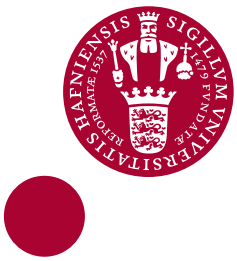

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The loss of mixotrophy in *Alexandrium pseudogonyaulax*: Implications for trade-offs between toxicity, mucus trap production, and phagotrophy

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Abstract

Two important competitive traits in protists, particularly in harmful algal bloom forming species, are lytic toxicity and mixotrophy. This study focuses on *Alexandrium pseudogonyaulax*, a dinoflagellate species that is both toxic and mixotrophic, and in addition uses a mucus trap to immobilize and capture potential prey. A single strain of *A. pseudogonyaulax* was subcultured and grown under two different conditions; one subculture was grown autotrophically, while the other was periodically offered algal prey. After 3 yr, the substrain that was fed remained an avid mixotroph, while the autotrophically grown substrain almost completely lost its ability to feed. The emergence of these two "substrains," with different nutritional strategies, allowed us to investigate possible trade-offs between lytic toxicity, mixotrophy, and mucus trap production. Both substrains were still capable of producing mucus traps, and successfully captured other cells. Although the nonfeeding substrain lost its ability to feed, it was more lytic than the feeding substrain, which may suggest a trade-off between lytic toxicity and feeding ability. However, this increased toxicity was not enough to outcompete other faster growing competitors such as *Teleaulax acuta* and *Heterocapsa rotundata*. In contrast, the feeding substrain *was* able to inhibit the growth of these same competing species. The results indicate that for *A. pseudogonyaulax*, the benefits of phagotrophy far outweigh those of lytic toxicity or the mucus trap alone. The nonfeeding strain used here provides evidence of the loss of mixotrophy under culture conditions, highlighting one of the obstacles in the study of mixotrophic protists.

The concept of trade-offs is ubiquitous in ecology and evolutionary biology. As selection pressures or constraints are applied, trade-offs between different traits arise resulting in alternative survival strategies, which allow for the magnificent diversity of the natural world. A trade-off occurs when a trait that is beneficial for one function becomes a disadvantage for other functions (Litchman and Klausmeier 2008). In phototrophic marine protists, trade-offs between functional traits can explain species distribution across environmental gradients, drive seasonal succession, and ultimately define phytoplankton community structure. This supports the coexistence of diverse species competing for the same resources (Margalef 1978; Litchman et al. 2007). For this reason, phytoplankton lend themselves exceptionally well to the popular trait-based modeling approach of community ecology. This type of modeling has great potential for predicting how protist community structure will alter with impending climate change or increased eutrophic conditions and could be particularly useful for understanding the dynamics of harmful algal blooms (HABs) in order to successfully predict or mitigate HABs. However, the success of this approach relies on accurate understanding of the costs and benefits of certain traits and their overall importance for the ecological processes under study.

Marine protists are under intense competition for limiting nutrients and one of the fundamental constraints they face is the diffusion limitation of nutrients (Munk and Riley 1952; Pasciak and Gavis 1974), resulting in various strategies of nutrient acquisition and utilization. These different strategies have been used to explain the distribution of major functional groups in phytoplankton (Litchman et al. 2007). For example, diatoms are faster growing and succeed in high nutrient, high turbulent environments, whereas dinoflagellates have relatively slower growth rates and low nutrient affinities (Margalef 1978). Despite being such poor competitors for inorganic nitrogen uptake and growth, dinoflagellates use other strategies to increase their competitive fitness, such as the ability to retrieve nutrients by swimming, removing competitors with the use of toxins (allelopathy), as well as taking up other

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forms of nitrogen by using mixotrophic nutritional strategies (Smayda 1997).

Traditionally, phytoplankton were considered phototrophs and protozooplankton were considered heterotrophs, but there is now increasing evidence that most marine protists can simultaneously do photosynthesis, while consuming prey through phagotrophy in a nutritional strategy known as mixotrophy. Rather than a dichotomy of either phototroph or heterotroph, nutritional mode is now understood as more of a spectrum, with the majority of planktonic protists capable of some form of mixotrophy (e.g., Flynn et al. 2013). This trophic continuum contains varying degrees of mixotrophy, and includes species with built-in chloroplasts as well as species that retain functional chloroplasts from their prey (Mitra et al. 2016). In spite of this diversity in mixotrophy and these different combinations and conditions in which mixotrophy resides, mixotrophy should be considered the norm, rather than the exception when discussing nutritional mode in plankton (Flynn et al. 2013). Previously, there had been a huge underestimation of the role of mixotrophs in the planktonic food web resulting in an inaccurate representation of the biological carbon pump (Mitra et al. 2014). With the imposing threat of climate change, and the necessity of producing reliable and accurate models, it is crucial to include mixotrophy as a functional type in models of phytoplankton growth and succession.

Mixotrophic species may have a competitive edge over strict heterotrophs or autotrophs, partly because mixotrophy allows for nutritional flexibility. Interestingly, the majority of HAB forming species are now proven mixotrophs (Burkholder et al. 2008), and thus the role of mixotrophy in bloom dynamics may be significant, contributing to the success of these organisms (Hansen 2011). While the benefits of being mixotrophic under certain conditions (light, nutrients) have been well documented, not as much is known about the costs of having both photosynthetic and heterotrophic machinery. Raven (1984, 1997) argued that the synthesis costs of the photosynthetic machinery may be up to 50% of the cell's total energy budget, whereas phagotrophic machinery makes up < 10%. Synthesizing both photosynthetic and phagotrophic machinery would of course result in the greatest costs.

Allelopathy, that is, the release of lytic compounds (toxins) into the surrounding media, which kill or inhibit the growth of competing protists, is also considered a competitive strategy which many of the HAB species exhibit (Granéli and Hansen 2006). However, the concept of allelopathy as an adaptive strategy for phytoplankton has received criticism. The producing algae may not always have direct benefits of allelochemical production (Lewis Jr 1986; Jonsson et al. 2009) due to the relatively high threshold cell density required to cause bulk toxicity (Jonsson et al. 2009). Cell-level benefits, like toxin-assisted mixotrophy which has been shown in *Prymnesium parvum* (Skovgaard and Hansen 2003; Tillmann 2003) and suggested for *Alexandrium pseudogonyaulax* (Blossom et al. 2012), would help explain the evolution of allelochemical compounds (Jonsson et al. 2009; Driscoll et al. 2013). Perhaps the benefits of lysing co-occurring cells extend beyond simply the removal of competitors as lytic algae may utilize the nutrients liberated from the lysed cells (Lindehoff et al. 2010). This concept has been suggested as one of the direct benefits of lytic toxicity, but there is no experimental evidence yet (Gross et al. 2012).

Species in the notorious HAB genus *Alexandrium*, which are both highly allelopathic and mixotrophic (Table 1), are responsible for extremely disruptive blooms and are the cause of the life-threatening paralytic shellfish poisoning syndrome (Anderson et al. 2012). Some species have even been implicated as the causative organism in destructive fish kills, that is, *A. tamarense* (Cembella et al. 2002) and *A. monilatum* (Gates and Wilson 1960; Ray and Aldrich 1967). The allelochemicals of *Alexandrium* spp., which are still not well characterized chemically, are released into the surrounding medium. Here, they can negatively affect competitors (Tillmann and Hansen 2009) and grazers (Hansen 1989; John et al. 2015; Xu et al. 2017), typically through immobilization followed by cell lysis. The lytic effects of the allelochemicals on other algae are quite target species specific, but *Alexandrium* cell concentrations > 25 cells mL^{-1}, are usually required to affect other algal species (Tillmann and Hansen 2009).

Alexandrium species are considered facultative mixotrophs because they have built-in chloroplasts and can grow photoautotrophically, but they are steadily being proven to be capable of phagotrophy and food vacuoles inside cells of *Alexandrium* spp. have often been observed in natural samples (*see* Table 1). Nevertheless, it has been difficult to induce mixotrophy in the laboratory perhaps due to the use of nutrient replete growth medium or specific prey preferences (Lim et al. 2015; Blossom et al. 2017). At present, only 7 out of ~ 35 species in the genus have been shown to consume prey (Table 1).

The mixotrophic strategy of the lytic *A. pseudogonyaulax* sets this species apart from other *Alexandrium*. Despite nutrient replete conditions, *A. pseudogonyaulax* feeds on a variety of prey organisms and exhibits optimal growth as a mixotroph (Blossom et al. 2012). *A. pseudogonyaulax* uses a compelling prey capture strategy, the mucus trap (Blossom et al. 2012), which is thus far unique among *Alexandrium* species (Blossom et al. 2017). The mucus trap of *A. pseudogonyaulax* is so effective and nonspecific in its capture of potential prey cells that many of the surrounding protists which are caught, are never consumed, and remain in the trap for hours or even days (Blossom et al. 2017). This suggests that the mucus trap may offer extra competitive advantages over other prey capture mechanisms.

Despite the urgent drive to recognize the significance of mixotrophy, and to accept that mixotrophy is the default nutritional mode of planktonic protists (Flynn et al. 2013), these organisms continue to be maintained under exclusively

Table 1. *Alexandrium* species with published evidence of phagotrophy and/or lytic compounds, and examples of references. FV = food vacuoles observed, V = video evidence of prey engulfment, MG = maximum growth with mixotrophic nutrition, x = attempt made to feed, but no evidence of phagotrophy.

Alexandrium spp.	Evidence of phagotrophy	Phagotrophy reference	Lytic	Lytic toxicity reference
A. affine	x	Lee et al. (2016)	Yes	Lee et al. (2016)
				Tillmann and John (2002)
A. andersonii	FV, V, MG, diverse prey	Lee et al. (2016)	Yes	Lee et al. (2016)
A. catenella[*]	FV, *Synechococcus*	Jeong et al. (2005*b*)	Yes	Arzul et al. (1999)
	FV, *Skeletonema costatum*	Yoo et al. (2009)		Tillmann et al. (2008)
A. fraterculus	x	Lee et al. (2016)	Yes	Lee et al. (2016)
A. fundyense[*]	—	—	Yes	Hattenrath-Lehmann and Gobler (2011)
A. insuetum	x	Blossom et al. (2017)	—	—
A. leei	—	—	Yes	Tang et al. (2007)
A. lusitanicum	—	—	Yes	Tillmann et al. (2008)
A. margalefii	x	Blossom et al. (2017)	Yes	*Unpublished data*
A. minutum	FV, *Synechococcus*	Jeong et al. (2005*b*)	Yes	Arzul et al. (1999)
				Fistarol et al. (2004)
				Yang et al. (2010)
				Tillmann et al. (2008)
A. monilatum	—	—	Yes	Ray and Aldrich (1967)
A. ostenfeldii	FV, field samples	Jacobson and Anderson (1996)	Yes	Tillmann and John (2002)
		Gribble et al. (2005)		Tillmann et al. (2007)
A. pohangense	FV, V, MG, specific prey *Cochlodinium polykrikoides*	Lim et al. (2015)	Yes	Lim et al. (2015)
A. pseudogonyaulax	FV, V, MG, diverse prey	Jacobson (1999)	Yes	Tillmann and John (2002)
		Blossom et al. (2012)		Blossom et al. (2012)
		Blossom et al. (2017)		
A. tamarense[*]	FV, diverse prey	Jeong et al. (2005*a*)	Yes	Arzul et al. (1999)
	FV, *Skeletonema costatum*	Yoo et al. (2009)		Tillmann and John (2002)
				Fistarol et al. (2004)
				Tillmann et al. (2008)
A. tamatum	—	—	Yes	*Unpubl. data*
A. taylorii	x	Blossom et al. (2017)	Yes	Tillmann and John (2002)
				Tillmann et al. (2008)
				Emura et al. (2004)

[*]Species name assigned here matches the reference and may not be the most recent species designation.

phototrophic conditions, in nutrient replete medium, without intraspecific interactions. Under these conditions, there is no selective pressure to maintain the phagotrophic ability. The establishment of unialgal laboratory cultures forces the algae to adjust to environmental conditions so drastically different from their natural environment. This culturing environment may cause such strong directional selection pressure that it will lead to significant evolutionary change of the cells in the culture over time, reviewed by Lakeman et al. (2009). There is even evidence of algae losing key traits after extended time in culture, as traits with an energetic cost, but no adaptive advantage in culture conditions, would be selected against. Examples include the loss of the eyespot in the dinoflagellate *Kryptoperidinium foliaceum* (Moldrup et al. 2013), and the loss of toxicity in *A. lusitanicum* (Martins et al. 2004). Mixotrophic capabilities could also be susceptible for selection against and could be an explanation for why field samples have food vacuoles, but cultures of *Alexandrium* rarely feed (Blossom et al. 2017).

These examples inspired us to grow a mixotrophic *A. pseudogonyaulax* strain under two different conditions for 3 yr (one grown mixotrophically with *Heterocapsa rotundata* as prey, and the other grown photoautotrophically). *A. pseudogonyaulax* exhibits three potentially costly, but also highly beneficial traits: the mucus trap, allelopathic (lytic) toxicity, and phagotrophy, all of which could be susceptible to change under culture conditions. The aim of this study was (1) to compare the key traits of phagotrophy, mucus trap production, prey capture, and lytic toxicity in the two substrains, (2) to identify trade-offs between these traits by growing the two sub-strains with other algae as potential prey and/or competitors, (3) to determine if *A. pseudogonyaulax* directly benefits

from the lysed cell material available due to the lytic compounds, and finally (4) to study the potential recovery of lost mixotrophic capabilities in the substrain that had grown photoautotrophically for 3 yr.

Methods

Strains and culture conditions

Cultures used in these experiments originated from Denmark and were obtained from the Scandinavian Culture Collection for Algae and Protozoa (SCCAP). The original *A. pseudogonyaulax* strain used was strain K-1344, isolated in 2009 from Limfjorden, Denmark. *Heterocapsa rotundata* (K-0483) and *Teleaulax acuta* (K-1486) were used as target cells; they were considered competitors in the mixed growth experiments, but referred to as "prey" in the mucus trap and feeding experiments. *T. acuta* was used as the target algae in the lytic toxicity assay. Cultures were grown in f/2 medium with salinity of 30. They were maintained at 15°C with an irradiance of between 90 and 120 μmol photons m^{-2} s^{-1} kept on a light : dark cycle of 14 : 10 h.

Development of two substrains

Three years prior to the start of the experiments presented here, the stock culture of *A. pseudogonyaulax* was separated into two subcultures. To one of the subcultures, a small inoculum of *H. rotundata* (< 100 cells mL^{-1}) was added as potential prey. Every ~ 6 weeks, about 1 mL from these two stock cultures of *A. pseudogonyaulax* was transferred to fresh f/2 medium. At this time, the stock culture that had received prey also got a fresh inoculum of prey *H. rotundata* (approximately < 100 cells mL^{-1}). After 3 yr, the two stock cultures were examined for mixotrophy, and were thereafter referred to as substrains F and NF, for the feeding and nonfeeding substrains, respectively.

Potential for feeding recovery of the nonfeeding substrain

In order to ensure that the "nonfeeding" substrain was really not feeding anymore, and that there was no recovery of feeding ability, the NF substrain was kept with sufficient prey (greater than 5×10^3 cells mL^{-1}) for at least 30 d and checked periodically for an increase in percentage of cells with food vacuoles. However, the percentage with food vacuoles actually declined, and thus it was considered "nonfeeding" over the course of the following experiments.

Mixotrophy and mucus trap production

The two substrains were tested for mixotrophic capabilities by mixing cultures of known cell concentration in triplicate wells of a 24-well plate. Each well (2 mL) contained 0.2×10^3 and 20×10^3 cells mL^{-1} of *A. pseudogonyaulax* and *T. acuta*, respectively (predator : prey ratio of 1 : 100). After 4 and 24 h of incubation at culture conditions, samples were first examined under a light microscope to estimate and compare mucus trap production by counting the number of mucus traps present in each well. The samples were then fixed with 2% glutaraldehyde (final concentration), and collected onto black polycarbonate filters with 5 μm pore size (Poretics®), and mounted on a glass slide. These were then examined under a light microscope (Olympus BX50) with epifluorescence using an Olympus U-MWG wide band green fluorescence filter cube with excitation wavelength of 510–550 nm. The *T. acuta* consumed by *A. pseudogonyaulax* were clearly visible as food vacuoles due to their orange autofluorescence which contrasted with the red autofluorescence of *A. pseudogonyaulax*. All *A. pseudogonyaulax* cells were counted, and the percentage of cells with food vacuoles was calculated.

Lytic toxicity

Toxicity in this study refers to the lytic toxicity of released extracellular toxins, otherwise known as allelochemicals, and not the toxicity of intracellular compounds. The released toxins of *A. pseudogonyaulax* have yet to be chemically identified, therefore lytic toxicity was measured using the *T. acuta* bioassay described in Blossom et al. (2014). This measures toxicity as lytic effects, and quantifies mortality, through cell lysis, of *T. acuta*. Cell-free supernatant of *A. pseudogonyaulax* cultures was necessary to ensure that mortality of *T. acuta* was due to cell lysis from the toxins, rather than because of consumption by *A. pseudogonyaulax*. *A. pseudogonyaulax* cultures of known cell concentration were centrifuged at $3000 \times g$ for 15 min to obtain cell-free supernatant. This is a gentle method and *A. pseudogonyaulax* survives the process, therefore we know cells are not ruptured, and thus no additional toxins were released. The supernatant was then diluted in triplicate 20 mL glass vials with f/2 medium in nine different dilutions. *T. acuta* culture was added to each of these vials along with a control in f/2 medium (in triplicate) for a final concentration of approximately 5×10^3 cells mL^{-1} with a total volume of 3 mL. These vials were incubated at 15°C at low light (< 20 μmol photons m^{-2} s^{-1}), for 3 h, and thereafter the relative fluorescence (using relative fluorescence units; RFU) was measured on a Turner® Trilogy Laboratory Fluorometer. These values were converted to percent intact *T. acuta* cells using the following equation:

$$\frac{\text{RFU value of sample} - \text{Positive control RFU}}{\text{Negative control RFU} - \text{Positive control RFU}} \times 100$$

where the Positive control RFU is the value obtained when 100% of the *T. acuta cells were lysed, and the Negative control RFU is the RFU value obtained when T. acuta cells were only placed in f/2 medium and no cell lysis occurred (100% intact). The different dilutions of supernatant were converted to A. pseudogonyaulax concentration (cells mL^{-1}) based on the initial concentration of the A. pseudogonyaulax culture as well as the volume of supernatant used for dilutions. This was done for both substrains. The median lethal concentration (LC_{50}; A. pseudogonyaulax concentration required to lyse 50% of the T. acuta cells) was calculated for each strain using a two-*

parameter log-logistic function where the upper and lower limits were fixed at 1 and 0, respectively, with the following formula:

$$f(x) = \frac{1}{1 + \exp(b(\log(x) - \log(e)))}$$

where b is the slope and e is the LC_{50}. This was done in R with the add-on package drc.

Mucus trap experiments

In order to compare mucus trap production between the F and the NF substrains, an experiment was set up comparing the number of traps produced and how many prey cells were caught. Single cells of both substrains of *A. pseudogonyaulax* were isolated using a micropipette and placed individually into wells of a 96-well plate containing 100 μL of f/2 medium. Thirty-two cells of each substrain were isolated. After 2 h of acclimation, 200 μL of *H. rotundata* (at approximately 5×10^4 cells mL^{-1}) were added to half of the wells (16 wells) and 200 μL of *T. acuta* (at approximately 5×10^4 cells mL^{-1}) were added to the other half as prey cells. The number of traps formed, the number of prey cells in each trap, and the number of *A. pseudogonyaulax* that were actively attached to a trap, were enumerated after 4 and 24 h. Only traps which had entrapped prey cells could be visualized and thus counted. Traps with lysed prey cells were still counted as traps, but the number of prey cells in these traps could not be accurately counted. This was only evident after 24 h of exposure with *T. acuta* as prey. Thus, the number of prey cells per trap and total prey caught were only calculated for *H. rotundata*. The final number of individuals observed in this experiment at 4 h was 20 for both substrains, while the number of cells observed after 24 h of exposure was 24 for the NF substrain and 26 for the F substrain, respectively, after pooling the treatments with two different prey species. Nonmotile *A. pseudogonyaulax* cells were not included in the analysis at 24 h.

A one-way ANOVA was performed to test for significant differences between the two substrains in terms of the number of traps formed per *A. pseudogonyaulax* cell, the number of prey per trap, and the total number of prey caught per *A. pseudogonyaulax* cell. Test assumptions for ANOVA were validated by visual inspection of Q-Q and residual plots, as well as the Levene's test for homogeneity of variance. In some cases, these assumptions were not met, therefore a log-transformation was done prior to the analysis (log + 1 in the case where zeros were present; i.e., traps per *A. pseudogonyaulax* cell). A chi-square analysis was used to determine any significant difference between the two substrains in the percentage forming a trap, and the percentage attached to a trap.

Mixed growth experiments

To quantify the trade-offs of mixotrophy, mucus trap production, and toxicity, two mixed growth competition/feeding experiments were set up to compare the F and NF substrains of *A. pseudogonyaulax*. For each substrain, growth was measured under three conditions: (1) as a monoculture in f/2 medium (control), (2) with the addition of whole cell prey/competitor, and (3) with the addition of lysed prey cells. The experiment was performed twice, first with *T. acuta* as prey/competitor, and second with *H. rotundata* as prey/competitor. The differences between these two experiments were the method of lysing prey cells, the initial concentrations, and the length of the experiment.

Mixed growth experiment 1: *T. acuta* as prey/competitor

The initial concentration of *A. pseudogonyaulax* in all treatments was 100 cells mL^{-1}. In the mixed treatment with whole cell prey, the initial concentration of *T. acuta* was 6×10^3 cells mL^{-1}. The treatment with lysed cell material had the equivalent of 6×10^3 cells mL^{-1} *T. acuta* cells. For each substrain, exponentially growing *A. pseudogonyaulax* cultures were added to three 270 mL tissue culture flasks. F/2 was added and filled to capacity for the monoculture treatment, and exponentially growing *T. acuta* were added for the whole cell prey treatment. For the treatment with lysed cell material, the supernatant of a dense culture of *A. pseudogonyaulax* (NF) was added to *T. acuta* in a glass vial. The number of lysed *T. acuta* cells used corresponded to the same amount used for the whole cell prey treatment. Before being added to the lysed cell treatment flask, this mixture (*A. pseudogonyaulax* supernatant and *T. acuta* cells) was incubated at 15°C under low light for 3 h, after which all of the *T. acuta* cells were completely lysed (visually inspected). Each flask for the three treatments, were then poured into triplicate 65 mL tissue culture flasks, placed on a vertically rotating plankton wheel, and kept at 15°C, 60 μmol photons m^{-2} s^{-1}. Every 3–4 d, subsamples of 2 mL were taken from each experimental flask and fixed with 2% Lugol's iodine for cell counts. At each sampling, the pH of each flask was measured. Cell counts were performed immediately to determine prey concentrations and dilution volumes required. All or at least 200 cells were counted using either a 1 mL Sedgewick rafter chamber or a 2 mL settling chamber with an inverted light microscope. After sampling, all flasks were diluted to their initial *A. pseudogonyaulax* cell concentration of 100 cells mL^{-1}. In order to maintain constant prey availability in the whole cell prey treatment, when *T. acuta* concentrations fell below 5×10^3 cells mL^{-1}, *T. acuta* was added to reach a concentration of 5×10^3 cells mL^{-1}; when this was the case, an equivalent amount of lysed *T. acuta* was added to the corresponding lysed cell treatment. If *T. acuta* cell concentrations exceeded 5×10^3 cells mL^{-1} in the whole cell treatment, no additional *T. acuta* was added; however, the equivalent of 5×10^3 cells mL^{-1} lysed *T. acuta* was added to the corresponding lysed cell treatment. *T. acuta* cells were lysed in the same way as previously stated in the beginning of the experiment. All bottles were then filled to capacity with f/2 medium and returned to the plankton wheel.

Growth rates were calculated using the linear part of a semi-log plot of cumulative cell number and time. 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 and calculated as*:

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

where N_1 is the cell concentration at time t_1 and N_0 is the initial cell number at time t_0. Calculated cumulative cell numbers are presented in the figures, rather than the enumerated cell count. Differences in growth rate were tested for significance using a one-way ANOVA. Test assumptions for ANOVA were validated by visual inspection of Q-Q and residual plots to test for normality, and by Levene's test to test for homogeneity of variance.

Mixed growth experiment 2: *H. rotundata* as prey/competitor

This experiment was done exactly as stated above, with the following exceptions. The initial concentration of *A. pseudogonyaulax* in all treatments was approximately 110 cells mL^{-1}. In the mixed treatment with whole cell prey, the initial concentration of *H. rotundata* was 5×10^3 cells mL^{-1}. The treatment with lysed cell material had the equivalent of 5×10^3 cells mL^{-1} *H. rotundata* cells. The method for lysing *H. rotundata* was different from that of *T. acuta*, because *H. rotundata* are much more resilient to lytic compounds of *A. pseudogonyaulax*. Therefore, the *H. rotundata* cells were sonicated using a probe tip sonicator (Bandelin Sonopuls, Buch & Holm, Denmark) and sonicated at pulse intervals for 2 min. The length of this experiment was 2 weeks rather than 3 weeks.

A mid-experiment feeding test showed that 60.5% and 0.7% of the F and NF substrain ate. This was done for the experiment with *H. rotundata* as prey just to evaluate whether the substrains could indeed be considered "feeding" and "nonfeeding."

Results

Potential for feeding recovery of the nonfeeding substrain

Despite a few individual cells containing food vacuoles in the NF substrain (maximum of 5.3% of cells contained food vacuoles; Fig. 1A), over time, this strain never fully recovered its ability to feed. After 30 d with constantly available prey, the percentage of cells with food vacuoles in the NF substrain did not increase, but declined. This confirms that the NF substrain had lost its ability to maintain mixotrophic capabilities.

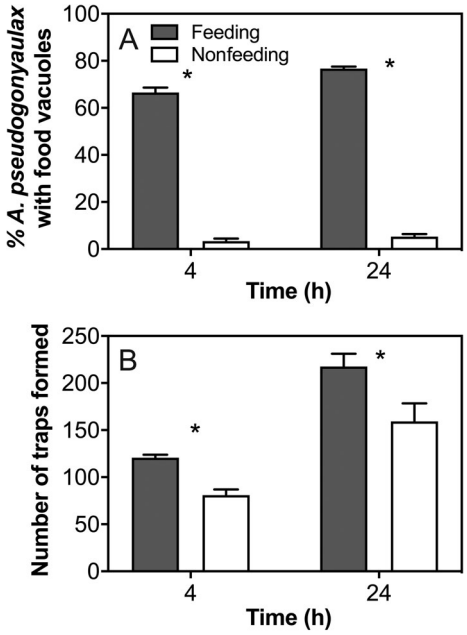

Fig 1. (**A**) Percentage feeding of the F and NF substrains, based on the presence of food vacuoles in individual cells after 4 and 24 h exposure to *T. acuta* prey. (**B**) Number of traps formed by the F and NF substrain immediately prior to fixation to determine the percent feeding shown in (**A**). Gray bars represent the F substrain and white bars represent the NF substrain. This was done in triplicate wells of a 24-well plate with approximately 200 *A. pseudogonyaulax* cells per well. Error bars represent standard deviation, n = 3. * indicates significant differences between the substrains.

Mixotrophy and mucus trap production

The two substrains differed greatly in their mixotrophic capabilities after being separated for 3 yr. The F substrain that had been fed *H. rotundata* every 1–2 months for 3 yr, maintained its ability to feed, while the NF substrain mainly lost its ability to feed, although a few cells were still capable of feeding. Thus, 66.4% and 3.4% of the N and NF substrains had food vacuoles after 4 h of exposure to prey, respectively (Fig. 1A). This percentage of cells with food vacuoles increased slightly to 76.7% and 5.3% of the F and NF substrains after 24 h of exposure to prey, respectively (Fig. 1A). Both substrains were capable of producing mucus traps. However, the F substrain produced significantly more traps than the NF substrain at both 4 h (p = 0.0006) and 24 h (p = 0.0126; Fig. 1B). Cultures of the F and NF substrains produced 120 and 81 visible traps, respectively, after 4 h exposure to prey. After 24 h, the number of traps had increased to 217 and 159, for the two treatments, respectively (Fig. 1B).

Lytic toxicity

Cell-free supernatants of both substrains caused cell lysis of the target algae, *T. acuta*. The NF substrain was more lytic than the F substrain with an LC_{50} of almost half (586 cells mL^{-1}) of that of the F substrain (1.05×10^3 cells mL^{-1}; Fig. 2).

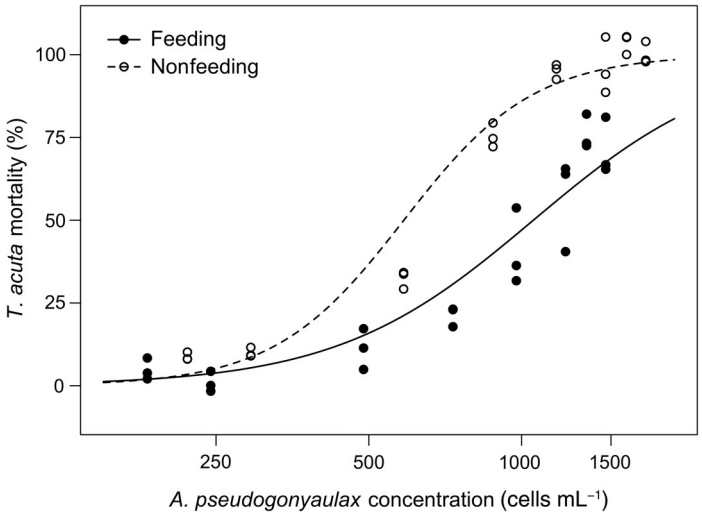

Fig 2. Fitted dose-response curves of the two *A. pseudogonyaulax* substrains used to calculate their relative toxicities, or lytic effects on *T. acuta*. The NF substrain (open circles) is more toxic (LC_{50} of 586 cells mL^{-1}) than the F substrain (black circles; LC_{50} of 1054 cells mL^{-1}). Note the x-axis is in logarithmic scale, but shown in actual cell concentrations.

Mucus trap experiments

When taken individually, some differences in mucus trap production between the two substrains were observed, but in most cases they behaved similarly. For example, there was no significant difference between substrains in the number of cells capable of producing traps. At 4 h, 75% and 65% of the F and the NF substrain had formed a trap (chi-square; $p = 0.4902$; Fig. 3A), respectively. The percentages increased to 92% and 83% of the F and NF substrains after 24 h, respectively (chi-square; $p = 0.3293$; Fig. 3A). A significant difference between the substrains in the number of traps formed per *A. pseudogonyaulax* cell was only observed after 24 h where the F and the NF substrain produced 4.65 and 2.46 traps per *A. pseudogonyaulax* cell, respectively ($p = 0.0032$; Fig. 3B).

There was a significant difference between the number of prey cells in each trap (26.3 prey cells per trap in the F substrain compared to 15.8 in the NF substrain; $p = 0.0200$; Fig. 3C) after 4 h. However, no significant differences were observed between the two substrains, after 24 h ($p = 0.664$; Fig. 3C). The total number of *H. rotundata* prey caught per *A. pseudogonyaulax* cell was significantly greater for the F substrain at 4 h ($p = 0.0107$; Fig. 3D), but there was no significant difference between the two substrains after 24 h with an average of 122 and 93 prey cells caught by the F and NF substrains, respectively ($p = 0.2040$; Fig. 3D). *T. acuta* was not included in this calculation, because after 24 h, many of the *T. acuta* that had been caught in a trap were lysed, and could not be properly counted. This was the case for both substrains. The F substrain stayed attached to their traps, especially at

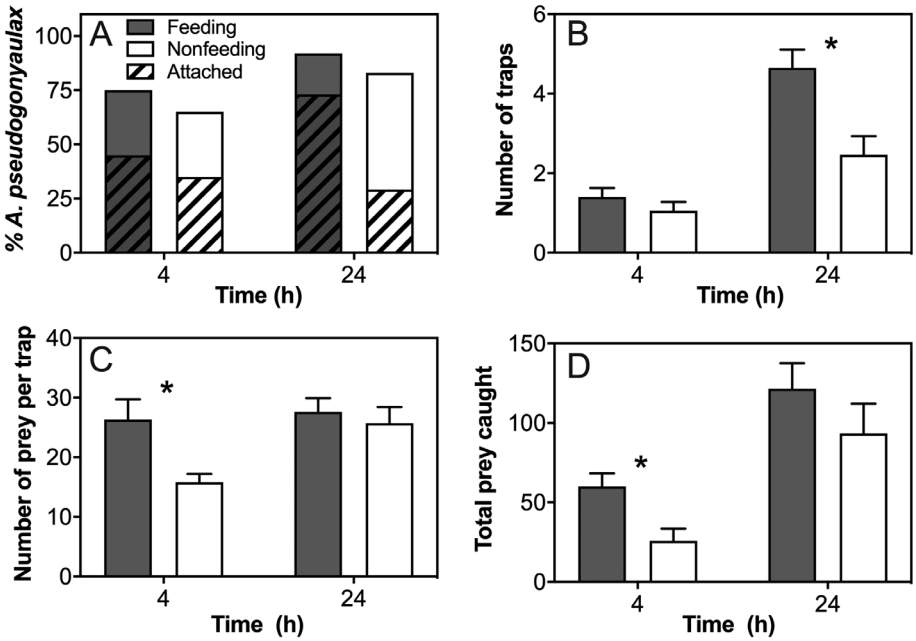

Fig 3. Mucus trap experiments: comparisons of mucus trap formation between the F substrain (gray bars) and the NF substrain (white bars) after 4 and 24 h exposure to prey cells. (**A**) Percentage *A. pseudogonyaulax* capable of forming a trap. The percentage of *A. pseudogonyaulax* cells also attached to their trap is indicated with diagonal line pattern. (**B**) Number of traps formed per individual *A. pseudogonyaulax* cell. (**C**) Number of prey cells caught in each trap formed. (**D**) Total number of prey cells caught per individual *A. pseudogonyaulax* cell. * indicates a significant difference between the two substrains. Data from both *H. rotundata* and *T. acuta* as prey cells were pooled for all these calculations except in (**C**) and (**D**); these show data only including *H. rotundata* as prey. Many *T. acuta* were lysed after 24 h and it was impossible to accurately count the number of *T. acuta* prey cells in the traps.

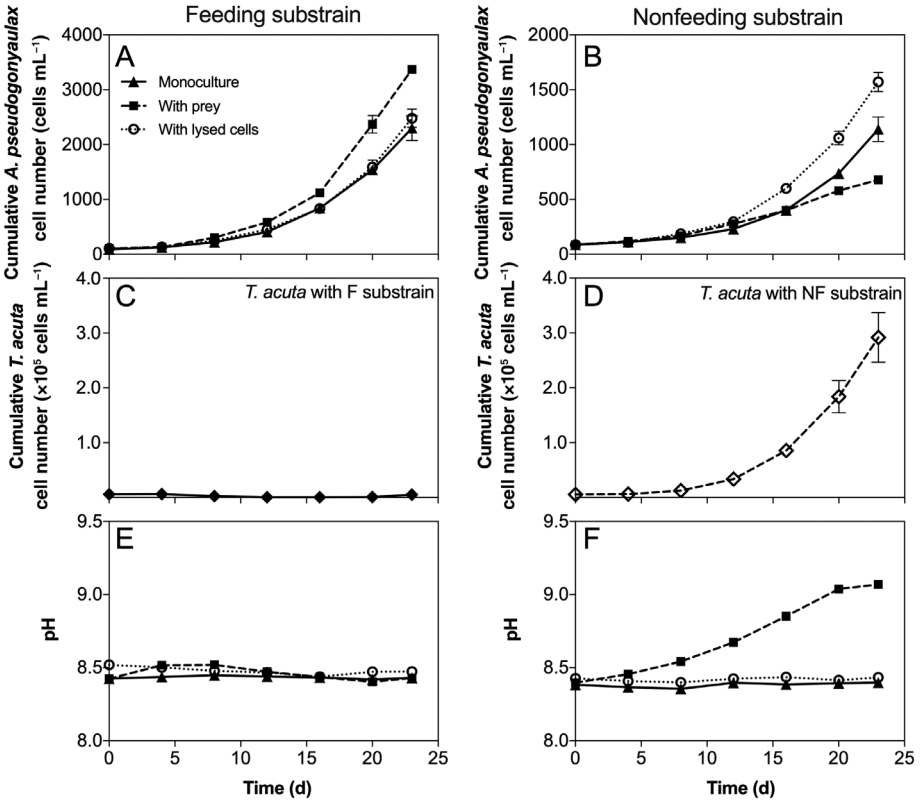

Fig 4. Mixed growth experiments with *T. acuta* as prey. Cumulative cell numbers of *A. pseudogonyaulax* grown by itself as a monoculture (triangles and solid line), with the addition of whole cell prey (squares and dashed line), and with the addition of lysed prey cell material (open circles and dotted line). (**A**) Growth of the F substrain. (**B**) Growth of the NF substrain. (**C**) Cumulative prey cell numbers (*T. acuta*) during the mixed growth experiments, mixed with the F substrain. (**D**) Cumulative prey cell numbers (*T. acuta*) during the mixed growth experiments, mixed with the NF substrain. (**E**) pH during the growth of the experiment shown in (**A**) and (**C**) with the F substrain. (**F**) pH of the cultures during the experiment shown in (**B**) and (**D**) with the NF substrain. Error bars represent standard error; $n = 3$.

24 h, with 73% of the individual F substrain cells attached compared to only 30% of the NF substrain cells ($p = 0.0019$; Fig. 3A).

Mixed growth experiments

Experiment 1: Growth of A. pseudogonyaulax *on* T. acuta *as prey/competitor*

The F substrain grew significantly faster when fed *T. acuta* compared to a monoculture without prey as well as the treatment with lysed cell material (ANOVA, $p = 0.01$; Tukey's post-hoc analysis, $p = 0.0206$ prey treatment vs. monoculture treatment and $p = 0.0135$ for prey treatment vs. lysed cell treatment; Fig. 4; Table 2). Furthermore, Tukey's post hoc analysis revealed that the monoculture treatment was not significantly different from the treatment given lysed cell material ($p = 0.9241$; Fig. 4A; Table 2). A one-way ANOVA test revealed an overall significant difference between growth rates in the treatments with the NF substrain (ANOVA, $p = 0.0022$). A post hoc analysis using Tukey's test of the same data showed that this was due to the significantly slower growth rate of the prey treatment ($p = 0.0020$ for the prey treatment vs. the lysed cell treatment; $p = 0.0118$ for the prey vs. monoculture treatment). No significant difference between the growth rate of the treatment offered lysed cell material, and the monoculture treatment was observed (Tukey's post hoc analysis, $p = 0.2345$). However, a significant difference in final cell concentrations in all treatments for the NF substrain (ANOVA, $p = 0.0008$; and Tukey's post hoc analysis) was observed with the treatment containing lysed cell material reaching the highest cell densities in the NF substrain. The highest cell densities of the F substrain were reached in the treatment including whole-cell prey, which were significantly different from both the monoculture and lysed cell material treatments (ANOVA, $p = 0.0084$).

The difference between the growth of *T. acuta* grown with the F substrain compared to the NF substrain was very pronounced. The F substrain completely controlled the growth of *T. acuta* in the mixed culture, and *T. acuta* could not grow at all during the course of this experiment (Fig. 4C). On the other hand, the NF substrain could not control the growth of *T. acuta* at all, and *T. acuta* completely took over the culture during the experiment (Fig. 4D).

Table 2. Growth rates of *A. pseudogonyaulax* during the mixed growth experiments, d^{-1} ± SE, $n = 3$.

	Experiment 1		Experiment 2	
Treatment	***T. acuta* as prey/competitor**		***H. rotundata* as prey/competitor**	
Substrain	**Feeding**	**Nonfeeding**	**Feeding**	**Nonfeeding**
Monoculture	0.16 ± 0.002	0.13 ± 0.053	0.09 ± 0.006	0.13 ± 0.011
With whole cell prey	0.18 ± 0.005	0.09 ± 0.009	0.07 ± 0.010	No growth
With lysed cell material	0.15 ± 0.005	0.14 ± 0.004	0.09 ± 0.002	0.13 ± 0.009

Not much variation in pH between treatments was observed in the experiment with the F substrain (Fig. 4E); however, the pH of the treatment with whole cell prey increased steadily during the experiment with the NF substrain (Fig. 4F).

Experiment 2: Growth of A. pseudogonyaulax *on* H. rotundata *as prey/competitor*

Both the F and the NF substrains when mixed with whole cell *H. rotundata* suffered from elevated pH due to rapidly growing *H. rotundata*, and consequently the monocultures of *A. pseudogonyaulax* grew better than the mixed cultures (Fig. 5A, B). No significant differences were observed between growth rates for any of the treatments for the F substrain ($p = 0.1035$; Table 2). The F substrain was still able to maintain growth, but the NF substrain could not control the growth of *H. rotundata*, and after the first 3 d, the NF *A. pseudogonyaulax* did not grow at all (Fig. 5B).

The F substrain was able to control the growth of the competitor cells much better than the NF substrain (Fig. 5B,C). Although *H. rotundata* still grew when mixed with the F

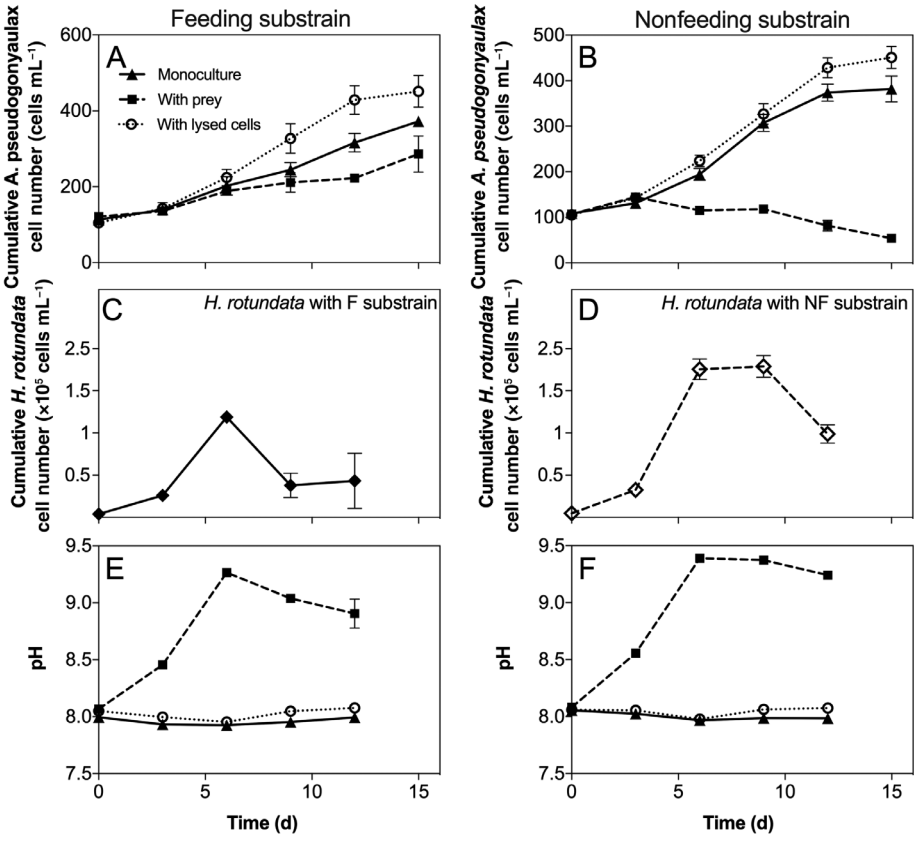

Fig 5. Mixed growth experiments with *H. rotundata* as prey. Cumulative cell numbers of *A. pseudogonyaulax* grown by itself as a monoculture (triangles and solid line), with the addition of whole cell prey (squares and dashed line), and with the addition of lysed prey cell material (open circles and dotted line). (**A**) Growth of the F substrain. (**B**) Growth of the NF substrain. (**C**) Cumulative prey cell numbers (*H. rotundata*) during the mixed growth experiments, mixed with the F substrain. (**D**) Cumulative prey cell numbers (*H. rotundata*) during the mixed growth experiments, mixed with the NF substrain. (**E**) pH during the growth of the experiment shown in (**A**), with the F substrain. (**F**) pH of the cultures during the experiment shown in (**B**), with the NF substrain. Error bars represent standard error; $n = 3$.

substrain (Fig. 5C), leading to elevated pH (Fig. 5E), the F substrain was still able to reduce the growth of *H. rotundata* more than the NF substrain (Fig. 5D).The elevated pH in the mixed treatments for both the F and the NF substrain (Fig. 5E,F) is due to the high abundance of *H. rotundata* cells.

Discussion

Mixotrophy

The two *A. pseudogonyaulax* substrains differed greatly in their mixotrophic capabilities after just 3 yr of growth under different nutritional regimes. The selection pressures applied by submitting one strain to photoautotrophic nutrition and the other to the presence of prey shows just how important mixotrophy is to *A. pseudogonyaulax* as a competitive strategy and allowed us to investigate the potential trade-offs involved.

Aside from anecdotal evidence that mixotrophy can be lost in culture, this is the first time such a pronounced loss of mixotrophy has been shown in a relatively controlled manner. These results have important implications for how we use laboratory cultures, especially when interpreting a sensitive trait like mixotrophy, and relating results to what happens in the natural environment. This loss of mixotrophy supports the strain concept of Lakeman et al. (2009) that "a phytoplankton strain is not a static snapshot of a natural algal population, but rather a dynamic, ever-changing laboratory population." This does not necessarily mean the use of cultures should be avoided, but rather that with awareness we can use this to our advantage, as was done here. Other studies have shown that spontaneous mutations, which leads to adaptation, in phytoplankton can occur at rates observable in the laboratory (Collins and Bell 2004). Furthermore, Collins and Bell (2006) showed that the evolutionary responses of *Chlamydomonas* sp. to elevated CO_2 found in the laboratory corresponded to what was seen in isolates from the natural environments. Although evolution in the laboratory is not as easy to control, and can be unpredictable, it does happen, and it can be used to understand responses to environmental changes.

Mucus trap formation

Alexandrium pseudogonyaulax uses an attached mucus trap to capture prey (Blossom et al. 2012, 2017). Because the mucus trap functions as a prey capture mechanism, a loss of mucus production would have been an excellent explanation for why the NF substrain was no longer feeding, and we expected a greater difference between the two substrains in this respect than what we observed. However, the NF substrain maintained its ability to produce mucus, and no significant difference between the percentages of individuals capable of forming a trap in the two substrains was observed (Fig. 3A). The F substrain were more actively involved with their traps compared to the NF strain; they were more frequently attached to a trap (Fig. 3A), had produced almost double the number of traps after 24 h of exposure than the NF substrain (Fig. 3B), and had caught more prey cells (Fig. 3D). This all suggests that the capture efficiency of the mucus trap was greater for the F substrain, and that the amount of mucus produced or the frequency of release was reduced for the NF substrain (Fig. 3B,C).

This apparent difference in efficiency or production of mucus could signify that the NF substrain was beginning to lose its ability to make a trap. Blossom et al. (2017) demonstrated that a 17-year-old *A. pseudogonyaulax* strain (CAWD-54) had lost its ability to feed and also to form mucus traps, while a 10-year-old strain of *A. pseudogonyaulax* was still able to produce mucus traps and feed. The fact that the NF substrain in the present study still produced a lot of mucus that could trap prey means it is not the prey capture mechanism that has been lost, but rather its ability to feed.

Lytic toxicity in the two substrains

The NF substrain was more lytic than the F substrain (Fig. 2), as the F substrain required twice as many cells to exhibit the same lytic effect as the NF substrain (lysis of 50% of the target algae). This was somewhat unexpected if lytic toxicity is involved in prey capture. One of the suggested roles of released lytic toxins in phytoplankton is toxin-assisted mixotrophy. *P. parvum* is a clear example of this, which can only consume motile prey after immobilization with lytic compounds (Skovgaard and Hansen 2003; Tillmann 2003), and it had also been suggested for *A. pseudogonyaulax* (Blossom et al. 2012). However, distinguishing between the effect of the toxin and the effect of mucus is difficult and requires further scrutiny. Additional advantages of lytic toxicity may expand beyond prey capture, for example, if lysis of the prey cells increases local nutrient availability for the lytic algae.

Competitive inhibition

Even though the NF substrain was more lytic, it could not inhibit the growth of either *T. acuta* or *H. rotundata*. In contrast, despite being less lytic, the *A. pseudogonyaulax* which could feed (F substrain), completely outcompeted *T. acuta* (Fig. 4), and was able to maintain positive growth when mixed with *H. rotundata*. It is clear that the removal of competitors through phagotrophy is extremely important for the competitive success of *A. pseudogonyaulax*. This is supported by the mucus trap experiments as well, since the number of prey cells caught after 24 h was not significantly different between the two substrains (Fig. 3C,D); however, these numbers do not include prey that had been caught and subsequently removed through phagotrophy, and thus the overall effect of the two substrains actually lies within the phagotrophic component. Our results of the mixed growth experiments together with the comparisons between toxicity and mucus trap production show that the F substrain is a better competitor thanks to its

ability to feed, and not because of lytic toxicity or mucus trap production.

The release of lytic metabolites by phytoplankton has been widely accepted as a competitive inhibition, known as allelopathy (Legrand et al. 2003; Granéli and Hansen 2006). But here we show that food uptake is much more effective as a competitive inhibition than allelopathy for the competition between phototrophic organisms, at least at the concentrations used here. Though at higher *A. pseudogonyaulax* concentrations, allelochemicals may play a more important role. The elimination of predators or competitors through mixotrophy is one of the ecological advantages of this nutritional mode (Stoecker et al. 2006). Phagotrophy provides cell-level benefits whereas benefits of allelopathy may only surface after threshold concentrations are reached. Some authors suggest that because of this threshold concentration, which is often only reached during blooms, allelopathy may not be responsible for HAB formation (Jonsson et al. 2009). Since mixotrophy is so prevalent in HAB species (Burkholder et al. 2008), it may be mixotrophy/phagotrophy that plays a greater role than allelopathy in bloom formation under most natural conditions.

Trade-off between phagotrophy and toxicity?

Even though the more lytic NF substrain could not outcompete the competitors with the concentrations used in the experiments shown here, there may be other benefits that could justify an increase in lytic toxicity exhibited by the NF substrain and explain a potential trade-off between toxicity and mixotrophy. The lysis of the other algae in the mixed culture will lead to increased bacterial concentrations and possible remineralization (Weissbach et al. 2012). Could the NF substrain of *A. pseudogonyaulax* exploit that? There is limited evidence to suggest that *Alexandrium* spp. are bacterivorous to any great extent. A few studies suggesting bacterial uptake of *Alexandrium* species have been published but uptake rates are low and no benefits of bacterial additions have been found so far (Nygaard and Tobiesen 1993; Jeong et al. 2005*b*). Another benefit could simply be that the increased dissolved organic matter (DOM) released from lysed cells offers a way for the NF substrain to compensate for their inability to consume whole cell prey, and improves the growth. We did not find a significant difference between growth with lysed cells and growth in the monoculture; however in the NF substrain, final *Alexandrium* cell concentrations were highest in the lysed cell treatment (with *T. acuta* as prey/lysed prey), compared to the monoculture (Fig. 4B). Although further investigation is necessary, it seems that there could in fact be a trade-off between toxicity and mixotrophy, which would make sense and could explain the increased toxicity of the NF substrain. It could be valuable in the future to investigate if increased DOM released from lysed cells would affect the growth of *A. pseudogonyaulax* under nutrient limited conditions. Furthermore, a deeper understanding of the pathways that have been up- or down-regulated, particularly in the NF strain, would be crucial to understand exactly how this potential trade-off may work. It would be speculation to conclude about what precisely is lost in the NF substrain, but a physiological change has certainly occurred.

The use of well-established cultures when studying mixotrophy

One of the major implications of this study is that an algal culture can easily and relatively quickly lose key defining characteristics, like phagotrophy, if grown in a way that selects against it. The very nature of culturing algae may exert extreme selective pressures, which are completely absent in their natural environment. For example, *Heterocapsa triquetra* cultures older than 10 yr had higher pH tolerance than more recently isolated strains because the high pH of a stationary phase culture prior to transfers into fresh medium conveys a selective pressure favoring pH tolerance (Berge et al. 2012). Under culture conditions, the loss of certain functions can be adaptively advantageous if there is a lack of selective pressure to keep an energetically costly trait. Loss of function traits have been shown in other dinoflagellates, for example, loss of toxicity (Martins et al. 2004), loss of bioluminescence (Latz et al. 2008), and loss of phototaxis (Moldrup et al. 2013) among others. Selective pressures influencing mixotrophy may be easy to manipulate, either by offering prey or not, as done here; without prey, there is no selective pressure to maintain feeding abilities. This could also explain why it has been relatively difficult to show food uptake under laboratory conditions with cultures of *Alexandrium* spp. in the past (*see* Blossom et al. 2017 for discussion).

But how quickly do these selection pressures or lack thereof, result in genetic adaptation? The time frame of our study of 3 yr does not seem like a long time, especially compared to the age of strains in culture collections, some of which are now over 50 yr old. However, if the originally separated *A. pseudogonyaulax* strain used here had a growth rate of ~ 0.1 d^{-1} (μ) after 3 yr, it would have gone through ~ 150 generations, which could have allowed for significant changes. Goho and Bell (2000) demonstrated that it took just ~ 100 generations of *Chlamydomonas* grown on either liquid or solid growth medium to exhibit adaptations toward either environment. Adaptation to higher levels of CO_2 has also been shown for the cyanobacteria, *Trichodesmium*, that had been exposed to high levels of CO_2 for a few hundreds of generations (Hutchins et al. 2015). Of course, this greatly complicates laboratory experimental design, and unfortunately most of our knowledge on mixotrophic physiology of phytoplankton actually comes from culture experiments, due to the problematic nature of obtaining reliable results on this matter in the field (Stoecker et al. 2017). This is not to say that we should completely stop using strains that have been in culture in our experiments, but rather that we have to be aware of these potential changes, and to use them to our advantage to learn more about the role of certain traits in protists. It could,

however, help to maintain mixotrophic species as mixotrophs, or try to use recent isolates. If the default conditions that test organisms are maintained in is photography, we may be far from understanding the true impact of mixotrophic strategies, as so many other studies might be biased for phototrophic growth.

Potential for feeding recovery

The small fraction of phagotrophic cells remaining in the NF substrain (Fig. 1A) raised the question of the possibility of a full recovery of phagotrophy over time if the culture was re-fed and consistently given prey. Just as removing prey from the culture's growth conditions selects for phototrophy, adding prey may do the reverse, and select for mixotrophy in this same culture. With abundant prey, it would seem likely that the phagotrophic cells would increase in frequency, simply because the feeding cells then would grow faster and gradually take over the nonfeeding cells. However, we found little evidence of recovery, at least in the months following these experiments. This is similar to other loss of function traits, where evidence that these traits can be recovered once adaptation occurs is lacking (Lakeman et al. 2009 and references therein). For example, changes in nitrogen fixation ability in *Trichodesmium* has proven to be irreversible after hundreds of generations grown under elevated CO_2 levels (Hutchins et al. 2015).

Conclusions

The *A. pseudogonyaulax* F substrain successfully coped with the rapid growth of *H. rotundata* and completely controlled the growth of *T. acuta* in mixed growth experiments, by consuming these competitors through phagotrophy. In contrast, the NF substrain of *A. pseudogonyaulax* could neither compete nor control the growth of either *H. rotundata* or *T. acuta*, despite significant mucus trap production, and lytic toxicity double that of the F substrain. This shows that phagotrophy, rather than allelopathic toxicity or immobilization through capture in mucus traps, offers the greatest benefit to *A. pseudogonyaulax*. Lysing of competitors does not appear to provide immediate direct benefits to *A. pseudogonyaulax*, at least when nutrients are not limited, and in the concentration ratios used here.

Our results clearly justify recent requests to fully appreciate mixotrophy and its role in marine ecosystems (Mitra et al. 2016; Caron 2017; Stoecker et al. 2017) as it may have a huge impact on the success of this species. We must carefully consider interpretations about mixotrophs that have not been given prey regularly, in order to obtain accurate results most closely resembling what may be found in nature. The fact that mixotrophy was lost in an otherwise voracious mixotroph, *A. pseudogonyaulax*, within a time span of just 3 yr, has important implications for how we study mixotrophy in the lab.

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Conflict of Interest

None declared.

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