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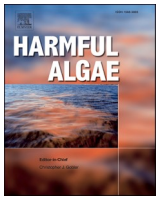

Toxic effects of the emerging *Alexandrium pseudogonyaulax* (Dinophyceae) on multiple trophic levels of the pelagic food web

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ABSTRACT

The dinoflagellate *Alexandrium 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 substances (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.

1. 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 ($< 200\ \mu m$) and mesozooplankton ($> 200\ \mu m$) 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

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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 bioactive extracellular compounds (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. *Alexandrium pseudogonyaulax* is further known for producing goniodomins (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 ≈ 75 %), while GDA-sa was the predominant extracellular GD congener (2–9 pg cell^{-1}, usually ≈ 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, *in prep.*). 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 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 (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.

2. Material & methods

2.1. 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), *A. 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 μmol L^{-1} NO_3^-, 25 μmol L^{-1} NH_4^+). The original K-medium recipe was modified by replacing the organic phosphorus source with 3.62 μmol L^{-1} 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).

2.1.1. 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. *P. 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 h 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}).

2.1.2. 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.

2.2. 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 h) 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 h, 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.

2.3. 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 × *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 × *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 × *g*. The resulting filtrates were transferred to HPLC vials and stored at −20 °C until mass spectrometric analysis. Cellular GD contents (mass $cell^{-1}$) of *Alexandrium* spp. utilized in this study were quantified and subsequently converted to molar toxin quotas (molar mass $cell^{-1}$) and normalized to the molar carbon content per cell (GDA C^{-1}). 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 × *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 → 733.5 with a three to four point (5–1,000 pg μL^{-1} GDA) external calibration curve ($R^2 = 0.99$). 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).

2.4. 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 h 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* (4-5 sampling points) and the number of *P. kofoidii* cells (7 sampling points) 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 h 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.

2.5. 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 h 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.

2.6. Effects of A. pseudogonyaulax on A. tonsa

2.6.1. 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.

2.6.2. 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_1-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.

2.6.3. 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 (≈ 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.

2.7. Effects of copepodamide exposure on the toxin cell content of A. pseudogonyaulax

Alexandrium 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 h 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 1,500 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, with a ratio of 86:14 copepodamides:dihydrocopepodamides in a total concentration of 28.8 μmol L^{-1} (Grebner et al., 2019). After 24 h, 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.

2.8. 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 h 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 h, 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 (CyQUANT™, 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.

2.9. Effects of Alexandrium spp. and goniodomins on 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 approximately 3–3,000 cells mL^{-1}) or GDs (0.2–180 pg μL^{-1}) for 3, 24 or 48 h 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.

2.10. 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 ($\alpha = 0.05$, 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 (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). 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).

3. Results

3.1. 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 h, 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 ($H_1 = 3.2$, $p > 0.05$). No significant differences for the other prey combination were found ($H_1 = 0.1–2.7$, $p > .05$) at any time points.

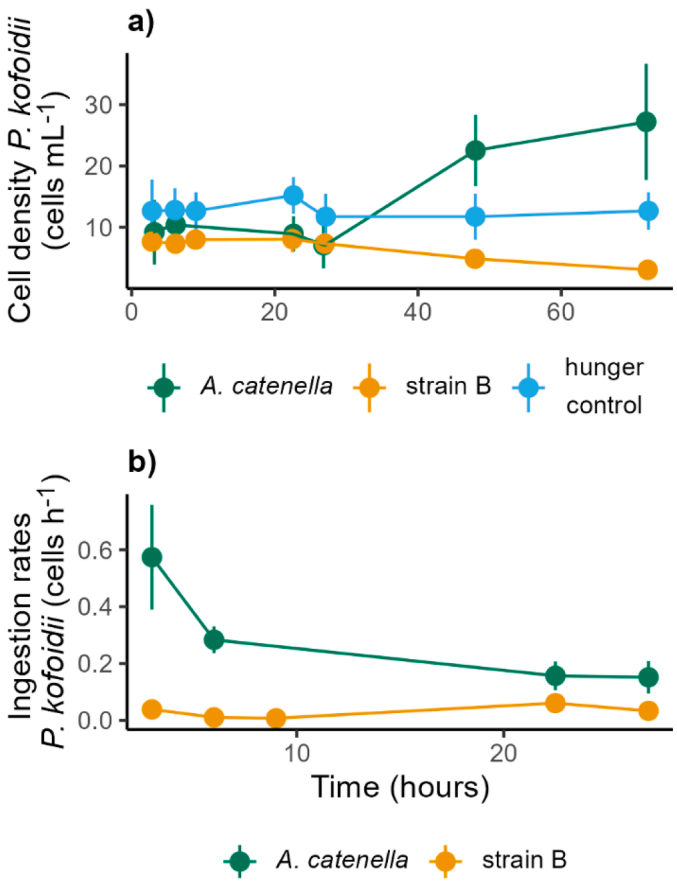

Fig. 1. a) Cell densities (cells mL^{-1}) over time of *P. kofoidii* feeding on monoalgal prey (*A. catenella* or *A. pseudogonyaulax* strain B) including an additional hunger control; points represent mean with 95 % confidence intervals from 3 – 6 biological replicates; b) corresponding ingestion rates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

3.2. Effects of A. pseudogonyaulax on A. tonsa

All three *A. pseudogonyaulax* strains (A, B, C) were consumed by all three life-stages of *A. 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). 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 pg GDA $cell^{-1}$ (Fig. 2). Even though the value of 45.7 pg GDA $cell^{-1}$ lies outside of the 95 % confidence interval, it was not detected as an outlier by the dixon test. After 24 h, significant differences within the toxin quotas (GDA $cell^{-1}$) of all three *A. pseudogonyaulax* strains were found (A: $F_{3,24} = 348.7$, $p < .001$; B: $F_{3,20} = 93.1$, $p < .001$; 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 mortality rate > 98 % 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

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

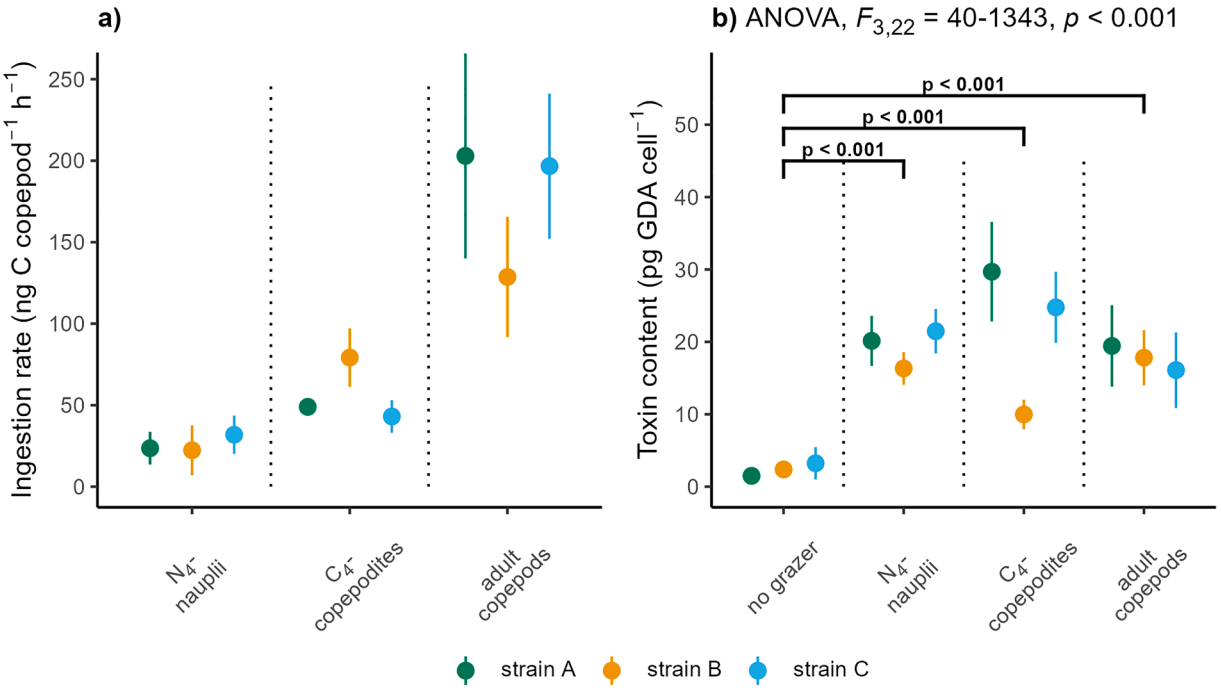

Fig. 2. a) Ingestion rates (ng C copepod^{-1} h^{-1}) of three developmental stages of *A. tonsa* (N_4-nauplii, C_4-copepodites, adult copepods) feeding on *A. pseudogonyaulax* strains A-C and b) corresponding toxin content (pg GDA cell^{-1}) of *A. pseudogonyaulax* both with 95 % confidence intervals from 3 – 10 replicates; ingestion rates were calculated according to Frost et al. (1972) and converted to ingested carbon according to the carbon content of *A. pseudogonyaulax*.

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	/	/	/	/	/	0, 0, 0, 0, 0, 0

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 rate 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 the egg hatching rate of *A. tonsa*. No significant differences in the hatching rate between the three examined strains of *A. pseudogonyaulax* were identified.

3.3. 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

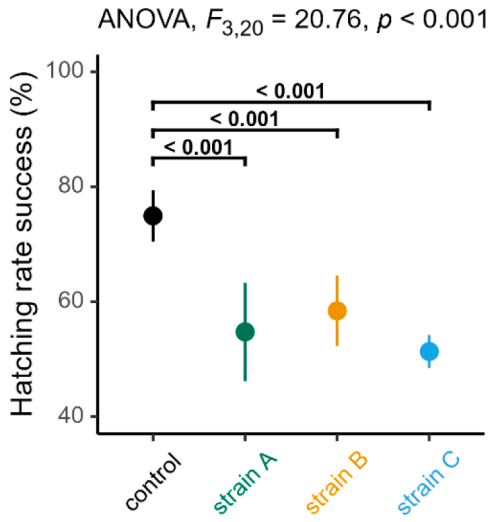

Fig. 3. Mean hatching rate success with 95 % confidence intervals of *A. tonsa* eggs exposed to the cell-free supernatant of *A. pseudogonyaulax* strains A-C with the control being filtered seawater, test statistics and pairwise comparisons correspond to an ANOVA test and to selected Tukey Honest Significant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

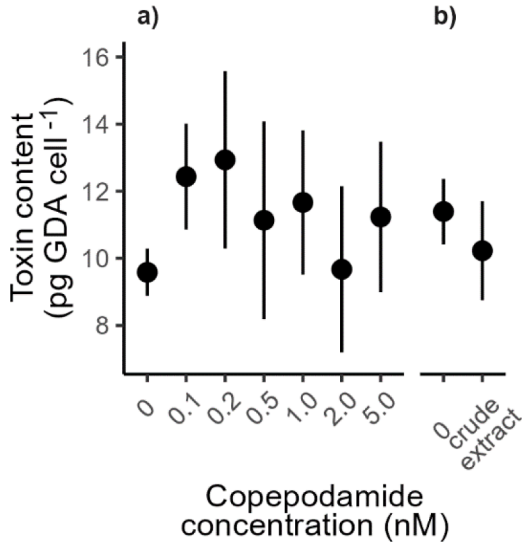

Fig. 4. Toxin content (pg GDA cell^{-1}) of *A. pseudogonyaulax* cells after exposure to copepodamides from a) *C. finmarchicus* or b) *A. tonsa*; points represent mean with 95 % confidence intervals from six replicates.

A. pseudogonyaulax (Fig. 4).

3.4. 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 (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

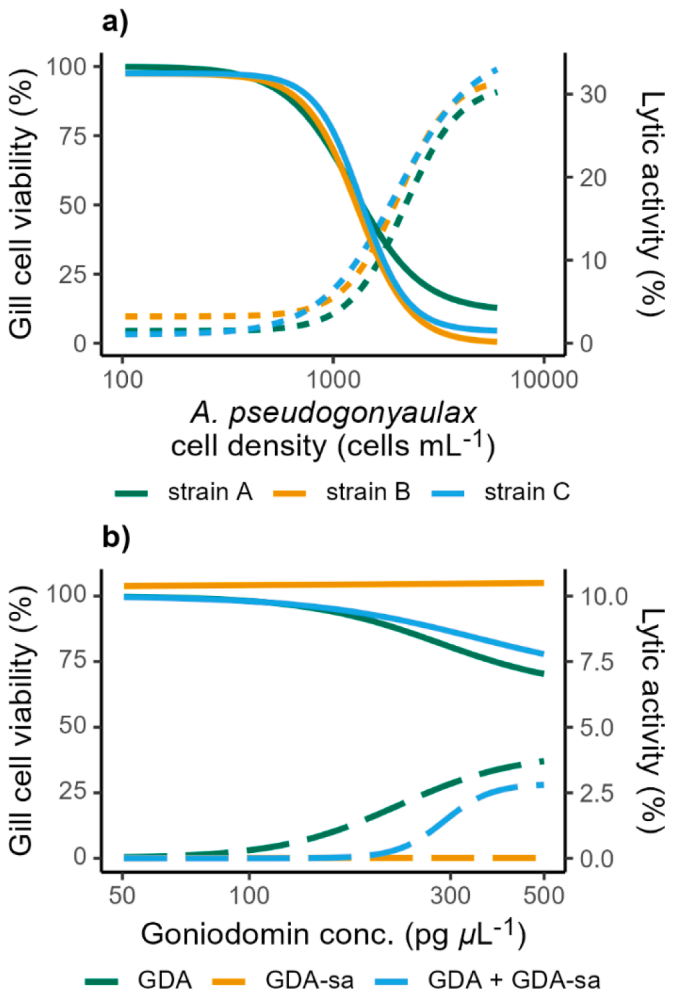

Fig. 5. Dose response curves of the gill cell viability (%, left y-axis) and lytic activity (%, right y-axis) of RTgill-W1 fish cells conducted with a) cell-free supernatants of *A. pseudogonyaulax* strains A-C and b) purified goniodomins; all data presented encompass a minimum of four biological gill 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.

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.

3.5. Effects of Alexandrium spp. and goniodomins on R. salina

Rhodomonas salina cells exposed to supernatants of *A. pseudogonyaulax* or *A. monilatum* were completely lysed after 3 or 24 h (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 h (Fig. 6, Table 3, Möller et al., 2024f). In addition, no synergistic interaction between GDA and GDA-sa was observed. *Alexandrium 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* were 240

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, and purified goniodomins.

Treatment	Strain/substance	EC_{50}-values viability / lytic activity	Time (h)	Total loss of viability?	Assay
cell-free supernatant *A. pseudogonyaulax*	strain A	1,246 / 2,178 cells mL^{-1}	24	yes	RTgill-W1
	strain B	1,308 / 2,029 cells mL^{-1}	24	yes	RTgill-W1
	strain C	1,360 / 2,003 cells mL^{-1}	24	yes	RTgill-W1
pure substance dissolved in K/2-medium	GDA	225 / 282 pg μL^{-1}	24	no	RTgill-W1
	GDA-sa	no effect	24	no	RTgill-W1
	GDA + GDA-sa	294 / 350 pg μL^{-1}	24	no	RTgill-W1
cell-free supernatant *A. monilatum*	strain YRK2007	60–454 cells mL^{-1}	3	yes	*R. salina*
	strain YRK2007	40–322 cells mL^{-1}	24	yes	*R. salina*
cell-free supernatant *A. pseudogonyaulax*	strain A	1,229 cells mL^{-1}	24	yes	*R. salina*
	strain B	1,260 cells mL^{-1}	24	yes	*R. salina*
	strain C	520 cells mL^{-1}	3	yes	*R. salina*
		240–461 cells mL^{-1}	24	yes	*R. salina*
pure substance dissolved in K/2-medium	GDA	no effect	3, 24, 48	no	*R. salina*
	GDA-sa	no effect	3, 24, 48	no	*R. salina*
	GDA + GDA-sa	no effect	3, 24, 48	no	*R. salina*
	GDB	no effect	3, 24, 48	no	*R. salina*
	GDC	no effect	3, 24, 48	no	*R. salina*

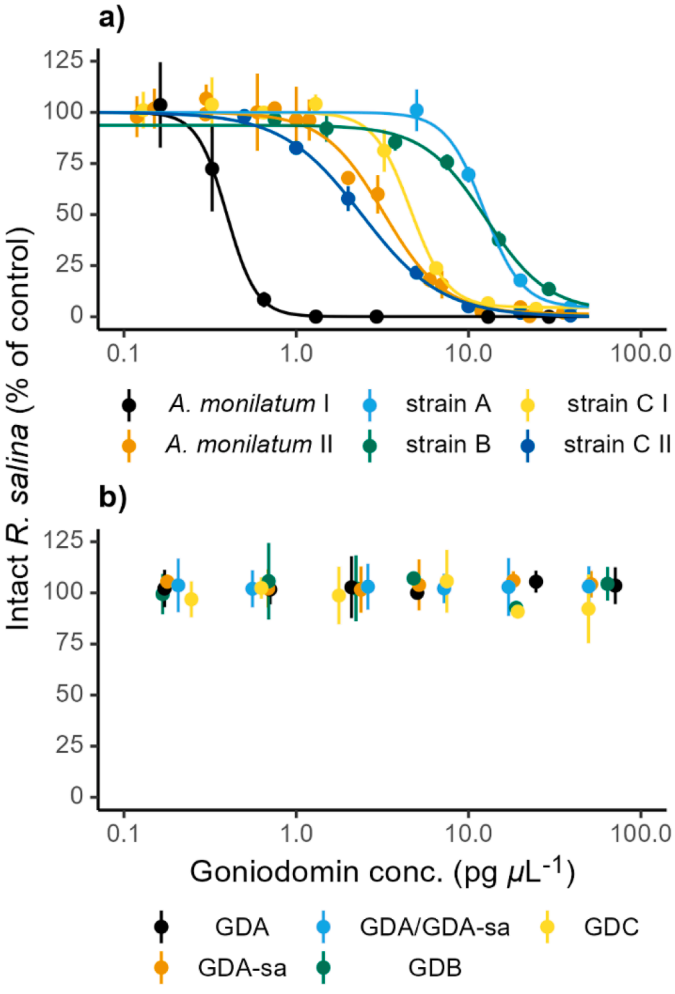

Fig. 6. Dose response curves of the *R. salina* bioassays over 24 h with a) cell-free supernatants of *A. monilatum* strain YRK2007 and *A. pseudogonyaulax* strains A-C and b) purified goniodomins; points represent mean with 95 % confidence intervals from three replicates, while lines represent modelled 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 4.

and 1,260, as well as 40 and 454 cells mL^{-1}, respectively.

4. 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 bioactive extracellular compounds (BECs) and not by goniodomins (GDs).

4.1. 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 previously 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.

4.2. *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* produce 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 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.

4.3. *Loss of metabolic activity and lysis of fish gill cells is 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 ($\approx$ 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.

4.4. *Lytic effect of Alexandrium spp. on microalgae is 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 h. In contrast, exposure to various GD congeners with concentrations up

to 180 pg μL^{-1} induced no lysis after up to 48 h. The dissolved GD concentrations of the cell-free supernatants ($\approx$ 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 and 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.

5. 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 increase 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.

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.

CRediT authorship contribution statement

Kristof Möller: Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization. **Urban Tillmann:** Resources, Writing – review & editing, Methodology, Investigation, Conceptualization. **Magdalena Pöchhacker:** Writing – review & editing, Investigation, Formal analysis. **Elisabeth Varga:** Writing – review & editing, Supervision, Resources, Conceptualization. **Bernd Krock:** Writing – review & editing, Supervision, Resources, Conceptualization. **Francesco Porreca:** Investigation. **Florian Koch:** Writing – review & editing, Supervision, Conceptualization. **Thomas M. Harris:** Writing – review & editing, Resources. **Cédric L. Meunier:** Writing – review & editing, Supervision, Conceptualization.

Declaration of competing interest

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

Data availability

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).

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