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The fish-killing dictyochophyte *Pseudochattonella farcimen*: Adaptations leading to bloom formation during early spring in Scandinavian waters

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ABSTRACT

Since 1998, the genus *Pseudochattonella* has formed massive blooms off the coasts of Norway, Sweden and Denmark, causing comprehensive kills of cultured and wild fish. Isolates from a bloom of *Pseudochattonella* sp. in 2001 were found to be a new heterokont flagellate *Pseudochattonella farcimen* (Dictyochophyceae). Since then recurrent blooms of *Pseudochattonella* sp. have appeared both in the early and late spring in Scandinavian waters. *P. farcimen* is the only species of *Pseudochattonella* documented to bloom in the cold waters and low light conditions characterizing winter and early spring. This study aims at determining why *P. farcimen* is able to bloom under these environmental regimes. The effect of temperature and irradiance on the growth of *P. farcimen* cells, isolated from a bloom in 2009, was examined. Furthermore a possible allelopathic effect of *P. farcimen* on the growth of coexisting algae (i.e. *Heterocapsa triquetra*, *Chaetoceros decipiens* and *Chaetoceros diadema*) was examined. Growth of *P. farcimen* was observed at irradiances as low as 2 $\mu E\ m^{-2}\ s^{-1}$. Growth rates increased with temperature from 1 to 6 °C, while a further increase in growth was not seen when the temperature was elevated from 6 to 10 °C. At the highest tested temperature (10 °C), dense cultures of *P. farcimen* were observed to develop large multinucleate cells. Highest growth rates were observed at the pH range 7.7–8.4, while growth rates decreased above pH 8.4, until cultures stopped growing at pH 8.9–9.0. As pH increased and growth declined, the average shape of *P. farcimen* cells changed from elongated to round. This shape could serve as an indicator on the growth condition of the alga. *P. farcimen* did not affect the growth of any of the tested phytoplankton in the present experiment but the growth rate of *P. farcimen* was in the same range as the diatoms. Thus, this study has shown that *P. farcimen* is adapted to successfully compete and grow in a low light and temperature regime that it is exposed to in the late winter and early spring, making it able to form blooms under these conditions.

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1. Introduction

Since spring 1998, *Pseudochattonella* sp. (initially named *Chattonella* aff. *verruculosa*) has caused recurrent massive blooms in the North Sea and Skagerrak. This phytoflagellate has been linked to mortality of both farmed and wild fish (Andersen, 2008; Edvardsen et al., 2007). In February–March 2001, a massive bloom of *Pseudochattonella* sp. in the Kattegat and Skagerrak, caused the death of 1100 tons of farmed salmon along the south coast of Norway (Andersen, 2008; Naustvoll et al., 2002). Cultures isolated from the bloom were found to be a new species *Pseudochattonella farcimen* (Dictyochophyceae) Eikrem, Edvardsen et Throndsen (Edvardsen et al., 2007; Eikrem et al., 2009). Since then, blooms of *P. farcimen* have occurred in Denmark in 2006, 2007 (Andersen, 2008). It bloomed again in January to March in 2009 (own obs.) All blooms have been associated with the death of caged fish causing severe economic losses to fish farmers.

In the late winter and early spring the temperature and light levels, in Danish coastal waters, are low, while nutrients are readily available. Many diatoms are adapted to these conditions, making them the dominating group in the phytoplankton community in early spring. To be able to bloom under these circumstances, *P. farcimen* has to be able to successfully compete with this group. Compared to diatoms, flagellates tend to have lower nutrient uptake affinities and growth rates (Banse, 1982; Smayda, 1997). Therefore they are not ideal competitors for nutrients. One way to overcome this is to produce and release secondary metabolites that negatively affect the growth of other organisms. Thus, the ability to produce allelopathic compounds could give an advantage of *P. farcimen* to outcompete other phytoplankton for nutrients. Many bloom forming phytoplankton species among several different groups, e.g. cyanobacteria, dinoflagellates, prymnesiophytes and raphidophytes have previously been shown to possess allelopathic effects on other microalgae (e.g. Fistarol et al., 2004; Granéli and

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Hansen, 2006; Legrand et al., 2003). To the best of our knowledge it has never been tested if silicoflagellates have the ability to form allelopathic compounds that inhibit the growth of other microalgae.

Low temperatures in the period where *P. farcimen* blooms (2–5 °C), are an obstacle for many phytoplankton species. Temperature is known to have a positive effect on the growth rate of phytoplankton (Eppley, 1972) as it greatly affects all enzyme-catalyzed reactions (Falkowski and Raven, 1997). Contrary, a decrease in temperature reduces enzyme activity. Low temperatures further reduce membrane fluidity and electron chain transfer, thereby limiting photosynthesis, respiration, nutrient uptake and thus the growth of algal cells (Raven and Geider, 1988). To thrive at low temperatures a few adaptive mechanisms are known. These include modifications of key enzyme kinetics and regulation of fluidity of the cell membrane (Davison, 1991; Morgan-Kiss et al., 2006). This allows cold water species to achieve growth rates of 0.24–0.45 d^{-1} at temperatures around 1–4 °C (Jahnke, 1989; Tiselius and Kuylenstierna, 1996).

Phytoplankton exposed to low irradiance levels is usually observed to increase their chlorophyll content (Dubinsky and Schofield, 2010; Perry et al., 1981; Smayda, 1997). As a result species adapted to low light are characterized by having low compensation and saturation points, together with high growth efficiency (Smayda, 1997). Estimates of compensation and saturation points are variable depending on species, but spring blooming diatoms are shown to have compensation points as low as 0.1–1.8 $\mu E\ m^{-2}\ s^{-1}$ (Falkowski and Owens, 1978; Marra, 2004; Smayda, 1997), while growth is saturated around 30 $\mu E\ m^{-2}\ s^{-1}$ (van Hilst and Smith, 2002).

Previous studies have shown that the shape of *P. farcimen* cells is highly variable. Edvardsen et al. (2007) observed that cells had an elongated "carrot like" shape when growing exponentially in the laboratory, but oval and spherical cell shapes were also observed in seawater samples. They noted that *P. farcimen* appear to change shape in response to growth phase, but the cause was not explained. If this change in shape is a response to growth during suboptimal conditions, this could serve as a growth indicator for *P. farcimen*.

As *P. farcimen* is a relatively newly described species, very little is known about its ecology. It blooms in a period generally unfavorable for most phytoplankton and its presence is associated with fish kills. Therefore it is essential to study its adaptation to the environmental settings characteristic of winter and early spring. Hence, in the present study we aim to determine: (1) a possible allopathic effects of *P. farcimen* on co-existing phytoplankton, (2) the effects of irradiance and temperature on the growth of *P. farcimen* and (3) which factors affect the overall shape of *P. farcimen* cells.

2. Materials and methods

2.1. Isolation and culturing of P. farcimen

Surface water from the Great Belt, Denmark (55°28′50N, 11°05′25E) was sampled in spring 2009, after mortality and stressed behavior of cultured rainbow trout (*Oncorhynchus mykiss*) in the area. *P. farcimen* was subsequently isolated and grown in f/20-Si medium (Guillard, 1972), 17 ± 1 psu based on autoclaved 35 psu seawater diluted with tap water and autoclaved at 95 °C for 90 min. Cultures were maintained at 15 °C for 2 months and thereafter at 4 °C at 80 $\mu E\ m^{-2}\ s^{-1}$.

2.2. Experimental conditions

Cultures were kept at a 10:14 h light:dark cycle due to the diurnal cycle at the isolation spot of *P. farcimen*. All experiments were carried out with a single algal strain in triplicates and samplings were conducted at intervals of maximum 4 days. Prior to each experiment, cultures were kept in exponential growth. Subsamples of 3 ml were fixed in Lugol's iodine (final concentration 1%) and cells were enumerated using a 1 ml Sedgewick-Rafter chamber. A minimum of 400 cells were counted.

Light was provided by cool white fluorescent tubes and irradiance was measured using a Li-Cor®, LI-1000 radiation sensor equipped with a spherical probe. Exponential growth rates (μ) were calculated assuming exponential growth (a minimum of 3 sampling points was included in the calculations) using the following equation:

$$\mu\ (d^{-1}) = \frac{(\ln N_t - \ln N_0)}{\Delta t} \quad (1)$$

where N_t and N_0 are the number of cells at t_1 and t_0, and Δt is the length of the time (d). Growth rates for 2 consecutive sampling dates (Figs. 4 and 7) was also calculated using Eq. (1).

To assure that the growth of *P. farcimen* was not nutrient limited, the cellular carbon and nitrogen content was determined and compared with the amount of nutrients added to the growth media. The size and volume of *P. farcimen* was measured in a light microscope (n = 100 cells). Cell volumes were estimated from linear dimensions assuming the average shape of a growing *P. farcimen* to be spheroid. The alga had a mean length of 12.4 (±1.6) μm, a mean width of 6.0 (±1.0) μm and a mean volume of 233.7 μm^3. Cellular carbon content was then calculated assuming C:Vol relationship equal to:

$$\mu g\ C\ cell^{-1} = 0.216 \times V^{0.939} \quad (2)$$

where V is the volume (Menden-Deuer and Lessard, 2000). Nitrogen content of the algae was determined assuming a mass C:N relationship of 41:7 (Redfield, 1958). Calculations using the maximum cell concentrations of *P. farcimen* in the experiments and the calculated cellular carbon content of the algae (3.07×10^{-5} μg C $cell^{-1}$) resulted in an estimated nitrogen uptake of 19–55% (=16–48 μM) of the nitrogen available in the f/20 media. Furthermore maximum pH of *P. farcimen* cultures in f/10 and f/20 media did not differ when *P. farcimen* entered stationary phase, meaning that *P. farcimen* are not nutrient limited in the f/20 medium. As all experiments were performed in closed systems, pH increased over time, caused by the removal of CO_2 during photosynthesis. To determine if increasing pH affects the growth of the cultures, the pH was measured after each sampling, using a Sentron 2001 pH meter with a Red Line electrode and a 2 point calibration.

2.3. Allelopathy

To test for possible allelopathic attributes of *P. farcimen*, mixtures of the strain C_t4 and three target algal species were prepared. A dinoflagellate, *Heterocapsa triquetra* (K-0482 Ehrenberg and two diatoms, *Chaetoceros decipiens* (H7St1) Cleve and *Chaetoceros diadema* (A3N) (Ehrenberg) Gran. The species are ubiquitous in temperate waters and may co-occur with *P. farcimen*. The strain of *P. farcimen* was randomly selected out of six strains, since screening results with *C. decipiens* showed no difference in the effect on the target algae. Target cultures were provided by The Marine Biological Section and The Scandinavian Culture Collection of Algae and Protozoa, University of Copenhagen, Denmark. All cultures were kept in exponential growth prior to the experiments.

P. farcimen and each target algae were tested with initial concentrations of 2000 and 200 cells ml^{-1}, respectively (10:1). Monocultures of each species were run as controls. In order to keep the species in suspension, the bottles were mounted on a plankton wheel (1 rpm) at 4 ± 1 °C at an irradiance of 50 $\mu E\ m^{-2}\ s^{-1}$. After each sampling, bottles were refilled with f/20 medium and the bottles

were remounted on the plankton wheel. The experiments were stopped after 11 days.

2.4. Temperature

A possible effect of temperature on the growth of *P. farcimen* was tested. Experiments were performed at 1, 3, 6 and 10 ± 1 °C. At each temperature *P. farcimen* were acclimated 14 days prior to the experiment, which was carried out at 20 μE m^{-2} s^{-1} in 65 ml tissue-bottles. Initial concentrations were 1000–2000 cells ml^{-1}.

As previous studies have shown that the overall morphology of *P. farcimen* is variable and seems to change in response to growth (Edvardsen et al., 2007; Lotocka, 2009), the shape of the cells were categorized as either elongated or round (n = 100) after each sampling in the temperature and light experiments.

2.5. Irradiance

The growth rate of *P. farcimen* as a function of irradiance was studied at 8 irradiances: 1, 2, 3, 10, 20, 30, 50 and 90 μE m^{-2} s^{-1}. The algae were acclimated to the respective irradiance at least 14 d prior to each experiment. The experiments were carried out in 285 ml tissue-culture bottles at 4 ± 1 °C at initial concentrations of about 1000 cells ml^{-1}.

2.6. Multinucleate cells

Cultures of *P. farcimen* grown at 10 °C were observed to develop large forms (width up to 100 μm) over time. These were always found at the bottom of the culture vessels.

Similar observations have been reported for another silicoflagellate, *Dictyocha speculum* (Henriksen et al., 1993). To test for a possible effect of pH and density on the development of these forms, cultures of *P. farcimen* were established at initial concentrations of about 1000, 4000 and 8000 cells ml^{-1}. The experiments were carried out in 285 ml tissue-bottles at 10 ± 1 °C on a 10:14 light:dark cycle at an irradiance of 70 μE m^{-2} s^{-1}. Subsamples consisted of 13 ml, of this 10 ml were used to enumerate multinucleate cells in a petri dish divided in squares, while normal cells were enumerated using a 1 ml Sedgewick-Rafter chamber. Subsamples were fixed in Lugol's iodine (final concentration 1%). A minimum of 400 cells were counted. The experiment lasted until >40% of the cells had multinucleate forms. Multinucleate cells were subsequently fixed with glutaraldehyde (final concentration 3%) and stained with DAPI, working solution 0.1 μg ml^{-1}; added in a volume ratio of 1:10 stain:sample (Sherr et al., 1993). Images of DAPI stained cells 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).

2.7. Statistics

Analysis of variance (ANOVA) and Tukey's Range Test was used to determine significant difference in growth due to temperature, irradiance and allelopathy on the growth rates of *P. farcimen* and the target species. These tests were used after data passed the Shapiro Wilk Normality Test and an Equal Variance Test. The computer application Sigmaplot 11.0 for Windows (Systat Software, Inc., 2008), was used for all statistical analysis.

2.8. Molecular identification of strains of P. farcimen

A 10 ml aliquot of each of the six exponentially growing clonal cultures was harvested by centrifugation for 10 min at 2500 rpm (=1201 × g). Cell pellets were transferred to 1.5 ml Eppendorf tubes and kept frozen at −18 °C until extraction of total genomic DNA using the CTAB (hexadecyltrimethyl-ammonium bromide) method as described in Daugbjerg et al. (1994). Extracted DNA was used as template to amplify ca. 1300 base pairs of the nuclear-encoded SSU rDNA gene in the 6 strains of *P. facimen* using primers ND2 and ND6 (see Ekelund et al. (2004) for primer sequences). PCR conditions were as follows: one initial cycle of denaturation at 95 °C for 1 min followed by 40 cycles each with a denaturation at 95 °C for 5 s, annealing at 65 °C for 5 s and an extension at 72 °C for 20 s. A final extension for 60 s at 72 °C ended the PCR temperature profile used. We used the Phire Plant Direct PCR kit (Thermo Scientific, Finland) for amplification in 50 μl reactions. DNA fragments were loaded on a 1.5% agarose gel with ethidium bromide and run at 150 V for 15 min. The gel was placed under ultraviolet illumination and to ensure that the amplified DNA fragments had the anticipated length we used as a molecular marker øX174 HaeIII. For PCR purification we used the NucleoFast 96 PCR Kit (Macherey-Nagel GmbH & Co.) following the manufactures recommendations. PCR products with a final concentration of 500 ng were air-dried and together with sequencing primers sent to the sequencing service at Macrogen (Korea). The SSU rDNA gene was determined in both directions using the amplification primers.

2.9. Sequence comparison

In order to verify the identification of the six Danish isolates of *P. farcimen* we compared the SSU rDNA sequence with a sequence of *P. farcimen* from Norway (strain UIO 110) available in Genbank ID: AM075624. Alignment and sequence comparisons were performed using CLC Main Workbench (vers. 6.1.1).

3. Results

3.1. Identity of Danish isolates of P. farcimen

To confirm the identity of *P. farcimen* isolated from Danish waters partial SSU rDNA (1295 base pairs) were determined from six strains and compared to the sequence of an isolate from Skagerrak (Norway). The percentage difference varied between 99.2 and 99.9%. Hence, the Danish isolates are genetically identical to *P. farcimen*.

3.2. Allelopathy

Growth rates of *C. diadema*, *C. decipiens* and *H. triquetra*, did not differ between monoculture and mixed cultures with *P. farcimen* (Fig. 1A–C). Lowest growth rates were observed in all three species in the beginning, indicating a short lag phase. After day 2, growth rates increased to their maximum levels for *C. diadema* and *H. triquetra*, while highest growth rates for *C. decipiens* were achieved after day 4. A significant reduction in the growth rate over time was not observed during the experimental period of 11 days in any of the cultures (one-way ANOVA, *C. diadema*: p = 0.069, *C. decipiens*: p = 0.114, *H. triquetra*: p = 0.080).

Exponential growth rates of *C. diadema* and *H. triquetra* were calculated from day 2 to 11, while the day 4 to 11 were used for *C. decipiens*. The exponential growth rate for *P. farcimen* was also estimated in this experiment from day 0 to 7 (data not shown). Exponential growth rates (±SD) of the target species were 0.18 d^{-1} (±0.01) for *H. triquetra*, 0.32 d^{-1} (±0.01) for *C. decipiens* and 0.45 d^{-1} (±0.02) for *C. diadema*, while *P. farcimen* had an exponential growth rate of 0.35 d^{-1} (±0.01).

The pH increased faster in mixed cultures compared to monocultures for all species, (Fig. 1D–F). As growth rates did not differ between monocultures and mixed cultures during the experiment period, pH had no effect on the growth of the three target algae in the mixed cultures.

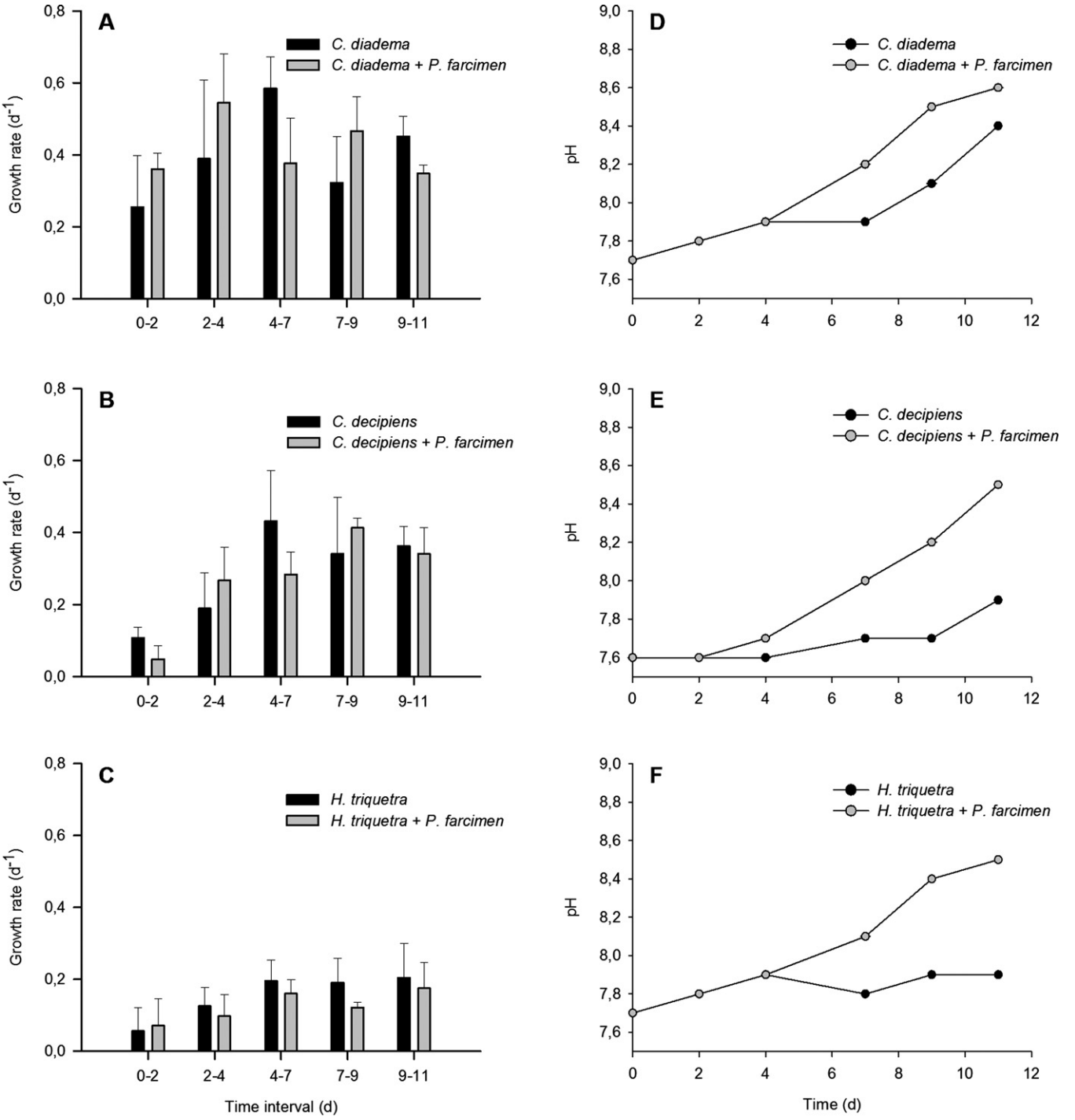

Fig. 1. Mean growth rates (d^{-1}) and pH (±SD, $n = 3$) of target species in mono and mixed cultures with *P. farcimen*. (A, D) *Chaetoceros diadema*, (B, E) *Chaetoceros decipiens*, (C, F) *Heterocapsa triquetra*.

3.3. Effects of temperature on growth and cell shape of P. farcimen

P. farcimen grew at all tested temperatures in the range of 1–10 °C (Fig. 2A–D). The algae sustained growth at 1 and 3 °C for the duration of the experiments, while a reduction in growth was observed at day 17 at 6 °C and day 9 at 10 °C. Growth stopped after day 22 and 15 at temperatures of 6 and 10 °C, respectively. Treatments at 6 °C reached maximum cells densities of 4.3×10^4 cells ml^{-1} (Fig. 2C), while the concentration of *P. farcimen* cells at 1 and 10 °C, never exceeded 2×10^4 cells ml^{-1} (Fig. 2D).

Exponential growth rates were calculated from day 5 to 20 and 24 for the 1 and 3 °C treatments, respectively, day 0 to 8 for the 6 °C treatment and day 0 to 9 for the 10 °C treatment. Lowest growth rates (±SD) were observed at 1 (0.09 ± 0.006) and 3 °C (0.16 ± 0.003), while growth rates did not differ significantly between 6 and 10 °C (0.25 ± 0.01 and 0.26 ± 0.01), respectively (Tukey Test, $p = 0.432$) (Fig. 3).

Round (Fig. 8A–C) and elongated (Fig. 8D–F) cell shapes could easily be distinguished. At all temperatures 4–8% of *P. farcimen* cells had a "round" shape in the start of the experiments. The

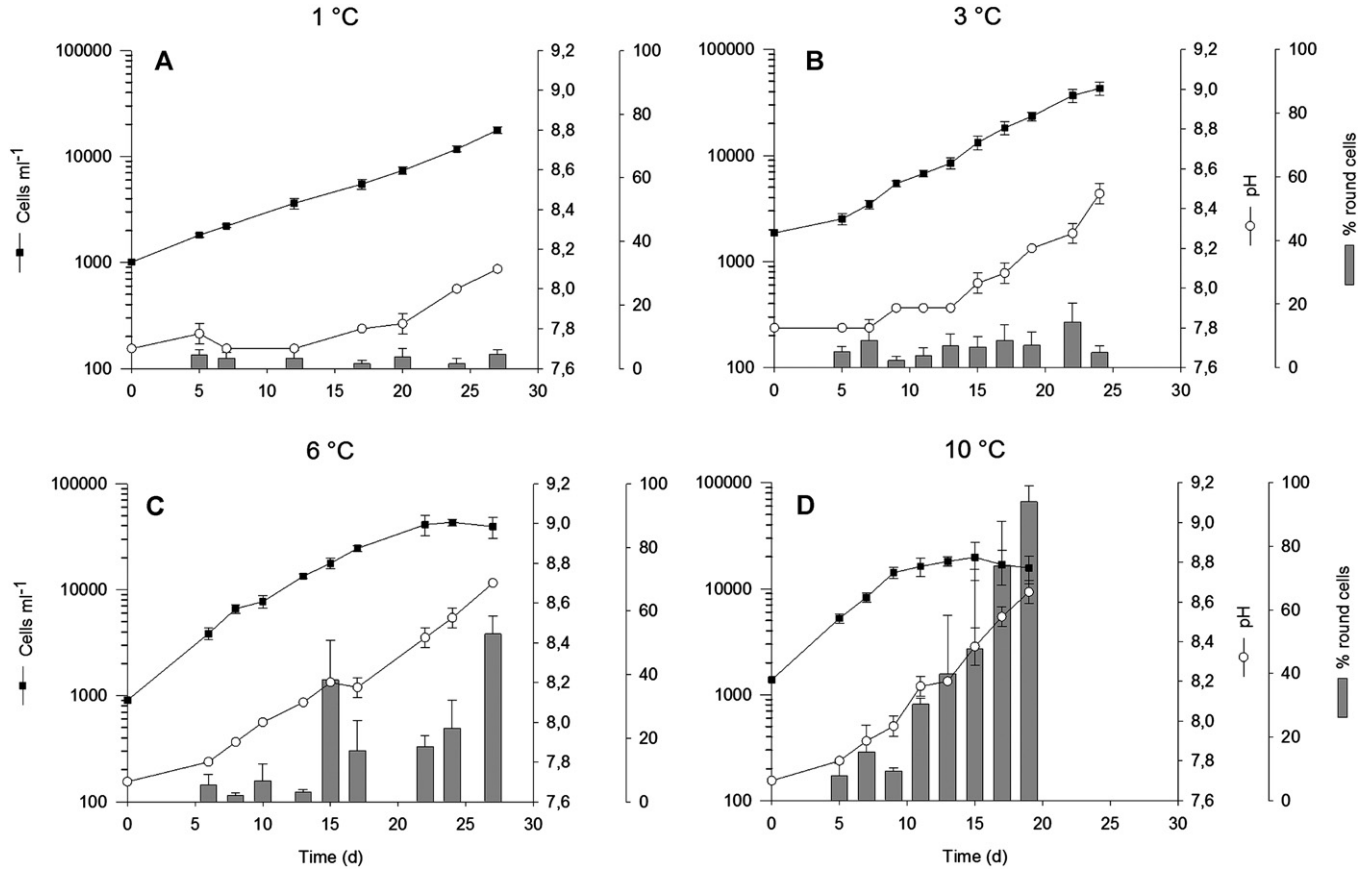

Fig. 2. Average cell concentration (cells ml^{-1}), pH and fraction of round cells (%) of *P. farcimen* at 4 different temperatures (means ± SD), $n = 3$. (A) 1 °C, (B) 3 °C, (C) 6 °C, (D) 10 °C.

fraction of round cells increased with time at 6 and 10 °C. At the end of the experiments the mean (±SD) fraction of round cells was highest at 10 °C (94 ± 4.97%) while the mean (±SD) fraction of round cells only reached 52.8 ± 5.56%, 4.7 ± 2.06% and 4.5 ± 1.29% at the 6, 3 and 1 °C treatments, respectively (Fig. 2A–C).

The pH increased over time during all temperature treatments (Fig. 2A–D). All four temperature experiments were initiated at a pH of 7.7–7.8. In the 6 and 10 °C treatments pH reached 8.7 at the end of the experimental period after 27 and 19 days, respectively.

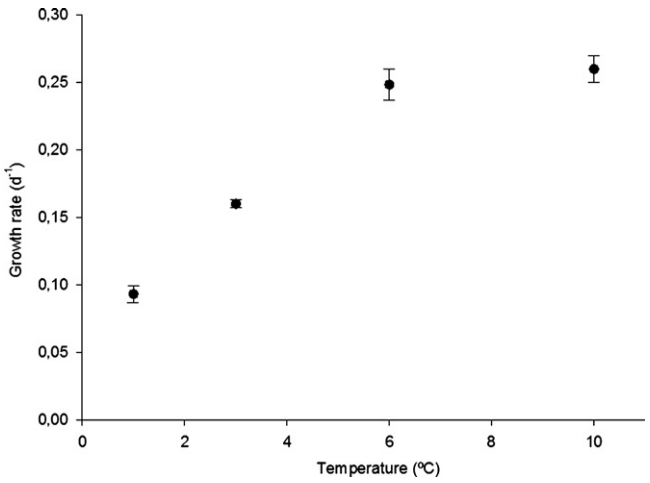

Fig. 3. Exponential growth rates (d^{-1}) of *P. farcimen* as a function of increasing temperature (means ± SD), $n = 3$.

In the 3 °C treatment, pH did not increase above pH 8.5, while a pH of 8.1 were reached in the end of the 1 °C treatment.

At 6 and 10 °C, a tendency between high pH, lower growth rate and round shape of *P. farcimen* was seen (Fig. 4A and B). At 6 °C the highest growth rates were observed at pH 7.8–7.9, above this pH level, growth rates slowly decreased until the growth of *P. farcimen* ceased above pH 8.5. At pH 7.8–8.1, when growth rates were high <6% of the cells had the round shape, above this pH level the fraction of round cells slowly increased as growth declined, until about 50% of the cells had a round shape above pH 8.5 (Fig. 4A). At 10 °C, the highest growth rates were observed at pH levels from 7.8–8.0. A further increase of 0.2 pH units resulted in a decline of 40% in the growth rate of *P. farcimen*, while growth apparently ceased at pH 8.4 (Fig. 4B), even though pH continued to increase. Similar to experiments at 6 °C, the lowest fraction of round *P. farcimen* cells at 10 °C, were observed at pH levels were growth rates were highest. As the growth rates decreased above pH 8.0, the fraction of round cells increased, at pH 8.4 about 50% of the cells were round, while about 95% of the counted cells had a round shape at pH 8.7 (Fig. 4B).

3.4. Effects of irradiance on growth and cell shape of P. farcimen

At 3 °C growth of *P. farcimen* was observed at irradiances ranging from 2 to 90 $\mu E\ m^{-2}\ s^{-1}$, while the algae were not able to sustain growth at 1 $\mu E\ m^{-2}\ s^{-1}$ (Fig. 5A–H). At irradiances of 30 and 50 $\mu E\ m^{-2}\ s^{-1}$, the experiments were continued until the growth of the cultures had stopped. A decline in the growth of *P. farcimen* cultures was observed after days 8 and 15 at irradiances of 50 and 30 $\mu E\ m^{-2}\ s^{-1}$, respectively, and growth stopped around

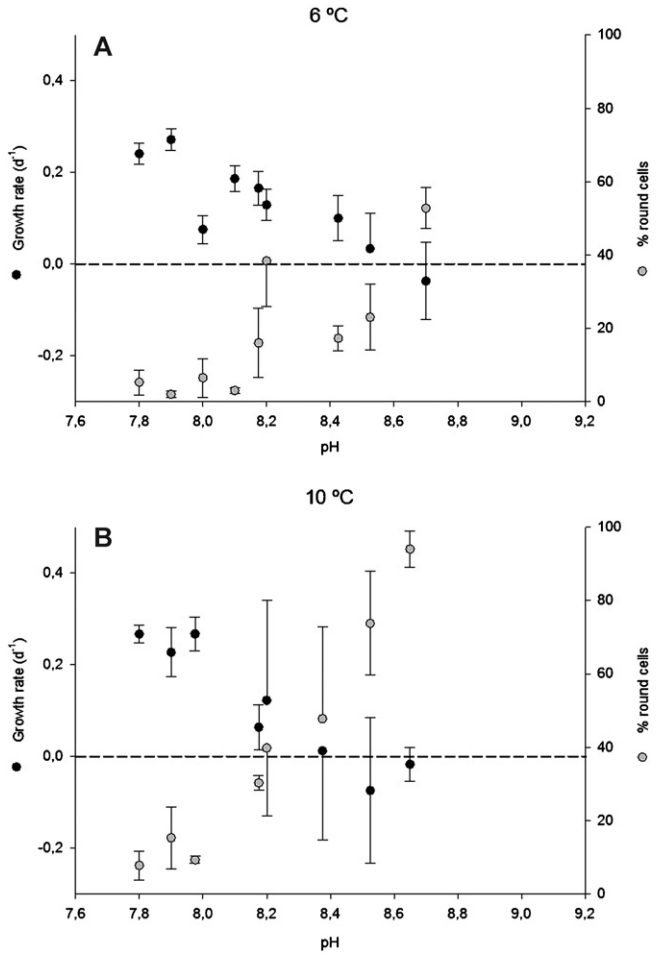

Fig. 4. Mean growth rates (d^{-1}) and fraction of round cells (%) (±SD, $n = 3$) of *P. farcimen* as a function of pH at temperatures of (A) 6 and (B) 10 °C.

day 25 in both treatments reaching cell densities of *P. farcimen* around 1.1×10^5 cells ml^{-1}. At an irradiance of 90 μE m^{-2} s^{-1}, a reduction in growth was observed after day 10.

Exponential growth rates were calculated from day 0 to 15 at irradiances of 3, 10 and 20 μE m^{-2} s^{-1} and day 0 to 24 at 1 and 2 μE m^{-2} s^{-1} (Fig. 6). All growth rates are obtained at a temperature of 3 °C.

Exponential growth rates at the irradiances 30, 50 and 90 μE m^{-2} s^{-1} were only observed for the first 13, 8 and 10 days, respectively. *P. farcimen* grew with a low rate of (mean ± SD) 0.002 ± 0.005 d^{-1} at an irradiance of 2 μE m^{-2} s^{-1}, while a negative growth rate of -0.007 ± 0.003 d^{-1} were observed at 1 μE m^{-2} s^{-1}. Growth rates increased with irradiances from 3 to 90 μE m^{-2} s^{-1}, where the highest growth rate was reached (0.34 $d^{-1} \pm 0.01$). The relationship between growth rate and irradiance were fitted by the Michaelis–Menten equation ($r^2 = 0.94$). The compensation irradiance was subsequently estimated to be around 1.3 μE m^{-2} s^{-1}.

At irradiances of 3, 10 and 20 μE m^{-2} s^{-1}, a maximum 4% of the cells had the round shape during the duration of the experiments (Fig. 5A–H). The fraction of round cells were initially higher, making up for 7 ± 1.73 and 17 ± 2.65% of the populations at irradiances of 30 and 50 μE m^{-2} s^{-1}, respectively. Moreover, a considerably increase in round cells was observed at the end of the experiments, making up for 36 ± 4.58 and 56 ± 2.89% at irradiances of 30 and 50 μE m^{-2} s^{-1}, respectively. An initial high fraction of 93 ± 1.52 and 76 ± 11.36% round cells were observed at 1 and 2 μE m^{-2} s^{-1}, respectively. At 1 μE m^{-2} s^{-1} the high fraction of round cells were maintained during the length of the experiment, while a decrease over time were observed at 2 μE m^{-2} s^{-1}.

An increase in pH was observed at irradiance levels above 3 μE m^{-2} s^{-1} during the course of the experiments (Fig. 5A–H). At irradiances of 1 and 2 μE m^{-2} s^{-1} a slight decrease of 0.2 pH units were observed at the end of the experimental period, while a pH of 7.7–7.8 was maintained at 3 μE m^{-2} s^{-1}. At 10 and 20 μE m^{-2} s^{-1}, only minor pH increases were observed from 7.8 to 7.9 and 8.1, respectively. At the higher irradiances, 30 and 50 μE m^{-2} s^{-1}, pH had increased to 8.9 and 9.0 at the end of the experiments, respectively. At 90 μE m^{-2} s^{-1} pH increased from 7.8 to around 8.5.

A plot of the fraction of round cells and growth rate as a function of pH in the culture medium, indicate that pH has an effect on both cell shape and growth rate (Fig. 7A and B). At irradiances of 30 and 50 μE m^{-2} s^{-1}, both growth rate and the fraction of round cells were unaffected by pH, until pH had increased to 8.4 and 8.2, respectively, although the fraction of round cells were higher at 50 μE m^{-2} s^{-1}. At both irradiances the growth stopped when pH reached ~8.9–9.0 and the fraction of round cells had increased to ~50–58%.

3.5. Multinucleate cells

Observations of DAPI-stained *P. farcimen* cells showed that the large cells observed after some time at 10 °C contained more than one nucleus (Fig. 8I–L). These multinucleate cells were not found in exponential growing cultures of *P. farcimen* at a temperature of 10 °C. With a starting concentration of 1000, 4000 and 8000 cells ml^{-1} the multinucleate cells started developing after ca. 10 days (Fig. 9). The length of the exponential growth phase of single cells of *P. farcimen* in these experiments was 6, 4 and 2 days, respectively. Growth of single cells stopped after day 8 in the experiments initiated at 1000 cells ml^{-1} and after day 6 in the experiments initiated with 4000 and 8000 cells ml^{-1}. Thereafter, a decline in the cell concentration of single cells was observed in all three treatments for the remaining experimental period. In all three experiments, densities between 4×10^4 and 5×10^4 cells ml^{-1} were reached before the decline in cell concentrations.

The first multinucleate cells were observed after day 6 in the experiments initiated with 4000 and 8000 cells ml^{-1}, coinciding with an apparent stop in growth, and after day 8 in the incubation initiated with 1000 cells ml^{-1}. The following 7–9 days of the incubations the fraction of multinucleate cells increased to 43–58% in all three experiments.

At all three initial concentrations the pH increased during the first 10 days of the experimental period. All experiments were initiated at pH 7.6. After 6 days, the cultures reached pH 8.3–8.6, coinciding with a reduction in the growth of the single-celled stage of *P. farcimen* and an increase in the fraction of multinucleate stages. Levels of pH never exceed 8.7–8.8 in any of these experiments and stayed quite stable for the duration of the experiments.

4. Discussion

4.1. Allelopathy

No effects of *P. farcimen* were observed on the growth of the three target species tested in mixed growth experiments. The target species were not randomly picked but species, which co-occur with *P. farcimen* in Danish waters in winter or early spring. Previous studies have shown that allelopathic effects may first be seen with high ratios between the harmful and target algae (Tillmann et al., 2009; Tillmann and Hansen, 2009). In the present

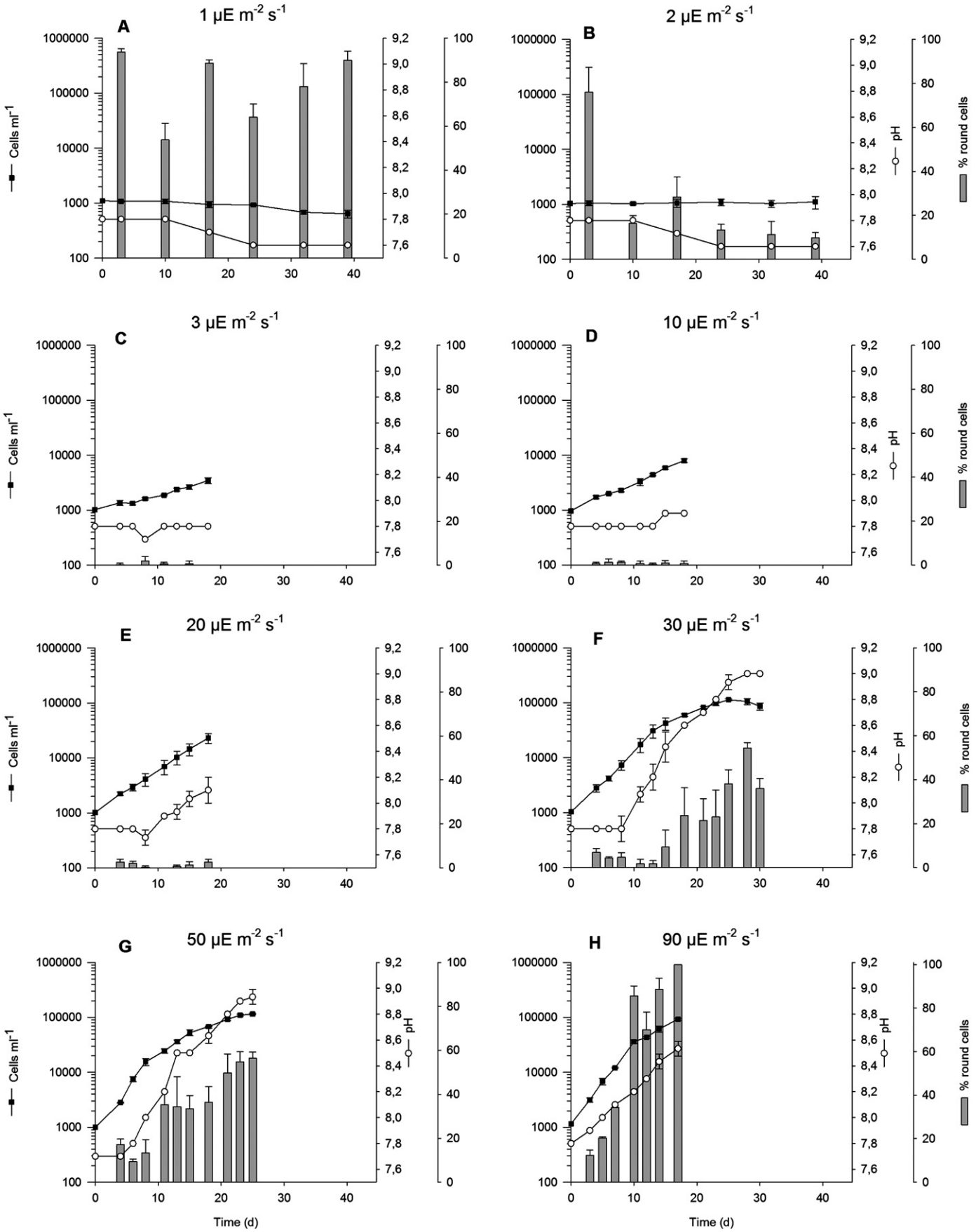

Fig. 5. Average cell concentration (cells ml^{-1}), pH and fraction of round cells (%) of *P. farcimen* at 8 different light levels (means ± SD), $n = 3$. (A) 1 µE m^{-2} s^{-1}, (B) 2 µE m^{-2} s^{-1}, (C) 3 µE m^{-2} s^{-1}, (D) 10 µE m^{-2} s^{-1}, (E) 20 µE m^{-2} s^{-1}, (F) 30 µE m^{-2} s^{-1}, (G) 50 µE m^{-2} s^{-1}, (H) 90 µE m^{-2} s^{-1}.

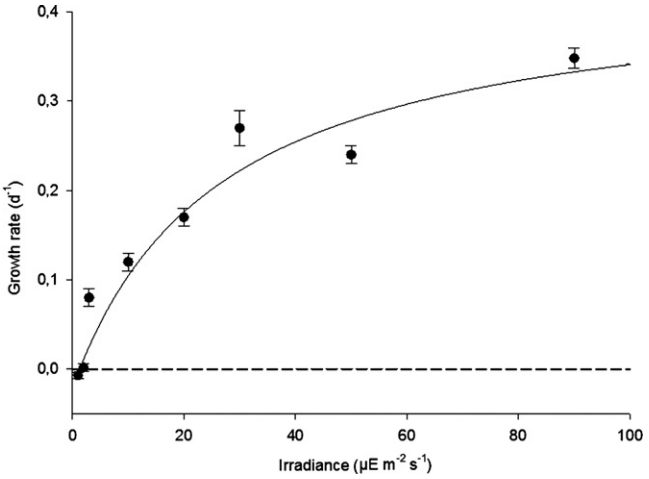

Fig. 6. Exponential growth rates (d^{-1}) of *P. farcimen* as a function of increasing irradiance (means ± SD). Temperature 3 °C, $n = 3$.

study, high cell concentrations of *P. farcimen* did not lead to inhibition of the other algae in the mixed culture experiments. Some algal species may be quite resistant or even completely unaffected by some allelochemicals (Fistarol et al., 2003; Schmidt and Hansen, 2001; Tillmann et al., 2007). However, none of the used target species are known to possess this ability.

All experiments were carried out at excess nutrient concentrations. This could possibly have affected the outcome of the experiments, as nutrient limitations may lead to increased production of allelochemicals (Graneli and Johansson, 2003). However, many species with allelopathic capabilities investigated so far are indeed allelopathic even in nutrient rich medium. It is also well known that allelopathic potency may vary considerably among different strains of the same algae (Tillmann et al., 2009; Tillmann and Hansen, 2009). In the present experiment we screened 6 strains of *P. farcimen*, none of these showed allelopathic effects on the target species. Skjelbred et al. (2011) tested the effect of extracts from 5 different *P. farcimen* strains on the metabolism of Chinook salmon embryo cells. This experiment showed that only some strains had an effect, while others did not. Thus, it cannot be ruled out that some strains of *P. farcimen* can produce allelochemicals against other microalgae.

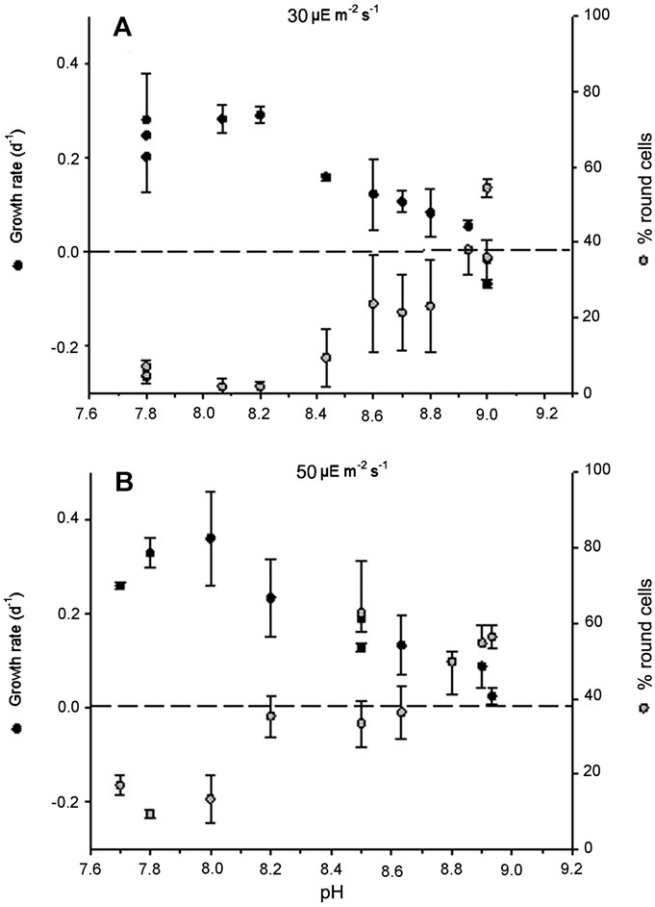

Fig. 7. Mean growth rates (d^{-1}) and fraction of round cells (%) (±SD, $n = 3$) of *P. farcimen* as a function of pH at irradiances of (A) 30 and (B) 50 µE m^{-2} s^{-1}.

4.2. Effects of temperature on growth rate of P. farcimen

Blooms of *Pseudochattonella* sp. in Scandinavian waters takes place during February and March, where the water temperatures ranges from 1 to 5 °C (Andersen, 2008; Naustvoll et al., 2002; Riisberg and Edvardsen, 2008) and April to May where the water temperature ranges from 5 to 10 °C (Andersen, 2008). However, the Danish blooms occurring in 2009 and 2011 took place in February and March when the water temperature was around 1–2 °C and at a salinity around 17 psu. We studied growth of *P. farcimen* at 1, 3, 6 and 10 °C. At 3 °C, *P. farcimen* grew with a rate as high as 0.16 d^{-1}, while a positive growth rate of 0.09 d^{-1} was observed at 1 °C. Temperature experiments were carried out at an irradiance of 20 µE m^{-2} s^{-1}, which is below the saturation irradiance of *P. farcimen* (Fig. 6). Thus, the growth rates obtained at the four different temperatures are not maximum rates.

The growth rate of *P. farcimen* increased when the temperature was elevated from 1 to 6 °C, but a further increase was not seen when the temperature was elevated from 6 to 10 °C. Thus, it seems that 10 °C is close to the upper temperature limit of *P. farcimen*. Edvardsen et al. (2007) noted that Norwegian isolates of *P. farcimen* grew optimal at temperatures between 5 and 10 °C, and did not tolerate temperatures >15 °C. Bloom events of *P. farcimen* in Scandinavian waters have therefore occurred at temperatures well below its maximum growth rate. The ecological advantage of *P. farcimen* might be that a low water temperature impairs the development of potential zooplankton grazers and thus allows for very high concentration of *P. farcimen*.

Of the target species the dinoflagellate *H. triquetra* grew poorly at 4 °C, while the highest growth rates were observed in the two diatoms *C. diadema* and *C. decipiens*. This coincides with the literature on phytoplankton growth and thus confirms that diatom growth rates are generally higher than the values for flagellates (e.g. Banse, 1982; Egge and Aksnes, 1992; Furnas, 1990; Smayda, 1997). This verifies one of the reasons why diatoms usually dominate the phytoplankton community in the early spring in temperate coastal waters. The exponential growth rate of *P. farcimen* exceeds that of the diatom *C. decipiens*. Combining this with the ability of *P. farcimen* to swim and therefore the ability to access nutrients and optimal light throughout the water column by vertical migration; *P. farcimen* is, through its growth rate in cold waters, a potential competitor to coexisting diatoms in cold waters. This is especially so in periods with low turbulence.

The obtained growth rates of *P. farcimen* are also comparable to other cold water blooming phytoplankton. A spring bloom in Gullmar fjord on the west coast of Sweden, recorded diatoms as the dominant group, with growth rates ranging from 0.26 to 0.46 d^{-1} at temperatures of 1–3 °C (Tiselius and Kuylenstierna, 1996). These findings are consistent as growth rates of *P. farcimen* reached similar values at 4 °C in the present experiment. Information on flagellate growth at low temperatures (<5 °C) at temperate areas is scarce, but as flagellates usually bloom in water temperatures

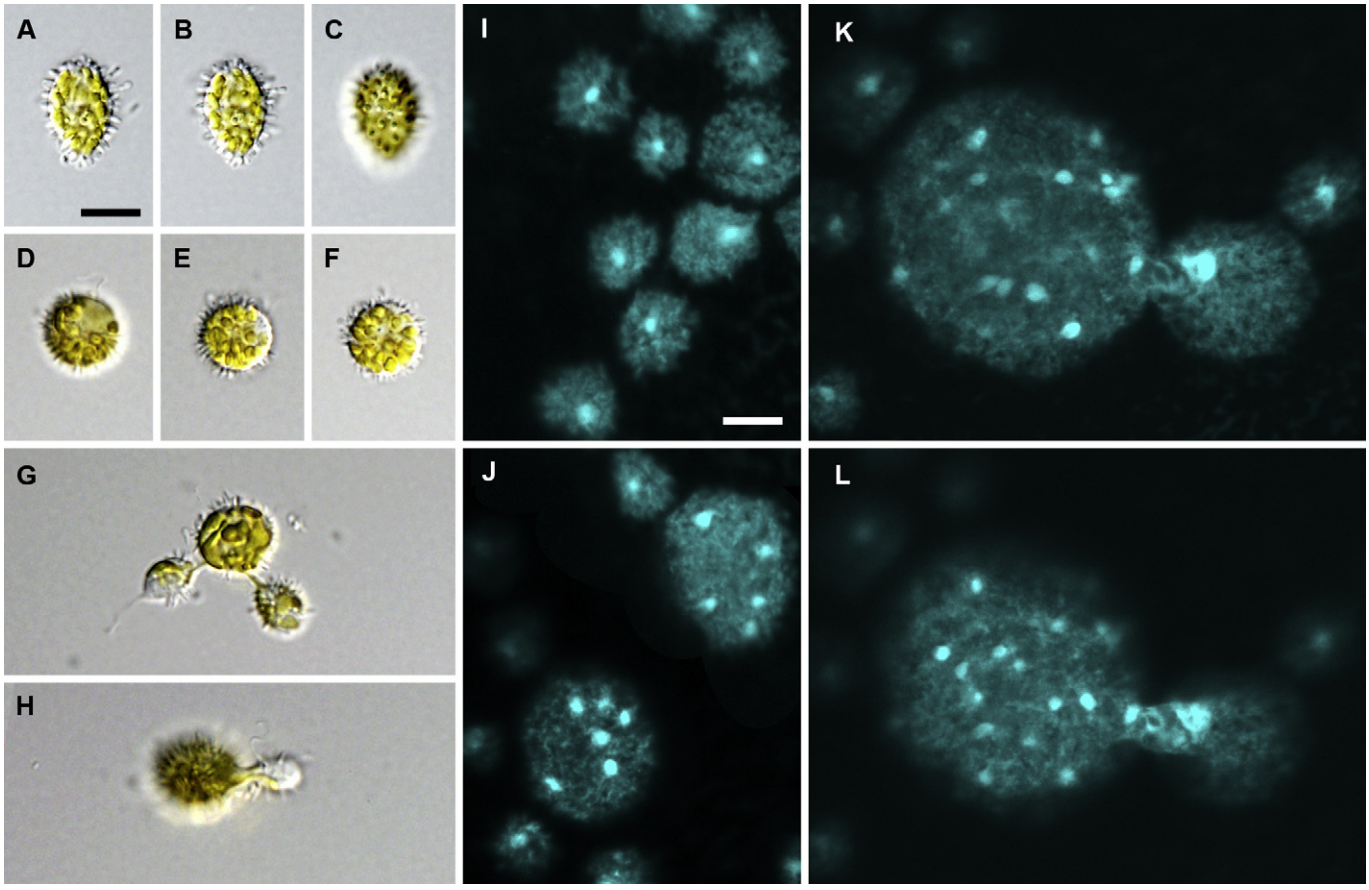

Fig. 8. Light micrographs (DIC) of live cells of *Pseudochattonella farcimen* (A–H). Elongate stage, same cell in three focal levels (A–C). Round stage, same cell in three focal levels (D–F). Cell fusion stage (G and H). Epifluorescence of DAPI-stained cells of *Pseudochattonella farcimen* (I–L). Vegetative cells each with one nucleus (I). Multinucleate stages with 5 (upper) and 6 (lower) nuclei, respectively (J). Two focal levels of a multinucleate stage. Notice the connection between the different sized stages. The large stage to the left possesses at least 17 nuclei (K and L). Scale bar = 10 μm (A–H, I–L).

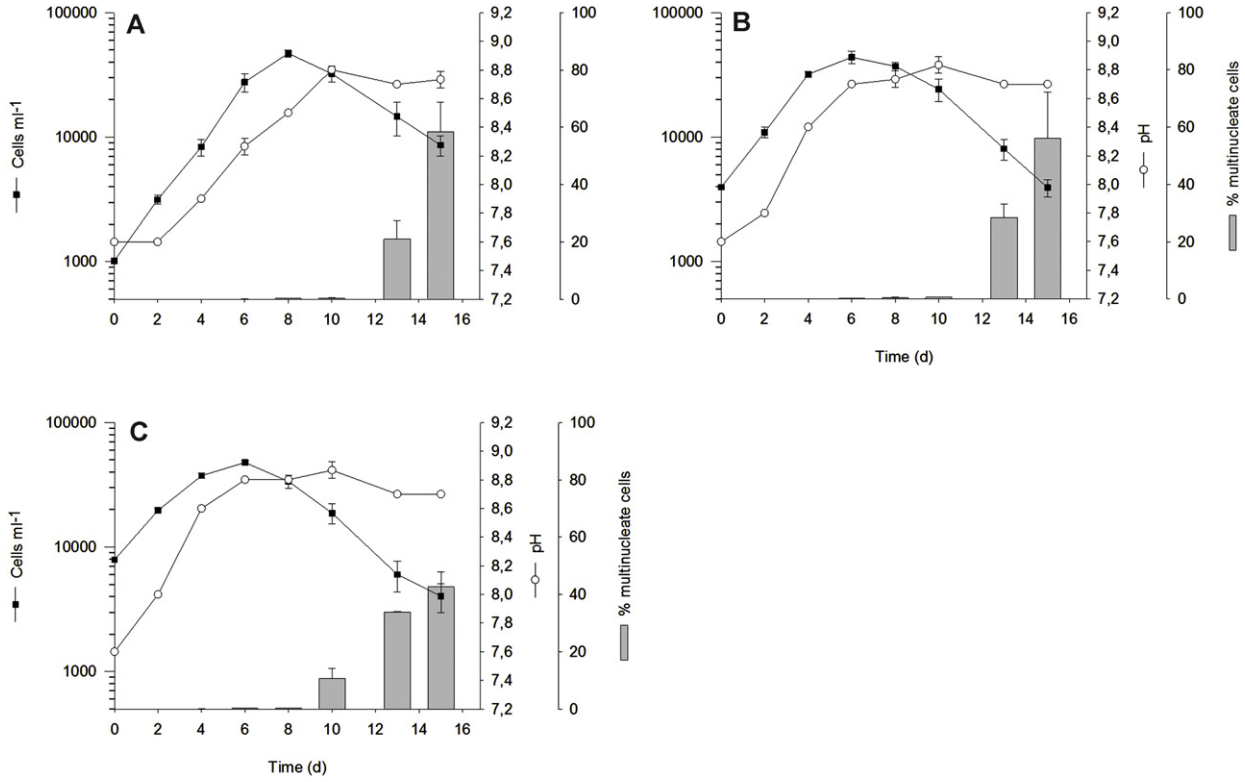

Fig. 9. Average cell concentration (cells ml^{-1}), pH and fraction of multinuclear cells (%) of *P. farcimen* at 3 different initial concentrations (means ± SD), $n = 3$. (A) 1000 cells ml^{-1}, (B) 4000 cells ml^{-1}, (C) 8000 cells ml^{-1}.

above 10 °C, they generally seem to have a higher temperature optimum than diatoms (Jensen, 2008; Yamaguchi et al., 1991, 2010). One exception is the colony forming prymnesiophyte, *Phaeocystis pouchetii*, which is one of the most frequently occurring species in spring blooms at the northern Norwegian coast and the Barents Sea (Degerlund and Eilertsen, 2010). This alga has a temperature tolerance of −2 to 14 °C, and growth rates of about 0.24–0.30 d^{-1} at temperatures between 1.5 and 4 °C (Jahnke, 1989), making it able to compete with co-existing diatoms. The chrysophyte *Dinobryon balticum* has also been observed to dominate plankton communities at low water temperatures (McKenzie et al., 1995), but this species support its photosynthesis with phagotrophy. Hence, it is not as dependent on light as *P. farcimen*. Mixotrophy has generally been observed in many harmful flagellates (Burkholder et al., 2008) and has also been observed in cold water during Antarctic blooms (Moorthi et al., 2009). It is unknown if *P. farcimen* can support its carbon uptake with phagotrophy, but so far it has not been observed. To our knowledge *P. farcimen* is one of the few free-living phototrophic flagellates able to grow at moderate rates and bloom at sea temperatures as low as 1–3 °C.

4.3. Effects of irradiance on growth rate of P. farcimen

Light in the late winter and early spring is limited, particularly caused by the low solar angle and short average day length. In this study we observed positive growth of *P. farcimen* at irradiances as low as 2 μE m^{-2} s^{-1}, and compensation irradiance were found to be around 1.3 μE m^{-2} s^{-1}. Few studies of phytoplankton growth have been conducted at the low irradiance levels of around 1–20 μE m^{-2} s^{-1} (Dubinsky and Schofield, 2010). Thus, values for the compensation irradiance in laboratory populations are few and highly variable depending on species (Marra, 2004). Light compensation points have been estimated in vivo for some common cold water to temperate diatoms such as *Chaetoceros socialis*, *Skeletonema costatum*, *Thalassiosira nordenskioeldii*, *Thalassiosira gravida*, *Thalassiosira hyaline* and *Thalassiosira antarctica* var. *borealis* (Eilertsen and Degerlund, 2010). These species are all associated with spring blooms in Northern Europe and Denmark (Degerlund and Eilertsen, 2010; Fenchel, 1992). All species have compensation irradiances ranging from 0.25 to 1.56 μE m^{-2} s^{-1} (Eilertsen and Degerlund, 2010; Langdon, 1987). As *P. farcimen* has a light compensation point within these values, it must be regarded as a successful competitor to co-occurring phytoplankton at low light levels.

Considering the low compensation irradiance, this study shows that *P. farcimen* requires little light for growth at 4 °C. The cold water blooming *Phaeocystis antarctica*'s dominance in the south central Ross Sea has been attributed to superior photosynthetic abilities at low irradiance levels (Arrigo et al., 1999). This also seems be a possible explanation of the success of *P. farcimen* in the late winter and early spring.

4.4. Effect of pH on growth of P. farcimen

Experiments with different light and temperature regimes showed that the growth rate of *P. farcimen* declined over time in batch cultures as the cultures grew dense. As nutrients were in excess pH was the growth limiting factor (Hansen, 2002; Hansen et al., 2007). Experiments done with f/10-media verified this, as growth stopped at the same pH level even though the amount of nutrients was doubled (data not shown). At 4 °C *P. farcimen* did not grow at a pH exceeding 8.9–9.0. The sensitivity to high pH is known to be species dependent. Hansen (2002) compared the upper pH growth limits for 35 phytoplankton species, which ranged from pH 8.4 to 10.2. At a pH above 9.2 only half of the investigated species could sustain growth. This suggests that *P. farcimen* is moderately tolerant to high pH.

In this study we observed growth of *P. farcimen* at pH ranging from 7.7 to 8.9–9.0. It appears to grow optimally below 8.2–8.4, as this is where exponential growth was observed. The pH range of *P. farcimen* is similar to that of another bloom forming dictyochophyte, *D. speculum*, which has an upper pH limit for exponential growth of 8.3 and stops growing at 8.8 (Schmidt and Hansen, 2001). Information of pH during *P. farcimen* blooms is almost completely lacking, but during a bloom in the gulf of Gdańsk in 2001, pH was recorded to range from 8.01 to 8.27 (Lotocka, 2009). This indicates that *P. farcimen* was not pH limited in its growth during that particular bloom.

4.5. Morphology of P. farcimen cells

Earlier observations report variation in the overall morphology of *P. farcimen* (Edvardsen et al., 2007; Lotocka, 2009). When the light experiments (only above 3 μE m^{-2} s^{-1}) and temperature experiments were initiated, about 90% of all *P. farcimen* cells were elongated (Fig. 8A–C), but over time *P. farcimen* cells started to turn round (Fig. 8D–F) and in some cases up to 100% of the cells became round. This morphological change from elongated to round cells appears to be a response to the growth condition of *P. farcimen*. As pH increases above the optimal range, the cell shape turned round as a reaction to growth under sub-optimal conditions. This could also explain why 80–90% of the cells were round at 1 and 2 μE m^{-2} s^{-1} initially. This low irradiance possibly served as a sub optimal factor.

Morphological responses are known to exist when algae are exposed to stressful situations, e.g. being confronted with a strong grazing pressure from zooplankton. In adapting to deter predators, phytoplankton species have increased in size, formed large chains and colonies, or grown spines (e.g. Tillmann, 2004). Many dinoflagellate species have been observed to change shape as an effect of shifting environmental conditions, generally to modify swimming and sinking behavior (Smayda, 2010). It is unknown if the change in shape of *P. farcimen* cells have any functional significance. Edvardsen et al. (2007) reported that elongated *P. farcimen* cells possess many chloroplasts (30–35), while round cells only contain few. This could imply that instead of producing costly chloroplasts, all resources are diverted to maintain the cell under stressful conditions. As the shape of *P. farcimen* is correlated to its growth conditions, we propose that morphological observations may be used as an indicator of the growth phase of a *P. farcimen* bloom.

Large multinucleate cells develop when the growth of the single cells had stopped in the 6 and 10 °C experiments (Fig. 8J and K). The formation of these multinucleate stages developed quickly and turned up when the pH in the culture was 8.3–8.6. At the same time the amount of single cells (Fig. 8I) decreased. Thus, the upper pH growth limit of *P. farcimen* at these temperatures appeared to be lower than at treatments at 4 °C. But as *P. farcimen* reacted to these temperatures by changing from single to multinuclear cells, the pH at which growth of single cells stopped was not an upper pH growth limit. As the single cells rapidly declined in cell number, multinucleate forms showed up. This could indicate that single cells actually merge to form these multinucleate stages. Connecting cells supporting the fusion theory have been seen in our cultures (Fig. 8G–H and K–L). The formation of multinucleate cells in another silicoflagellate, *Dictyocha fibula*, has earlier been described as nuclear division without cytokinesis (Van Valkenburg and Norris, 1970). These authors do not account for the number of single nucleated cells in their culture or other factors that could indicate that it in fact, is nuclear division and not merging of cells that creates the multinucleate cells. Independent of the initial

concentration in our experiments, growth stopped when cultures reached about the same density of single cells (4.3–4.7 $\times$ 10^4 cells ml^{-1}). So, it might be that *P. farcimen* reacts to the stressful condition of high temperature and growth limitation (in this case high pH) at a certain cell density, and form multinuclear stages.

A similar observation has been made in another dictyochophyte, *D. speculum* (Henriksen et al., 1993). They observed large multinucleate cells when the bloom forming *D. speculum* entered the stationary growth phase. As in our study the large cells were always found on the bottom of the culture bottles. How to interpret this? Why did such growth forms suddenly turn up, what is the adaptive significance? Henriksen et al. (1993) argued that the large multinucleate cells would be carried below the euphotic zone, as sinking rates of spherical cells increase with cell size (Walsby and Reynolds, 1980). These multinucleate stages could most likely function as a resting stage of *P. farcimen* in unfavorable periods, i.e. when the water temperatures increase above 6 °C. This hypothesis can also explain why no multinuclear cells were observed at 4 °C even though cell densities got high and growth definitely stopped. The theory of the multinucleate cells being a resting stage are further plausible since we have observed that *P. farcimen* cultures can be established by lowering the temperature of a single multinucleate cell from 10 to 3 °C. This reoccurring of single nucleated cells from a multinucleated cell has not been seen for other silicoflagellates (Henriksen et al., 1993; Van Valkenburg and Norris, 1970). But these authors did not try to lower the temperature of a multinucleate cell and thereby imitating the possible natural life cycle. Additional studies on this subject are strongly needed.

In April 2001 a bloom of *P. farcimen* was observed for the first time in the southern part of the Gulf of Gdańsk (Lotocka, 2009). An increase of *P. farcimen* cells was observed in 7 days, after which the number of cells rapidly declined over the next 7 days. The temperature in the period ranged from 7.2 to 9.8 °C. A possible explanation for the decline in *P. farcimen* cells could be the change from single cells to multinuclear, induced by the unfavorable rising temperatures.

5. Conclusion

P. farcimen is well adapted to the low-irradiance low-temperature conditions observed in Danish waters in late winter and early spring. Temperature seems to be the more important of the two physical factors tested. Although *P. farcimen* is capable to reach moderate growth rates at low temperatures it appears to grow optimal at temperatures higher than the temperatures it is exposed to when blooming (2–5 °C). At higher temperatures, *P. farcimen* reacts over time by forming large multinucleate cells when the culture becomes dense. Probably this stage functions as a resting stage during summer. Our isolates of *P. farcimen* lack an allopathic effect on other phytoplankton. However, *P. farcimen* is able to grow equally as fast as diatoms at low temperatures. Combining this with the ability to swim and thereby seek optimal light and nutrients makes it unnecessary for *P. farcimen* to produce a costly allelopathic compound to form dense blooms in cold waters.

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