Microb Ecol (2017) 73:259–270
DOI 10.1007/s00248-016-0856-z

MICROBIOLOGY OF AQUATIC SYSTEMS

Interspecific Competition Study Between *Pseudochattonella farcimen* and *P. verruculosa* (Dictyochophyceae)—Two Ichthyotoxic Species that Co-occur in Scandinavian Waters

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Received: 9 June 2016 / Accepted: 5 September 2016 / Published online: 19 September 2016

Abstract The genus *Pseudochattonella* has become a frequent component of late winter-early spring phytoplankton community in Scandinavian waters, causing extensive fish kills and substantial economic losses. One of currently two recognised species, *P. farcimen*, is often abundant prior to the diatom spring bloom. Recent field studies have revealed that *P. farcimen* and *P. verruculosa* have a period of overlap in their temperature ranges and thus their seasonal occurrences. Using laboratory cultures, we investigated the seasonal succession and growth of *P. farcimen* and *P. verruculosa* in both mono- and mixed-culture using the recently developed *Pseudochattonella* 'qPCR subtraction method', which for the first time allowed the simultaneous enumeration of these morphologically indistinguishable species in mixed assemblages. We examined how these species interacted over four different temperatures (5, 8, 11 and 15 °C). The observed growth rates and cell yields varied with temperature revealing their preferred temperature optima. *P. farcimen* was able to achieve positive net growth over all temperatures, while *P. verruculosa* failed to grow below 11 °C. Growth responses were statistically different between mono- and mixed-cultures with the outcome of these interactions being temperature-dependent. Nutrients (nitrate and phosphate) and pH levels were also measured throughout the growth experiments to better understand how these factors influenced growth of both species. *P. verruculosa* was shown to be less sensitive to high pH as growth ceased at pH 9.1, whereas *P. farcimen* stopped growing at pH 8.4. Understanding the influence of abiotic factors (e.g. temperature, pH and competition) on growth rates allows for a better understanding and prediction of phytoplankton community dynamics.

Keywords Interspecific competition · Fish-killing algae · *Pseudochattonella farcimen* · *Pseudochattonella verruculosa* · pH tolerance · Temperature tolerance

Introduction

Since 1998, the then called *Chattonella* aff. *verruculosa* [1] and the newly discovered closely related *Verrucophora farcimen* [2], now referred to as *Pseudochattonella verruculosa* and *P. farcimen*, respectively [3, 4], have been observed on an almost annual basis in Scandinavian waters [5]. In some years, blooms have caused extensive fish mortality events and substantial economic losses for the aquaculture industry [2, 6].

Pseudochattonella farcimen commonly forms blooms in very cold waters (2–5 °C) in the Kattegat and Skagerrak area between January and March [2, 5, 7]. The capability to bloom during colder conditions indicates a realised niche at lower temperatures. This may give a competitive advantage over other species e.g. utilisation of nutrients prior to the occurrence of competing species that have higher and additional nutrient requirements such as diatoms, as well as reduced grazing pressure from herbivores by blooming before the emergence of the zooplankton species [8]. *Pseudochattonella verruculosa* is considered more of a warm water species compared to *P. farcimen*. The latter species typically forms blooms between April and May when water temperatures can reach a maximum of 16 °C [6, 23].

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Communities of marine phytoplankton are shaped by a suite of abiotic and biotic interactions such as temperature, salinity and pH tolerance levels, nutritional modes, life histories and grazing pressure. Annual succession patterns are often well established, where changes in species composition are accompanied by significant seasonal oscillations in the physicochemical environment [9]. Temperature appears to be one of the main factors influencing phytoplankton succession patterns both through its direct effects on physiological factors such as growth rate as well as the indirect effect on water column stability [10, 11]. Phytoplankton communities in temperate waters often experience a 'void' in winter [12, 13], followed by a diatom bloom in spring when conditions turn favourable and a dinoflagellate and diatom bloom in late summer early autumn. Understanding phytoplankton responses to abiotic and biotic factors allows us to better predict succession, turnover, productivity and community dynamics enabling greater forecasting abilities.

Environmental conditions in late winter-early spring are characterised by low light levels but plentiful nutrients. Species adapted to low light levels commonly have low compensation and saturation points combined with high growth rates [14, 15]. Such conditions favour the diatoms, which typically have low compensation points of between 0.1 and 1.8 μmol photons m^{-2} s^{-1} and saturation points up to 30 μmol photons m^{-2} s^{-1} [16]. Compensation points for *P. farcimen* have been shown to range between 4.2 and 14.9 μmol photons m^{-2} s^{-1} and saturation irradiances between 18.3 and 51 μmol photons E m^{-2} s^{-1} in laboratory-based experiments [8].

To be able to bloom under such conditions, *P. farcimen* must be able to successfully compete against the diatoms despite having low growth rates [5]. The actual mechanism allowing *P. farcimen* to actively compete against other phytoplankton species is yet unknown. One way to overcome the ecophysiological differences is to release secondary metabolites that negatively affect the growth of competing species. Many other bloom forming species have been shown to have allelopathic effects on other phytoplankton species including *Alexandrium* [17] and *Prymnesium* [18]. It is well known that allelopathic activity is not usually connected to 'normal' toxin synthesis [19] and *Pseudochattonella* is thought to lose its harmful impact very quickly in culture with ichthyotoxic effects only being observed when they possess mucocysts [20].

Until recently, it was suspected that due to the distinct autoecological differences between *P. verruculosa* and *P. farcimen*, blooms that developed earlier in the season were formed by *P. farcimen*, whereas blooms that developed later were exclusively *P. verruculosa* [5, 8, 21]. However, it was not clear until the new qPCR assay was designed that a period of overlap of temperatures favourable to growth between the two species was confirmed [7]. Following a small bloom of *P. farcimen* in March–April 2015 in Danish waters off the east coast of Jutland, *P. verruculosa* was observed alongside *P. farcimen* in April when water temperatures reached 8.8 °C. This temperature is below the optima described previously for *P. verruculosa* (12–20 °C) [8, 22]. However, it has been known that some species including *Pseudochattonella* spp. have higher temperature optima in culture compared to when they are found in the environment [8].

The purpose of this study was to further examine the conditions that favour the growth and proliferation of the two *Pseudochattonella* species in isolation and in mixed-culture using the recently developed quantitative PCR assay that allows distinction between the two *Pseudochattonella* species [7]. Experiments were conducted with the species maintained at a range of environmentally relevant temperatures to simulate the succession of temperatures from late winter to early summer in Danish waters, which can range from 2 °C in February to 15 °C in late June [23]. This allowed us to assess the succession pattern of these two closely related species. Nutrient concentrations (NO_3^- and PO_4^{3-}) and pH levels were also measured during the course of the experiment to evaluate if these factors had influenced the growth of *Pseudochattonella* spp.

Materials and Methods

Cultured Material

The following microalgal cultures were used: *P. farcimen* was obtained from the Scandinavian Culture Collection of Algae and Protozoa, Copenhagen, Denmark (strain SCCAP K-1809), and *P. verruculosa* was kindly provided by Bente Edvardsen (strain JG8). Both strains were grown in L1 media with a salinity of 30. *P. verruculosa* was grown at 15 °C and *P. farcimen* at 5 °C, both under a light intensity of 100 μmol photons m^{-2} s^{-1}. Cultures were grown in 250-ml flasks in batch mode and acclimated to four different temperatures to represent winter to early summer conditions in Danish waters: 5, 8, 11 and 15 °C. Non-axenic cultures were grown both separately in mono-culture and together in mixed culture. The inoculum cell density for all experiments was ≈2000 cells ml^{-1} with the mixed culture experiments containing a 50:50 mix of both species. Cells were allowed to grow in batch mode until the post-stationary phase of the culture became established.

Sampling for Cell Counts and DNA Extraction

Every second day, pH of the culture media was measured using a Jenway 3510 pH meter before sub-samples were removed aseptically from each flask. A 10-ml sample was removed and filtered through a 0.2-μm filters (28 mm Minisart, CA filter) and the supernatant frozen for analysis of total NO_3^-

and total PO_4^{3-}. A further aliquot (1.2 ml) was removed and preserved with Lugol's iodine solution (1 % final concentration) and enumerated using a 1-ml Sedgewick-Rafter counting chamber. Growth rate (μ) was calculated as divisions per day. A further 1 ml was removed for DNA analysis. DNA samples were centrifuged (6000×*g*, 10 min) and the supernatant removed. Cell pellets were then washed in 1× PBS buffer, centrifuged (6000×*g*, 10 min) and the supernatant removed. Cell pellets were stored at −20 °C until extraction of total genomic DNA.

Growth Rates

The duration of the exponential growth phase was determined by calculating the maximum achievable R^2 when fitting straight lines to logged plots of cell density. At least five data points were used for the calculations of growth rate. The maximum growth rate was calculated for each replicate, and the mean of the growth rates of the three replicates was taken as the maximum growth rate.

The empirical growth rate (μ) (Eq. 1) was defined as the number of cell divisions per day and defined as:

$$\mu = \frac{In\left(\frac{N_2 \cdot t_2}{N_1 \cdot t_1}\right)}{t_2 - t_1} \quad (1)$$

where Nt_1 and Nt_2 are the abundances at time 1 (t_1) and time 2 (t_2), respectively. Statistical comparisons of growth rates and cell densities over the duration of the exponential phase were carried out using the Minitab 14 software package.

Standard Curves

Standard curves were obtained by amplifying the DNA extracted from the 10-fold serial dilutions (10^1–10^5 total cell number) of known cell concentrations in triplicate measurements. Reactions were carried out with a no-template control containing only double distilled water (ddH_2O).

Extraction of Total Genomic DNA

Samples for DNA extraction (i.e. 519 from the experiments and 30 standards) were thawed and cell pellets re-suspended in 10 μl of ddH_2O before being transferred to the powersoil-htp bead plate supplied with the Powersoil-htp 96-well soil DNA isolation kit (MO BIO) according to the manufacturer's recommendations. The 'centrifugation protocol' was followed with one exception; an aliquot (4 μl) of internal DNA extraction control (Primer Design, UK) was added prior to extraction. For all experiments, extractions were carried out using this kit. The storage tube was then given a further two to three washes using 10 μl of ddH_2O to ensure that all material had been transferred. *Pseudochattonella* cells are notoriously sticky (personal observation) and care should be taken to ensure that all cells are transferred. Loss in per cent was calculated from absolute qPCR values generated from the internal DNA control before and after extraction. Cell numbers used to generate calibration curves were reduced by the same percentage. Total elution volume of DNA was 100 μl and samples were stored at −20 °C until analysis.

Primers and Hydrolysis Probe for qPCR

The primers and hydrolysis probe were previously described in Eckford-Soper and Daugbjerg [7]. The qPCR amplicon (97 base pairs) was identical for strains of *P. farcimen* and *P. verruculosa*, respectively. Optimised conditions for qPCR were with 20-μl reactions with 4 μl of HOT $FIREPol_1$ Probe qPCR Mix Plus (no ROX) (1× final concentration) (Solis BioDyne), 2 μl of template, 0.5 μl of each appropriate primer, 1 μl of probe and 12 μl ddH_2O. The cycling parameters for all reactions were 15 min at 95 °C for activation of the polymerase, followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C.

Nutrient Analysis

After defrosting, the filtered samples were analysed for total nitrate (NO^{3-}) and dissolved reactive phosphate (PO_4^{3}). Phosphate was measured as liberated orthophosphate with the standard molybdenum blue technique [24, 25]. Nitrate was measured using a nitrate-nitrogen test kit (LaMotte, MD, USA), which uses the cadmium reduction method for the analysis or nitrate nitrogen. The sample absorbance was analysed using a Triology Laboratory fluorometer (Turner Design, Sunnyvale, CA, USA) using the appropriate modules. The detection limit for phosphate was 0.1 μmol L^{-1} and 0.2 μmol L^{-1} for nitrate.

Results

Growth of Mono-Cultures

P. verruculosa and *P. farcimen* were studied in batch culture experiments at 5, 8, 11 and 15 °C. Each of the temperature experiments was conducted in triplicate (Fig. 1). *P. verruculosa* failed to achieve positive net growth at 5 and 8 °C (Fig. 1a, b) but grew successfully at 11 and 15 °C (Fig. 1a, d), whereas *P. farcimen* was able to grow at all temperatures examined (Fig. 1a–d). The length of the exponential growth phase differed between species and temperatures. The longest duration for exponential growth was observed for *P. verruculosa*, which lasted 3 weeks at 11 °C (Fig. 1c) and only 2 weeks at 15 °C (Fig. 1d). The length of the exponential

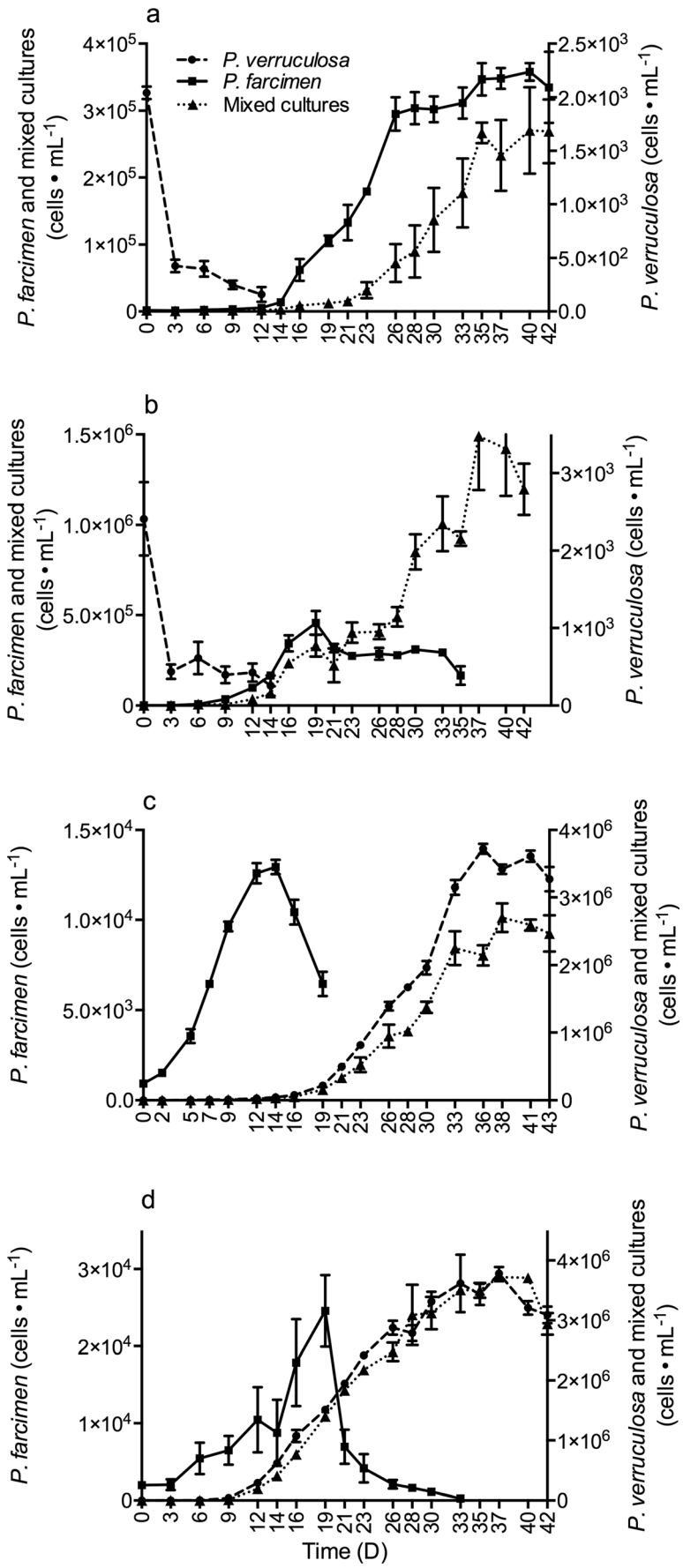


Fig. 1 Number of cells per millilitre calculated by light microscopy (LM) for *P. verruculosa* (*circle*), *P. farcimen* (*square*) in single-culture and mixed culture (*triangle*) at 5 (**a**), 8 (**b**), 11 (**c**) and 15 °C (**d**). All results are means of triplicate flasks and *error bars* are standard error of the mean (SEM) ($n = 3$)

growth phase for *P. farcimen* lasted 2 weeks at 5, 8 and 15 °C (Fig. 1a, b, d) but only 1 week at 11 °C (Fig. 1c).

The linear phase of logarithmic plots of cell abundances was examined, and the mean maximum cell specific growth rates was calculated (Fig. 2a). Both species displayed a difference in temperature optima. The highest maximum specific growth rate for *P. farcimen* was 0.39 div day^{-1} at 8 °C, whereas *P. verruculosa* had a maximum growth rate of 0.56 div day^{-1} at 15 °C. The maximum specific growth rates for both species were found to be significantly different between temperatures (ANOVA, $p < 0.05$). Growth rates were significantly different between species at all temperatures except at 11 °C (Fig. 2a), where growth rates were not significantly different (one-way ANOVA, $p > 0.05$).

Cell Yields

P. farcimen achieved the highest maximum cell densities of 3.9×10^5 and 4.6×10^6 cells ml^{-1} at 5 and 8 °C, respectively (Fig. 2b). With increasing temperatures, maximum cell densities declined to 12,949 cells ml^{-1} at 11 °C and 27,100 cells ml^{-1} at 15 °C. *P. verruculosa* achieved significantly higher maximum cell densities compared to *P. farcimen* at 11 and 15 °C where it reached 3.7×10^6 cells ml^{-1} at 11 °C and 4.0×10^6 cells ml^{-1} at 15 °C, (Fig. 2b) (ANOVA, $p < 0.05$). All numbers listed above for maximum cell densities are averages of triplicate experiments.

While the extracellular nutrients PO_4^{3-} and NO_3^- decreased through cell uptake, they were never completely eliminated from the media for either species (Fig. 3). Due to differences in biomass, *P. verruculosa* consumed more nutrients compared to *P. farcimen* at 11 and 15 °C. Figure 4 displays peak cell yield normalised per micromolar of NO_3^- and PO_4^{3-} utilised. *P. verruculosa* was able to create a cell yield approximately four times greater per unit resource compared to *P. farcimen*. Total cell yield per unit resource was significantly greater for *P. farcimen* at 4 and 8 °C compared to 11 and 15 °C ($p < 0.05$).

pH

A Spearman Rank correlation analysis found a statistically significant linear correlation between cell densities and pH for *P. verruculosa* at 11 °C ($r = 0.674$, $p = 0.002$, $r = 0.744$, $p = 0.00$) and 15 °C ($r = 0.533$, $p = 0.028$, $r = 0.694$, $p = 0.002$) and for *P. farcimen* at 5 °C ($r = 0.867$, $p = 0$, $r = 0.790$, $p = 0.00$) and 8 °C ($r = 0.589$, $p = 0.021$, $r = 0.789$, $p = 0.00$) in single and mixed culture respectively (Fig. 5). A

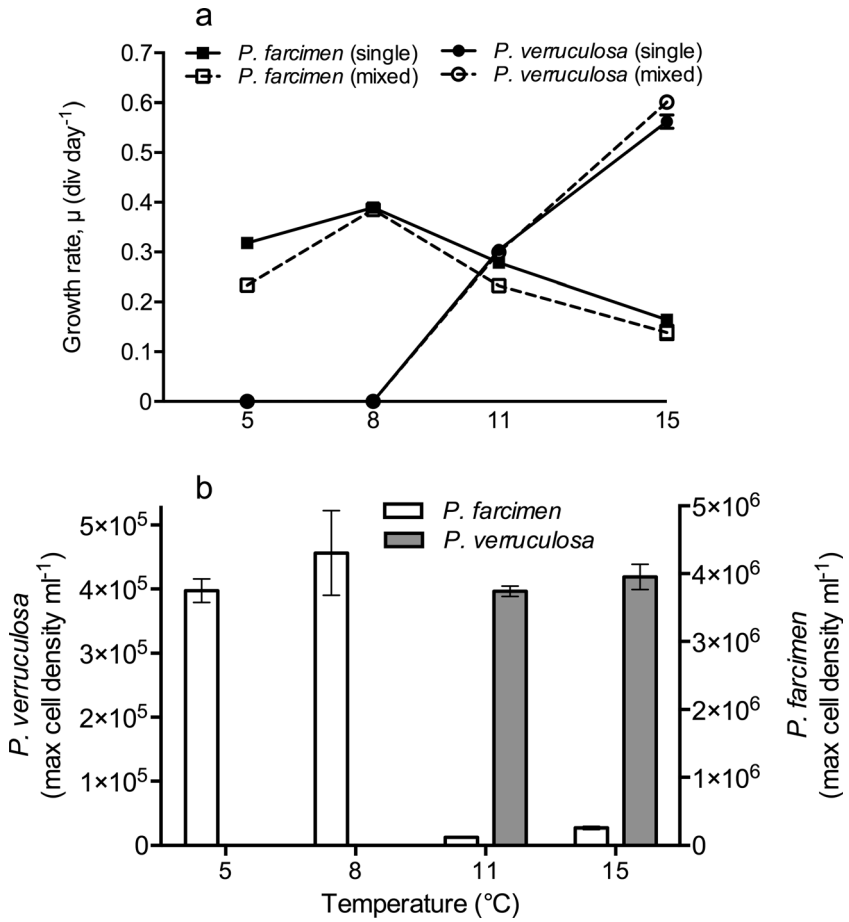
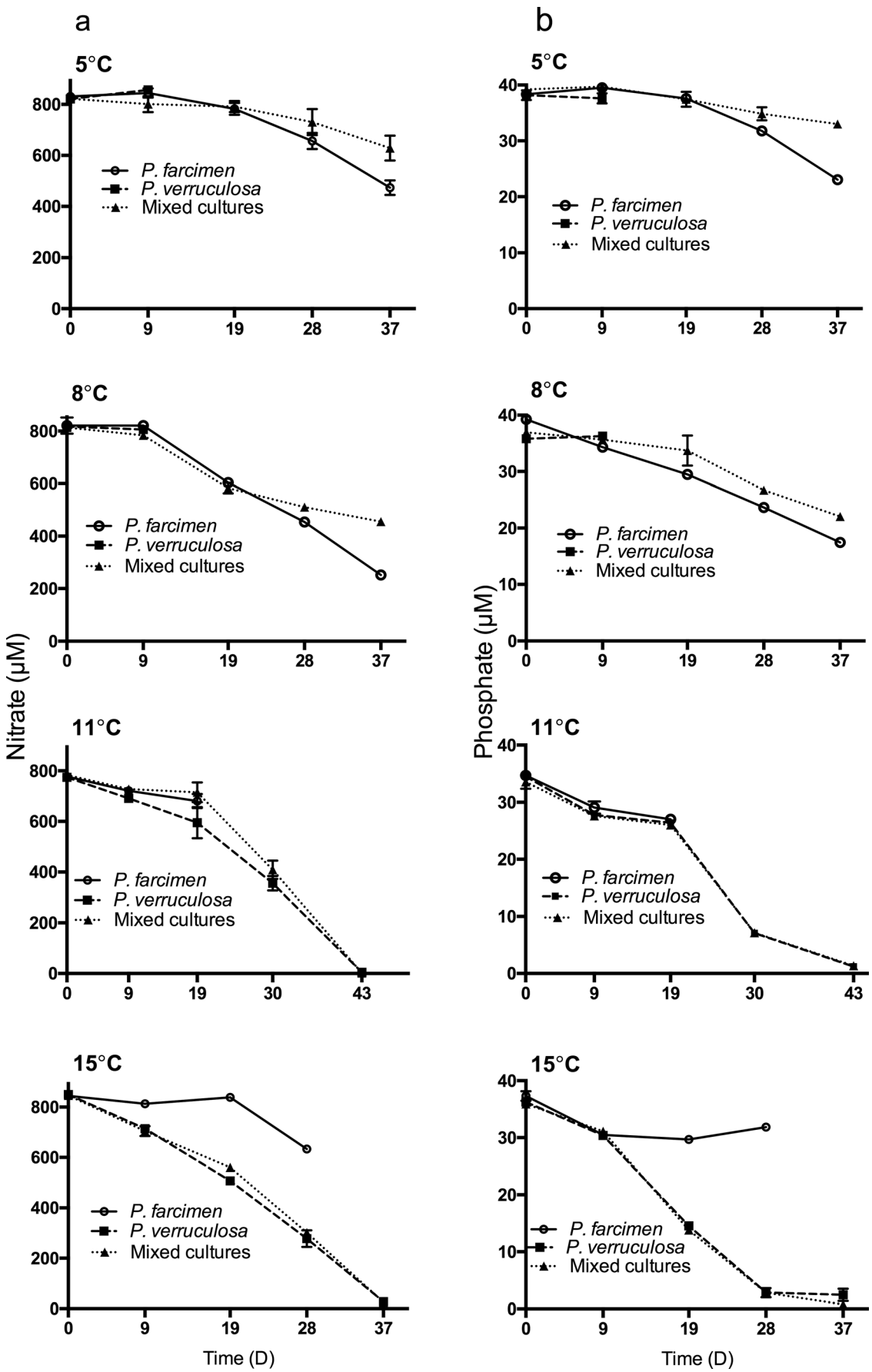


Fig. 2 Maximum specific growth rates (div day^{-1}) for *P. farcimen* in single (*black square*) and mixed culture (*white square*) and for *P. verruculosa* in single (*black circle*) and mixed culture (*white circle*) at 5, 8, 11 and 15 °C (**a**) and maximum cell densities (cells ml^{-1}) for *P. farcimen* (*left axis*, *no fill*) for *P. verruculosa* (*right axis*, *grey fill*) at 5, 8, 11 and 15 °C in single culture (**b**). All results are means of triplicate flasks and *error bars* are SEM ($n = 3$)


a
b
5°C
8°C
11°C
15°C
P. farcimen
P. verruculosa
Mixed cultures
Nitrate (µM)
Phosphate (µM)
Time (D)
0
9
19
28
30
37
43
200
400
600
800
10
20
30
40

◀ **Fig. 3** Residual NO_3^- (**a**) and PO_4^{3-} (**b**) in the media (μM) at 5, 8, 11 and 15 °C for *P. verruculosa* (*circle*), *P. farcimen* (*square*) in single-culture and mixed culture (*triangle*). All results are means of triplicate flasks and *error bars* are SEM ($n = 3$)

significant linear correlation between cell densities and pH was not observed for *P. farcimen* at 11 and 15 °C.

Mixed Culture

Figure 6 shows the dynamics of the combined abundance of *P. farcimen* and *P. verruculosa* in mixed-culture from the cell counts calculated using qPCR. Peak cell densities achieved by *P. farcimen* varied with temperature with a maximum cell density of 2.4×10^5 cells ml^{-1} at 5 and 8 °C. Densities declined by three orders of magnitude to just 6.4×10^3 cells ml^{-1} at 11 °C and 9.4×10^3 at 15 °C. No net growth of *P. verruculosa* occurred at 5 or 8 °C. *P. verruculosa* dominated in terms of cell numbers at 11 and 15 °C, where the peak cell yield for *P. verruculosa* was ≈300 % (2.4×10^6 cells ml^{-1}) and 360 % (3.3×10^6 cells ml^{-1}) higher than *P. farcimen*. At all temperatures, the mean peak cell density was statistically significantly different between the species (ANOVA, $p < 0.01$).

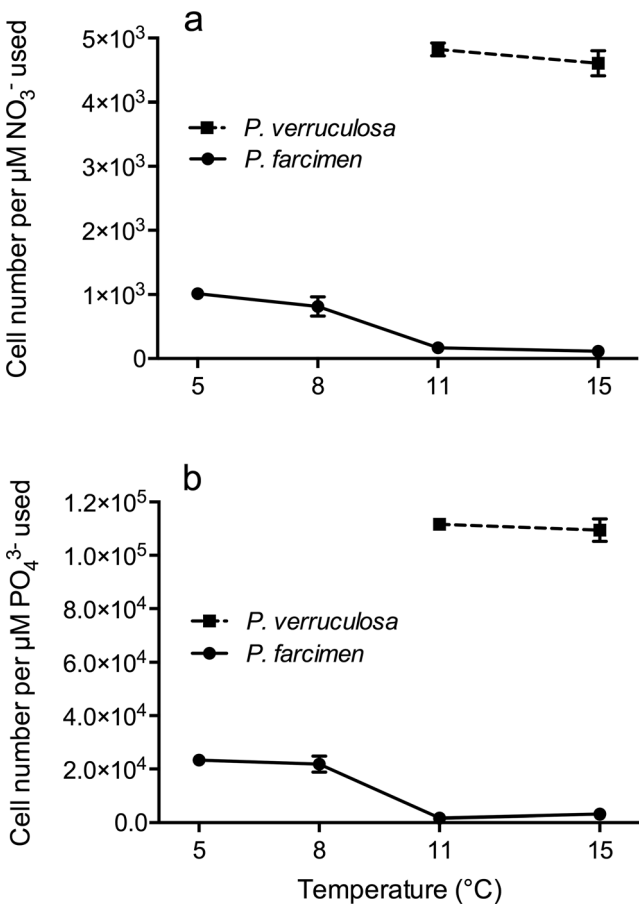


Fig. 4 Cell number per millilitres per μM of NO_3^- utilised for *P. verruculosa* (*circle*), *P. farcimen* (*square*) at 5, 8, 11 and 15 °C (**a**) and cell number per millilitres per micromolars of PO_4^{3-} utilised for *P. verruculosa* (*circle*), *P. farcimen* (*square*) at 5, 8, 11 and 15 °C (**b**). All results are means of triplicate flasks and *error bars* are SEM ($n = 3$)

Maximum specific growth rates for both species exhibited a similar response to the mono-culture growth rates (Fig. 2a). For *P. farcimen*, growth rates were significantly lower in the mixed-culture compared to the mono-culture experiments at 5, 11 and 15 °C (ANOVA $p < 0.05$), but similar at 8 °C. Growth rates for *P. verruculosa* were similar between the mono and mixed-culture treatments at 11 and 15 °C (ANOVA $p > 0.05$).

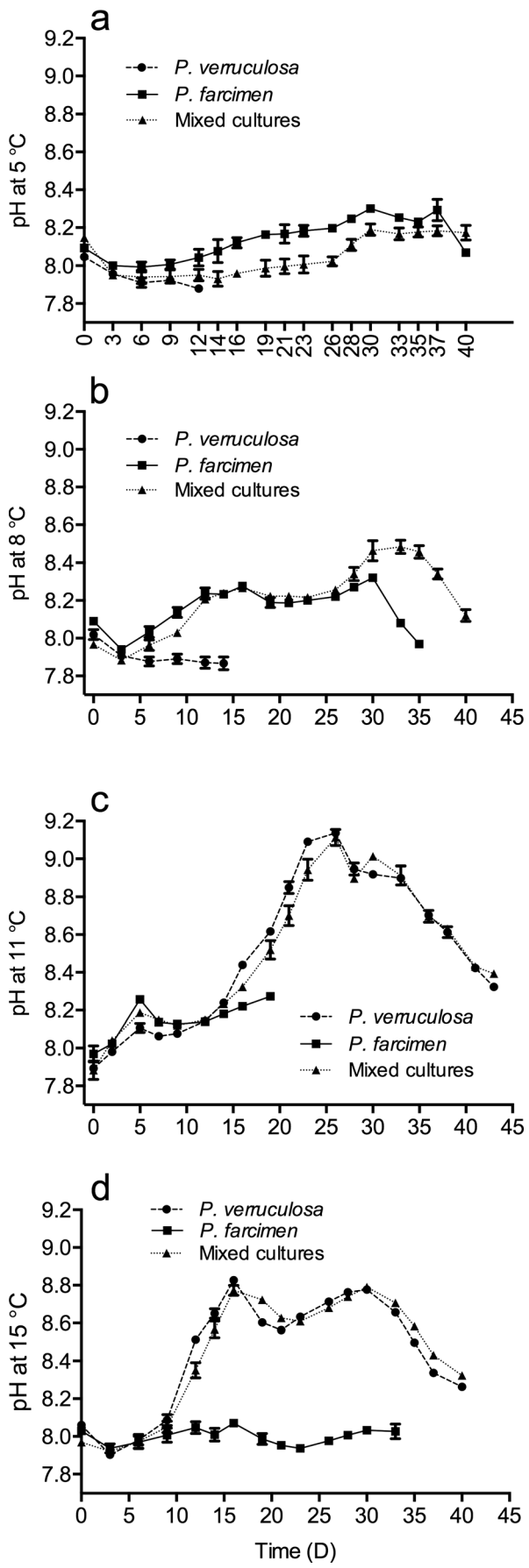
Discussion

Until recently, it was impossible to separate *P. farcimen* from *P. verruculosa* in environmental samples by either light microscopy or molecular methods. As a consequence, little is known about these species interactions in the environment. Species-specific molecular-based detection methods have previously been tested for *Pseudochattonella* spp. with varying degrees of success, including microarray, dotplot hybridisation, PCR and qPCR [26, 27]. With the use of the now called 'qPCR subtraction method' [7], community and laboratory experiments can now be conducted. Hence, we were able to accurately separate and quantify *Pseudochattonella* spp. in mixed assemblages in time course laboratory culture studies.

A recent field study revealed *Pseudochattonella verruculosa* to appear in low densities (≈16–160 cell ml^{-1}) alongside *P. farcimen* (1600–18,000 cell ml^{-1}) when the water temperatures reached 8–8.5 °C [7]. This illustrates that a time period of co-occurrence does exist between the two *Pseudochattonella* species. In this study, growth and competition were examined over a wide range of temperatures to simulate the changing temperature conditions from late winter to early spring in Scandinavian waters [23].

Both species were isolated from European waters, *P. farcimen* from the Skagerrak and *P. verruculosa* from German waters during bloom conditions. Origin is one of the most important features for strain designation. Here, we assume that the strains represent the natural population from which they were isolated. Strains can diversify in culture; either by selection, genomic drift or the high growth rates and population densities could lead to more spontaneous mutations occurring. To minimise the effect of the previous history of the culture, the cultures were acclimated until the growth rate had stabilised so that the response was due to the treatment conditions and not the plastic physiological response to the immediate history [28].

Our results from laboratory experiments go against the general understanding that the blooms that developed earlier in the season are solely formed by *P. farcimen* [5, 8] and later blooms more exclusively consist of *P. verruculosa* [22].



◀ **Fig. 5** pH for *P. verruculosa* (*circle*), *P. farcimen* (*square*) in single-culture and mixed cultures (*triangle*) at 5 (**a**), 8 (**b**), 11(**c**) and 15 °C (**d**). All results are means of triplicate flasks and *error bars* are SEM ($n = 3$)

Rather, we were able to confirm the field observations in Eckford-Soper and Daugbjerg [7], where *P. verruculosa* was identified to co-exist in low (≈160 cell ml^{-1}) densities during a minor *P. farcimen* bloom. Here, *P. farcimen* and *P. verruculosa* had different tolerance ranges and temperature optima. *P. farcimen* favoured conditions, representative of late winter-early spring conditions but was able to grow over the full range of temperatures. Contrary, *P. verruculosa* achieved positive growth only at higher temperatures.

Optimal growth for *P. farcimen* occurred at 8 °C with a maximum specific growth rate of 0.39 div day^{-1}. A decline in growth rate occurred on either side of this optima. Jakobsen et al. [5] observed growth rates of 0.09 div day^{-1} at temperatures as low as 1 °C and a maximum specific growth rate similar to the one observed here of 0.35 div day^{-1}. Unlike Jakobsen et al. [5] and Edvardsen et al. [2] reporting an upper temperature limit for *P. farcimen* of 10 °C, we observed a broader tolerance range with positive growth up to 15 °C. Skjelbred et al. [8] even observed positive growth as high as 20 °C. Skjelbred et al. [8] calculated the temperature optima for five *P. farcimen* strains, all of which exhibited very little intraspecific variability (i.e. temperature optima overlapped between strains). The growth of relatively few strains of *P. verruculosa* has also been described by Skjelbred et al. [8] when they examined a Japanese strain NIES670 and the same strain used in this study (JG8). JG8 was originally isolated from German waters. Skjelbred et al. [8] noted growth rates up to 0.61 div day^{-1} for the Japanese strain, whilst our observed growth rates for the German strain of 0.56 div day^{-1} were similar to the values obtained by Skjelbred et al. [8] which were 0.51 div day^{-1}. As the maximum growth temperature in this study was only 15 °C and as *P. verruculosa* has been observed in water temperatures of 18 °C or higher in Scandinavian waters [6, 29, 30], a higher maximum specific growth rate could possibly have been achieved at a higher temperature. The failure of *P. verruculosa* to grow at the lowest temperatures (5 and 8 °C) shows that temperature represents a strong autecological barrier for survival of this species in cold waters.

Differences in maximum cell yield occurred between the species at all temperatures. *Pseudochattonella verruculosa* clearly outcompeted at 11 and 15 °C in terms of biomass, where peak cell densities far exceeded those of *P. farcimen*. Cultures of *P. verrculosa* reached a maximum of 3.8×10^6 cell ml^{-1} at 15 °C, a density that may have caused light limitation due to self-shading [31]. From visual observation, the cultures of *P. verruculosa* consisted of only small spherical cells, whilst *P. farcimen* had a much broader range of cell

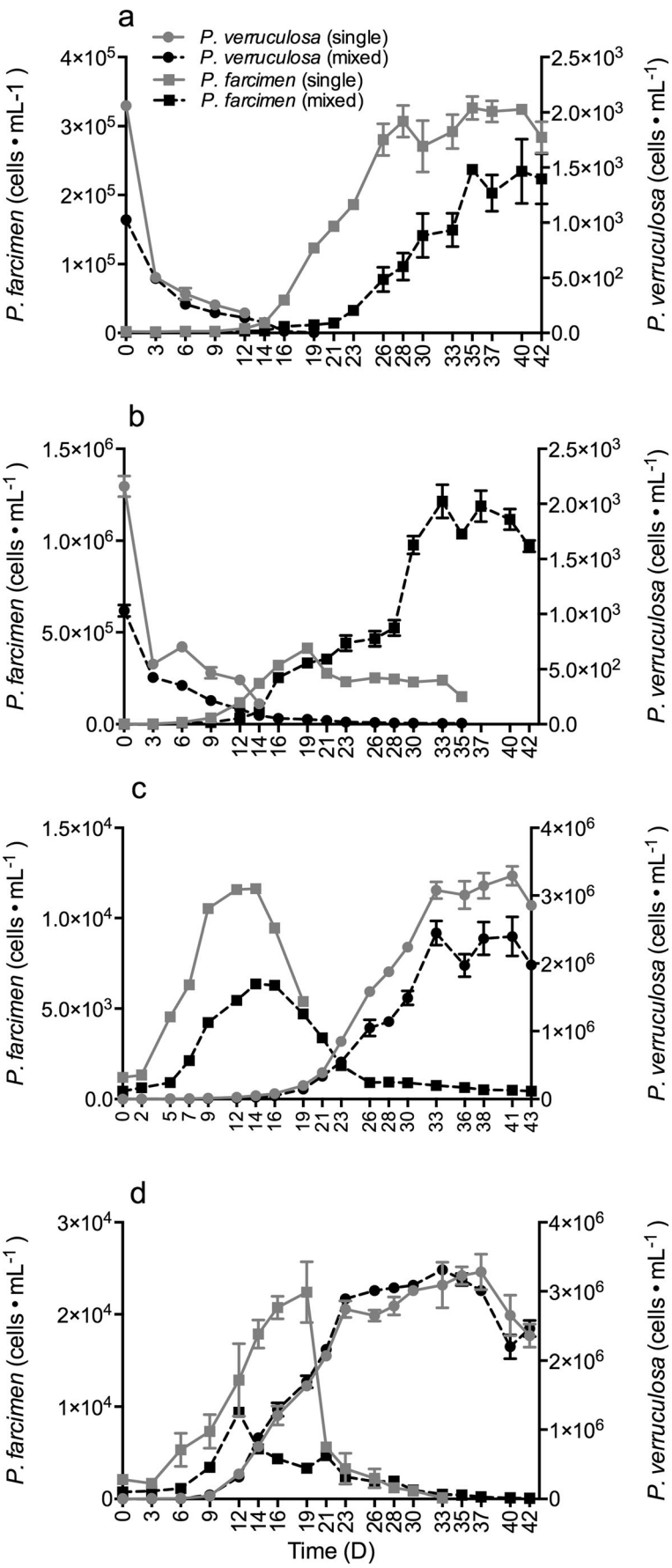


Fig. 6 Comparison of cell counts per millilitres calculated by qPCR for *P. verruculosa* (●, *right axis*) and *P. farcimen* (■, *left axis*) in single cultures (*grey scale*) and *P. verruculosa* (*circle*, *right axis*) and *P. farcimen* (*square*, *left axis*) in mixed cultures (in *black*) at 5 (**a**), 8 (**b**), 11 (**c**) and 15 °C (**d**). All results are means of triplicate flasks and *error bars* are SEM ($n = 3$)

shapes from large elongated forms to small spherical cells. These observations are consistent with observations on cell size and appearance described by other authors for *P. farcimen* [2, 5] and *P. verruculosa* [2, 32]. Along a temperature gradient, species replacement is commonplace with the most dominant species having the highest carrying capacity and highest growth rate [11]. It is common in protists that an increase in temperature often occurs alongside a reduction in body size [33]. It has been hypothesised that if nutrient inputs remain fixed, the increased metabolic demand due to warmer temperatures will be compensated through either a reduction in abundance or a reduced body size [11]. The smaller body size gives a competitive advantage over larger cells with regard to nutrient uptake and growth. As *P. verruculosa* cells are typically smaller than *P. farcimen*, it could explain their greater maximum growth rates and greater nutrient utilisation. It has also been suggested that nutrient pulse rates can be related to phytoplankton size classes as infrequent nutrient pulses might select for small species and more frequent nutrient pulses for larger cells [34]. As *P. farcimen* occurs earlier when nutrients are more plentiful, it may in part explain the size differences between the two species. A reduced body size has also been observed to reduce the grazing pressure from copepods, which would act as an advantage during the emerging zooplankton bloom [35].

While the extracellular nutrients PO_4^{3-} and NO_3^- decreased through uptake, they were never completely eliminated from the media for either species. When peak cell yield was normalised per micromolar of PO_4^{3-} and NO_3^- utilised, *P. verruculosa* was able to create a cell yield approximately four times greater per unit resource compared to *P. farcimen*. This suggests either a more efficient utilisation of resources or that smaller cells created by this species equalled the same total biomass in terms of carbon as the larger *P. farcimen* cells.

Growth was also clearly inhibited by pH. Both species had differing pH tolerances, as *P. farcimen* was highly sensitive to pH with no growth occurring above pH 8.4. Jakobsen et al. [5] observed a higher pH tolerance of 8.9–9 for this species. This pH tolerance is also in the range of the often co-occurring species *Dictyocha speculum*, which has an upper tolerance of pH 8.81 [36]. In comparison, *P. verruculosa* had a higher tolerance to pH with growth ceasing at pH 9.1. A significant linear correlation between cell densities and pH was observed for *P. verruculosa* at 11 and 15 °C and for *P. farcimen* at 5 and 8 °C. This suggests that temperature and not pH was the limiting factor for growth of *P. farcimen* at 11 and 15 °C.

Mixed Culture

Clear interactions between the two species were observed in the mixed culture treatments. As with the mono-culture experiments, *P. verruculosa* was limited by temperature and failed to achieve positive growth at 5 and 8 °C. During the experimental period equal cell abundances of either species were not observed at any temperatures. At 5 and 8 °C, there was a significant linear correlation between cell densities and pH for *P. farcimen*. At 5 °C, *P. farcimen* did not perform as would have been expected from its relative performance in mono-culture. The maximum specific growth rate and maximum cell density were significantly lower in the mixed-culture treatment. Growth ceased when the culture reached pH 8.19 compared to 8.3 in mono-culture, suggesting that conditions were not optimal and the pH tolerance has been lowered. At 8 °C, *P. farcimen* reached a greater maximum cell density in mixed-culture compared to mono-culture, with growth declining at pH 8.23 in both instances. At 11 and 15 °C, *P. farcimen* also had a lower maximum cell density and growth rates compared to those in mono-culture, but here, cell numbers were not significantly correlated with pH, again suggesting temperature had a greater overall effect on growth. One other possibility affecting their performance is the production of growth inhibiting substances. These substances or allelochemicals can affect other organisms (hetero-inhibition) or themselves (auto-inhibition) [37]. The release of these extracellular substances could be a mechanism for the successful competition against species and groups such as diatoms, which are more adapted to low temperatures and light conditions that occur in late winter and early spring in temperate regions. Allelopathic effects were not observed between *P. farcimen* and the dinoflagellate *Heterocapsa triquetra* and the diatoms *Chaetoceros decipiens* and *C. diadema* in mixed growth experiments [5]. Despite this, species-specific differences in phytoplankton sensitivity are common [38]. Therefore, further assessments on the allelopathic ability of both species of *Pseudochattonella* on a number of co-occurring phytoplankton species should be carried out.

Temperature has previously been shown to influence the outcome of competition between phytoplankton species in the laboratory and outdoor cultures [39, 40] with competition affecting the total biomass yields. As *P. farcimen* exhibited a lower maximum cell density and growth rate at three out of the four temperatures with the exception being 8 °C, this suggests that changes of just a few degrees can have a huge impact on species interactions and overall community structure. At genus level high inter and intra-specific variations and adaptations exist which are caused by many factors including light and temperature. However, Boyd et al. [41] noticed that parameterising growth differences at the species level is much easier as there is a broad similarity in the shape of a strain's reaction norms within a species. Ideally, multiple strains should be examined to explore intraspecific variations in growth rate; unfortunately, only one strain of each was available to us. Additional strain diversity should be included in future studies as well as higher and intermediate temperatures.

The results of this study and a single set of field data [7] suggest that there is a period of co-occurrence between the two

species caused by an overlap in their temperature niches, but temperature acts as physical barrier to either species being in direct competition with each other, with neither species being capable of proliferating to bloom densities at the same time. Yet, the presence of each species could potentially give rise to a seed population when suitable conditions arise.

Acknowledgments We thank Herdis Preil Damberg (Danish Meteorological Institute) for helping to provide the SST data. We also thank Jim Grover and two anonymous referees for their comments that helped to improve the manuscript. This study was funded by the Danish Strategic Research Council (project HABFISH).

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