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Toxic mucus traps: A novel mechanism that mediates prey uptake in the mixotrophic dinoflagellate *Alexandrium pseudogonyaulax*

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ABSTRACT

The functional role of harmful substances (i.e. toxins) produced by marine planktonic algae is still, in many cases, unknown. This study describes a novel mechanism by which the phototrophic dinoflagellate *Alexandrium pseudogonyaulax* secretes a toxic mucus trap where prey items are caught and immobilized prior to ingestion. Prey cells remain entrapped and immobile in the mucus trap, but most stay intact, readily available as whole-cell prey. It is shown that food uptake by *A. pseudogonyaulax* increases its growth rate considerably even in nutrient-replete, high-light conditions. The increase in growth rate was more enhanced in light-limited treatments and *A. pseudogonyaulax* grew significantly faster when fed *Heterocapsa rotundata*, than when fed *Teleaulax acuta* under both light conditions. For comparison, strains of *Alexandrium catenella* and *Alexandrium minutum* were studied for their mixotrophic capabilities. None of these strains were mixotrophic under the conditions provided. In addition, the toxic effects on various protistan targets of these *Alexandrium* strains as well as *Alexandrium tamarense* and *Alexandrium ostenfeldii* were compared to that of *A. pseudogonyaulax*. *A. tamarense* and *A. catenella* did immobilize and lyse target cells through substances leaked directly into the water, differing from all the strains of *A. pseudogonyaulax* studied. Results show that the toxic effect of *A. pseudogonyaulax* is non-specific causing nearly 100% immobilization of a variety of protistan targets at relatively low cell concentrations (500 cells ml^{-1} of donor cell). A critical donor cell density was not required as only one *A. pseudogonyaulax* cell was able to cause immobilization of target cells. For the first time, the connection between excreted toxins and phagotrophy is evident in an *Alexandrium* species and this particular strategy has the potential to severely impact competing phytoplankton communities.

1. Introduction

Mixotrophy, i.e. the simultaneous use of photosynthesis and particulate food uptake is widespread among marine phytoflagellates (Stoecker et al., 2006; Burkholder et al., 2008; Jeong et al., 2010; Hansen, 2011). Food uptake can contribute substantially to the total carbon uptake and many mixotrophic species grow faster as mixotrophs than solely autotrophically (Skovgaard, 1996; Li et al., 1999; Hansen et al., 2000) while others only consume prey during nutrient limitation (Urabe et al., 1999; Li et al., 2000). Some species are obligate mixotrophs, and require prey to provide necessary growth factors for phototrophic growth (Kimura and Ishida, 1989; Skovgaard, 2000). One ecological advantage of mixotrophy is simply the elimination of predators or competitors (Li et al., 1999; Tillmann, 2003; Jeong et al., 2004; Stoecker et al., 2006). The nutritional flexibility which mixotrophy provides, may offer the competitive edge over strict phototrophs or heterotrophs particularly under variable environmental conditions, despite any energetic costs involved in maintaining mixotrophic capabilities. Historically, mixotrophy and its ecological impact may have been underestimated in the phytoplankton community, as mixotrophy is now understood to be a significant mode of nutrition for many species originally assumed to be exclusively phototrophic (Burkholder et al., 2008).

Mixotrophy in dinoflagellates has been suggested as a strategy to compensate for relatively slower growth rates and lower nutrient affinities compared to other co-occuring phytoplankton (Smayda, 1997). Little is known about the mixotrophic abilities of the otherwise well known PSP producing dinoflagellate genus *Alexandrium*. Yet there is now evidence of mixotrophy in *Alexandrium catenella*, *Alexandrium minutum*, *Alexandrium ostenfeldii* and *Alexandrium tamarense* (Jacobson and Anderson, 1996; Jeong et al., 2005a,b; Yoo et al., 2009). Proven prey species have included the cyanobacteria *Synechococcus* sp. (Jeong et al., 2005a) and the diatom *Skeletonema costatum* (Yoo et al., 2009). *A.*

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tamarense is also able to eat small (<12 μm ESD) prymnesiophytes, cryptophytes, raphidophytes and dinoflagellates. It does this by direct engulfment through the sulcus, and thus without the use of a tow filament, like most other dinoflagellates (Jeong et al., 2005b). Food vacuoles have been seen in field samples of *A. ostenfeldii* (Jacobson and Anderson, 1996; Gribble et al., 2005) and one particular food vacuole was identified to contain *Dinophysis* sp. (Jacobson and Anderson, 1996).

Another competitive strategy used in dinoflagellates is the use of allelopathy, i.e. production and release of secondary metabolites (allelochemicals) to the surroundings that inhibit the growth or physiology of competing organisms. Allelopathy may have evolved in phytoplankton as an adaptation that provides a competitive advantage either by deterring predation (Legrand et al., 2003; Adolf et al., 2007), or reducing the number of competitors, allowing increased access to limiting resources, such as nitrogen and phosphorus (Legrand et al., 2003; Granéli and Hansen, 2006).

As more allelopathic algae are now proving to be mixotrophic (Stoecker et al., 2006; Hansen, 2011), it is speculated that toxins previously known as allelochemicals, may have a different purpose other than what is typically seen in allelopathy (deterring predators or removing competitors through killing or growth inhibition), and in fact, may be involved in prey capture in various ways. For example, toxins of the haptophyte *Prymnesium parvum* excreted into the medium have been shown to cause prey immobilization allowing the algae to catch otherwise motile prey cells (Skovgaard and Hansen, 2003; Tillmann, 2003).

In other cases, toxins are not exuded to the surroundings (and thus cannot really be considered allelochemicals), but instead are injected into the prey upon capture. Karlotoxins produced by *Karlodinium veneficum* have recently been shown to function in this way (Adolf et al., 2006; Sheng et al., 2010). We suggest that the current definition of allelopathy does not cover toxic substances released or injected for the purpose of immobilizing potential prey cells. As more toxic species become known as mixotrophs the role of toxicity in prey capture may become clearer and the distinction between allelopathy and prey capture mechanisms will be better understood.

Strains of several species of *Alexandrium* are well known to show allelopathic effects including *A. catenella*, *A. ostenfeldii* and *A. tamarense* (Hansen, 1989; Hansen et al., 1992; Arzul et al., 1999; Tillmann and John, 2002; Tillmann et al., 2007; Tillmann and Hansen, 2009). These species are the same ones that have shown evidence of mixotrophy. However no direct link between mixotrophy and toxin production has previously been resolved.

Initial screening experiments for mixotrophy in various *Alexandrium* species prior to this study revealed that one species, *Alexandrium pseudogonyaulax*, caused localized immobilization of various prey cells surrounding the *A. pseudogonyaulax* cells. Since food vacuoles were also observed in this species it appeared that some negative response (resembling an allelopathic response) of prey species preceded consumption by *A. pseudogonyaulax* and required additional investigation. Furthermore, observations of a field sample taken from Helsingør Harbor, Denmark during summer 2010, showed similar localized immobilization of various phytoplankton cells surrounding *A. pseudogonyaulax* cells. This indicates that this unique response of co-occurring cells is also found in nature, and therefore may have an important ecological relevance.

The goal of the present study was to describe and quantify this novel food capture strategy of *A. pseudogonyaulax* in comparison to other allelopathic *Alexandrium* spp. through microscopic observations and short-term experiments investigating the negative effects of these species on other protists. Furthermore, we attempt to quantify the mixotrophic capabilities of *A. pseudogonyaulax* through mixed growth experiments and to find a link between the seemingly allelopathic strategy and the ability for mixotrophy in this species. In this way we distinguish between allelopathy used to kill predators or competitors from the release of toxic substances which aid in prey capture and uptake.

2. Materials and methods

2.1. Algal cultures

Algal cultures were provided by the Scandinavian Culture Collection for Algae and Protozoa (SCCAP), the Marine Biological Laboratory (MBL) in Helsingør, Denmark, and the Alfred Wegener Institute in Germany (Table 1). Cultures were maintained in f/2 medium with a salinity of 30 at 15 °C and an irradiance of 90 μmol photons m^{-2} s^{-1} using cool white fluorescent light on a 16:8 h light:dark cycle. All experiments were performed under these conditions except for the irradiance in mixed growth experiments, which had varying light levels.

2.2. Molecular identification

To verify the identification of the three Danish clones of *A. pseudogonyaulax* we determined their nuclear-encoded SSU rDNA sequences. The methods used are outlined in Hansen et al. (2007). The SSU rDNA sequences (1744 base pairs for K-1344 and MBL-AP2 and 1556 base pairs for K-1345; data not shown) were compared to two reference sequences of *A. pseudogonyaulax* available in Genbank (viz. AB088302 and JF521638).

2.3. Microscopic observations

Initial observations of mixed cultures of *Alexandrium* spp. with various other target species were made using an inverted

Table 1
Algal species used in the experiments, SCCAP clone number (K-strains), isolation place, and isolation date. Species with MBL clone number were obtained from the Marine Biological Laboratory in Helsingør, Denmark. Scottish clones were provided by the Alfred Wegener Institute in Germany.

Species	Clone	Isolation place	Isolation date
A. pseudogonyaulax	K-1344	Limfjorden, Denmark	July 2009
A. pseudogonyaulax	K-1345	Limfjorden, Denmark	July 2009
A. pseudogonyaulax	MBL-AP2	Helsingør, Denmark	August 2010
A. ostenfeldii	K-1354	Helsingør, Denmark	July 2009
A. minutum	K-0992	Korsør Nor, Denmark	June 2001
A. tamarense	Alex5	Scotland	2009
A. tamarense	Alex2	Scotland	2009
A. catenella	K-1490	Saanich Inlet, Canada	November 2010
Heterocapsa rotundata	K-0483	Denmark	December 1988
Heterocapsa triquetra	K-1134	Copenhagen, Denmark	October 2008
Scrippsiella trochoidea	K-1110	Koljo Fjord, Sweden	April 2008
Mesodinium rubrum	MBL-DK2009	Helsingør, Denmark	September 2009
Teleaulax acuta	MBL-TA1	Nivå, Denmark	August 2009

microscope (BX40 Olympus, Japan) to detect any allelopathic effects and/or evidence of mixotrophy. Species used were *A. pseudogonyaulax*, *A. catenella*, *A. tamarense*, *A. ostenfeldii*, and *A. minutum* with target species/potential prey species *Heterocapsa rotundata*, *Heterocapsa triquetra*, *Teleaulax acuta*, *Scrippsiella trochoidea* and *Mesodinium rubrum.* Whole-cell cultures were mixed directly in a 24-well plate to a final volume of 2.5 ml at a ratio of about 1:5, *Alexandrium* spp.: target species, typically with an initial concentration of 100:500 cells ml^{-1}. Observations were done immediately using an inverted microscope, and after 24 and 48 h, taking note of any immobility, cyst formation or cell lysis as well as the presence of food vacuoles and phagotrophy. Target cell response was compared to observations of the target cells mixed with f/2 medium in the same 24-well plates.

All images were taken of cultures with the same initial ratios, 2–4 h after mixing in a clean 3 ml settling chamber. Pictures were taken using a motorized inverted microscope (Olympus IX81, Japan) equipped with a digital camera (Canon EOS 5D Mark II, Canon, Japan).

The prey species *M. rubrum* and *T. acuta* both have different autofluorescent properties from *Alexandrium* species, making it easier to see evidence of phagotrophy under epifluorescence. Mixed cultures with these two prey species were fixed in 2% gluteraldehyde and mounted on a black filter for use under the epifluorescence microscope. Images of these slides were taken using a black and white digital camera (Soft Imaging System FViewII), mounted on an inverted microscope (Olympus IX81, Japan) equipped with a disk-spinning unit (DSU).

2.4. Immobilization and cell lysis experiments (1–4)

2.4.1. Experiment 1. Effect of exposure time to Alexandrium pseudogonyaulax and A. catenella on Heterocapsa rotundata motility and cell lysis

A timed experiment was carried out using *A. pseudogonyaulax* (K-1344) and *A. catenella* to determine the effect of time on the motility and cell lysis of *H. rotundata* cells exposed to *A. pseudogonyaulax* compared to a known lytic *Alexandrium* species. In this experiment 0.2 ml *A. pseudogonyaulax* cell suspension (500 cells ml^{-1}) or *A. catenella* cell suspension (5000 cells ml^{-1}) was mixed with 0.1 ml *H. rotundata* cell suspension (5000 cells ml^{-1}) in triplicates in a 96-well multi-dish plate (well volume 330 μl). The control consisted of the same amount of *H. rotundata* cells mixed with f/2 medium. The number of non-motile *H. rotundata* cells lying at the bottom of the well was enumerated using an inverted microscope (Olympus BX40, Japan) at different exposure times: 10 min, 0.5, 1, 2, 3, 4, and 5 h. The suspensions were then fixed in 2% Lugol's iodine and counted after all cells settled to obtain total remaining *H. rotundata* cells in the wells at each time interval. This cell number was then compared to the number of *H. rotundata* cells in the control at the end of the experiment to calculate the percentage motile and intact (i.e. not lysed) target cells. Cells were counted directly in the well of the plates, using the entire area of the well. Initial observations of the mixed cultures revealed the formation of distinct aggregates or clumps of target cells around *A. pseudogonyaulax* cells. To investigate how these aggregations form, the number of aggregates of *H. rotundata* cells and the number of cells in each group was enumerated during each time point, after fixation with 2% Lugol's iodine.

2.4.2. Experiment 2. Effect of Alexandrium pseudogonyaulax and A. catenella cell density on Heterocapsa rotundata motility and cell lysis

This experiment followed the same protocol as described above except for different concentrations of *A. pseudogonyaulax* (K-1344) and *A. catenella* and only one observation after 4 h. The initial cell densities of *A. pseudogonyaulax* used were 10, 25, 50, 100, 200, 300, and 500 cells ml^{-1}. At only 500 cells ml^{-1} there was no effect of *A. catenella* after four hours on motility or cell lysis. Thus, initial cell densities of *A. catenella* were higher: 500, 2000, 3000, and 5000 cell ml^{-1}. Cell densities were obtained by diluting cultures of *A. pseudogonyaulax* or *A. catenella* with f/2 medium.

2.4.3. Experiment 3: Screening for toxic effects (target cell immobilization and cell lysis) of other Alexandrium species and strains on a variety of target cell species

Screening of toxic effects was done for several other species and strains of *Alexandrium* with five different target species to determine if other strains of *A. pseudogonyaulax* or other *Alexandrium* species exhibit the same negative effects of *A. pseudogonyaulax* strain K-1344. Furthermore, we wanted to determine if this effect is consistent with different target species. These experiments followed the same protocol as the previous immobilization and cell lysis experiments. *Alexandrium* species used for screening were *A. pseudogonyaulax* (K-1344), *A. pseudogonyaulax* (K-1345), *A. pseudogonyaulax* (MBL-AP2), *A. tamarense* (Alex2), *A. tamarense* (Alex5), *A. catanella* (K-1490), *A. minutum* (K-0992), and *A. ostenfeldii* (K-1354) (Table 1). Target species used were *H. rotundata* (K-0483), *T. acuta* (MBL-TA1), *H. triquetra* (K-1134), *S. trochoidea* (K-1110), and *M. rubrum* (MBL-DK2009) (Table 1). The concentration of all *A. pseudogonyaulax* strains was 500 cells ml^{-1} and for all other *Alexandrium* species it was 3000 cells ml^{-1} (excluding *A. ostenfeldii*, which did not reach high concentrations so an average of 1000 cells ml^{-1} was used). The initial concentration of each target species was 5000 cells ml^{-1}.

2.4.4. Experiment 4: Effects of the supernatant of Alexandrium pseudogonyaulax and A. catenella cultures compared to the effects of whole-cell cultures on the motility and cell lysis of two target cell species

To investigate further into the nature of the negative effects of *A. pseudogonyaulax* compared to other known allelopathic *Alexandrium* species, experiments using the supernatant instead of whole-cell cultures were performed. The supernatant of a culture of *A. pseudogonyaulax* (K-1344) (500 cells ml^{-1}) and *A. catenella* (3000 cells ml^{-1}) was obtained by centrifugation at 3000 × *g* for 10 min. This was then immediately mixed with target cells, *H. rotundata* and *T. acuta* (5000 cells ml^{-1}), in wells of a 96-well plate, following the same protocol as above.

2.5. Mixed growth experiments

Mixed growth experiments were carried out with three *Alexandrium* species: *A. catenella*, *A. minutum* and *A. pseudogonyaulax* using the dinoflagellate *H. rotundata* and the cryptophyte *T. acuta* as prey under light-limiting conditions (i.e. low light, defined here as 17 μmol photons m^{-2} s^{-1}). In the case of *A. pseudogonyaulax*, additional experiments were carried out under high-light conditions (defined here as 120 μmol photons m^{-2} s^{-1}) as well as in the dark. Initial concentrations were 100 cells ml^{-1} of predator cells and 5000 cells ml^{-1} of prey cells. Another experiment was carried out using a higher initial concentration of *A. catenella* (1000 cells ml^{-1}) to determine if it grows faster at concentrations that may have negative effects on potential prey cells. In all cases, the growth of *Alexandrium* monocultures was monitored as a control.

Initial concentrations were established by inoculating the same amount of exponentially growing culture with known cell concentrations to a 270 ml tissue culture flask, one for the monoculture and one for each mixed culture. Flasks were then filled to capacity with f/2 medium. Each of these was mixed and

aliquoted into triplicate 65 ml tissue culture flasks and mounted on a vertically rotating plankton wheel.

Every 2–3 d (or in lower light conditions, 5–7 d) 5 ml subsamples were taken from each flask and fixed with Lugol's iodine (2% final concentration) for enumeration. The pH of each triplicate was measured and recorded at this time using a pH meter (Sentron, Netherlands). Cells were counted in a Sedgewick-Rafter chamber or a 2 ml settling chamber in order to count at least 200 cells. After every sampling, flasks were then diluted to the initial *Alexandrium* concentration of 100 cells ml^{-1} and prey species were added, if necessary, to the initial prey concentration of 5000 cells ml^{-1} to ensure a relatively constant food supply.

The exponential growth rate μ (d^{-1}) was calculated for each replicate using linear parts of semi-log plots between cumulative cell number and time. Differences in mean growth rates between treatments were tested using a one-way ANOVA. The growth rate for two consecutive sampling dates was calculated as:

$$\mu = \frac{\ln(N_1/N_0)}{t_1 - t_0}$$

where N_1 is the cell concentration at time t_1 and N_0 is the initial cell number at time t_0. Cumulative cell numbers were calculated based on cell counts assuming exponential growth as:

$$C_1 = C_0{}^{\mu\times(t_1-t_0)}$$

where C_1 is the cumulative cell number at time t_1 and C_0 is the cumulative cell number at time t_0 and μ is the growth rate between two consecutive days.

3. Results

3.1. Danish isolates of Alexandrium pseudogonyaulax

Species identification of the three recently established clones of *A. pseudogonyaulax* from Danish waters was confirmed at the genotypic level. The three SSU rDNA sequences determined were identical and comparing these to two available SSU rDNA sequences of *A. pseudogonyaulax* from Genbank it was shown that they were 100% identical to JF521638 from Olso Fjord (Norway) and 99.7% identical to AB088302 from Japanese waters (pairwise comparisons not shown).

3.2. Effects of Alexandrium spp. on motility and cell lysis of target cells

3.2.1. Observations on the negative effects of Alexandrium pseudogonyaulax on target cells compared to that of other Alexandrium species

Among the *Alexandrium* species investigated, *A. pseudogonyaulax*, *A. catenella*, and *A. tamarense* (Alex2) caused immobilization and/or cell lysis in at least some of the target species. *A. catenella* and *A. tamarense* (Alex2) lysed some of the target species, whereas *A. pseudogonyaulax* caused immobility of all target cells investigated. *A. pseudogonyaulax* also caused cell lysis in some target species, mainly *T. acuta* and *M. rubrum*, but only after immobilization and clumping in what appears to be a mucus material (Video 1). The other *Alexandrium* species used in this study, *A. minutum*, *A. tamarense* (Alex5) and *A. ostenfeldii* did not appear to have any negative effects in initial observations, as the target cells showed no particular differences from monocultures in terms of motility, cell shape and concentration.

The immobilized target cells form dense clumps surrounding some of the *A. pseudogonyaulax* cells indicating that this species differs considerably from the other species tested (Fig. 1A, C–J). Typically, within 30 min (depending on the density of target cells), distinct aggregates of target species begin to form around cells of *A. pseudogonyaulax* (but not necessarily all individuals). Many of the target cells are still capable of flagellar movement but are unable to swim out of the mucus material keeping them aggregated. Hence, they appear stuck or trapped in the vicinity of *A. pseudogonyaulax* cells (Video 1). All the target cells screened during this experiment (i.e. *H. rotundata*, *H. triquetra*, *T. acuta*, *M. rubrum*, and *S. trochoidea*), exhibited this same response (Fig. 1C–G). Other species of *Alexandrium* (*A. tamarense*, *A. catenella*, and *A. minutum*) also showed this response when mixed with *A. pseudogonyaulax* (Fig. 1H–J). The formation of target species into distinct clumps was completely absent in any target species monocultures, and when mixed with cultures of other *Alexandrium* spp. (Fig. 1B).

3.3. Immobilization and cell lysis experiments

3.3.1. Effect of time on the negative effects of Alexandrium pseudogonyaulax and A. catenella on Heterocapsa rotundata

In initial experiments, *A. pseudogonyaulax* began to show negative effects on the motility of *H. rotundata* cells after as little as 30 min exposure with 50% immobility after 60 min, and with no apparent cell lysis of the target cells (Fig. 2A). *A. catenella* also caused 50% immobility after 60 min. However, 50% cell lysis occurred between 1 and 2 h of exposure and 80% were lysed (i.e. not intact) after 5 h (Fig. 2B). In contrast, almost all *H. rotundata* cells mixed with *A. pseudogonyaulax* were non-motile but remained completely intact (i.e. not lysed) after 5 h (Fig. 2A).

3.3.2. Formation of target cell aggregates over time in Alexandrium pseudogonyaulax

In the case of *A. pseudogonyaulax*, immobility of *H. rotundata* cells occurred within 30 min of exposure. However, the formation of noticeable aggregates did not happen until after 2 h. After 5 h of incubation approximately 50% of the non-motile target cells were found in clumps (Fig. 2C). The average number of cells per clump did not change over time but remained between 8 and 10 *H. rotundata* cells per clump. The number of clumps (Fig. 2C) and the maximum number of cells in one clump increased steadily over time (data not shown). The formation of target cells into dense clumps or aggregations was unique to treatments with *A. pseudogonyaulax*. In all cases with *A. catenella* and in the control, target cells were evenly distributed in the well (Fig. 1B).

3.3.3. Effect of Alexandrium pseudogonyaulax and A. catenella cell concentration on Heterocapsa rotundata

Lysis of *H. rotundata* cells did not occur at all in the four hours of incubation with *A. pseudogonyaulax*. Immobility of *H. rotundata* cells began to occur with as little as 25 cells ml^{-1}, with 50% motile *H. rotundata* cells at 50 cells ml^{-1} and nearly 100% immobility with as little as 300 cells ml^{-1} of *A. pseudogonyaulax* (Fig. 3A). In contrast, the effect of *A. catenella* was highly dependent on cell concentration, causing more immobilization and cell lysis in *H. rotundata* cells at higher *Alexandrium* concentrations (Fig. 3B). At 500 cells ml^{-1}, *A. catenella* was not lytic and did not cause any immobility of target cells, but at 3000 cells ml^{-1}, it caused 50% immobility of *H. rotundata* cells. Also, the number of intact *H. rotundata* cells and the number of motile *H. rotundata* cells were nearly identical for *A. catenella* treatments, showing that the remaining *H. rotundata* cells in the wells were in fact motile.

3.3.4. Screening of negative effects of other strains of Alexandrium pseudogonyaulax and other species of Alexandrium on various target species

We tested three strains of *A. pseudogonyaulax* all of which were able to immobilize target cell species without substantial cell lysis,

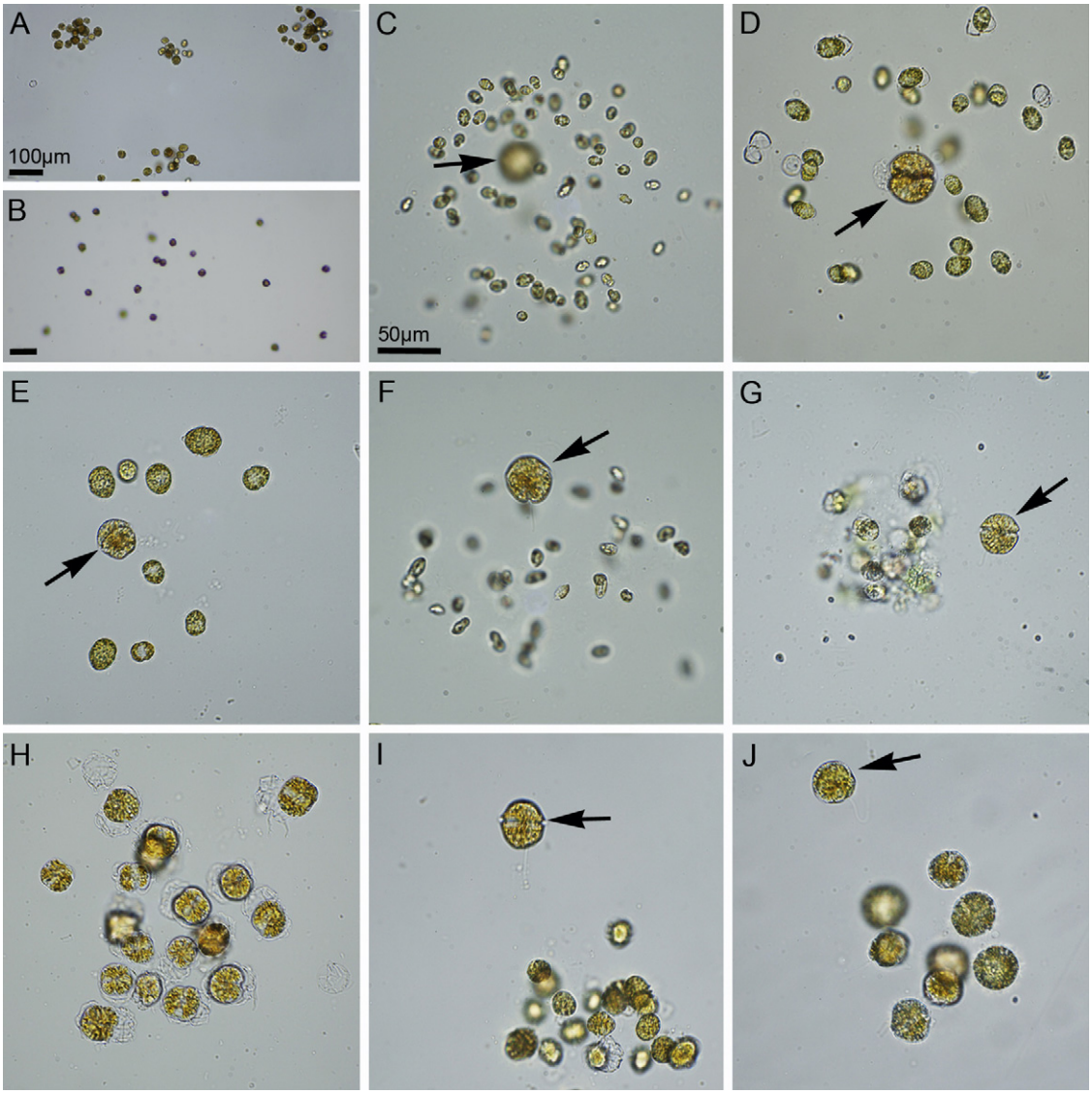

Fig. 1. Light micrographs (Nomarski interference contrast) of mixed cultures of *Alexandrium pseudogonyaulax* (K-1344) showing aggregations of *A. minutum* (A). Examples of uniform distribution of a monoculture, here *A. catenella* (B). Light micrographs of *A. pseudogonyaulax* (K-1344) (concentration of 500 cells ml^{-1}) mixed with various target cell species (concentrations of 5000 cells ml^{-1}) after ca. 4 h with *H. rotundata* (C), *H. triquetra* (D), *S. trochoidea* (E), *T. acuta* (F), *M. rubrum* (G), *A. catenella* (H), *A. minutum* (I) and *A. tamarense* (Alex2) (J). Scale bar represents 100 µm (A–B), and 50 µm (C–J). Arrows indicate cells of *A. pseudogonyaulax* (C, E–G, I–J) and a dividing cyst of *A. pseudogonyaulax* (D).

keeping the majority of target cells intact (Fig. 4A–C). This effect was observed in all target species tested here. In addition, lysis of some target cells by *A. pseudogonyaulax* was observed with some prey species (i.e. *T. acuta* and *M. rubrum*).

The ability to immobilize target species to the extent seen in *A. pseudogonyaulax* was lacking in the other *Alexandrium* species including the lytic species. *A. catenella* and *A. tamarense* (Alex2) were both lytic towards *T. acuta* (Fig. 4D–E). The lytic effects on *H. rotundata*, however, were less pronounced. *M. rubrum* was only slightly affected and *H. triquetra* was completely unaffected. *S. trochoidea* formed temporary cysts when exposed to both *A. catenella* and *A. tamarense* (Alex2), therefore many of the intact target cells were non-motile (Fig. 4D–E).

The three other *Alexandrium* species used in this study, *A. minutum*, *A. ostenfeldii* and *A. tamarense* (Alex5) did not show short-term negative effects on any of the target species (Fig. 4F–H).

3.3.5. *Effect of cell-free supernatant of Alexandrium pseudogonyaulax and A. catenella cultures on Heterocapsa rotundata and Teleaulax acuta*

Cell-free supernatant of *A. pseudogonyaulax* caused significantly less immobilization of both *H. rotundata* and *T. acuta* cells than when *A. pseudogonyaulax* cells were present (Fig. 5A; ANOVA: *H. rotundata* $p = 0.0013$, *T. acuta* $p = 0.0002$). The effect on *T. acuta* was more pronounced with only 8% motile cells after 4 h of exposure when *A. pseudogonyaulax* cells were present compared to about 82% motile cells with cell-free supernatant. The effect on *H. rotundata* was also stronger when the *A. pseudogonyaulax* cells were present, with an average of 38% motile *H. rotundata* cells when exposed to cell-free supernatant after 4 h compared to only 4% when *A. pseudogonyaulax* cells were present.

The percentage of intact target cells was not dependent on the presence of *A. pseudogonyaulax* cells. There was no difference in percent intact *H. rotundata* cells in the cell-free supernatant

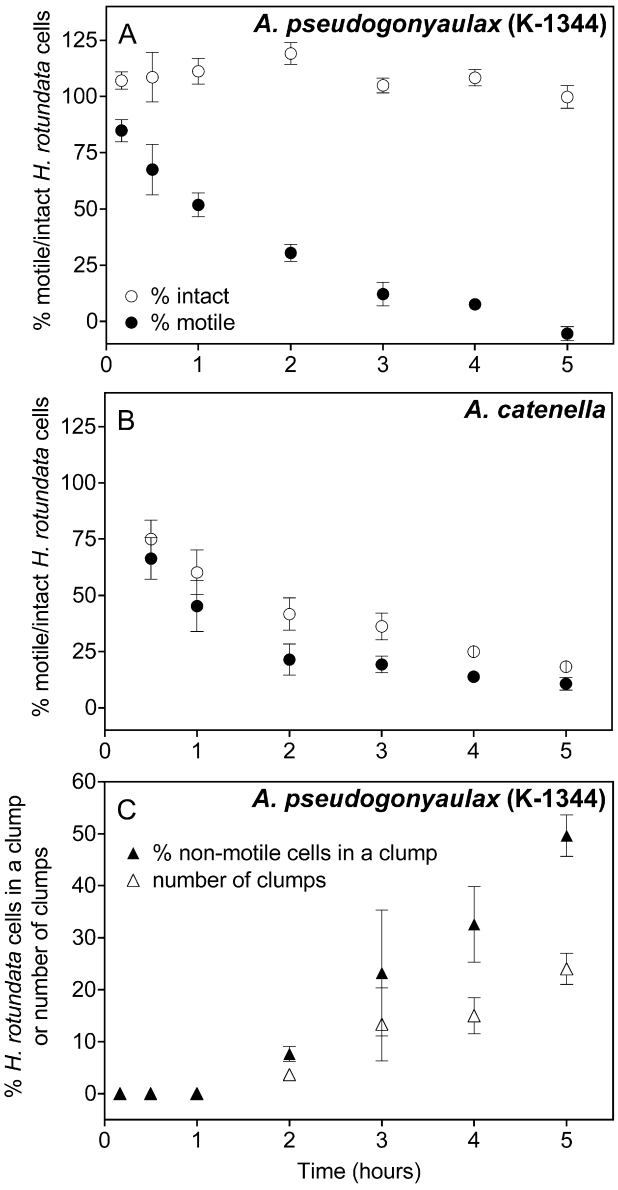

Fig. 2. Percentage of motile (filled circles) and intact (i.e. not lysed; open circles) *Heterocapsa rotundata* cells as a function of exposure time (h) to *Alexandrium pseudogonyaulax* (K-1344) (A) and *A. catenella* (B) compared to control values. Control values could be lower than in treatments due to enumeration or inoculation variation, hence the greater than 100% values. Formation of aggregations (clumps) of *H. rotundata* cells over time (C). Percentage of non-motile *H. rotundata* cells that are in a clump (filled triangles) and the total number of clumps at each time point (open triangles). Error bars represent standard deviation ($n = 3$).

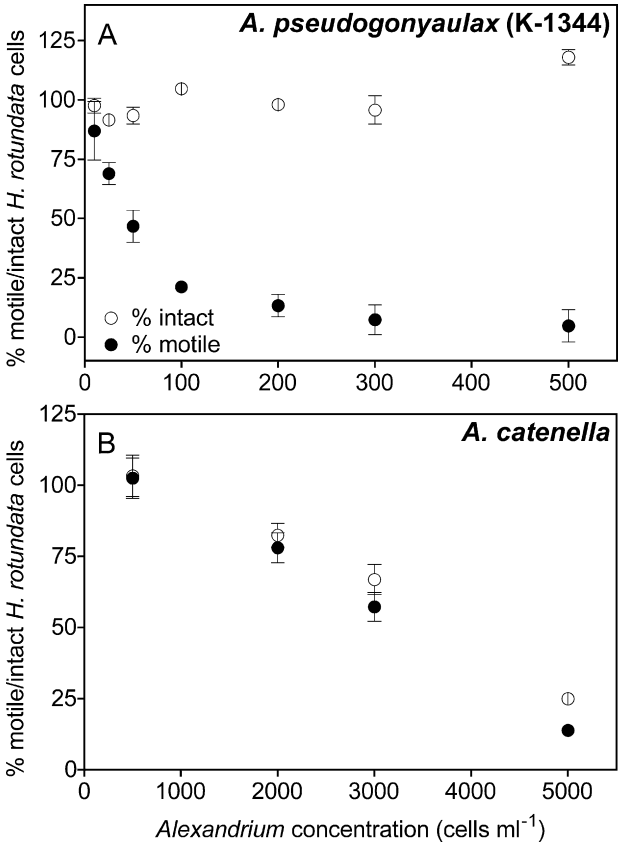

Fig. 3. Percentage of motile (filled symbols) and intact (i.e. not lysed; open symbols) *Heterocapsa rotundata* cells as a function of *Alexandrium* concentration after 4 h mixed with *A. pseudogonyaulax* (A) or *A. catenella* (B). Error bars represent standard deviation ($n = 3$).

compared to the culture (Fig. 5A; $p = 0.118$). The presence of *A. pseudogonyaulax* cells caused slightly more cell lysis of *T. acuta* cells than the cell-free supernatant (71% intact *T. acuta* cells compared to 87% in the cell-free supernatant; $p = 0.0316$; Fig. 5A).

The cell-free supernatant of *A. catenella* caused significantly more immobility and cell lysis in *H. rotundata* cells than the *A. catenella* whole-cell culture (Fig. 5B; $p = 0.0012$ for percent motile and 0.0014 for percent intact). However, the ratio of motile to intact *H. rotundata* cells was the same regardless of the presence of *A. catenella* cells (Fig. 5B). There was no difference in the effect of the presence of *A. catenella* cells on the motility and survival of *T. acuta* cells, with both cell-free supernatant and whole-cell culture causing nearly 100% immobility and lysis (Fig. 5B).

3.4. Mixotrophy in Alexandrium spp.

3.4.1. Observations on the presence of food vacuoles and evidence of mixotrophy in Alexandrium spp.

A. pseudogonyaulax was observed consuming *M. rubrum* and *H. rotundata* through phagocytosis (Fig. 6A–H; Video 2). Food vacuoles were present in *A. pseudogonyaulax* cells when mixed with *M. rubrum* (Fig. 6I) and *H. triquetra* (Fig. 6J). Under epifluorescence food vacuoles were clearly visible when *M. rubrum* (Fig. 6K) and *T. acuta* (Fig. 6L) were offered as prey. The only prey species offered which we did not see evidence of being consumed by *A. pseudogonyaulax* was *S. trochoidea*. No food vacuoles or evidence of phagotrophy were directly observed under the microscope in any of the other *Alexandrium* species used in this study. We did not see any evidence of *A. pseudogonyaulax* consuming other *Alexandrium* spp. when mixed, although this was not an exhaustive search.

3.4.2. Feeding mechanism

The first evidence of prey consumption in *A. pseudogonyaulax* was observed about 30 min after mixing with a potential prey species. After several prey cells have been captured in the mucus trap, the *A. pseudogonyaulax* cell moves close to one of the prey cells and starts to oscillate around it. When surface contact is established, the *A. pseudogonyaulax* positions itself so that the prey cell is right by the sulcus. The sulcus then appears to open slightly and the prey cell is taken in. The *A. pseudogonyaulax* cell then continues to move back and forth gathering cells and reeling in its mucus trap. The process of making contact with a prey cell can take several minutes. However once the prey cell is attached to the

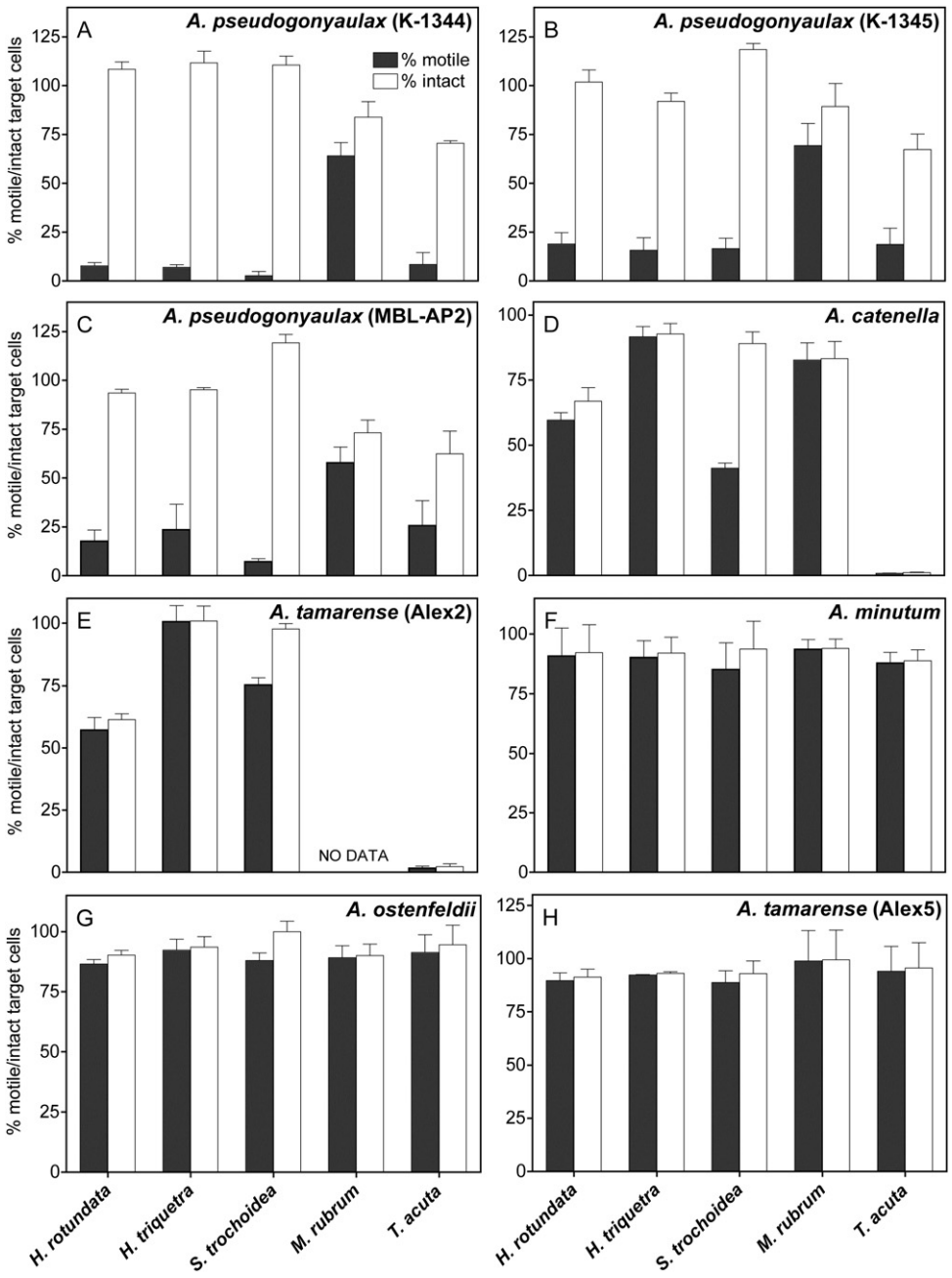

Fig. 4. Screening of toxic effects of different *Alexandrium* species on various target cell species. Percent motile (black bars) and intact (i.e. not lysed; white bars) target cells after 4 h of exposure to different *Alexandrium* species and strains compared to controls. *Alexandrium pseudogonyaulax* (K-1344) (A), *A. pseudogonyaulax* (K-1345) (B), *A. pseudogonyaulax* (MBL-AP2) (C), *A. catenella* (D), *A. tamarense* (Alex2) (E), *A. minutum* (F), *A. ostenfeldii* (G), *A. tamarense* (Alex5) (H). Values above 100% are due to enumeration and inoculation variation amongst treatments and controls. Error bars represent standard deviation ($n = 3$).

surface of the *A. pseudogonyaulax* cell, it is able to consume the prey in less than 15 s (Fig. 6A–H; Video 2). There was no indication that *A. pseudogonyaulax* eats prey cells that are not already caught in a mucus trap.

The mucus trap is attached to the *A. pseudogonyaulax* cell via the longitudinal flagellum (Videos 1 and 3). Occasionally, the ends of the flagella of two *A. pseudogonyaulax* cells were stuck together (Video 3), providing further evidence that some sticky material is attached to the tip of the longitudinal flagellum.

The *A. pseudogonyaulax* cells appear to be in complete control of the cohesive aggregate as they are able to move around with it attached, but can leave it at any time, and thus do not always carry a mucus trap. The mucus, with the entrapped target cells, remains after the *A. pseudogonyaulax* cell detaches from it and leaves the aggregate (Video 4). Not all individual *A. pseudogonyaulax* cells will form mucus traps when mixed with potential prey species. Because the number of traps (i.e. the number of *A. pseudogonyaulax* cells with a trap) increases over time (Fig. 2C) it seems likely that they release the traps when stimulated by the presence of potential prey and do not always have them.

3.4.3. Mixed growth experiments

A. pseudogonyaulax grew significantly faster when prey food was available compared to monoculture growth, in both high and low light experiments (Fig. 7A–B; ANOVA, high light: $p < 0.0001$; low light: $p = 0.0001$). *A. pseudogonyaulax* grew significantly faster

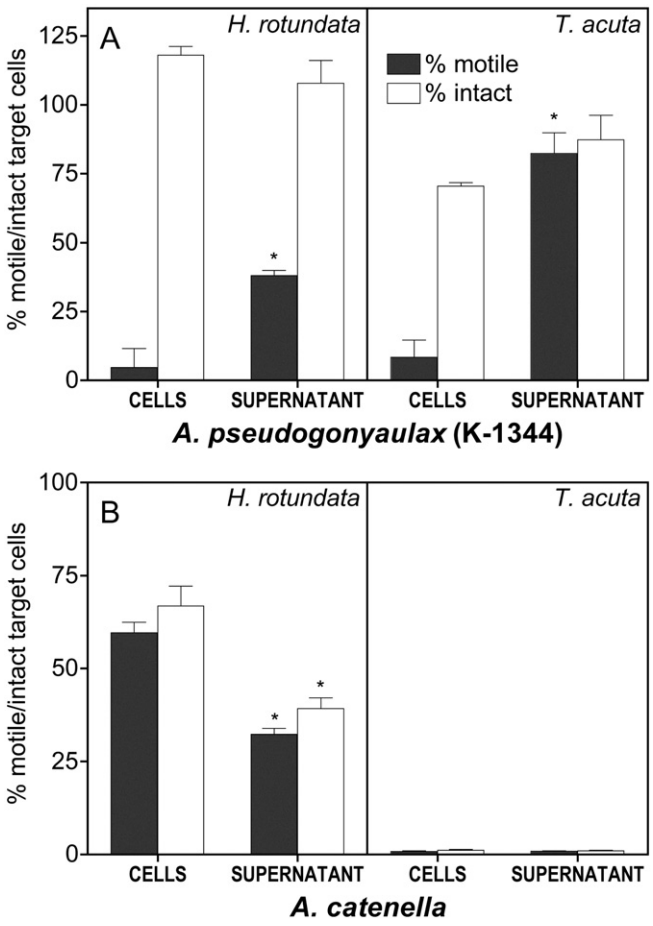

Fig. 5. Effect of cell-free supernatant of *A. pseudogonyaulax* (A) and *A. catenella* (B) on *T. acuta* and *H. rotundata* compared to that of whole-cell cultures. Black bars represent percent motile target cells and white bars represent percent intact (i.e. not lysed) target cells. Asterisks above bars indicate supernatant values significantly different from whole-cell culture values (ANOVA, $p < 0.01$). Error bars represent standard deviation ($n = 3$).

with *H. rotundata* as prey compared to *T. acuta* as prey in both high and low light conditions (Fig. 7A–B; student's *t*-test, high light: $p = 0.0021$; low light: $p = 0.0112$). In the dark, *A. pseudogonyaulax* could not survive for more than three weeks, regardless of the availability of prey (Fig. 7C). However, the decline was not as pronounced when prey was offered. Growth rates of *A. pseudogonyaulax* under all conditions can be found in Table 2.

A. minutum grew fastest in monoculture, without any other species. *H. rotundata* inhibited the growth of *A. minutum* to a much greater extent than *T. acuta* (Fig. 8A; Table 2). At a low initial cell density of 100 cells ml^{-1} *A. catenella* grew significantly slower with *H. rotundata* ($p = 0.0018$), than with either *T. acuta* or in monoculture, while there was no difference in growth rates between monoculture and in mixed cultures with *T. acuta* ($p > 0.05$, student's *t*-test; Fig. 8B). At high initial cell densities (1000 cells ml^{-1}), *A. catenella* grew significantly faster in monoculture than when mixed with either *H. rotundata* or *T. acuta* (Fig. 8C; $p = 0.0111$). The growth rate of *A. catenella* was not dependent on the initial cell concentrations (Table 2). The initial *A. catenella* concentrations did have an effect on the success of the prey species, which showed positive growth only when *A. catenella* was at lower cell densities.

Growth of the prey species in mixed culture with *A. pseudogonyaulax* was never observed. Under high light the amount of *H. rotundata* remaining at each sampling point was quite consistent at around 3000 cells ml^{-1}, whereas the amount of *T. acuta* declined steadily over the course of the experiment (Fig. 9B). Under low light conditions, the concentration of *H. rotundata* at each sampling point was less than under high light, whereas the number of *T. acuta* cells at each sampling was greater under low light than under high light, until the end of the experiment at which time very few *T. acuta* cells were found at both light levels (Fig. 9).

4. Discussion

4.1. Mixotrophy in Alexandrium spp.

Alexandrium species have previously been shown to be capable of mixotrophy (Jacobson and Anderson, 1996; Jeong et al., 2005a,b; Yoo et al., 2009), yet the extent to which *Alexandrium* species may rely on mixotrophy for nutrition is largely unknown. Here we show for the first time that a species of *Alexandrium* (viz. *A. pseudogonyaulax*) clearly benefits from mixotrophic nutrition, as its growth rate is substantially higher when prey species are offered at various irradiances and in nutrient replete inorganic growth medium. Mixotrophic *Alexandrium* species (e.g. *A. ostenfeldii* and *A. tamarense*) contain permanent peridinin-containing chloroplasts but do not require prey and are typically grown phototrophically in culture. *A. pseudogonyaulax* appears to fall within Type I of the two types of mixotrophic dinoflagellate species with permanent chloroplasts as identified by Hansen (2011). Typically, red tide dinoflagellates fall within Type I and are characterized by only a marginal increase in growth rates with increasing prey concentration. Although *A. pseudogonyaulax* increases its growth rate more than most of these species, particularly at low irradiances, it does not quite fit in with the Type II species, which include obligate mixotrophs that are able to increase growth to a large extent when feeding mixotrophically.

The other two *Alexandrium* species selected for the mixed growth experiments, *A. minutum* and *A. catenella* were not mixotrophic. However, strains of these species, as well as strains of *A. tamarense* and *A. ostenfeldii* have been previously shown to be capable of food uptake (Jacobson and Anderson, 1996; Jeong et al., 2005a,b; Yoo et al., 2009). It is possible that the experimental conditions in this study were not conducive to mixotrophy in *A. minutum* or *A. catenella*. In some cases, dinoflagellates only feed under nutrient limitation (Legrand et al., 1998), and our experiments were done with nutrient replete medium. There is also evidence of strain specificity to mixotrophy (Yoo et al., 2009), and perhaps these strains were simply not mixotrophic. Although the *A. catenella* strain used here was recently isolated, the *A. minutum* strain is 10 years old and it is likely that strains kept as phototrophs have lost their feeding abilities over time.

4.1.1. Effect of prey species on the mixotrophic capacity of Alexandrium pseudogonyaulax

The two prey species *T. acuta* and *H. rotundata* were used for mixed growth experiments, because of their different response to the toxic substances excreted by *A. pseudogonyaulax*. Nearly 100% of *H. rotundata* cells remained intact, while many *T. acuta* cells lysed (about 60% intact; Fig. 4A–E), after exposure to the same concentration of *A. pseudogonyaulax*. Interestingly, *A. pseudogonyaulax* grows faster when fed *H. rotundata* than when fed the same concentrations of *T. acuta* perhaps because there are more *H. rotundata* cells intact and available for consumption. It is also possible that these two prey species offer different nutritional value. On average, there were a couple thousand less *T. acuta* cells available than *H. rotundata* cells at each sampling, although the supply of prey was not exhausted even for *T. acuta*, until the end of the experiments (Fig. 9).

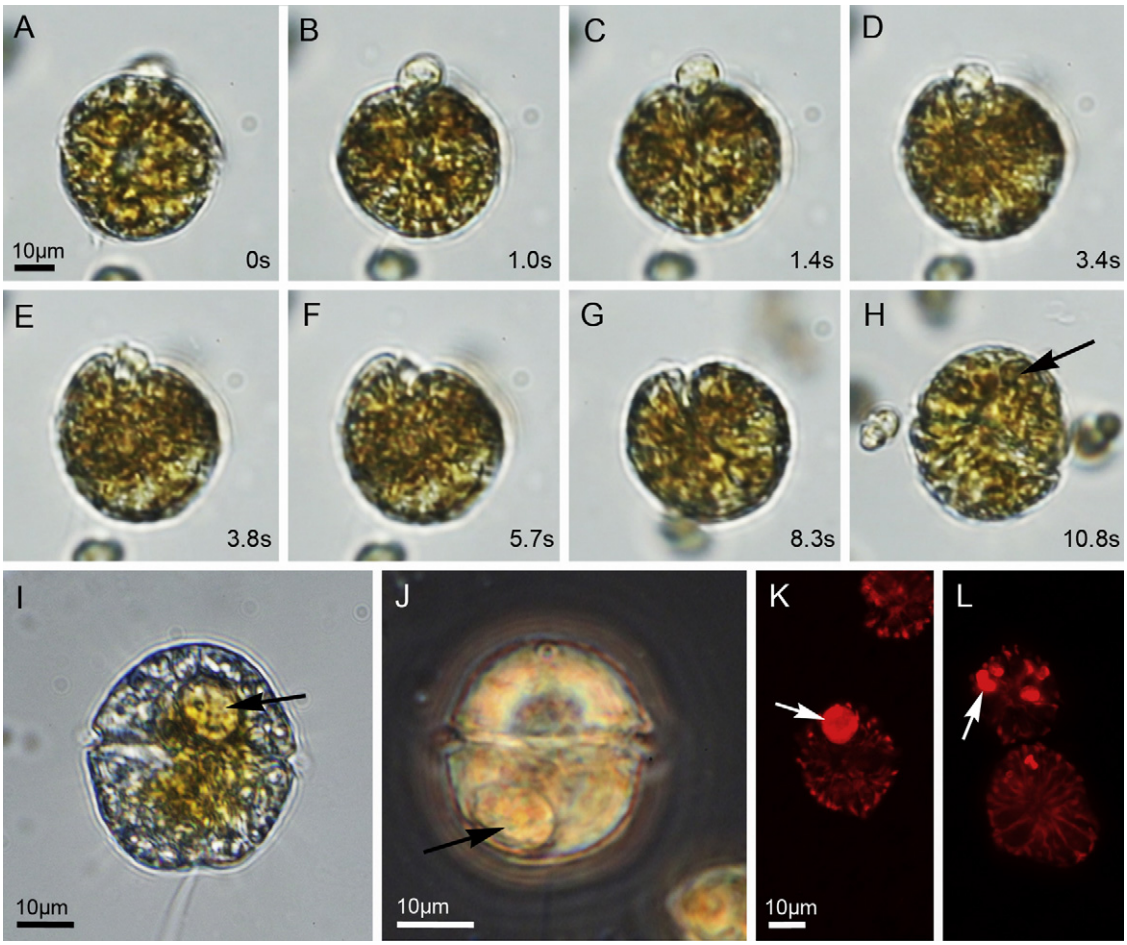

Fig. 6. Evidence of phagotrophy in *Alexandrium pseudogonyaulax*. Sequential images of *A. pseudogonyaulax* cell engulfing a *Heterocapsa rotundata* cell through the sulcus (A–H). *A. pseudogonyaulax* with a food vacuole containing *M. rubrum* (I). Phase-contrast image of *A. pseudogonyaulax* mixed with *Heterocapsa triquetra* with a visible food vacuole inside resembling *H. triquetra* (J). Epiflourescence micrographs of *A. pseudogonyaulax* mixed with *M. rubrum* (K) and *Teleaulax acuta* (L). The solid red structures in the *A. pseudogonyaulax* cells are food vacuoles and several *T. acuta* are visible in the top cell (L). Arrows indicate food vacuoles.

4.1.2. Effect of light on the mixotrophic growth of Alexandrium pseudogonyaulax

To gain a better understanding of how environmental factors affect mixotrophy in *A. pseudogonyaulax* we performed mixed growth experiments at three irradiance levels (including no light). *A. pseudogonyaulax* is an obligate phototroph and cannot grow in the dark. Although some species are able to survive in the dark using mixotrophy (e.g. *Fragilidium subglobosum*; Skovgaard, 1996), most mixotrophic dinoflagellates known so far are not able to, regardless of prey availability (Stoecker et al., 2006; three out of 36 known mixotrophic dinoflagellates can grow in the dark with prey; reviewed in Hansen, 2011). Light stimulates phagotrophy in many mixotrophs (Hansen and Nielsen, 1997) and in many cases it is required to induce feeding and maintain phagotrophic activity (Stoecker et al., 1997; Li et al., 1999, 2000). For *A. pseudogonyaulax*, light is not required for phagotrophy because food vacuoles of *T. acuta* were visible under epifluorescence in dark treatments. This is also the case for *Karlodinium armiger*, which can feed in complete darkness, but still cannot support positive growth (Berge et al., 2008).

The addition of prey increases the growth rate of *A. pseudogonyaulax* to a greater extent in low light (17 µmol photons m^{-2} s^{-1}) than in high light (120 µmol photons m^{-2} s^{-1}), which seems to be the case for other mixotrophic dinoflagellates as well (Skovgaard, 1996; reviewed in Hansen, 2011). *A. pseudogonyaulax* increases its growth by 267% when fed (with *H. rotundata* as food; 0.11 d^{-1}) compared to with just phototrophic growth (0.03 d^{-1}) under light-limiting conditions. For comparison, growth only increased by 45% at high light conditions (with *H. rotundata* as food; 0.32 d^{-1} mixotrophic growth compared to 0.22 d^{-1} phototrophic growth). A reliance on mixotrophic nutrition by *A. pseudogonyaulax* thus plays a much bigger role, relatively, when light is limited.

4.1.3. Feeding mechanism in Alexandrium pseudogonyaulax and production and release of a toxic mucus trap

A. pseudogonyaulax differs from other studied *Alexandrium* species in that target cells become immobilized and get trapped in a mucus trap, forming a dense aggregate of cells surrounding the *A. pseudogonyaulax* cell. *A. pseudogonyaulax* cells consume whole cell prey through the sulcus, but they lack any mechanism to capture individual, swimming, motile prey cells. They were able to consume a number of the target species in this study, only because they were already completely immobilized and stuck in the mucus trap produced by *A. pseudogonyaulax*. In most cases the target cells do not lyse right away in these traps and thus can stay intact and alive, with moving flagella, for quite some time (hours–days), but completely unable to swim away, making them susceptible to grazing by *A. pseudogonyaulax*.

The only other known case of a dinoflagellate that uses mucus to catch prey is that of *Noctiluca scintillans* (Kiørboe and Titelman, 1998). In *N. scintillans*, which has mucocysts and trichocysts (Gaines and Elbrächter, 1987), the mucus is attached to a tentacle,

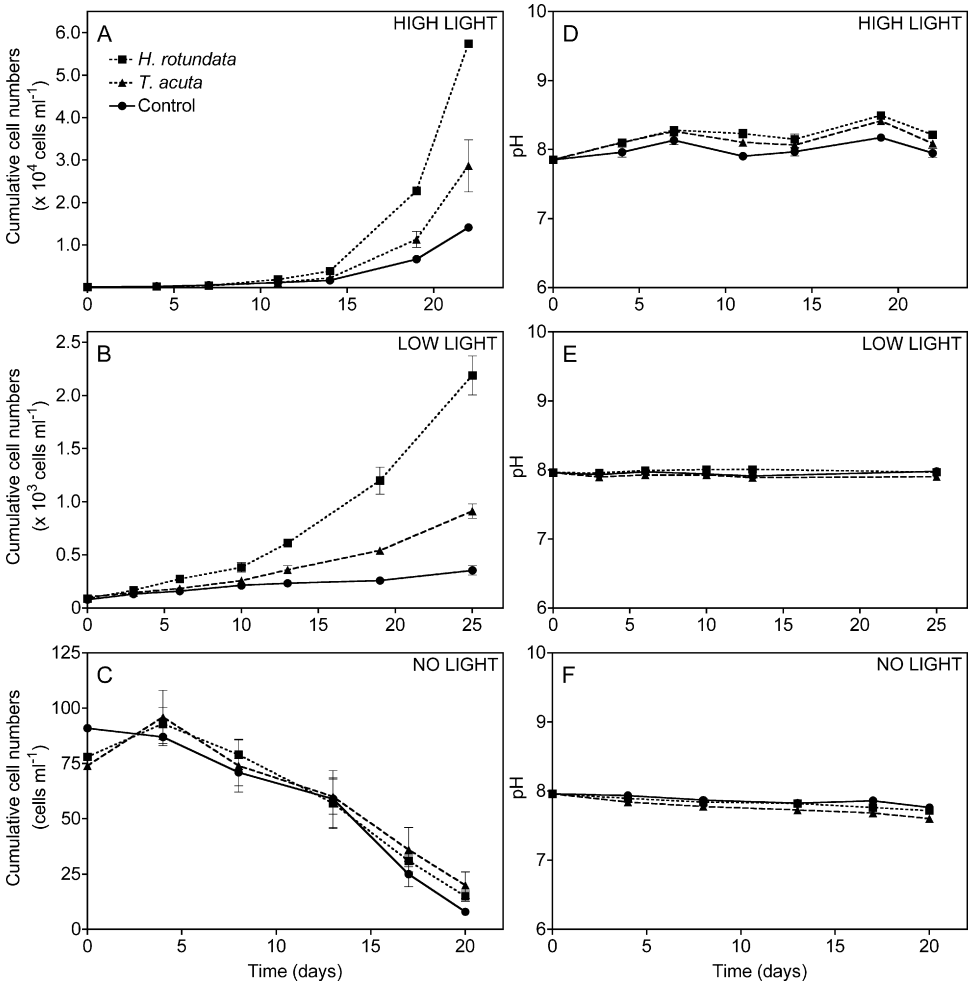

Fig. 7. Cumulative growth rates of *Alexandrium pseudogonyaulax* grown with either *Teleaulax acuta* (triangles), *Heterocapsa rotundata* (squares), or alone as a monoculture (circles), in high light (120 μmol photons m^{-2} s^{-1}) (A), low light (17 μmol photons m^{-2} s^{-1}) (B), and no light (C), with corresponding pH values during growth in high light (D), low light (E), and no light (F). Error bars represent standard deviation ($n = 3$).

not a flagellum, which sweeps through the water column, adhering to sinking particles and capturing potential prey species (Omori and Hamner, 1982; Kiørboe and Titelman, 1998). *A. pseudogonyaulax* does not have any visible physical structures for capturing motile prey, like trichocysts or other capture filaments and therefore must rely on making the prey non-motile prior to capture and consumption.

As opposed to *N. scintillans*, which has a rudimentary flagellum that is not used for motility (Kiørboe and Titelman, 1998), *A. pseudogonyaulax* is able to swim quite efficiently. However, individuals that have released the mucus traps once prey cells are added do not typically swim, but stay stationary, reeling in impaired target cells (i.e. cells which are still motile, but completely unable to swim as usual) that drift or swim into the mucus (Video 5). Many of the *A. pseudogonyaulax* cells are still able to swim though, in which case they drag the mucus traps behind them (Video 4).

The mucus is beyond doubt formed by the *A. pseudogonyaulax* cells themselves, as aggregations of target cells were completely absent in experiments using cell-free supernatants. However, effects of cell-free supernatants from dense *A. pseudogonyaulax* cell suspensions on the motility were still observed in the experiments with *H. rotundata* as target cells indicating that excreted toxic substances are involved in the feeding strategy of *A. pseudogonyaulax* and do have a negative effect on potential prey species. These excreted toxins could be considered allelochemicals based on their effects, but because they are ultimately used to immobilize cells for prey uptake we refer to them as "capture compounds" instead. Assuming the excreted capture compounds remain in the mucus and accumulate there, as they are likely continually released, the concentration of toxins in the mucus may be higher than in the surrounding medium. Some immobility and swimming impairment (Video 4) does precede the formation of dense clumps because the first recognizable clumps surrounding *A. pseudogonyaulax* cells occur after 2 h, at which point already about 75% of the cells are already immobile (Fig. 2A and C). The delay in the formation of distinct clumps is partly an artifact of the target cell concentration and with a higher initial concentration the encounter rate of target cells with the mucus will be higher. With more target cells they do get caught earlier and form recognizable aggregates earlier than two hours. The capture compounds are clearly working together with the mucus to ensure ample immobile prey for the *A. pseudogonyaulax* cells.

4.1.4. Use of toxins as a mechanism to capture prey

Alexandrium species are well known to produce extracellular toxins having lytic/immobilizing effects on other protists (which have been collectively known in the literature as allelopathic effects), and these same groups of toxins have recently been suggested as a mechanism to capture prey in some dinoflagellates and haptophytes. The haptophyte *P. parvum* is an omnivorous feeder, which cannot catch motile prey, and thus cannot exploit

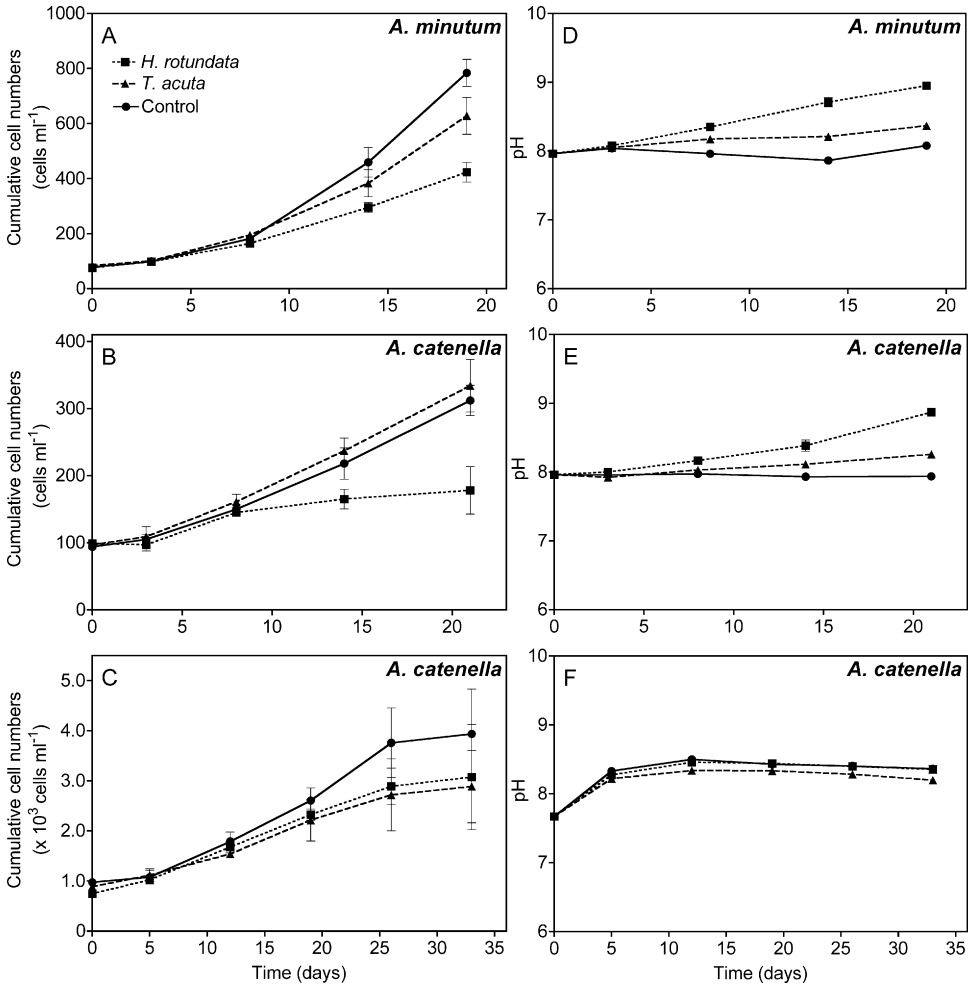

Fig. 8. Cumulative growth rates of *Alexandrium minutum* with an initial cell concentration of 100 cells ml^{-1} (A), *A. catenella* with an initial cell concentration of 100 cells ml^{-1} (B), and *A. catenella* with an initial cell concentration of 1000 cells ml^{-1} (C), grown in low light (17 μmol photons m^{-2} s^{-1}) with either *Teleaulax acuta* (triangles), *Heterocapsa rotundata* (squares), or alone as a monoculture (circles). Corresponding pH values for *A. minutum* (D), and two initial cell densities of *A. catenella* (E–F). Error bars represent standard deviation (n = 3).

this potential food source unless the prey is immobilized. In this species toxins are exuded into the surrounding water, which will immobilize otherwise motile cells, provided a certain *P. parvum* cell density. Above this critical cell density the feeding frequency of *P. parvum* increases with increasing cell density, due to increased exudation of toxins (Tillmann, 2003; Skovgaard and Hansen, 2003).

Toxins involved in prey capture that are not exuded into the surrounding water, depend upon direct cell contact. One of the best-studied examples of this is *K. veneficum*; the non-toxic strains are unable to feed (Adolf et al., 2008), whereas strains producing karlotoxins are capable of mixotrophy (Adolf et al., 2006; Sheng et al., 2010). In this case, and with other species of *Karlodinium*, cell contact is likely necessary for immobilization of prey cells (Adolf et al., 2006), because the toxin is suggested to be directly injected or released into the target cell once contact is established (Adolf et al., 2006; Berge et al., 2008). There is evidence that karlotoxins may be implicated in immobilization of swimming prey cells (Adolf et al., 2006). However, in this species little toxin is released under normal conditions (Deeds et al., 2002) without stimulation and about 95% remains inside the cell (Bachvaroff et al., 2008). This

Table 2
Growth rates (d^{-1} ± SD) of *Alexandrium* species in monocultures and in mixed cultures with *Teleaulax acuta* or *Heterocapsa rotundata* as prey. Initial concentrations of *A. catenella* are indicated, for all other *Alexandrium* species the initial concentration was 100 cells ml^{-1}. "–" refers to no growth.

	Monoculture	With *T. acuta*	With *H. rotundata*
Low light (17 μmol photons m^{-2} s^{-1})			
A. catenella (100 cells ml^{-1})	$0.06 \pm 6 \times 10^{-3}$	$0.06 \pm 4 \times 10^{-3}$	$0.03 \pm 4 \times 10^{-3}$
A. catenella (1000 cells ml^{-1})	$0.07 \pm 5 \times 10^{-3}$	$0.04 \pm 1.2 \times 10^{-2}$	$0.05 \pm 4 \times 10^{-3}$
A. minutum	$0.13 \pm 6 \times 10^{-3}$	$0.11 \pm 1.1 \times 10^{-2}$	$0.09 \pm 5 \times 10^{-3}$
A. pseudogonyaulax	$0.03 \pm 7 \times 10^{-3}$	$0.08 \pm 4 \times 10^{-3}$	$0.11 \pm 5 \times 10^{-3}$
High light (120 μmol photons m^{-2} s^{-1})			
A. pseudogonyaulax	$0.22 \pm 1.0 \times 10^{-2}$	$0.28 \pm 8 \times 10^{-3}$	$0.32 \pm 2 \times 10^{-3}$
Dark			
A. pseudogonyaulax	–	–	–

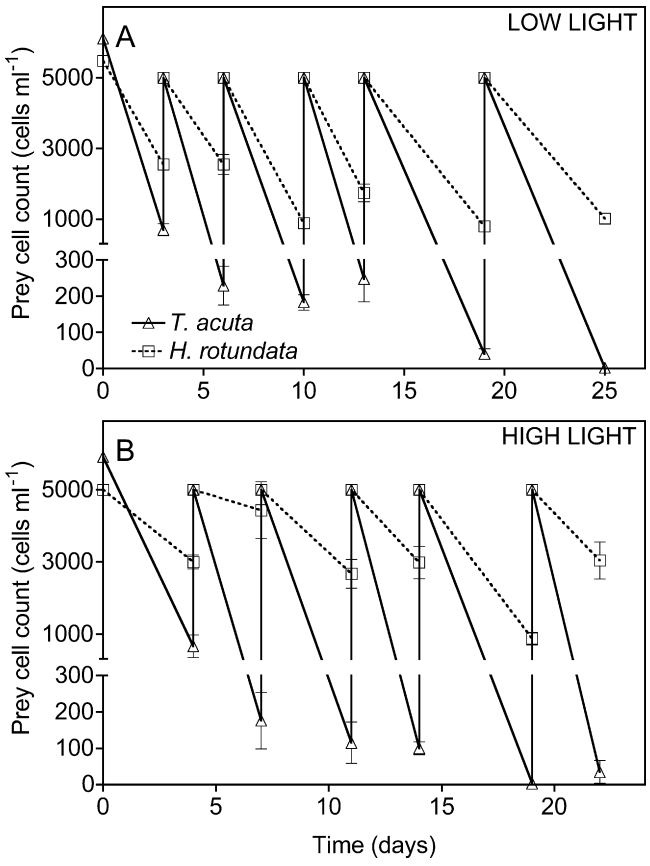

Fig. 9. Prey cell counts throughout the mixed growth experiments with *Alexandrium pseudogonyaulax* in low light (17 μmol photons m^{-2} s^{-1}) (A) and high light (120 μmol photons m^{-2} s^{-1}) (B) with *Teleaulax acuta* (triangles) and *Heterocapsa rotundata* (squares) as prey. Error bars represent standard deviation (n = 3).

requires very high concentrations of *K. veneficum* (Adolf et al., 2006) or very close proximity (Sheng et al., 2010) to exhibit growth inhibition or immobilization in surrounding cells.

Another case in which growth inhibition and ultimately death of surrounding phytoplankton is completely dependent on direct cell contact is that of the dinoflagellate *Heterocapsa circularisquama* (Uchida et al., 1995, 1999; Yamasaki et al., 2011). In this case, it is suggested that a growth-inhibiting substance is not secreted but that cell contact is what kills other species. This may therefore not be considered an example of allelopathy (Uchida et al., 1999), but rather considered "contact immobilization" (Smayda, 1997). Unfortunately, mixotrophy was not addressed at all in these studies and it is not known if the strategy described is used for prey acquisition.

4.2. Allelopathy versus prey capture in Alexandrium spp.

4.2.1. Effect of Alexandrium cell concentration on immobilization and cell lysis of Heterocapsa rotundata

A. pseudogonyaulax has a unique feeding mechanism, where it uses a mucus trap to immobilize, gather, and retain prey species. Although used for a different purpose, this has an ultimate effect identical to that of typical allelopathy. Therefore we used short-term immobilization and cell lysis experiments to compare these negative effects to that of other members of the genus *Alexandrium*, a genus notorious for its allelopathic strengths.

Typically, there is a critical cell density in which an algal species can show allelopathic effects and the amounts of allelochemicals released are proportional to the densities of the donor cells (Hansen, 1989; Schmidt and Hansen, 2001; Tillmann, 2003; Legrand et al., 2003). In some instances when two species are allelopathic and either can inhibit the growth of the other, the species that limits the other is the one able to reach high cell densities first (Yamasaki et al., 2011). This does not apply to *A. pseudogonyaulax* mixed with other allelopathic *Alexandrium* species. *A. pseudogonyaulax* will immobilize and kill *A. catenella*, for example, even though the initial concentration of *A. catenella* was approximately 5 times that of *A. pseudogonyaulax* (data not shown). Furthermore, the initial cell density of donor cells is a crucial component applied to mathematical models of allelopathy in phytoplankton, directly affecting the outcome of the model (Solé et al., 2005).

This critical initial cell density has been a major criticism to the theory that allelopathy may be used by harmful algal bloom (HAB) species during bloom formation (Jonsson et al., 2009). It could not be concluded that allelopathy is what allows harmful algae to form blooms based on the concentrations used in previous studies, just that it may help in prolonging the bloom once it has already begun. However, in *A. pseudogonyaulax*, a necessary critical concentration does not seem to exist, as immobility of target cells can occur when there is only one *A. pseudogonyaulax* cell present. The cell concentration of *A. pseudogonyaulax* required to induce a significant negative response on the target cells was much lower than what has been typically seen in other *Alexandrium* spp. in short-term experiments (Tillmann et al., 2007, 2008) and in the current study (Fig. 3). Strains of both *A. catenella* and *A. tamarense* have been shown to exhibit allelopathic effects, but again, usually at fairly high concentrations (Arzul et al., 1999). In our study, the initial cell concentration causing 50% immobility of *H. rotundata* cells was 60 times higher for both *A. tamarense* (data not shown) and *A. catenella* than it was for *A. pseudogonyaulax*. It is possible that the toxic substances produced by *A. pseudogonyaulax* are much more effective than the allelochemicals of *A. tamarense* and *A. catenella* or at least of a different chemical nature. Perhaps *A. pseudogonyaulax* is simply able to use its secreted toxins more efficiently by concentrating them in the surrounding mucus trap. This strategy shows the ability of *A. pseudogonyaulax* to utilize toxins at low, pre-bloom cell concentrations, rather than only at concentrations reached once a bloom is already established. Additionally, it questions the applicability of current allelopathy models relying on initial cell densities as a crucial input.

4.2.2. Target species responses to strains of Alexandrium pseudogonyaulax and other Alexandrium species

The toxic substances produced by *A. pseudogonyaulax* were non-specific, and within the target cells examined here, all *A. pseudogonyaulax* strains exhibited much more consistent effects on target cells compared to *A. catenella* or *A. tamarense* (Fig. 5) and what has been previously shown for *Alexandrium* spp. (Fistarol et al., 2004; Tillmann et al., 2007; Tillmann and Hansen, 2009). Variability in the response of different target cells has been shown and appears to be typical of allelochemicals (Kubanek et al., 2005; Tillmann et al., 2007; Tillmann and Hansen, 2009). Certain target cell species can also be completely resistant and unaffected by allelochemicals (Tillmann et al., 2007). However, *A. pseudogonyaulax* seems to produce and excrete a universally debilitating, paralyzing toxin. At a relatively low concentration, *A. pseudogonyaulax* (K-1344) causes nearly 100% immobility in three dinoflagellates and one cryptophyte and 60% immobility of a ciliate. The dinoflagellates remain intact while about 30% of the cryptophytes, and 20% of the ciliates lyse (Fig. 4A). Interestingly, *A. pseudogonyaulax* also causes complete immobilization of other *Alexandrium* species, both lytic and non-lytic strains. This suggests that the immobilizing toxin of *A. pseudogonyaulax* is something completely different from that of other *Alexandrium* species and it can

outcompete other toxic algae regardless of initial cell concentrations.

4.2.3. Mucus as a mechanism to capture prey by concentration of exuded toxins

We suggest that the toxins released by *A. pseudogonyaulax* are released with the mucus and are maintained there. This is a much more efficient and effective way of using toxins than simply releasing them into the environment or releasing or injecting toxins upon direct cell contact. Leaking toxins into an aqueous environment hardly seems efficient when considering turbulence, chemical diffusion and cell motility as it reduces the bulk concentration of the toxins, and cannot directly target specific cells. A direct cell-contact release of the toxin like that of *Karlodinium* spp. and *H. circularisquama* is an effective alternative interaction, and may make more sense in terms of energy efficiency: releasing allelochemicals only when necessary, rather than at all times (Uchida et al., 1999). However, this strategy is more limiting, only affecting one cell at a time, and requiring close proximity (Sheng et al., 2010) and high encounter probabilities (Jonsson et al., 2009).

By using a toxic mucus trap mechanism to concentrate released toxins, *A. pseudogonyaulax* maintains high concentrations of the toxins in the vicinity of the producing cell, which is the most efficient use of excreted toxins known so far. The production of a mucus substance by *A. pseudogonyaulax* coupled with excreted toxins creates spatial stability and works on an individual level. Turbulence and therefore dilution of excreted toxins, as well as motility and donor cell concentrations become irrelevant making current modeling systems of allelopathy much less applicable. Jonsson et al. (2009) points out that with previously known allelopathy examples in phytoplankton the producing cell will disperse away from the released toxins and other cells could benefit from it, making this trait unlikely to be favored through natural selection. By the use of a non-specific mucus trap, the *A. pseudogonyaulax* cell does not disperse away from the toxins produced. In addition, there is no evidence that another, non-producing "cheater" cell would benefit as well because all target cells investigated here were affected, and entrapped, including the highly lytic *Alexandrium* species.

Jonsson et al. (2009) suggests that the reported allelopathic effects may be a non-adaptive side effect of compounds released to aid in prey capture. In the case of *A. pseudogonyaulax*, the toxins definitely mediate prey capture and therefore can be known as capture compounds rather than allelochemicals. This particular strategy allows *A. pseudogonyaulax* to keep its potential prey cells intact in order for them to be available later for phagotrophy. It is very likely that other mixotrophic protists that have allelopathic capabilities are actually using this strategy for prey capture and mixotrophy.

4.3. Is Alexandrium pseudogonyaulax unique amongst all Alexandrium spp.?

In addition to differing in allelopathic strategies and feeding mechanisms *A. pseudogonyaulax* differs genetically from other species of its genus (e.g. MacKenzie et al., 2004). In phylogenetic analyses based on nuclear-encoded ribosomal genes and internal transcribed spacers, *A. pseudogonyaulax* groups together with members of the subgenus *Gessnerium* (Balech, 1995) including *Alexandrium taylori*, *Alexandrium hiranoi*, *Alexandrium satoatum* (Touzet et al., 2006; Tang et al., 2007; Penna et al., 2008), and *Alexandrium monilatum* (Rogers et al., 2006). This suggests that these species comprise a monophyletic group (Kim et al., 2005), which diverges earlier than the other members of the genus *Alexandrium* (Rogers et al., 2006).

Members of the *Gessnerium* group do not produce PSP (Balech, 1995), but *A. pseudogonyaulax*, *A. hiranoi* and recently *A. monilatum*, a known fish-killer, have been shown to produce the unique toxin goniodomin A, a novel antifungal polyether macrolide similar to the spirolide toxins of *A. ostenfeldii* (Murakami et al., 1988; Hsia et al., 2006). The morphologically similar *A. taylori* (Penna et al., 2008) produces a proteinaceaous hemolytic toxin that is secreted into the medium (Emura et al., 2004). We need to determine the chemical structure of the toxins released by *A. pseudogonyaulax* and if other species use similar capture compounds for mixotrophy before we can suggest a wider distribution of these toxins and their ecological significance. Perhaps the puzzling phylogeny within *Alexandrium* is closely linked to the divergent evolution of toxins involved in mixotrophy.

5. Conclusions

A. pseudogonyaulax is unique among the tested *Alexandrium* species. It exhibits an extraordinary prey capture technique, which allows it to collect a large variety of prey items, increasing its growth rate through mixotrophic nutrition. The uptake of motile prey seems only to be possible after immobilization due to excreted toxins and subsequent capture of prey cells in mucus traps. The potential impact that this species has on the surrounding phytoplankton community is immense. With highly effective, non-specific toxins and a ravenous appetite, *A. pseudogonyaulax* can render all surrounding prey cells immobile within 4 h, even at low *A. pseudogonyaulax* cell concentrations, and exhibit a nearly 4-fold increase in growth rates by utilizing mixotrophic nutrition at both low and high light conditions. Based on the present study, *A. pseudogonyaulax* is unique among its genus and this study provides insights into the complex relationship between toxicity and nutritional modes, clearly of high importance in the ecology of HAB species and the dynamics of harmful algal blooms.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.hal.2012.02.010.

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