52	1977-2024	6	9.20e+02	16.1-19.2	17.6-23.1	2015-2021	7-8
RIB1510007	46	coastal	55.34	8.35	1989-2024	12	3.72e+03	15.7-19.7	30.2-32.7	2015-2020	6-10
BRKBMPK2, BY5 Bornholmsdj, 35E Gudhjem, TF0213	47	open	55.25	15.99	1995-2023	4	2.53e+02	15.9-19	7.6-7.6	2014-2022	6-8

Table S1: *Station parameters and characteristics of stations that passed the filtering criteria; 'open' corresponds to open water stations.*

Station characteristics						Characteristics of present observations of *A. pseudogonyaulax*					
Combined stations	Station number	Assigned water body	Latitude (DD)	Longitude (DD)	Sampling period	Number of present observations	Max cell density (cells L^{-1})	Temperature range (°C)	Salinity range	years	months
BY2 ARKONA, TF0109	48	open	55.00	14.08	1995-2023	7	1.52e+03	16.1-20	7.9-8.1	2016-2023	7-9
DMU444, BY1, TF0069	49	open	55.00	13.30	1995-2024	13	2.78e+04	11.4-20.2	5.1-8.5	2016-2020	7-10
FYN6300043	50	open	55.00	10.16	1975-2024	17	2.70e+03	6.7-19.3	12.9-19.2	2004-2020	6-12
Kieler Bucht, TF0360	51	open	54.60	10.45	1995-2022	12	1.13e+04	16.7-21.2	11.8-15.8	2010-2022	7-8
TF0046	52	open	54.47	12.22	1995-2022	17	9.36e+03	4.8-19.5	8.4-18.7	2011-2022	3-11
Mecklenburger Bucht, TF0012	53	open	54.32	11.55	1995-2022	24	1.18e+04	8.8-21.4	8.5-16.3	2011-2022	7-11
Heiligendamm	54	coastal	54.15	11.84	1998-2020	86	4.72e+03	8-20.8	7.6-16.7	2010-2020	5-11

Chapter 5

Synthesis

5.1 Discussion of the major findings

In this last chapter, the major findings of this thesis are discussed, and the presented results are put into perspective of the undergoing expansion of *A. pseudogonyaulax* in Northern European waters. Finally, new research directions are suggested to extend the knowledge of factors determining the toxicity of GD-producing *Alexandrium* species and to increase the spatiotemporal monitoring of HABs.

5.1.1 Bottom-up controls of *A. pseudogonyaulax*

Nitrogen, light, temperature and the ecophysiology of *A. pseudogonyaulax*

Publication I investigated the influence of N and light, two important drivers of HAB development, on primarily growth and toxin content of *A. pseudogonyaulax*. The results of this study can be incorporated into ecological models and may help in understanding the ongoing expansion of this dinoflagellate in Northern European waters.

The findings of publication I suggest that dissolved urea is not the primary driver of the proliferation of *A. pseudogonyaulax*, especially in eutrophic waters such as the Danish Limfjord. This was surprising, since urea represents a significant proportion of the dissolved organic N pool, especially in coastal systems (Sipler and D. A. Bronk 2015) and high levels of anthropogenic urea input from intensive agricultural farming in Denmark (Glibert et al. 2005, Carlsson et al. 2009). Nowadays, most globally utilized N fertilizers are based on urea instead of ammonium primarily due to safer transportation and storage, as ammonium is highly explosive (Glibert et al. 2006). Increasing urea usage, along with high N:P ratios, have been linked to favourable conditions for HABs (J. Li et al. 2010, Glibert et al. 2014). This is of particular importance for the Baltic Sea and the southeast continental coast of the North Sea, which remain highly eutrophicated (J. H. Andersen et al. 2017). However, large uncertainties in the composition and magnitude of coastal DOM pools, including urea, hinder an accurate assessment of the interplay between urea and HAB development (Davidson et al. 2012).

In Northern Europe, substantial regulatory efforts to reduce N and P pollution partially alleviated eutrophication (Carstensen et al. 2006, Skogen et al. 2014), yet it is unlikely that reduced nutrient inputs can revert marine systems back to historical reference values (Duarte et al. 2009). Most regions in which *A. pseudogonyaulax* has started to dominate the *Alexandrium* community (publication III) experience strong eutrophication, suggesting a link between nutrient enrichment and the expansion of this dinoflagellate. However, the Skagerrak region is not influenced by eutrophication (Skogen et al. 2014), yet *A. pseudogonyaulax* is still increasing in abundance and frequency (publication III). Additionally, nutrient pollution in Northern Europe peaked in the 1950s/1960s (Elmgren 2001) and thus the question remains why *A. pseudogonyaulax* was noticed or only ap-

peared decades later. Nevertheless, competitive advantages of *A. pseudogonyaulax* over other microalgae under nutrient replete conditions might have favoured its establishment. In fact, there are studies indicating that dinoflagellates have a competitive advantage over other microalgae when ammonium and urea are dominant, even under lower concentrations (Collos et al. 2007, R. M. Kudela et al. 2008). Additionally, urea uptake by HAB species, such as *Lingulodinium polyedrum* or *A. catenella*, can occasionally meet most of the cellular N-demand, despite lower concentrations of urea relative to other inorganic N sources (C. M. Solomon et al. 2010). Publication I showed that *A. pseudogonyaulax* is able to utilize urea initially, albeit not as sole N-source. Analysing the molecular regulation of genes associated with urea metabolism may help in explaining this temporary usage of urea.

Growth of two strains of *A. pseudogonyaulax* stagnated at low light but could be rapidly recovered by increasing the light intensity indicating that cells can overcome longer periods of light without decaying or forming resting cysts (publication I). This is particularly relevant for estimating the persistence of *A. pseudogonyaulax* blooms, as encystment removes vegetative cells from the water column and may thereby reduce the bloom intensity or lead to bloom termination (Cosgrove et al. 2014). Resting cysts of *A. catenella* from Cape Cod, USA, germinate only after a mandatory dormancy period of several month and are thus not sustaining but rather inoculating blooms in successive seasons (A. D. Fischer et al. 2018). In contrast, germination periods of *A. catenella* or *A. tamarense* from temperate waters are less than two month (Genovesi et al. 2009, G. Hallegraeff et al. 1998) suggesting similar dormancy requirements for *A. pseudogonyaulax* in Northern Europe. Resilience towards low light intensity and a low light compensation point (publication I) further indicate that *A. pseudogonyaulax* is well adapted towards environmental changes reducing light availability, such as coastal darkening (A. Garnier et al. 2023, Frigstad et al. 2023).

Coastal darkening associated reductions in light availability and increases in DOM concentrations are likely resulting in decreased benthic production (Pratt et al. 2014, Batt et al. 2015, Mangan et al. 2020, Striebel et al. 2023), as well as increased heterotrophy, especially bacterial production, relative to autotrophic primary production (Hitchcock et al. 2010, Wikner and Andersson 2012, Jonsson et al. 2017, Striebel et al. 2023). In coastal regions, this shift in lower pelagic food web structures might restrict the energy transfer to higher trophic levels, especially fish, which are dependent on benthic primary production (Batt et al. 2015, Christianen et al. 2017). Increased DOM concentrations might simultaneously favour osmotrophic mixotrophs (Stoecker et al. 2017, Burkholder et al. 2008), however uptake of dissolved organic substrates has not been explicably demonstrated for *A. pseudogonyaulax*. Additionally, coastal darkening has been associated with shifts from visual predators, such as planktivorous fish, to tactile predators, such as jellyfish (Sørnes et al. 2007, Ask et al. 2009, Sørnes and Aksnes 2004, Aksnes et al. 2009).

Jellyfish predation subsequently decreases zooplankton abundances favouring the proliferation of microalgae (Riisgård et al. 2012a, Riisgård et al. 2012b).

Publication I demonstrated that carbon acquisition of *A. pseudogonyaulax* at low light (20 μmol photons $m^{-2}s^{-1}$) is insufficient to meet carbon demands for cell division. Even highly mixotrophic species, such as *Karlodinium* spp. (Berge and P. J. Hansen 2016), are dependent on light to feed, however, the underlying causality is unclear. Ingested prey can substitute for cellular carbon demands, considering that *A. pohangense* has a lower light compensation point when grown under mixotrophic compared to phototrophic conditions (A. S. Lim et al. 2019b). Recently, it has also been demonstrated that several mixotrophic HAB species exhibited increased ingestion rates under nutrient- and light-depleted conditions (Mena et al. 2024). Phagotrophic feeding by *A. pseudogonyaulax* likely involves large carbon-based BECs (Blossom et al. 2012) and it may be speculated that their biosynthesis is competing with phototrophic driven cell division. This hypothesis is strengthened by evidence suggesting a potential trade-off between growth rate and lytic activity of *A. pseudogonyaulax* (Blossom et al. 2019) and by the fact that phagotrophic mixotrophs, when feeding, generally have higher growth rates (Mitra et al. 2014). Furthermore, photosynthetic efficiencies of two strains of *A. pseudogonyaulax* under low, medium and high light were equal (publication I) and hence, high electron transport rates were not translated into growth, but rather contributed to alternative electron pathways.

Altogether, resilience towards low light, substitution of cellular carbon demands through mixotrophy and increased DOM concentrations might have supported the expansion and establishment of *A. pseudogonyaulax* across Northern European waters. Mixotrophic feeding can also result in the acquisition of other essential nutrients, such as N or P. In fact, potentially limiting nutrients, such as P, can be more concentrated in bacteria or other prey of mixotrophs than in the dissolved phase (Vadstein 2000). This can be particularly important in the Kattegat and Baltic Sea that regularly experience P-limitation in autumn (M. E. Petersen et al. 2017, Savchuk 2018) coinciding with the main vegetative period of *A. pseudogonyaulax* (publication III). In the case of mixotrophs, it may thus be misleading to suppose nutrient limiting conditions when dissolved inorganic nutrients are low as they can satisfy their nutrient demands through phagotrophic feeding. Additionally, coastal darkening has been demonstrated to enhance ammonium efflux from the sediment, which can stimulate primary productivity (Pratt et al. 2014). Reduced N compounds, such as ammonia, are thought to favour dinoflagellates over diatoms (D. Bronk et al. 2007, Glibert 2016), which may consequently stimulate the proliferation of *A. pseudogonyaulax* as it grows well on ammonium (publication I). The findings of publication I further demonstrate that *A. pseudogonyaulax* is tolerant to high light (200 μmol photons $m^{-2}s^{-1}$) scenarios, which are regularly reached in shallow and stratified estuaries in Northern Europe, like the Danish Limfjord (Stæhr et al. 2020). Similarly, *A. catenella* can also grow over broad light ranges and is tolerant towards high light (Parkhill and A. D. Cem-

bella 1999, Etheridge and Roesler 2005, Laabir et al. 2011). In contrast, growth of one strain of *A. ostenfeldii* exhibited a sharp maximum at 100 μmol photons $m^{-2}s^{-1}$ followed by a rapid decline (Maclean et al. 2003). Hence, differences in light tolerance (publication I) might have contributed to the displacement of *A. ostenfeldii* yet cannot explain the analogous trend observed for *A. catenella* (publication III).

Altogether, the current information about the growth response of *A. pseudogonyaulax* to varying bottom-up factors suggests that this dinoflagellate has the capacity to further expand and establish itself in Northern European waters. Although not generated within publication I, it should be noted that Tulatz et al. (2024, *in prep.*) expanded the knowledge of the ecophysiology of *A. pseudogonyaulax* by analysing its growth response to temperature, salinity and CO_2. The findings demonstrated that *A. pseudogonyaulax* is well-adapted to grow on a wide range of salinities (10–45), temperatures (12–30 °C) and CO_2-concentrations (250–1,000 μatm). Water temperatures in Northern Europe, when the probability of observing *A. pseudogonyaulax* is highest, are around 18 °C, associated with the late summer blooming season (publication III). The microalgal growing season in Northern Europe has already expanded mainly due to longer summers, and the same can be hypothesized for the growing season of *A. pseudogonyaulax*. Further increasing water temperatures are unlikely to pose a problem to the vegetative life-cycle of this dinoflagellate considering that it still exhibits high growth rates at 25 °C (Tulatz et al. 2024, *in prep.*). The influence of warming on the germination success of *A. pseudogonyaulax* cysts remains speculative as the optimal temperature range for germination is unknown and because studies have reported different effects of chilling on dormancy and germination of *Alexandrium* cysts (A. D. Fischer et al. 2018, G. Hallegraeff et al. 1998). For instance, breaking dormancy and high germination rates of *A. catenella* cysts required preceding exposure to cold temperatures (A. D. Fischer et al. 2018). Thereafter, cysts germinated when temperatures exceeded 15 °C (A. D. Fischer et al. 2018). In contrast, cysts of *A. ostenfeldii* from the Baltic Sea have been shown to germinate when temperatures exceed 10 °C (Hjerne et al. 2019). Bloom initiations and terminations are tightly linked to coupling between pelagic and benthic life stages (Brosnahan et al. 2020). Consequently, it may be hypothesized that the rapidly increasing temperatures of the Baltic Sea (Belkin 2009) and the subsequent effects on encystment and germination rates of *Alexandrium* have played a role in the observed shift towards *A. pseudogonyaulax*. Additionally, the global distribution patterns of *A. catenella* suggest that it thrives in temperate to cold zones primarily driven by low sea surface temperatures and high oxygen levels (W. Hu et al. 2024). Hence, increasing temperatures and oxygen depletion across Northern European waters might have favoured the displacement of *A. catenella* by *A. pseudogonyaulax*. In contrast, *A. ostenfeldii* populations in the Baltic Sea also benefit from increasing water temperatures (Hakanen et al. 2012, Kremp et al. 2012) and it remains unclear whether *A. pseudogonyaulax* could be a competitor to *A. ostenfeldii* in the eastern Baltic Sea.

However, the strong salinity tolerance of *A. pseudogonyaulax* and observations as far east as Gotland at salinities just above 7 (publication III) generally support this notion.

Effects of bottom-up factors on the phycotoxin content of *A. pseudogonyaulax*

Estimating the toxic impacts of HAB species is important for assessing the potential damages of HABs and can enhance the explanatory power of ecological models. Although the toxicity of some HAB species seems to be little correlated with their phycotoxin content (Tillmann and John 2002, Flores et al. 2012, Shang et al. 2021, Wolf 2021), a synergistic role in the overall toxicity or grazer deterring properties of phycotoxins cannot be ruled out yet. Currently, significant uncertainties regarding the phycotoxin content of HAB species in response to varying environmental conditions exist. Different studies on the influence of N on the phycotoxin content of microalgae have shown strong, weak or no influence of N (Leong et al. 2004, T.-S. Li et al. 2011, J. Xu et al. 2012) and similarly direct, inverse or no systematic relationships between light and toxin content have been reported (Parkhill and A. D. Cembella 1999, Maclean et al. 2003, Laabir et al. 2013, D.-Z. Wang and Hsieh 2005). Publication I cannot resolve these uncertainties as no influence of N-source or light on the GD-content of *A. pseudogonyaulax* was found. In fact, GD-contents of *A. pseudogonyaulax* are rather driven by the growth stage, as they were always higher in the stationary than the exponential growth phase (publication I). This might imply that toxin production follows a constitutive mechanism leading to the accumulation of these substances in the stationary phase. Regardless of the underlying mechanism, the findings of publication I imply that GD-associated adverse effects of *A. pseudogonyaulax* likely increase towards the later stages of algal blooms. Consequently, this may function as a positive feedback loop sustaining the bloom, as GDs have been associated with grazer deterring properties (J. Xu et al. 2017).

Limitations of single-strain laboratory studies

Conducting monoclonal laboratory studies is important for generating information about the growth characteristics of microalgae. These characteristics also provide an essential basis for multi-species experiments (e.g. predator-prey or other algal competitors), as well as for analysing long-term trends in the plankton community with respect to changing environmental conditions. The absence of prey, however, represents one major limitation for the mixotrophic *A. pseudogonyaulax*, as it can substitute nutrient demands through phagotrophic feeding. This also represents one of the main competitive advantages of dinoflagellates, especially during light limiting conditions. Moreover, the ability to utilize urea, or to switch to mixotrophic feeding under low light, may only provide advantages evident in relation to other microalgae. Substantial differences between nutrient amendments in the form of a single nutrient dose or nutrient pulses have also been demonstrated

(Rothenberger and Calomeni 2016, Doucette and P. Harrison 1991). Although integrating all of these additional parameters into laboratory studies is likely more representative of an ecosystem setting, it also increases the amount of interacting and interfering factors. The subsequent data analysis requires an initial understanding of the growth response of only *A. pseudogonyaulax* to environmental parameters, such as N-sources and light. Consequently, the results of publication I could be utilized as a guidance in designing follow-up mesocosm experiments, which have the advantage of considering interactions between representative trophic levels. For instance, different cell densities of *A. pseudogonyaulax* could be added to natural microalgae communities in a microcosm or mesocosm system, monitoring the relative contribution of microalgal groups and *A. pseudogonyaulax* to the total biomass. Additionally, DON could be supplied in low pulses to mimic riverine input and observe whether it facilitates bloom development of *A. pseudogonyaulax*. For instance, river runoff has been associated with increased bloom formation of *A. tamarense* in the Gulf of St. Lawrence, Canada (Weise et al. 2002). Mesocosms have several disadvantages, such as high workload and therefore, small sample sizes and many interfering bottom-up and top-down factors complicating statistical analysis. However, they seem to represent a powerful experimental tool in analysing the population dynamics of mixotrophs, that can likely only manifest their competitive advantages relative to other microalgae.

5.1.2 Top-down effects: Interactions of *A. pseudogonyaulax* with higher levels of the pelagic food web

The proliferation of an organism in natural dynamic systems is dependent on the interplay between and the relative contributions of bottom-up and top-down factors, respectively. Consequently, the second part of this thesis investigated the interactions of *A. pseudogonyaulax* with higher levels of the pelagic food web. The main findings are summarised in Fig. 5.1 along with noteworthy literature information about the toxicity of GDs and GD/BEC-producers. Overall, publication II has demonstrated adverse effects of *A. pseudogonyaulax* on four representative trophic levels of the pelagic food chain, including algae, protistan microzooplankton, mesozooplankton and fish gill cells. The primary conclusion is that the harmful effects of *A. pseudogonyaulax* are driven by BECs and not by GDs.

Adverse effects of *A. pseudogonyaulax* across the lower marine food web

Copepods can selectively reject phycotoxin and/or BEC producing prey (Barreiro et al. 2006, Schultz and Kiørboe 2009, Ding et al. 2023). Consequently, model simulations have suggested that high grazing selectivity on non-toxic prey increases the likelihood of toxic bloom formation (Y. Zheng et al. 2022, Chakraborty et al. 2022). Xu et al. (2017) demonstrated short-term (4 h) adverse fitness effects of PSP- and GD-producing *Alexandrium* spp. on the filter-feeding copepod *Temora longicornis* yet lytic activity of prey

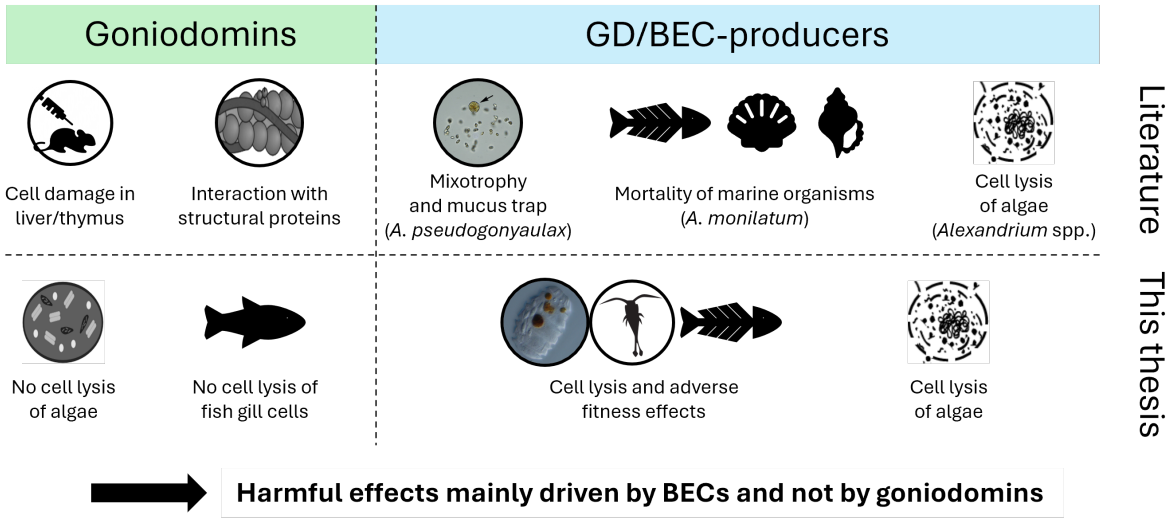

Fig. 5.1: *Main harmful effects of GDs and GD/BEC-producers in the literature and this thesis.*

had no influence on feeding behaviour. Moreover, a non-lytic GD-producing *A. pseudogonyaulax* strain from New Zealand was heavily rejected (≈ 90 %), much more often than PSP-producing *A. catenella*, providing first evidence that GDs may function as grazer deterrent (J. Xu et al. 2017). However, chemically uncharacterised BECs or other compounds may simultaneously be involved in prey rejection by copepods and only strain of *A. pseudogonyaulax* was analysed in that study (J. Xu et al. 2017). Hence, the potential prey rejection properties of GDs require confirmation and it would be useful to conduct similar copepod experiments with purified GDs. This could be further investigated by coating artificial microbeads or natural food particles with cell extracts of *A. pseudogonyaulax* or purified GDs and investigating the feeding response (Makino et al. 2008). This approach has the advantage of considering chemical interactions between predator and prey in the form of cell surface recognition (Roberts et al. 2011). Publication II also hinted towards GDs being involved in the prey rejection mechanism of protistan microzooplankton *P. kofoidii*, however the evidence is scarce and requires further investigations. The selective feeding capabilities of protozoan grazers (Irigoien et al. 2005, Verity 1991), and *P. kofoidii* (Jeong et al. 2001), have long been known and even cannibalism has been reported in absence of favourable prey items (Goldman and Caron 1985). Adaptation of copepods to PSP-producing *Alexandrium* species has also been demonstrated (Colin and Dam 2005, Colin and Dam 2007) and consequently, it may be hypothesized that *T. longicornis* was not adapted to GD-producing *Alexandrium*. However, *T. longicornis* was isolated from the Øresund in Denmark (J. Xu et al. 2017), where *A. pseudogonyaulax* occurs more frequently since 20 years (publication II). The zooplankton *Daphnia galeata* has been shown to evolve resistance to cyanobacteria blooms already within a decade (Hairston Jr et al. 1999) and both *Temora* and *Daphnia* species have similar generation cycles per year (<10, Dodson 1972, Dutz et al. 2010, Dudycha and Tessier 1999).

While it is not clear whether microzooplankton or mesozooplankton grazers across Northern European waters can adapt or already adapted to GD-producing *A. pseudogonyaulax*, decreased grazing by zooplankton can inhibit the energy transfer to higher trophic levels. For instance, HABs of the toxic dinoflagellate *Vicicitus globosus* strongly impacted the zooplankton community and consequently, reduced the energy transfer to higher trophic levels (Riebesell et al. 2018). Instead, HABs rather stimulate the microbial loop, especially upon bloom termination (Burkholder et al. 2018). Although this accelerates oxygen depletion, it also increases the concentrations of DOM, which may stimulate future HAB outbreaks (Burkholder et al. 2018). Additionally, HABs have been shown to decrease the biodiversity of zooplankton and microalgae, which is associated with disruptions of ecosystem functioning and altered energy flows (Burkholder et al. 2018, Amorim and Nascimento Moura 2021). Recently, Lee et al. (2024) suggested that *Acartia* spp. could eliminate up to 7 % of natural *A. pseudogonyaulax* populations per day, which the authors calculated by extrapolating grazing rates obtained in laboratory experiments to natural abundances of *Acartia* spp. and *A. pseudogonyaulax* in Korean waters. However, some shortcomings in this study suggest that this is a highly optimistic calculation and cast doubt on the potential of *Acartia* spp. to substantially reduce natural *A. pseudogonyaulax* populations. For instance, grazing rates were only obtained for adult copepods over a time-frame of 24–48 h and mortality of *Acartia* spp. was not compared to a non-toxic food control (M. J. Lee et al. 2024). While grazing rates were nearly identical to publication II, adverse effects of *Acartia* spp. only manifest themselves in the long-term due to reduced food intake and adverse effects on fecundity. Moreover, exposing juvenile *A. tonsa* nauplii to *A. pseudogonyaulax* resulted in lysis (publication II), which also points towards an involvement of BECs in nauplii impairment. This is especially important as nauplii are the most vulnerable life-stages towards environmental stressors (Hopp and Maier 2005) and the most abundant life stage of copepods that can have larger grazing impacts than adults (Turner et al. 2001, López et al. 2007). The selective feeding capability of zooplankton also impedes extrapolating grazing rates obtained from single prey laboratory experiments to the field. The results in publication II rather suggest that mesozooplankton, as well as microzooplankton, are not efficient grazers of *A. pseudogonyaulax* due to resulting adverse fitness effects. Altogether, the findings of publication II indicate that *A. pseudogonyaulax* blooms will alter food web dynamics likely reducing the energy flow to higher trophic levels, such as fish. This alteration has the potential to indirectly reduce the resilience of fish populations across Northern Europe, which are already negatively impacted by a variety of anthropogenic stressors, such as overfishing and eutrophication yet *A. pseudogonyaulax* may also directly impact aquaculture and fisheries.

Ichthyotoxicity of *A. pseudogonyaulax*

Ichthyotoxicological assays in publication II were conducted with the RTgill-W1 cell line from the rainbow trout *O. mykiss*. Predicting fish toxicity in vitro offers significant advantages to the conventional approach using juvenile and adult fish, including higher throughput of samples in less time, higher reproducibility, lower sample volume requirements and a potential mechanistic understanding of the analysed toxins (Dayeh et al. 2005). Additionally, cell based bioassays circumvent ethical constraints to sacrifice animals and are generally cheaper (Dayeh et al. 2005). However, the use of cell based bioassays can produce different results due to varying toxicological behaviour between whole organisms and fish cell lines with the latter being generally less sensitive (Kramer et al. 2009, Tanneberger et al. 2013). The difference in sensitivity between the RTgill-W1 bioassay and in vivo acute 'fathead minnow' toxicity was generally less than one order of magnitude, but could increase to 2–3 orders of magnitude for some chemicals (Kramer et al. 2009, Tanneberger et al. 2013). Large sensitivity differences were associated with the chemical's mode of action or with low bioavailability due to physiochemical properties, such as volatility or hydrophobicity (Kramer et al. 2009, Tanneberger et al. 2013). For instance, neurotoxic chemicals altering sodium or chloride channel dynamics were 2–3 orders of magnitude more toxic to fish than gill cells, likely due to differences in the enzymatic machinery (Tanneberger et al. 2013). In contrast, Mooney et al. (2011) reported that polyunsaturated fatty acids were more toxic to RTgill-W1 gill cells than to sheepshead minnow larvae and concluded that gills need to be well developed to be impacted by fatty acids. Similarly, Deeds et al. (2006) demonstrated that juvenile sheepshead minnow were more sensitive to karlotoxins than larvae. Thus, the interpretation of ichthyotoxicological assays needs to account for potential sensitivity differences in methodological approaches. Nevertheless, fish gill cells are still a good choice for the estimation of toxicological effects by microalgae, considering that gill epithelia are the primary uptake site of dissolved toxins (Tanneberger et al. 2013). Additionally, a high repeatability (in the same laboratory) and reproducibility (between laboratories) of the RTgill-W1 cell line has been reported, underlining the robustness of this methodological approach (M. Fischer et al. 2019) and making the RTgill-W1 cell line a promising candidate for international standardization of ichthyotoxicological analyses of HABs.

The results of publication II demonstrated that *A. pseudogonyaulax* is ichthyotoxic and thus confirm the hypothesis of similar toxicity to the closely related *A. monilatum*. Purified GDs did not cause any toxic effect towards fish gill cells relative to cell-free supernatants containing both GDs and BECs. Considering that GD-concentrations in the cell-free supernatants ($\approx$ 1 pg μL^{-1}) were two magnitudes lower than the highest tested GD-concentrations of 180 pg μL^{-1}, the findings in publication II suggest that ichthyotoxicity is primarily driven by the BECs. EC_{50}-values, i.e. the effective concentration at which

the half-maximal response is obtained (Lakshmanan et al. 2022), were 1,300–2,000 cells mL^{-1}. The obtained EC_{50}-values are similar to cell-free supernatants of other dinoflagellates (Table 5.1) from the *Alexandrium* or *Karlodinium* genus. In contrast, the EC_{50}-value of the haptophyte *Chrysochromulina leadbeateri*, which caused a massive fish-killing event in several fjords in northern Norway, was much higher with 2.9×10^6 cells mL^{-1} (X. Wang et al. 2024). However, this haptophyte is also significantly smaller (≈ 5 μm) and thus its biovolume is less than one percent of *A. pseudogonyaulax*, assuming a cell size of 28 μm for *A. pseudogonyaulax* (publication I). Additionally, it is unclear to what extent lytic activity increases when cells rupture or die off, for instance during bloom termination. Lysed or ruptured cells of some ichthyotoxic HAB species, including dinoflagellates *A. catenella* (J. I. Mardones et al. 2015) and *K. veneficum* (Dorantes-Aranda et al. 2011), as well as raphidophyte *Chattonella marina* (Mooney et al. 2011), caused increased cytotoxicity to RTgill-W1 gill cells in comparison to supernatants and whole cells. On the other hand, lytic activity to microalgae has been reported to be higher in cell-free supernatants than cell extracts (Van de Waal et al. 2015). While an important extracellular role of BECs is evident, it remains to be demonstrated if the compounds responsible for the lytic activity to microalgae and ichthyotoxicity are identical and whether concentrations are higher intra- or extracellular. Cell densities of *A. pseudogonyaulax* corresponding to the EC_{50}-values of ichthyotoxicity (publication II) have not been reached yet in Northern Europe (publication III). However, considering the lower sensitivity of the RTgill-W1 bioassay in comparison to whole fish bioassays (Tanneberger et al. 2013), natural bloom concentrations of *A. pseudogonyaulax* may still cause ichthyotoxic effects. In addition, dense patches with high cell densities and potential accumulation of BECs within the mucus of *A. pseudogonyaulax* could cause increased ichthyotoxicity. The mucus excreted by *A. pseudogonyaulax* may also exacerbate the ichthyotoxic effects by causing additional physical stress to the gills of fish. Notably, aquacultures are generally more susceptible to HAB induced damages than natural fisheries as encaged fish cannot escape either toxins or oxygen depletion (Brown et al. 2020). Considering that the pelagic food web in Northern Europe is already exposed to a variety of stressors, such as overfishing, eutrophication and climate change, it may be hypothesized that its resilience towards additional stressors, such as toxic *A. pseudogonyaulax* blooms, is low (Griffith and Gobler 2020). Altogether, it remains feasible that natural *A. pseudogonyaulax* blooms can damage aquaculture and fisheries in Northern Europe, and thus, it is important to elucidate its mechanism of ichthyotoxicity.

The environmental modulation of ichthyotoxicity of HAB species, such as *Alexandrium*, remains poorly resolved. Considering climate-induced changes, such as increasing temperatures and decreasing pH, research into the interplay between abiotic factors and the ichthyotoxicity of HAB species is urgently needed. For instance, the ichthyotoxic effect of *Cochlodinium polykrikoides* was inversely temperature-dependent with higher toxicity

at lower temperatures suggesting that blooms become more toxic upon transition from summer to fall (Griffith and Gobler 2016). This finding suggests that ichthyotoxins are less stable at higher temperatures, however, increased detoxification of the exposed organism due to higher metabolic activity is also conceivable. In contrast, haemolytic activity of *A. taylorii* was temperature-dependent, but showed higher activity with increasing temperatures (Emura et al. 2004), while lytic activity of *A. catenella* was not or only slightly affected by temperature (Ma et al. 2009, Fistarol et al. 2004). Low salinity increases the lytic activity of *A. ostenfeldii* (Martens et al. 2016), while ocean acidification likely reduces the ichthyotoxicity of *K. veneficum* (Müller et al. 2019). Nitrate and phosphate starvation only slightly influenced the lytic activity of *A. catenella* (Zhu and Tillmann 2012), and did not modify the lytic activity of *A. minutum* (I. Yang et al. 2011). Considering the detrimental effects of BECs towards the pelagic food web (publication II), factors modulating the lytic activity of *A. pseudogonyaulax* require further investigation.

Table 5.1: EC_{50}-values of RTgill-W1 cells exposed to cell-free supernatants of selective dinoflagellates.

Species	**EC_{50} (cells mL^{-1})**	**Reference**
A. catenella	$0.2 - 2 \times 10^3$	Mardones et al. 2015
A. catenella	$0.9 - 11.1 \times 10^3$	Wolf 2021
A. minutum	$3.7 - 4.4 \times 10^3$	
C. leadbeateri	2.9×10^6	Wang et al. 2024
A. pseudogonyaulax	1.3×10^3	Publication II

Many ichthyotoxic HAB species produce lytic compounds that can bind into the lipid bilayers of the sensitive gill cell membranes of fish, provoking irreparable damage and ultimately leading to death from suffocation (G. M. Hallegraeff et al. 2023). The precise mechanism of action remains poorly understood, however, recent progress has been made for karlotoxins (Deeds et al. 2015), karmitoxins (Rasmussen et al. 2017, Binzer et al. 2020) and prymnesins (Clinton et al. 2021, Varga et al. 2024). Recently, Tainter et al. (2020) showed that GDs form strong complexes with alkali ions, especially potassium, suggesting that ionophoric properties may be involved in their toxicity. Furthermore, Harris et al. (2021) demonstrated that GDA-sa forms strong complexes with sodium suggesting that GDA/GDA-sa could also function in maintaining the osmotic balance. Even though isolated GDs did not cause substantial adverse effects in *R. salina* or fish gill cells, the possibility of synergistic interactions between GDs and BECs cannot be ruled out. For instance, the lytic activity of BECs could penetrate the cell membrane, allowing GDs to infiltrate and potentially disturb the osmotic balance. This hypothesis was preliminary tested by exposing gill cells to varying concentrations of triton (0.01–0.1 mM), a commonly used detergent in biochemistry, and GDA or GDA-sa or to cell-free supernatants containing additional GDA or GDA-sa. These additional results (data not shown), however, showed no enhanced toxic effects relative to only triton or the cell-free

supernatant, respectively, strengthening the results in publication II that identified BECs as the primary driver of the ichthyotoxicity of *A. pseudogonyaulax*.

Altogether, these results have far-reaching implications for the toxicological analysis and risk assessment of *Alexandrium* species, especially from the *Gessnerium* clade. It has already been demonstrated for some *Alexandrium* species, including *A. catenella* (Tillmann and John 2002, Tillmann et al. 2009), *A. ostenfeldii* (Tillmann et al. 2007) and *A. leei* (Shang et al. 2021), that their toxicity does not correlate well with the PSP toxin content (or, in case of *A. ostenfeldii*, spirolide content) indicating that BECs are the main driver of their toxicity. Additionally, differences in the toxicity of BEC-containing *A. minutum* strains relative to non-BEC-containing strains towards the oyster *Crassostrea gigas* have been reported (Castrec et al. 2018). However, publication II is the first study showing a similar lack of correlation between phycotoxin concentrations and toxic effects for GD-producing *Alexandrium* species, in this case *A. pseudogonyaulax*. Considering that this trend has now been shown for GD-, spirolide-, and PSP-toxin producing *Alexandrium* species, it may be hypothesized that BECs are generally the main driver of toxicity towards marine organisms. Nevertheless, accumulation of GDs in marine invertebrates has been demonstrated (Harding et al. 2009) and the potential health risk to humans following consumption of intoxicated shellfish remains poorly resolved. It is likely that the lactone group of GDA hydrolyses at low pH (e.g. gastric acid in the stomach) or that GDA undergoes conversion to GDB/GDC, and thus toxicological studies should rather include these GD-congeners. Similarly, pectenotoxins get rapidly metabolized to their corresponding, less toxicologically active, seco acids and therefore oral toxicity of pectenotoxins is low (Miles et al. 2004, Sandvik et al. 2020). However, metabolization of GDs in shellfish and/or conversion during human consumption require further investigation to assess the oral toxicity of GDs. While phycotoxins still represent a risk to humans and occasionally elicit adverse effects in other marine organisms, the ecological role of phycotoxins remains mostly speculative. Many theories have been formulated, like functioning as signalling molecule (e.g. intracellular or as pheromone for sexual mating), nutrient storage or grazer deterrent (A. D. Cembella 2003, Cusick and Sayler 2013). However, the co-existence of toxic and non-toxic strains in the same population casts doubt on most theories. If phycotoxins confer an evolutionary advantage in the producing organism, why would some strains lose the ability to produce toxins? What seems to be certain is that the damage to higher trophic levels of the pelagic food chain or humans is collateral, rather than targeted. For instance, saxitoxin targets voltage-gated sodium channels (Llewellyn and Airs 2010, Cusick and Sayler 2013). However, this unlikely represents the ecological function of saxitoxin considering that genes involved in saxitoxin production emerged earlier than voltage-gated sodium channels (Murray et al. 2011).

Studies demonstrating costs of toxin production (Blossom et al. 2019, Park and Dam 2021, Park et al. 2023), raise the question of how slower growing toxin-producing strains

could prevail over time. If benefits are shared by non-toxic strains (i.e. 'public good') without having to spend energy (Driscoll et al. 2016, Ehrlich et al. 2022), why are they not outcompeting the slower growing toxin-producing strains? On the other hand, if benefits are not shared (i.e. 'private good'), how come the toxin-producing strains are not prevailing over time? These questions trace back to Darwinism and the occurrence of natural selection whenever there is a consistent fitness difference among 'individuals', which are the unit of natural selection (Futuyma 2009, Williams 1992). However, the answer may simply be that both benefit in certain situations, for instance, the non-toxin producing and faster growing strain would likely benefit in bottom-up and the toxic strain in top-down controlled environments, respectively. There are also mechanisms that restrict benefits to conspecifics as shown for *K. veneficum*, which protects itself from the pore-forming properties of karlotoxins through a unique composition of membrane sterols (Deeds et al. 2006, Deeds et al. 2015). Dense patches of conspecific algae with much higher cell densities than their surroundings (Durham et al. 2013, Breier et al. 2018) may represent another mechanism of ensuring that concentrations of dissolved toxins or BECs are high enough to cause adverse effects, yet localized to restrict benefits. Additionally, BECs have been demonstrated to protect non-BEC-producing cells from the same species suggesting a mutual benefit (John et al. 2015). Recently, Blossom et al. (2021) showed that a strain of *A. pseudogonyaulax* lost its phagotrophic abilities, while retaining lytic activity and forming mucus traps, while another strain of *A. pseudogonyaulax* (CAWD138) was reported as non-lytic (J. Xu et al. 2017) yet contained food vacuoles and formed mucus traps (Blossom et al. 2017). These examples highlight our limited understanding of the ecological roles of lytic BECs that may play a role in prey assimilation, but also grazer deterrence as suggested for GDs (J. Xu et al. 2017). However, damage to higher trophic organisms, such as fish, likely represents a side effect that got exacerbated by multiple anthropogenic stressors of natural ecosystems.

5.1.3 *A. pseudogonyaulax* is here to stay

After the first occurrences of *A. pseudogonyaulax* in Aalborg Bay and Aarhus Bay at the end of the 1990s, it took another decade until this dinoflagellate became a regular member of the *Alexandrium* community across the Kattegat, Skagerrak and the southern part of the Baltic Sea (Fig. 5.2 publication III). Although these represent the first occurrences of *A. pseudogonyaulax* in the long-term monitoring programs in Northern Europe, scarce reports about earlier sightings along the Norwegian coast (e.g. Flekkefjord in Southern Norway) in summer exist (Throndsen et al. 2007). For instance, in July and August 1984, Balech noted that *A. pseudogonyaulax* cells "were in the mucilaginous state and were abundant" (Balech 1995). Since then, *A. pseudogonyaulax* has become a prominent member of the *Alexandrium* community with observations as far east as Gotland at salinities

just above 7 (publication III). After 2007, a shift from *A. catenella* and *A. ostenfeldii* to *A. pseudogonyaulax* was observed in the Danish Limfjord, as demonstrated by Kremp et al. (2019), however, the former two re-appeared after 2017 (publication III). Today, *A. pseudogonyaulax* is appearing more frequently than other *Alexandrium* species but in lower cell densities (publication III). The general vegetative period of *A. pseudogonyaulax* is from May to September with occasional sightings throughout the winter. Modelling the yearly calculated probabilities of observing *A. pseudogonyaulax* suggests that the frequency of occurrences of this dinoflagellate are likely to increase across Northern Europe (Fig. 5.2), even though they may stagnate or even decrease in certain regions, e.g. the Danish Limfjord.

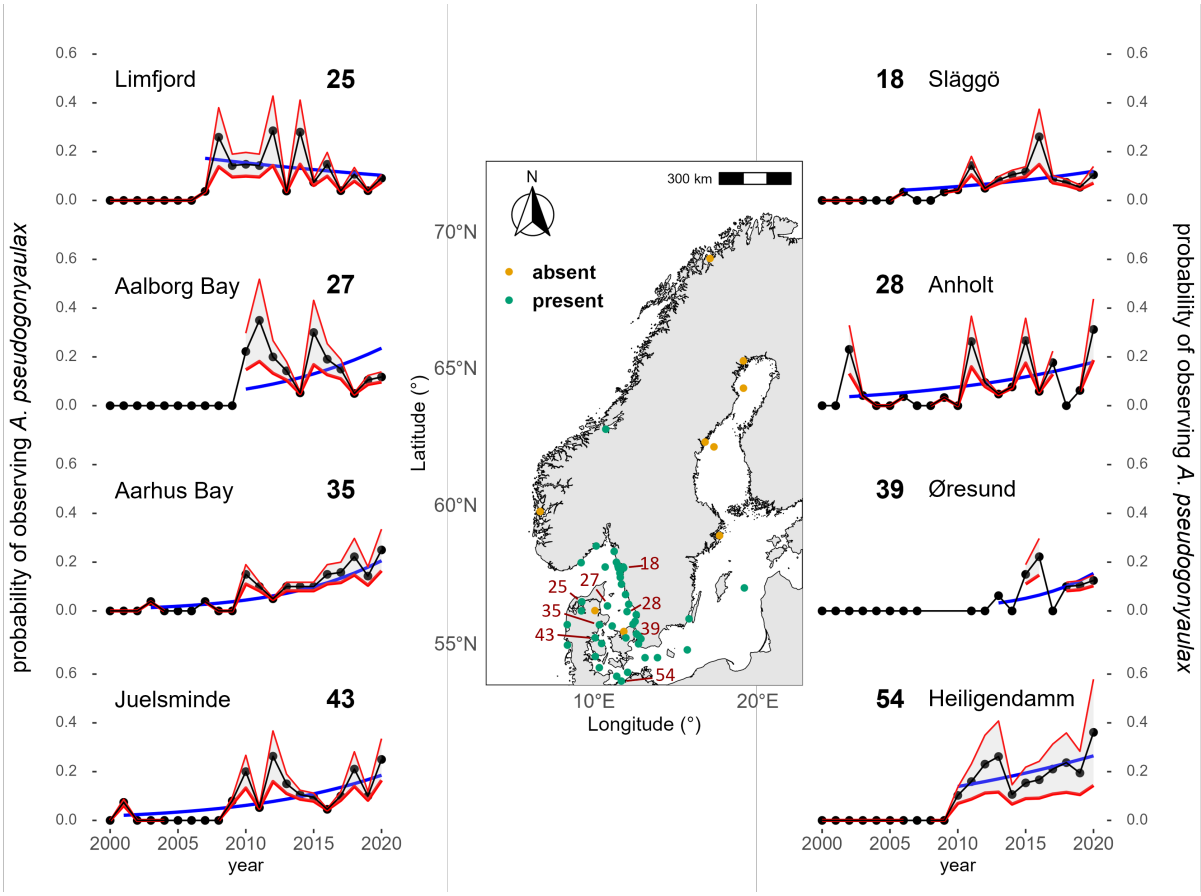

Fig. 5.2: *Probability of observing* Alexandrium pseudogonyaulax *across Northern European waters; black points and lines correspond to yearly calculated probabilities; red lines and grey ribbons to calculated 95 % confidence intervals and the blue line to a logistic regression model fitted to the yearly probability pattern.*

The expansion of *A. pseudogonyaulax* – case study: Danish Limfjord

A. pseudogonyaulax suddenly appeared in the Danish Limfjord in 2007 (Fig. 5.3, (1)) and already two years later displaced *A. ostenfeldii* and *A. catenella* (Kremp et al. 2019), but the latter two species reappeared one decade later (publication III). Simultaneously, the invasive ctenophore *Mnemiopsis leidyi* (Fig. 5.3, (1)) has been observed in the Limfjord for the first time (Riisgård et al. 2012a), likely introduced from the North Sea via the Thyborøn Kanal (Riisgård et al. 2012b). This inflow of water is driven by the South Jutland Current, which flows north along the Danish west coast carrying mixed water masses from the English Channel and southern North Sea (Gyllencreutz et al. 2006). Apart from the natural dispersal of microalgae through currents, transoceanic ships have long been associated with the expansion of invasive species due to the transport of ballast water and sediments that can contain vegetative cells or cysts (Shang et al. 2024). After introduction of *M. leidyi* in 2007, zooplankton abundances in the Limfjord were significantly reduced in 2008 and 2009 (Fig. 5.3, (2), (Riisgård et al. 2012b), which was primarily attributed to extensive grazing pressure by *M. leidyi* and another already established jellyfish *Aurelia aurita* (Riisgård et al. 2012b, Møller and Riisgård 2007). For instance, the half-life of copepods in the Limfjord was averaging only 1.6 days during June in 2009 (Riisgård et al. 2012b). The ctenophore *M. leidyi* is a generalist predator that utilizes an unselective feeding current and thus captures microalgae, microzooplankton, mesozooplankton and fish larvae (Sullivan and Gifford 2004, Colin et al. 2010). Eutrophication in the Limfjord likely benefits the fecundity of *M. leidyi* (McNamara et al. 2014), which can exhibit egg production rates exceeding 10^4 eggs d^{-1} (Jaspers et al. 2015) enabling rapid population increases. Jellyfish induced trophic cascades have been demonstrated to favour microalgal proliferation (Dinasquet et al. 2012, Tiselius and Møller 2017), which suggests that the positive feedbacks of enhanced jellyfish grazing outweigh the grazing pressure of jellyfish on microalgae. Consequently, it may be assumed that the predation pressure on *A. pseudogonyaulax* was low due to low abundances of grazers against whom it also shows remarkable resilience (publication II). Additionally, jellyfish blooms can cause high DOM concentrations due to sloppy feeding or excretion (Pitt et al. 2009, Condon et al. 2011), although this DOM has been shown to be rapidly metabolized by bacteria (Condon et al. 2011). Results from publication III indicate that *A. pseudogonyaulax* may also benefit of high carbon to chlorophyll *a* ratios indicating efficient usage of high DOM concentrations, which for example can stem from enhanced algal loss processes during the senescence of a bloom or extensive jellyfish grazing. Increased microalgal sedimentation rates in the absence of strong grazing pressure also exacerbate hypoxic conditions in the Limfjord (Riisgård et al. 2015, Møller and Riisgård 2007). Hypoxia enhances mortality of benthic filter feeders further reducing the grazing pressure on microalgae and also releases large amounts of nutrients from the sediment stimulating microalgal growth. Additionally, *M. leidyi* is more resistant towards hypoxic conditions than its predators and prey and con-

sequently hypoxic conditions may favour proliferation of jellyfish over planktivorous fish (Decker et al. 2004). However, hypoxia is also unfavourable for cyst germination (Persson and B. C. Smith 2022).

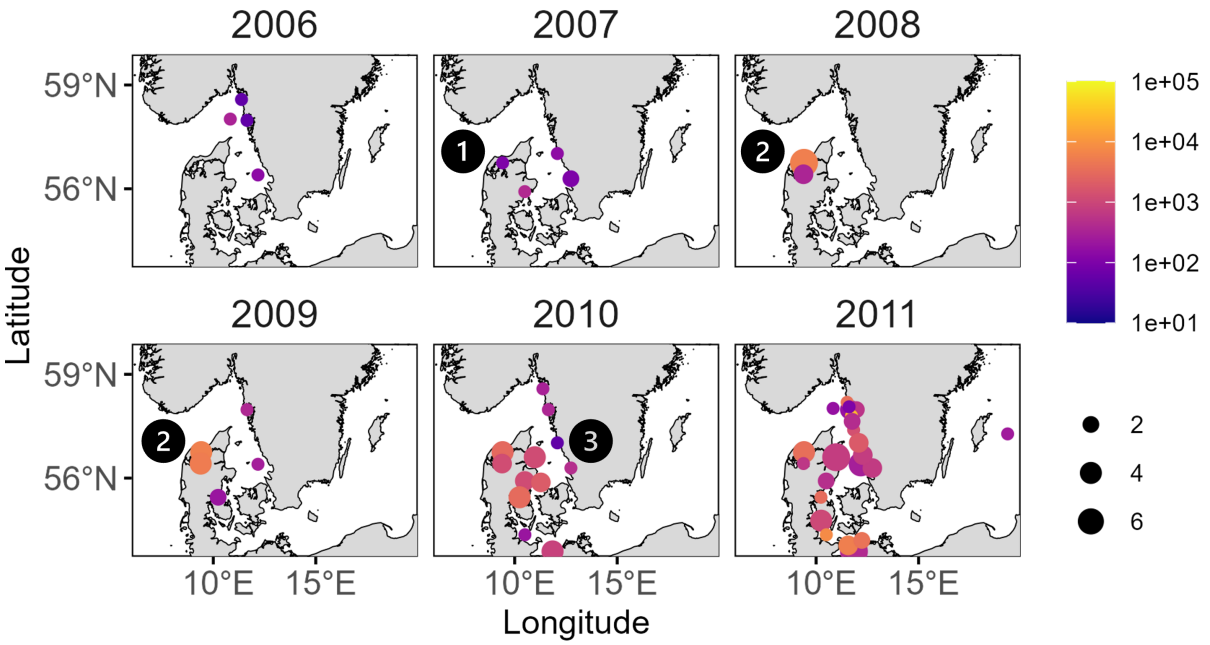

Fig. 5.3: *Expansion of* A. pseudogonyaulax *in Northern European waters and proposed establishment in the Kattegat; colour scheme corresponds to the cell densities (cells* L^{-1}*) and size to the number of present observations during the respective time-frame (Möller et al., in prep.); 1) Arrival of* M. leidyi *in the Danish Limfjord; 2) Trophic cascade initiated by predation of* M. leidyi *and* A. aurora *on zooplankton enables* A. pseudogonyaulax *to proliferate and form cyst beds; 3) Waterflow from the Limfjord into the Kattegat transports cysts that subsequently seed the local* A. pseudogonyaulax *populations.*

Even though *A. pseudogonyaulax* was already observed in the Kattegat in the 1990s, it only became a regular member of the microalgae community after 2009 (Fig. 5.3, publication III). Strikingly, high abundances of *A. pseudogonyaulax* in the Limfjord in 2008 and 2009 were preceding this expansion and co-occurred with the invasion of *M. leidyi*. The importance of pelagic and benthic life-cycle transitions in bloom dynamics of HABs is well-known (Brosnahan et al. 2017, Brosnahan et al. 2020) and hence, it may be hypothesized that large cyst beds in the Limfjord, established during 2008 and 2009, aided the subsequent expansion of *A. pseudogonyaulax* into the Kattegat (Fig. 5.3, (3)). In fact, sediment in the Limfjord, along with dinoflagellate cysts, is regularly resuspended by strong westerly winds, which also drive the water exchange between the Limfjord and Kattegat (Olesen 1996). Additionally, mussel dredging, along with eutrophication associated reductions in light availability, has led to a significant decline of eelgrass (Olesen

1996, Carstensen et al. 2013), which further reduces the stability of the sediment contributing to cyst resuspension (Carstensen et al. 2013). In 2016, cyst densities of *A. pseudogonyaulax* across the Limfjord were approximately 100 cysts cm^{-3} (Kremp et al. 2019). In comparison, cyst beds of *A. fundyense* in the Gulf of Maine and adjacent Bay of Fundy are usually higher than 1,000 cysts cm^{-3}, sometimes exceeding 5,000 cysts cm^{-3} (Martin et al. 2014, D. M. Anderson et al. 2014). Cyst beds of *A. catenella* in the Alaskan Arctic even reach densities exceeding 10^4 cysts cm^{-3}, although accumulation was likely caused by unfavourable conditions for germination (D. M. Anderson et al. 2021). Considering that *A. pseudogonyaulax* was a regular part of the microalgal community since 2007 regularly exceeding cell densities of 10^3 cells L^{-1} from June to October (publication III), cyst densities of 100 cysts cm^{-3} seem low. It may thus be hypothesized that the residence time of cysts in the Limfjord is short due to fast resuspension of the sediment. Altogether, the resilience of *A. pseudogonyaulax* towards grazers (publication II) along with jellyfish induced trophic cascades may have aided the permanent establishment of this toxic dinoflagellate in Northern European Waters (publication III). Additionally, the complex interplay between favourable conditions, such as jellyfish abundance, nutrients and temperature, in the Danish Limfjord might have catalysed the establishment of *A. pseudogonyaulax* in the Kattegat and Skagerrak.

5.2 Future perspectives & outlook

In this thesis, three manuscripts explored the interplay of bottom-up and top-down factors with the harmful dinoflagellate *A. pseudogonyaulax* and investigated its ongoing expansion in Northern European waters. This is of particular importance as *A. pseudogonyaulax* has been associated with ichthyotoxic characteristics. These properties were confirmed in this thesis and additional adverse effects on algae, protistan microzooplankton and mesozooplankton were demonstrated. However, several challenges remain, such as gaining a mechanistic understanding of the ichthyotoxicity of *A. pseudogonyaulax*, cost-effectively monitoring its expansion and mitigating the potential economic consequences of blooms.

5.2.1 Structural elucidation of BECs and the role of phycotoxins

The findings of this thesis support the notion that BECs produced by *Alexandrium* of the *Gessnerium* clade are the main drivers of their toxicity (publication II). While phycotoxins remain important due to their adverse effects in humans, BECs are more important in determining adverse effects on other marine organisms, especially fish. This raises the question if phycotoxin monitoring is an effective mitigation tool, especially for preventing fish death in aquacultures. However, the correlation between the GDA content and cell densities of *A. pseudogonyaulax* in plankton net tows is good (Fig. 5.4) and thus, monitoring of GDs as chemotaxonomic trait of *A. pseudogonyaulax* is still valuable in Northern Europe where no other GD-producing *Alexandrium* species occur. Monitoring of BECs would be the other option, which, however, currently remains elusive due to the absence of effective isolation and purification protocols, and therefore, analytical standards. Estimating the toxicity of natural *A. pseudogonyaulax* blooms without determination of BEC concentrations might be error-prone considering the high intraspecific variability in lytic activity of *Alexandrium* species (Long et al. 2021). Hence, establishment of isolation and purification methods of BECs is of critical importance for facilitating further toxicological studies and possibly monitoring programs. This is certainly no easy task considering the large size and chemical nature of BECs and requires collaborate efforts in toxicology, biology and chemistry. In the meantime, it may be conceivable to design a low-cost early warning HAB system for aquacultures based on measuring a behavioural response of a target organism. For instance, real-time behavioural analysis of mussels, such as *Unio tumidus*, is already used across Europe to continuously monitor water quality (Ferreira-Rodríguez et al. 2023). Cell lysis of other microalgae could be an indicator of HAB toxicity to aquacultures assuming that the ichthyotoxic effect of *A. pseudogonyaulax* is caused by the same BECs as the lytic effect towards *R. salina* (publication II).

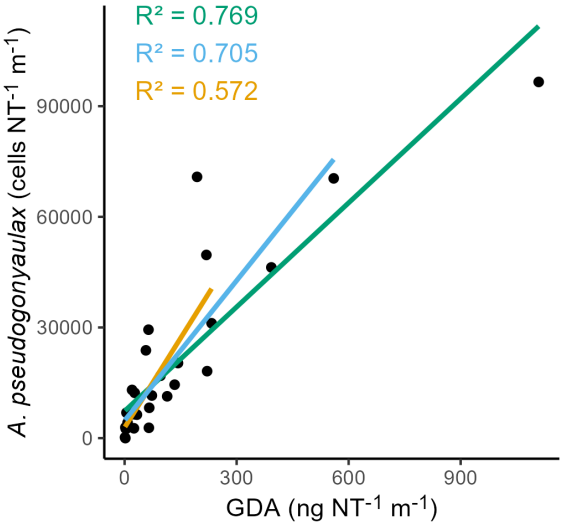

Fig. 5.4: *Linear correlation between the GDA abundance and microscopic cell counts of* A. pseudogonyaulax *in the 20-50 µm size fraction of vertical microalgal net tows (NT) during a research cruise across the North Sea, Limfjord and Baltic Sea in 2020; details regarding sampling and subsequent analysis protocols can be found in Krock et al. (2018).*

5.2.2 Novel microalgal detection methods to facilitate HAB monitoring

A major limitation in integrating HABs into ecological models, and thereby, enhancing the ability to forecast HABs, stems from the limited spatiotemporal coverage of monitoring studies. However, the increasing availability of real-time water quality sensors, such as those measuring salinity, temperature, dissolved oxygen or chlorophyll *a* fluorescence, facilitates the development of HAB early warning systems. All monitoring programs analysed in publication III still rely on enumeration by microscopy of microalgal samples. This analysis requires extensive taxonomic expertise and although the analysis itself is not inherently costly, high expenses arise from the required manual labour. While more rapid approaches, such as bulk water optical measurements (e.g. chlorophyll *a* fluorescence or light absorption), exist, they provide little information about taxonomic composition. A variety of novel detection methods are available, which, if integrated into long-term monitoring systems, could enhance spatiotemporal coverage of species- or group-specific abundance data. Four promising methodological approaches, suitable for economically efficient large-scale monitoring, are automated imaging techniques, remote sensing, autonomous ocean instruments, as well as molecular methods.

Automated imaging techniques (e.g. FlowCam, CytoSense, Imaging Flowcytobot IFCB) can sample natural microalgal assemblages over multiple months, providing nearly real-time data (Agarwal et al. 2023, Kenitz et al. 2023). Theoretically, systems like the IFCB can be deployed for much longer if equipped with an energy source, such as photovoltaic cells, although bio-fouling of analytical instruments remains a challenge (Ziccarelli et al. 2016). Analysis of the resulting images requires automated taxonomic classification using machine learning techniques, as the IFCB can capture up to 40,000 high resolution images per hour (Olson and Sosik 2007). Multiple studies have reported very good agreements between automated image analysis and manual enumeration (Sosik and Olson 2007, Rivas-Villar et al. 2021, Ayala et al. 2023). The accuracy of automated image analysis using machine learning techniques is directly related to the diversity and quality of training data. Thus, it is conceivable that these techniques will be applicable to distinguish morphologically similar *Alexandrium* species in the future, at least to some extent. The employment of imaging techniques has already demonstrated shifts from diatoms to dinoflagellates (A. D. Fischer et al. 2020) and has complemented early warning and forecasting system for HABs (Agarwal et al. 2023, Campbell et al. 2013, Harred and Campbell 2014).

Remote sensing techniques involving satellites offer another approach, capable of monitoring HABs with high spatiotemporal resolution. Typically, satellites measure chlorophyll *a* fluorescence as a proxy for microalgal biomass, but hyperspectral imaging techniques capturing a wider spectrum of light, such as UV-light, also exist (Blondeau-

Patissier et al. 2014). The latter enable the detection of specific pigment signatures, thereby, including more information about community compositions (Blondeau-Patissier et al. 2014, Uitz et al. 2006). For instance, data from satellite-derived UV measurements was utilized to track and identify a bloom of *L. polyedra* off Southern California on the basis of its special composition of UV-absorbing mycosporine-like amino acids (Kahru et al. 2021). However, the long history of *L. polyedra* blooms in this region (Shipe et al. 2008, Bialonski et al. 2016) and previous studies on UV-absorbing compounds in *L. polyedra* (Vernet and Whitehead 1996, Whitehead and Vernet 2000) likely facilitated the correct identification, which was also validated through IFCB and microscopy images (Kahru et al. 2021). UV-absorbing compounds are produced by a wide range of microalgae, yet with significant variability in composition (Llewellyn and Airs 2010). Hence, an extensive library of microalgal UV-absorbing compounds and pigments could be a powerful tool to track and potentially identify HABs.

Autonomous ocean instruments, including floats, gliders, and moorings have rapidly improved and are regularly employed today. For instance, usage of an ocean-wave powered mooring device equipped with an autonomous vertical depth profiling system recorded the 30-40 m vertical migration of the dinoflagellate *L. polyedra* and measured associated nitrate depletion in the sub-euphotic zone (B. Zheng et al. 2023). The most prominent example of floats is the Argo float, primarily used to measure temperature, salinity and currents, with nearly 4,000 floats deployed worldwide (Jemai et al. 2021). Once Argo floats include hyperspectral imagery similar to satellites, they may become crucial tools for detecting HABs (Dierssen et al. 2020).

Finally, rapid advances in molecular methods, such as quantitative real-time PCR (qPCR) or microarrays, provide promising alternatives to standard morphological species determination (Medlin and Orozco 2017). These methods offer faster and potentially more accurate means of monitoring species, that require less technical expertise and can even be utilized in the field. Quantification is ultimately dependent on the copy numbers of the targeted gene in the species of interest, which is often the small or large ribosomal subunit. For dinoflagellates, this copy number can be highly variable between species (Liu et al. 2021), but also within species of the same population (Ruvindy et al. 2023). For instance, Kremp et al. (2019) demonstrated that the 28S rRNA sequences, which are part of the large ribosomal subunit, of multiple strains of *A. pseudogonyaulax* from the Limfjord were nearly identical (> 99 %) and indistinguishable from global *A. pseudogonyaulax* strains. However, the genomic copy number of the same strains was estimated to range between 81,000 and 703,000 per genome and hence quantifying natural populations on the basis of the genomic copy number of single laboratory strains is prone to be erroneous. More consistent cell count estimations of PSP-producing *Alexandrium* species were reported by studies targeting genes associated with toxin biosynthesis (Murray et al. 2019, Stüken et al. 2015), which can also offer additional insights into the toxicity of

blooms. PSP-toxins are produced by eukaryotic dinoflagellates, as well as prokaryotic cyanobacteria, and the earlier discovery of a set of core genes of PSP-toxin production in cyanobacteria certainly facilitated the elucidation in dinoflagellates (Moustafa et al. 2009, Stüken et al. 2015). Genes involved in the biosynthesis of goniodomins or BECs remain to be discovered. Notably, Wohlrab et al. (2016) demonstrated that gene expression of a lytic relative to a non-lytic *A. catenella* strain differed in 5 % of all analysed genes, highlighting the complexity of elucidating the genes responsible for the biosynthesis of BECs. Although quantification in most methods is still more or less limited by the variability in gene numbers, qPCR based approaches represent valuable tools in the analysis of HABs. Molecular methods can also be used to quantify cysts in sediment samples from the genus *Alexandrium* (Kamikawa et al. 2007, Erdner et al. 2010).

Altogether, the rapid development and improvement of novel microalgal detection methods provide an exciting opportunity to realize HAB modelling and forecasting. These techniques are already complementing microscopic observations and in the near future might be able to replace microscopic enumeration as well.

5.2.3 Mitigation strategies

Several anthropogenic drivers of HAB proliferation, such as nutrient pollution or overfishing, have been discussed in the introduction of this thesis. Although it is crucial to minimise these alterations to reduce the risk of HAB development, exploitation of marine resources will remain an important ecological aspect of many countries. Approximately 40 % of the global population are living in coastal zones within 100 km of the coastline (Kennish 2023), and may be directly or indirectly affected by HABs. Consequently, it is useful to consider mitigation strategies for HABs in general, as well as for *A. pseudogonyaulax* considering its ichthyotoxic potential (publication II) and its ongoing expansion in Northern European waters (publication III). This is of particular importance since food fish production from aquacultures now exceeds capture fisheries and is projected to further increase (FAO 2024). Spatial and temporal planning of aquaculture sites can preventively reduce the risk of economic losses (Brown et al. 2020). The former includes the choice of site, e.g. offshore with high exposure to tides, winds and waves, where bloom development is generally suppressed, as well as impacts from nutrient enrichment due to water mixing. Additionally, adjacent shellfish and macroalgal culturing can mitigate blooms through filter-feeding, nutrient removal or the excretion of allelochemicals by macroalgae (Brown et al. 2020). Promising approaches combining these features are integrated multi-trophic aquacultures, which employ extractive species (e.g. bivalve filter-feeders or macroalgae) to reuse waste nutrients from the aquaculture ideally providing a self-sustaining food-web.

Mitigation strategies can be divided into physical, chemical and biological control methods. Physical methods consist of the use of barriers around aquaculture sites or the removal of HAB cells by e.g. filtering or flocculation (Brown et al. 2020, G. M. Hallegraeff et al. 2023, G. Hallegraeff et al. 2017). Cost effective preventive methods include water column mixing disrupting thermal stratification. Flocculation of algae is usually conducted with clay and after sinking and burial of algal cells, sediment can be dredged, treated to remove algal cells and discharged back to the removal site (Brown et al. 2020). Modified clays can have additional advantages, including killing of HAB cells (S. E. Beaulieu et al. 2005), accumulation of dissolved phycotoxins (Seger et al. 2015, Seger et al. 2017) and particulate nutrients (Yu et al. 2017). These mitigation strategies are already regularly employed in China, where economic damages caused by HABs are common (Sakamoto et al. 2021). The major drawback of flocculation techniques is the lack of specificity towards HAB cells, as all algal cells are sedimented during this approach. Chemical mitigation strategies consist of natural biosurfactants, biocides, biochemical extracts from macroalgae containing allelochemicals or synthetic chemicals, such as hydrogen peroxide (Brown et al. 2020). These substances can interfere with HAB species cell survival or reproduction. Similar to the flocculation techniques, the lack of specificity is the general set-back of these methods.

Finally, biological mitigation strategies are consisting of top-down population controls of HAB species (Pal et al. 2020), including mesozooplankton (e.g. copepods) or microzooplankton (e.g. ciliates, heterotrophic dinoflagellates) and pathogenic microorganisms (e.g. bacteria or viruses). For instance, ciliates of the genus *Favella* have been demonstrated to be unaffected by PSP-producing *A. catenella* (reported as *A. tamarense*, (Kamiyama et al. 2005) and bacterial algicides have been shown to lyse *A. catenella* (reported as *A. tamarense*, (B. Wang et al. 2010) and inhibit photosynthetic efficiency eventually leading to cell lysis of *A. minutum* (Gustafsson et al. 2009). The adverse effects of *A. pseudogonyaulax* towards *A. tonsa* and *P. kofoidii* (publication II) render these grazers unsuitable as potential top-down controls of A. pseudogonyaulax. Even though biological control mechanisms are generally assumed to be more environmentally friendly (Pal et al. 2020), impacts on other components and potential irreversible alterations of ecosystems have prevented field trials so far.

Altogether, multiple mitigation strategies controlling, preventing, or removing HABs are currently investigated. The dualism of economic benefits versus potential ecological damages resembles the ongoing debate about carbon dioxide removal techniques. It seems obvious that measures must be taken to guarantee marine food security in coastal systems highlighting the urgent need for research about short- and long-term ecological risks of mitigation strategies.

5.3 Conclusions

HABs of *Alexandrium* species have been a recurring problem across Northern European waters occasionally provoking ecological and economic damages. This dissertation demonstrates that *A. pseudogonyaulax* has established itself as a new toxic HAB species in Northern Europe with the potential to disrupt ecosystems and alter the energy flow between trophic levels. Additionally, the ichthyotoxic potential of *A. pseudogonyaulax* was uncovered suggesting similar toxicity for other GD-producers of the *Gessnerium* clade. The toxicological studies further highlight the prominent role of lytic BECs in the overall toxicity of *Alexandrium* species, although the understanding of the ecological roles of BECs and GDs remain largely elusive. However, toxicological results presented in this study are based on laboratory experiments and an assessment of the toxicity of *A. pseudogonyaulax* in natural ecosystem settings, along with the elucidation of its ichthyotoxic mechanism of action, are urgently required to formulate a risk assessment of this emerging dinoflagellate in Northern Europe. The findings of this study further highlight how anthropogenic impacts continue to shape microalgal communities with the potential to increase the likelihood of HAB formation and the introduction of invasive species. The importance of coastal ecosystems in maintaining food security, but also in recreational activities, call for interdisciplinary approaches to increase the spatiotemporal monitoring of HABs, to elucidate the mechanism of toxicity and to identify suitable mitigation strategies to avert economic and ecological damages.

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Contribution

Declaration on the contribution of the candidate to a multi-author article / manuscript. Contribution of the candidate in % of the total workload (up to 100 % for each of the following categories).

Chapter 2, Publication I

- Experimental concept and design: ca. 50 %
- Experimental work and/or acquisition of (experimental) data: ca. 95 %
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