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Harmful alga trades off growth and toxicity in response to cues from dead phytoplankton

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Abstract

Organisms are under selection pressure to recognize predators and assess predation risk to avoid becoming prey. In some cases, the presence of injured competitors alerts individuals to the likelihood that predators are nearby. Previous studies have shown that the marine dinoflagellate *Alexandrium minutum* responds to chemical cues from copepods by dramatically upregulating sodium channel-blocking toxins that appear to function as defenses against copepod grazing. However, it is unknown whether *A. minutum* uses other cues, such as damaged phytoplankton, some of which are its competitors, to assess predation risk and subsequently increase its resistance to predators. To investigate the role of dead phytoplankton cues in chemical defense plasticity, *A. minutum* was exposed for 3 days to chemical cues from six different phytoplankton. Chemical cues from dead, unrelated, historically co-occurring phytoplankton species induced toxin production in *A. minutum* coincident with a decrease in growth. In contrast, exposure to chemical cues from more closely related dead phytoplankton, either conspecific or congeneric, suppressed toxin production in *A. minutum* relative to their absence. This was coupled with a modest, yet significant, increase in growth. The consistent inverse relationship between toxin production and growth suggests that *A. minutum* experiences a trade-off. Together, these results reveal that relatedness of dead phytoplankton is important in how *A. minutum* utilizes resources for growth and defense.

Predation is an important factor controlling phytoplankton populations (Sherr and Sherr 1988; Weisse 1991; Strom 2008). Filter-feeding animals and zooplankton have been implicated in the termination of phytoplankton blooms (Alpine and Cloern 1992; Smayda 2008). It is estimated that 60–75% of phytoplankton mortality in the oceans is due to microzooplankton grazing (Calbet and Landry 2004). Thus, like organisms in other ecosystems, phytoplankton are under selection pressure to recognize predators and assess predation risk to avoid becoming prey (Lima and Dill 1990).

Organisms can detect predators using visual, auditory, mechanical, or olfactory stimuli (Bollens et al. 1994; Lass and Spaak 2003; Malavasi et al. 2008). After perceiving predators some organisms undergo dramatic changes in behavior, morphology, and defense in order to avoid attack (Pohnert et al. 2007; Hay 2009; Scherer and Smee 2016). Both the copepod *Acartia hudsonica* and the cladoceran *Daphnia magna* recognize diurnal predatory fishes and avoid them through diel vertical migration. *A. hudsonica* employs visual and mechanical cues (Bollens et al. 1994), whereas *D. magna* utilizes chemical cues (Loose and Dawidowicz 1994; Hahn et al. 2019). Other *Daphnia* spp. react to compounds excreted by midge fly larva, water fleas, and freshwater copepods, increasing their helmet size that allows them to avoid gape-limited predators (Laforsch and Tollrian 2004; Weiss et al. 2018). Compounds emitted by predators also trigger morphological and chemical defenses in single-celled organisms such as dinoflagellates and diatoms, which decrease chain length and increase toxin production after detecting copepods (Selander et al. 2006; Selander et al. 2011; Amato et al. 2018; Selander et al. 2019). Recently, toxin induction in *Alexandrium minutum* was attributed to a family of molecules called copepodamides excreted by copepods (Selander et al. 2015).

In addition to detection of predators, many organisms assess predation risk indirectly through feeding cues associated with predation events (Scherer and Smee 2016). For example, cues from grazing copepods stimulate greater toxin production in *A. minutum* than cues from starved copepods (Selander et al. 2006). This is likely due to increased copepodamide production when copepods are feeding (Selander et al. 2015) but may also be partly caused by cues released by grazed phytoplankton

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from sloppy feeding. For instance, *Alexandrium catenella* (previously *A. fundyense*) increases toxin production when exposed to lysed conspecifics (Senft-Batoh et al. 2015). Similarly, compounds released from macerated conspecifics, and even some heterospecifics, elicit growth in defensive helmets in several species of *Daphnia* (Laforsch et al. 2006). In fact, many organisms from diverse taxa use cues from dead competitors when assessing predation risk (Chivers et al. 1997; Hazlett and McLay 2005;Schoeppner and Relyea 2005; Dalesman et al. 2007).

When organisms react to cues from dead competitors, their response may be correlated with either the phylogenetic relatedness or the historical, geographic co-occurrence of the competitor. Since competitors often share predators, cues from more closely related competitors may trigger stronger antipredatory behaviors due to greater risk of being attacked (Schoeppner and Relyea 2005; Dalesman et al. 2007). Therefore, cues from dead, closely related competitors are expected to be a reliable indication of high predation risk. Alternatively, an organism might respond strongly to cues from dead competitors that they frequently encounter as an indication that there is a hungry predator nearby (Chivers et al. 1997; Scherer and Smee 2016). The effects of cues from dead competitors and the influence of relatedness and co-occurrence of the competitors has been relatively well studied in animals (Chivers et al. 1997; Hazlett and McLay 2005; Schoeppner and Relyea 2005; Dalesman et al. 2007; Scherer and Smee 2016); however, there have been almost no studies in single-celled organisms such as phytoplankton.

The goal of the current study was to investigate whether *A. minutum* uses cues from dead phytoplankton, some of which act as competitors, to assess predation risk and subsequently upregulate toxin production. Furthermore, we endeavored to explore the relative importance of phylogenetic relatedness and historical, geographic co-occurrence of the dead phytoplankton as cues for predation risk. We hypothesized that *A. minutum* uses cues from dead phytoplankton as an indication of imminent predation risk and consequently upregulates toxin production if the cues were derived from closely related and historical co-occurring phytoplankton. Alternatively, *A. minutum* might invest less in chemical defense and more in growth in response to cues from closely related phytoplankton if its toxins are ineffective at preventing predation.

Materials and methods

Phytoplankton cultivation

Cultures of the dinoflagellates *A. minutum* strain CCMP 113, *Prorocentrum lima* strain CCMP 686, *Prorocentrum micans* strain CCMP 688, and *Coolia monotis* strain CCMP 304 were grown in monoculture at 15°C with irradiance of 89–100 μmol m^{-2} s^{-1} and *Alexandrium pacificum* strain CCMP 1493 and *Alexandrium tamarense* strain CCMP 2023 were maintained as monocultures at 21°C with irradiance of 100–145 μmol m^{-2} s^{-1}. All cultures were acquired from NCMA Bigelow Laboratory and grew in filtered seawater from the Gulf of Maine (NCMA Bigelow Laboratory, 35 ppt) amended with full strength K media minus Si (Keller et al. 1987) in incubators set on a 12:12 h light:dark cycle. Cells were enumerated visually using at least two sets of 10 whipple disc grid fields of view and a minimum of 150 cells counted in a 125 μL Palmer-Maloney counting cell on an Olympus IX-50 inverted microscope after preservation with a 1% acidified Lugol's solution and by in vivo fluorescence using a Turner Design Trilogy fluorometer. Biomass for each phytoplankton species was calculated by first measuring their biovolumes, using 30 living cells visualized with an eyepiece reticle on an Olympus IX-50 microscope, noting the corresponding shapes for each phytoplankton species, as described by Hillebrand et al. (1999). Biomass for each phytoplankton was then calculated using the carbon to volume equation for all dinoflagellates from Menden-Deuer and Lessard (2000) (Table 1).

Table 1. Comparison of the cell size, biomass, and concentration of lysed phytoplankton added to *Alexandrium minutum* replicates for Experiment 1.

Lysed phytoplankton species	Biovolume (μm^3 $cell^{-1}$)	Biomass (pgC $cell^{-1}$)	Lysed phytoplankton to *A. minutum* ratio by biomass
Alexandrium minutum	6963	1064	1:3
Alexandrium pacificum	21,812	2719	2.6:3
Alexandrium tamarense	11,112	1565	1.5:3
Prorocentrum lima	14,158	1908	1.8:3
Prorocentrum micans	16,764	2192	2.1:3
Coolia monotis	15,001	2001	1.9:3

Experimental design

Preparation of chemical cues from dead phytoplankton

To investigate whether phylogenetic relatedness to *A. minutum* strain CCMP 113 and/or historical co-occurrence of a phytoplankton with *A. minutum* are important in determining the ability of *A. minutum* to use the damaged phytoplankton as an indication of predator presence, experiments were carried out similar to those conducted by Senft-Batoh et al. (2015). By this design, phytoplankton lysates were added to cultures of *A. minutum* CCMP 113 in order to simulate nearby phytoplankton being grazed upon, without introducing cues from the grazers themselves. The following phytoplankton were used: a conspecific (individuals of the same strain of *A. minutum*, CCMP 113), a co-occurring congener (*A. tamarense*, CCMP 2023), a non-co-occurring congener (*A. pacificum*, CCMP 1493), and three co-occurring less closely related dinoflagellates (*P. lima*, CCMP 686; *P. micans*, CCMP 688; *C. monotis*, CCMP 304). These phytoplankton were chosen because they come from coastal waters of northern Spain

(except *A. pacificum*) and are expected to share similar predators. Additionally, despite some of the species tending to be mostly benthic (*P. lima* and *C. monotis*), they should each directly compete at least occasionally with *A. minutum* for nutrients, particularly due to the regular oscillation of upwelling and downwelling in the region that results in large-scale mixing of nutrients (Álvarez-Salgado et al. 2000; Tilstone et al. 2000). For both Experiments 1 and 2, aliquots that contained appropriate concentration of cells, or biomass, were harvested from each phytoplankton monoculture when they were in exponential growth phase. The cells were then concentrated by centrifugation and the media decanted to bring the final volume of each aliquot to approximately 2 mL. The aliquots were stored at −80°C. Daily for 3 d, concentrated aliquots of phytoplankton cells were lysed via four freeze–thaw cycles with sonication via a bath (lysis was confirmed by microscopy) and added to *A. minutum* as described later.

Experiment 1

The first experiment was performed in six batches, each including a subset of the dead phytoplankton treatments as well as control cultures where *A. minutum* was grown with an equivalent volume of full strength K media added ("media control") instead of the addition of lysed cells (Table 2). The total replicate number for each treatment across the six batches was as follows: n = 43 for the media control, n = 12 for the conspecific treatment, n = 12 for the co-occurring congener treatment, n = 12 for the non-co-occurring congener treatment, and for the less related co-occurring phytoplankton treatment n = 16 for *P. lima*, n = 12 for *P. micans*, and n = 12 for *C. monotis*. Batches were initiated when *A. minutum* was in exponential growth phase at a population density of ~15–20,000 cells mL^{-1}. To initiate the experiment for each batch, a 1.0 mL aliquot of *A. minutum* was preserved from each replicate with 1% acidified Lugol's solution and visually enumerated. Phytoplankton lysates were prepared as described earlier and added daily for 3 d to cultures of *A. minutum* in a ratio of 1:3 lysed phytoplankton cells to *A. minutum* cells, as per Senft-Batoh et al. (2015), corresponding to ratios of lysed phytoplankton to *A. minutum*, by biomass, as reported in Table 1. On the fourth day, a 1.0 mL aliquot of *A. minutum* from each replicate was preserved with 1% acidified Lugol's solution and visually enumerated. The remaining *A. minutum* cells were harvested and extracted for intracellular toxin analysis, as described later. Percent growth was calculated using the cell counts from the start and end points of each *A. minutum* replicate.

Table 2. Distribution of treatments of chemical cues from dead phytoplankton and replicates across batches (Experiment 1).

Batch	Chemical cues from dead phytoplankton	Number of replicates
1	Media control	4
	A. minutum	4
	A. pacificum	4
	A. tamarense	4
2	Media control	4
	A. minutum	4
	A. pacificum	4
	A. tamarense	4
	P. lima	4
3	Media control	4
	A. minutum	4
	A. pacificum	4
	A. tamarense	4
	P. lima	4
4	Media control	8
	P. lima	8
5	Media control	11
	P. micans	12
6	Media control	12
	C. monotis	12

Experiment 2

Experiment 2 was conceived separately to test the effects of nutrients and concentration of dead phytoplankton cues on toxin production and growth of *A. minutum* using similar methods to Experiment 1 but with lysates from only one dead phytoplankton, the non-co-occurring congener *A. pacificum* (CCMP 1493). In this experiment, *A. minutum* was grown with either daily additions of 2 mL of full strength K media ("media control," n = 8), K media diluted with ammonia and nitrate in concentrations equivalent to the 2.6:3 treatment *A. pacificum* lysate ("dilute media control", n = 8), *A. pacificum* lysate at the same concentration ratio as Experiment 1 (1:3 lysed *A. pacificum* cells to *A. minutum* cells equivalent to 2.6:3 by biomass) ("2.6:3 treatment," n = 8), or *A. pacificum* lysate at a concentration ratio of 0.38:3 lysed *A. pacificum* cells to *A. minutum* cells (equivalent to 1:3 by biomass) ("1:3 treatment", n = 8) for 3 d. The *A. pacificum* lysates were prepared as described earlier. The experiment was initiated when *A. minutum* was at a population density of ~19,000 cells mL^{-1} and a 1.0 mL aliquot of *A. minutum* was preserved from each replicate for visual enumeration. On the first day, three additional aliquots of *A. pacificum* lysate were prepared for the 2.6:3 treatment for the purpose of having lysate available to measure ammonia and nitrate, which became the basis for the ammonia and nitrate concentrations in the dilute media controls. The cell debris from the lysates were pelleted by centrifugation and from the supernatant, 200 μL and 40 μL were collected for ammonia and nitrate analyses, respectively. Ammonia concentrations of the three *A. pacificum* lysate supernatants, three aliquots of seawater, and three aliquots of full strength K media were measured in duplicate (Table 3) using a Sigma-Aldrich Ammonia Assay Kit with samples transferred into a 96-well plate and read at 340 nm on a Thermo Scientific

Table 3. Measured nutrient concentrations used to calculate concentrations of ammonia and nitrate used for dilute media control in Experiment 2.

Sample	Nitrate concentration (μM)	Ammonia concentration (μM)
Seawater	1.1 ± 0.4	61 ± 6
K media in seawater	1120 ± 25	107 ± 6
A. pacificum cell lysate	672 ± 6	22 ± 6

Multiskan GO microplate spectrophotometer following the manufacturer's protocol (Sigma-Aldrich, St. Louis, MO, USA). For nitrate analysis, the three lysate supernatants were diluted 10-fold and three aliquots of full strength K media were diluted 50-fold with Cayman Chemicals Nitrate/Nitrite Assay Buffer. The nitrate concentrations of the *A. pacificum* lysates, K media, and three aliquots of seawater were measured in duplicate (Table 3) using a Cayman Chemical Nitrate/Nitrite Colorimetric Assay Kit read at 540 nm on a Thermo Scientific Multiskan GO microplate spectrophotometer following the manufacturer's protocol (Cayman Chemical, Ann Arbor, MI, USA). A bottle of modified K media was then prepared by diluting a full strength bottle, minus ammonia, 1.7-fold with seawater and adding in ammonia at a 4.9-fold dilution compared to full strength K media resulting in final concentrations of approximately 672 μM and 22 μM for nitrate and ammonia, respectively. This modified dilute K media was used in the "dilute media control" treatment, and the experiment proceeded as for Experiment 1. On the fourth day, a 1.0 mL aliquot of *A. minutum* from each replicate was preserved with 1% acidified Lugol's solution and visually enumerated. The remaining cells were harvested and extracted for intracellular toxin analysis, as described later. The cell pellet from one dilute media control replicate was lost during the freeze-drying process resulting in $n = 7$ for the dilute media controls for toxin analysis. Percent growth was calculated using the cell counts from the start and end points of each *A. minutum* replicate.

Intracellular toxin analysis

From the portion of each *A. minutum* replicate set aside for toxin analysis, cells were harvested by centrifuging at 3260 x *g* for 10 min and the supernatant was inspected via microscopy to ensure no cells remained. In Experiment 1, a 2.0 mL aliquot of the supernatant was stored at −20°C for later analysis of extracellular toxins. The cell pellets were then freeze-dried and suspended in 500 μL 1% aqueous acetic acid, and then subjected to four freeze–thaw cycles with sonication, via a bath, to lyse the cells and extract toxins (Anderson et al. 1990). After the final thaw step, the samples were centrifuged at 10,000 x *g* for 10 min to pellet debris and the toxin-containing supernatant was filtered through a 0.2 μm nylon filter to remove precipitate, in preparation for liquid chromatography/mass spectrometric (LC/MS) analysis (Harju et al. 2015). Samples were chromatographically separated using a 20 min isocratic elution of 60% aqueous acetonitrile with 0.1% formic acid on a TOSOH Bioscience 3 μm HILIC TSK-gel Amide-80 column (150 mm x 4.6 mm) with three rinses of the injection loop between samples on a Waters 2695 Separation Module attached to a Waters QDA mass spectrometer. Since the gonyautoxins (GTX) in standard solutions readily desulfonated in the QDA, toxin concentrations were calculated using the area of the desulfonated fragment ion (*m/z* 332 for GTX 1 and 4 and *m/z* 316 for GTX 2 and 3, respectively) and the molecular ion (*m/z* 412 for GTX 1 and 4 and *m/z* 396 for GTX 2 and 3, respectively) peaks, compared with standards solutions obtained by dilution of certified reference calibration solutions purchased from The National Research Council of Canada. A calibration curve of external standards at six concentrations for each of the toxins showed a linear relationship between toxin concentration and area of mass spectral peaks. Gonyautoxins 1–4 were chosen for the toxin analysis because they are the primary paralytic shellfish toxins produced by *A. minutum* (Hwang and Lu 2000; Grzebyk et al. 2003; Selander et al. 2015; Senft-Batoh et al. 2015).

Extracellular toxin analysis

The 2.0 mL aliquots of supernatant from experimental replicates in Experiment 1 were thawed and 100 μL from each sample was diluted with Abraxis Seawater Matrix Sample Diluent. Similarly, GTX 1–4 standards were prepared in Abraxis Seawater Matrix Sample Diluent from certified reference calibration solutions obtained from The National Research Council of Canada. Each sample and standard was analyzed using the Abraxis saxitoxin (PSP) ELISA microtiter plate assay read at 450 nm on a Hidex Sense plate reader following the saxitoxin in seawater sample analysis protocol (Abraxis LLC, Warminster, PA, USA). Unfortunately, following communication with the manufacturer these measurements were deemed unreliable due to the inconsistent response of the ELISA antibody to the GTX standards and its potential to cross-react with other components in the exudates. Therefore, 450 μL of the eight most toxic samples, as determined by ELISA results, were spiked with 5 μL glacial acetic acid, to create a 1% aqueous acetic acid sample solution, and subjected to LC/MS analysis for comparison of toxin concentrations as described above (Harju et al. 2015). When measured by LC/MS, the extracellular toxin concentrations were at or below the limits of detection. This limit of detection corresponded to concentrations which were 90% lower than those predicted by ELISA (data not shown), further reducing confidence in the use of ELISA for extracellular toxin analysis in these experiments. Therefore, the focus of the study was thereafter restricted to dynamics of intracellular toxins produced by *A. minutum*.

Statistical analysis

Cellular toxin concentration, growth, and bulk molar toxin concentration for Experiment 1 were analyzed using linear

mixed effect models (nlme, package nlme) in R (v 3.4.2) (Pinheiro et al. 2017; R Core Team 2017). Models were selected by testing linear and linear mixed effect models via likelihood ratio tests to obtain the optimal model. The optimal model in all cases was a random intercept mixed effect models with batch set as a random effect and phytoplankton treatments set as fixed effects. Significant differences between treatments were tested using a generalized linear hypothesis test (glht) and Tukey test for multiple comparisons of mixed effects models (package multcomp) (Hothorn et al. 2008). Effects of phylogenetic relatedness and historical, geographic co-occurrence of the phytoplankton on toxin production and growth relative to the media controls in Experiment 1 were analyzed using nested ANOVAs (aov, base stats package) in R (Pinheiro et al. 2017; R Core Team 2017). Additionally, the relationship between the mean cellular toxicity and mean growth for the media control and all phytoplankton treatment in Experiment 1 were analyzed using linear regression analysis (lm, base stats package) in R (R Core Team 2017). Cellular toxin concentration and percent growth for Experiment 2 were analyzed using a Welch's ANOVA with a Games-Howell post-hoc test (oneway, userfriendlyscience package), due to unequal sample sizes resulting in unequal variance, in R (Peters et al. 2018). Graphs depicting data associated with the nested ANOVAs, Welch's ANOVA, and linear regression were constructed using the ggplot2 package in R (Wickham 2016).

Results

When *A. minutum* was exposed to chemical compounds from lysed phytoplankton, significant species-specific effects were observed on cellular concentrations of GTX 1–4 and on growth (Fig. 1). Contrary to the primary original prediction, exposure to chemical cues from more closely related phytoplankton (one conspecific and two congeneric) suppressed *A minutum* toxin production by 41–61% compared to media controls, whereas chemicals from any of three other phytoplankton stimulated *A. minutum* toxin production by 32–86% (Fig. 1a). However, there were also finer-scale species-specific effects. Exposure to chemical cues from *A. pacificum* caused the greatest suppression of toxicity in *A. minutum* (61%) ($p < 0.001$). In contrast, cues from dead *P. lima* caused the greatest increase in cellular toxin production compared to media controls (86%) ($p < 0.001$), a significantly greater increase than was caused by *P. micans* and *C. monotis* at 32% ($p = 0.002$) and 39% ($p < 0.001$), respectively (Fig. 1a). In general, cues from the six different dead phytoplankton each resulted in a distinct pattern of altered cellular toxicity.

A consistent, inverse relationship was observed between cellular toxicity and growth when *A. minutum* was exposed to chemicals from six different phytoplankton (Fig. 1). Exposure to chemical cues from congeners, which suppressed toxin production increased *A. minutum* growth by 54–90% (Fig. 1b) compared to media controls ($p < 0.001$ for all phytoplankton).

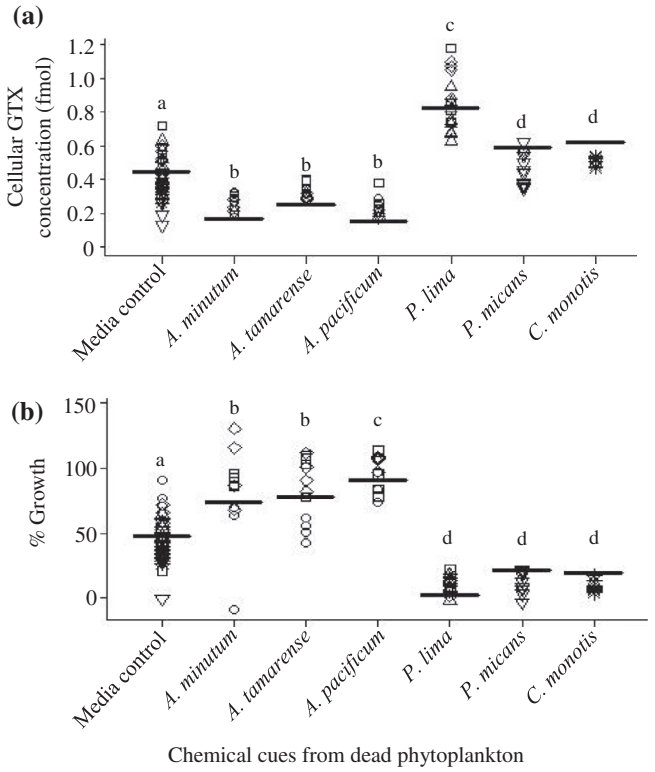

Fig. 1. Effects of chemical cues from dead phytoplankton (as an indication of predation risk) on **(a)** cellular toxin concentration of GTX 1–4 and **(b)** percent growth of *Alexandrium minutum* (Experiment 1). Data were analyzed using a random intercept model whereby the dark bars represent the line of best fit and the symbols show the contribution of each batch to the mean. The lower case letters show statistical differences between treatments via Tukey tests ($p \leq 0.05$).

In contrast, cues from the three less related phytoplankton that significantly induced cellular toxin production, suppressed *A. minutum* growth by 56–90% (Fig. 1b) relative to media controls ($p < 0.001$ for all phytoplankton). In all six cases, the cues from dead phytoplankton resulted in alteration of growth that was inverse to their effect on cellular toxicity, although the magnitude of differences were not generally equivalent. Additionally, when the mean growth and cellular toxicity for all treatments together are considered there was a significant inverse relationship ($p < 0.001$) (Fig. S1). These findings suggest that the dead phytoplankton cues trigger a population level trade-off between toxin production, or defense, and growth in *A. minutum*.

When toxicity was measured as bulk molar concentration instead of per cell, *A. minutum* exposed to chemical cues from lysed phytoplankton showed similar, but less extreme patterns, of toxin regulation (Fig. 2). Exposure to cues from more closely related phytoplankton significantly suppressed toxin production of *A. minutum* by 33–58% compared to media controls ($p < 0.001$ for all phytoplankton), whereas cues from the three less-related phytoplankton slightly stimulated *A. minutum* toxin

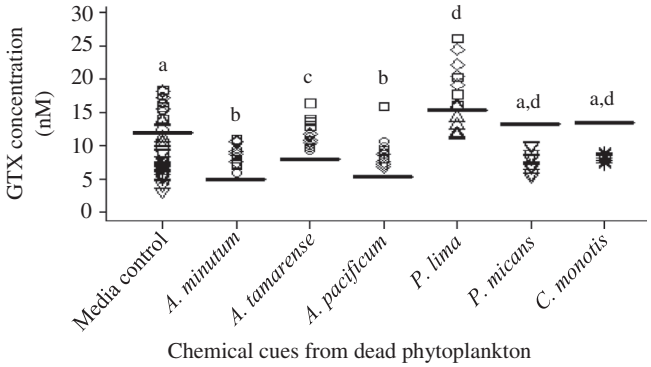

Fig. 2. Effects of chemical cues from dead phytoplankton (as an indication of predation risk) on bulk (molar) toxin concentration of GTX 1–4 of *Alexandrium minutum* (Experiment 1). Data were analyzed using a random intercept model whereby the dark bars represent the line of best fit and the symbols show the contribution of each batch to the mean. The lower case letters show statistical differences between treatments via Tukey post-hoc test ($p \leq 0.05$).

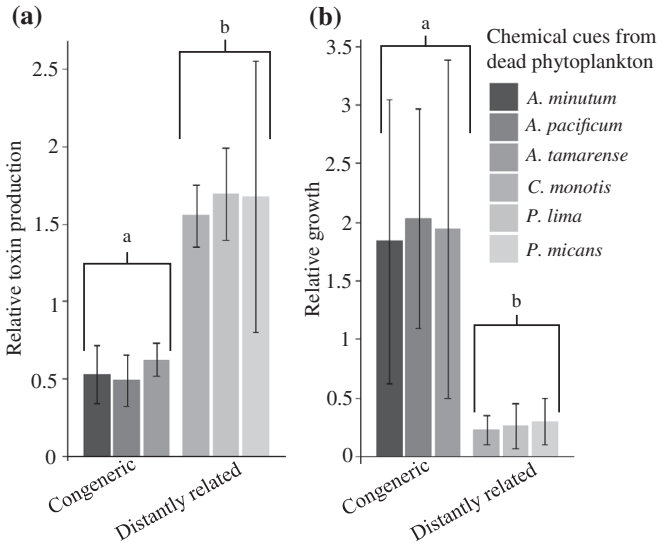

Fig. 3. Effects of phylogenetic relatedness on **(a)** cellular toxin production of GTX 1–4 and **(b)** growth of *Alexandrium minutum* relative to media controls in response to chemical cues from dead phytoplankton, as an indication of predation risk (Experiment 1). Data from exposure to chemical cues from *A. minutum*, *A. pacificum*, and *A. tamarense* are nested under "congeneric" and *C. monotis*, *P. lima*, and *P. micans* are nested under "distantly related." Data were analyzed using a nested ANOVA. Lower case letters show statistical differences between treatments ($p \leq 0.05$).

production by 12–28% (Fig. 2). Unlike cellular toxicity, bulk toxicity was only significantly increased relative to media controls when *A. minutum* was exposed to chemical cues from dead *P. lima* (28%) ($p = 0.003$); whereas, exposure to dead *P. micans* and *C. monotis* caused insignificant increases of 12% ($p = 1.0$) and 13% ($p = 0.30$), respectively (Fig. 2). Overall, this suggests that changes in cellular toxicity of *A. minutum* in response to cues from dead phytoplankton are mostly, although with subtle differences, a reflection of actual toxin production and not an artifact of possible dilution among an increased number of cells.

When the toxicity-altering effects of chemical cues from conspecifics and congeners were considered as a group relative to less related phytoplankton, effects on *A. minutum* toxicity and growth were strongly reinforced (Fig. 3). Upon exposure to chemical cues from dead, closely related phytoplankton considered as a group, *A. minutum* responded by decreasing its toxicity by 45% (Fig. 3a) while increasing growth by 94% (Fig. 3b) relative to media controls. Conversely, in response to cues from dead less related phytoplankton considered as a group, *A. minutum* increased toxin production by 64% (Fig. 3a) and reduced growth by 74% (Fig. 3b) compared to media controls. Overall, this suggests that phylogenetic relatedness of phytoplankton to *A. minutum* is a significant determining factor in how *A. minutum* responds to predation risk via cues from dead phytoplankton both in terms of toxin regulation ($p < 0.001$, $F_{1,4} = 143.8$) and growth ($p < 0.001$, $F_{1,4} = 74.4$).

There were no strong effects on *A. minutum* toxicity and growth associated with historical co-occurrence when, to eliminate the confounding factor of relatedness, only the conspecific and congeners were considered (Fig. 4). Surprisingly, *A. minutum* did not show a heightened sensitivity to cues from co-occurring phytoplankton relative to phytoplankton with no known historical overlap with the geographic distribution of *A. minutum* (Figs. 1a and 4). In fact, between the two sets of phytoplankton (historically co-occurring or not) there was no significant difference in toxin production ($p = 0.14$, $F_{1,1} = 2.2$)

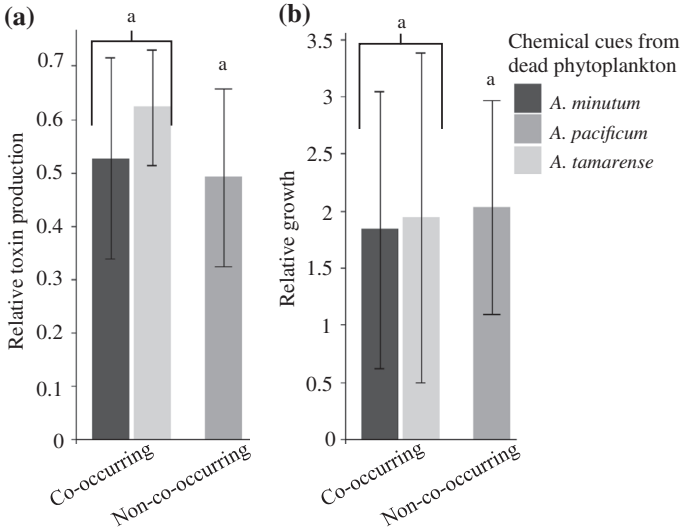

Fig. 4. Effects of historical, geographic co-occurrence on **(a)** cellular toxin production of GTX 1–4 and **(b)** growth of *Alexandrium minutum* relative to controls, in response to chemical cues from dead phytoplankton, as an indication of predation risk (Experiment 1). Data from exposure to chemical cues from *A. minutum* and *A. tamarense* are nested under "co-occurring" and *A. pacificum* is under "non-co-occurring." Data were analyzed using a nested ANOVA. Lower case letters show statistical differences between treatments ($p \leq 0.05$).

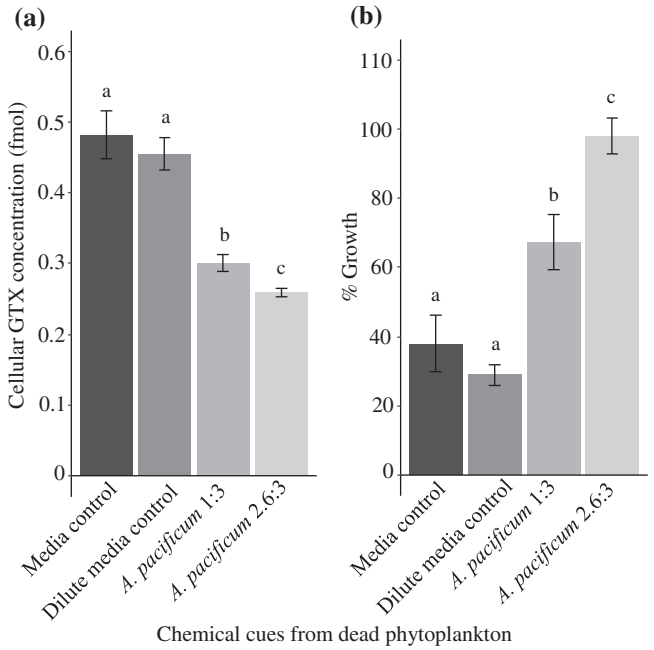

Fig. 5. Effects of nutrients and chemical cues from dead *A. pacificum* (as an indication of predation risk) on **(a)** cellular toxin concentration of GTX 1–4 and **(b)** growth of *Alexandrium minutum* (Experiment 2). *A. minutum* was exposed to *A. pacificum* lysate at a 1:3 and 2.6:3 ratio of *A. pacificum* lysate biomass to *A. minutum* biomass, equivalent to 0.38:3 and 1:3 ratio of *A. pacificum* lysate to *A. minutum* by cell number. Additionally, either full strength K media ("media control") or K media diluted to reflect the nitrate and ammonia concentration present in the 2.6:3 ratio of *A. pacificum* lysate biomass to *A. minutum* biomass ("dilute media control") were added to *A. minutum* as controls. Data were analyzed using a Welch's ANOVA. Lower case letters show statistical differences between treatments via Games-Howell post-hoc test ($p \leq 0.05$).

or growth ($p = 0.74$, $F_{1,1} = 0.10$) in *A. minutum* (Fig. 4). Instead, chemical cues from either co-occurring or non-co-occurring dead, related phytoplankton caused about a 50% reduction in toxin production (Fig. 4a) and a nearly 100% increase in growth (Fig. 4b) compared to media controls. Therefore, historical co-occurrence appears to not be an important factor when *A. minutum* is assessing potential predation risk in response to cues from dead phytoplankton.

When the experiment with *A. pacificum* was repeated to include a more realistic control ("dilute media control") in which ammonia and nitrate concentrations were manipulated to mimic concentrations in the lysed *A. pacificum* treatment, toxin suppression ($p < 0.001$, $F_{3,11.8} = 229.9$) and growth induction ($p < 0.001$, $F_{3,14.28} = 354.7$) patterns were again observed (Fig. 5). On the other hand, impacts on toxicity ($p = 0.30$, $F_{3,11.8} = 229.9$) and growth ($p = 0.063$, $F_{3,14.28} = 354.7$) of *A. minutum* did not vary significantly between the full strength media control and the dilute media control, which imitated the ammonia and nitrate levels of the *A. pacificum* lysate (Fig. 5). This suggests that chemical cues in the dead phytoplankton lysates, and not macronutrients from the lysates, are responsible for the changes in toxin production and growth observed in *A. minutum*.

The lower concentration of chemical cues in the "1:3 treatment" of Experiment 2 is likely still much higher than natural bulk concentrations of cell lysates from a sloppily feeding predator; however, it may still be ecologically relevant within short-lived hotspots of extremely high lysate concentrations created by actively feeding predators. The different concentrations of dead *A. pacificum* cue resulted in a graded response by *A. minutum* with the cues having a greater impact when applied at a higher concentration; yet, still causing significant toxin suppression ($p < 0.001$, $F_{3,11.8} = 229.9$) and growth induction ($p < 0.001$, $F_{3,14.28} = 354.7$) at a lower, more realistic, concentration (Fig. 5). Overall, this means that while the differences in the effective concentrations of the dead phytoplankton cues may have impacted the magnitude of the observed effects on toxin production and growth in *A. minutum* it likely did not impact the direction of the effects.

Discussion

The toxic marine dinoflagellate *A. minutum* responds to dead phytoplankton cues in a species-specific manner (Figs. 1a and 2). *A. minutum* responds to the presence of distantly related, but co-occurring, dead phytoplankton with enhanced chemical defenses, in the form of increased cellular toxin concentration (Figs. 1a and 3a). This is in agreement with recent findings that *A. catenella* increases toxin production in response to a lysed diatom and green alga (Griffin et al. 2019). However, cues from closely related phytoplankton suppressed toxin production in *A. minutum* (Figs. 1a, 2, 3, and 5a); therefore, the original hypothesis that close relatedness among phytoplankton results in upregulation of toxin production was rejected. Additionally, the observed reduction of toxin production due to exposure to cues from related dead phytoplankton contrasts with recent findings in other studies (Senft-Batoh et al. 2015; Griffin et al. 2019). In the study by Senft-Batoh and colleagues, *A. catenella* (previously *A. fundyense*) increased toxin production modestly, but significantly, in response to two strains of lysed conspecifics (Senft-Batoh et al. 2015); whereas, Griffin et al. found that the same *A. catenella* strain did not significantly change its toxin production in response to the same two strains of lysed conspecifics (Griffin et al. 2019). Consequently, responses to dead phytoplankton cues by *Alexandrium* species appear to be species-specific and not a conserved trait, given that some *Alexandrium* species respond to cues from conspecifics and congeners with decreased toxicity et al with either no change or enhanced toxicity. A distinction between the previous studies and the current one is that those experiments investigated the effects of cues from two conspecifics, whereas, the current study considered effects of a conspecific as well as two other congeneric species (plus three less-related dinoflagellates).

Many studies have found that organisms respond to cues of other dead organisms consumed by common predators

(Scherer and Smee 2016). However, Scherer and Smee reported that in more than 50% of studies analyzed, cues from conspecifics induced stronger defenses than cues from heterospecifics (Scherer and Smee 2016). For example, gray tree frog tadpoles decreased their activity level the most (Schoeppner and Relyea 2005) and pond snails spent the most time out of the water (Dalesman et al. 2007) in response to crushed conspecifics compared to other organisms that share a common predator. In contrast, in the current study toxicity in *A. minutum* was reduced by nearly 50% in response to cues from dead conspecifics; toxicity increased only in response to cues from less related phytoplankton (Fig. 3a). One explanation for this pattern is that for *A. minutum*, cues from dead conspecifics may be an indication of bloom senescence (a regular occurrence for bloom-forming dinoflagellates) rather than sloppy predation. In this scenario, increased toxin production would not be adaptive, such that it may be more advantageous for *A. minutum* to use nutrients released by lysed cells to grow to offset mortality instead of investing in increased toxin production.

We originally hypothesized phytoplankton prey to be under selection pressure to respond to cues from other phytoplankton that they more frequently encounter, since those cues should reliably indicate predation risk. However, *A. minutum*'s response differed from studies in several other systems in that historical, geographic co-occurrence did not affect how it responded to cues from dead phytoplankton; instead, cues from both co-occurring and non-co-occurring closely related phytoplankton suppressed toxicity and increased growth (Fig. 4). For example, both snails (Dalesman et al. 2007) and salamanders (Chivers et al. 1997) have been shown to engage in more antipredatory behavior in response to cues from dead closely related competitors and heterospecifics with which they cohabitate. In contrast, the marine crab *Heterozius rotundifrons* increased antipredatory posturing in response to crushed related crabs but not distantly related, co-occurring crabs (Hazlett and McLay 2005). Therefore, *A. minutum* and *H. rotundifrons* are similar in that genetic relatedness, rather than cohabitation, of the other organism is most important in determining how to respond to cues that an organism has died.

In the current study, there was an observed trade-off between growth and toxin production in response to dead phytoplankton cues (Figs. 1, 5, and S1). Due to the similar magnitudes but opposite effects of cues on growth vs. toxin production in *A. minutum*, the most parsimonious explanation is that lysed phytoplankton caused a change in growth, which in turn affected toxin concentration: If growth is suppressed, more resources are available for toxin induction; if growth is enhanced, existing toxins are diluted among daughter cells creating the appearance of toxin suppression. However, bulk toxin concentrations showed similar patterns to cellular toxin concentrations (Fig. 2). In the case of exposure to conspecific and congener cues, *A. minutum* produced less toxin, even taking into account that enhanced growth led to a greater number of cells containing these toxin molecules (Fig. 2). This means that toxins were not just diluted because *A. minutum* cells divided more. However, when *A. minutum* was exposed to cues from less related dead phytoplankton, with the exception of *P. lima*, the observed increase in toxin production could be accounted for by reduced growth, via more synthesized toxin molecules and build-up of more toxins within fewer cells.

Another alternative hypothesis is that the decrease in toxin production by *A. minutum* in response to cues from dead conspecifics and congeners results from the cues being used as a quorum sensing-like mechanism. If dense blooms of *A. minutum* are not grazed upon at a greater rate than *A. minutum* growing at low population density, the chance of any one individual being eaten would decrease with increasing population concentration, in which case it would be adaptive to downregulate (otherwise costly) toxin production during a dense bloom. One would then expect *A. minutum* to decrease toxin production the most in response to conspecific cues, which may be an indication of high population density, while decreasing toxicity less, if at all, in response to cues from congenerics. This is contrary to what was discovered during the current study: *A. minutum* decreased toxin production the most, not in response to conspecific cues, but in response to cues from dead *A. pacificum*, with which it does not co-occur (Fig. 1a). Therefore, the alternative hypothesis that *A. minutum* uses cues from dead congenerics for quorum sensing, suppressing toxin production, can likely be rejected.

Nutrients from phytoplankton lysates provided *A. minutum* with resources enabling toxin production and/or growth, but chemical cues likely enhanced the response. Phosphate limitation and high concentrations of nitrate and ammonia have previously been linked to toxin induction in *Alexandrium* species (John and Flynn 2000; Guisande et al. 2002; Leong et al. 2004; Selander et al. 2008). Conversely, nitrogen limitation has been linked to decreased toxin production (John and Flynn 2000). In the current experiment, when exposed to similar nitrate and ammonia conditions as *A. pacificum* lysate, but without phytoplankton chemical cues, *A. minutum* did not alter toxin production or growth relative to exposure to full strength media (Fig. 5, "dilute media control" vs. "media control"). Instead, only exposure to cue containing *A. pacificum* lysates reduced toxin production in *A. minutum* (Fig. 5). Although we did not measure phosphate in our controls or lysates, our diluted media control contained a 1.7-fold dilution of phosphate relative to the full strength media control; nevertheless toxins were neither induced nor suppressed when comparing *A. minutum* exposed to these two controls (p = 0.30). Therefore, it is likely that chemical cues in the lysate from dead congeners contributed to the observed suppression of toxin production.

A trade-off between defense and growth, as observed in this study, is in line with the optimal defense theory, which posits that defenses are expected to evolve in proportion to the risk of predation and inversely proportional to their cost (Rhoades 1979). Additionally, Rhoades proposed that defenses should

be maintained at low or nonexistent levels in the absence of predators and increase in the presence of predators (i.e., be inducible). During the summer when *A. minutum* blooms under nutrient-depleted conditions with high grazing pressure, a reduction in growth, associated with toxin induction, is worth the allocation cost if higher toxicity results in reduced grazing by copepods and a concomitant increase in the *A. minutum* population (Teegarden 1999; Selander et al. 2006). However, if copepods are even partially resistant to the toxins, the benefits of toxin production could be lost, as copepods would be capable of eating larger quantities of cells (Senft-Batoh et al. 2015) with little ill effect and *A. minutum* may not grow fast enough to offset the mortality from predation.

Overall this study shows that *A. minutum* experiences a global trade-off between growth and toxin production upon exposure to cues from dead phytoplankton. Cues from more distantly related phytoplankton led to toxin induction, the magnitude of which was effectively accounted for by the reduced growth in *A. minutum* (i.e., *A. minutum* cells were dividing more slowly and made proportionally more toxin molecules). On the other hand, exposure to cues from both dead conspecifics and congenerics enhanced *A. minutum* growth and dramatically suppressed toxin production beyond the extent expected by rapid cell division. Taken as a whole, these results reveal that *A. minutum* distinguished relatedness of dead phytoplankton, adjusting its growth and defense.

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Conflict of Interest

None declared.

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