

A QUANTITATIVE REAL-TIME PCR ASSAY FOR IDENTIFICATION AND ENUMERATION OF THE OCCASIONALLY CO-OCCURRING ICHTHYOTOXIC *PSEUDOCHATTONELLA FARCIMEN* AND *P. VERRUCULOSA* (DICTYOCHOPHYCEAE) AND ANALYSIS OF VARIATION IN GENE COPY NUMBERS DURING THE GROWTH PHASE OF SINGLE AND MIXED CULTURES¹

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The ichthyotoxic genus *Pseudochattonella* forms recurrent extensive blooms in coastal waters in Japan, New Zealand and Northern Europe. It comprises of two morphologically similar species, *P. verruculosa* and *P. farcimen*, which complicates visual species identification and enumeration of live and fixed material. Primers designed previously could not quantitatively distinguish species in mixed assemblages. To address this issue we developed two primer sets: one revealed itself to be genus specific for *Pseudochattonella* and the other species-specific for *P. verruculosa*. By subtracting cell estimates for *P. verruculosa* from combined results we could calculate cell numbers for *P. farcimen*. This approach has overcome the challenges posed by the very limited sequence availability and low gene variability between the two species. The qPCR assay was extensively tested for specificity, efficiency and sensitivity over an entire growth cycle in both single and mixed assemblages. Comparison of cell abundance estimates obtained by qPCR assay and microscopy showed no statistically significant difference until stationary and death phases. The assay was also tested on environmental samples collected during a small *Pseudochattonella* bloom in Denmark in March–April 2015. It was impossible to distinguish *P. farcimen* and *P. verruculosa* by light microscopy but qPCR showed both species were present. The two methods provided nearly identical cell numbers but the assay provided discrimination and enumeration of both species.

Key index words: cell enumeration; copy number of LSU rDNA; growth phase; *Pseudochattonella farcimen*; *Pseudochattonella verruculosa*; qPCR assay

Abbreviations: FISH, fluorescent in situ hybridization; LM, light microscopy; TEM, transmission electron microscope

Between April and May 1998 a heterokont flagellate bloom formed off the west coast of Germany, Denmark, Sweden and Norway killing 350 tons of farmed fish as well as some wild stocks (Riisberg and Edvardsen 2008). The organism responsible for this fish kill was initially identified as *Chattonella* aff. *verruculosa* due to its resemblance to a similar species that had been described from Japanese waters in the late 1980s (Hosoi-Tanabe et al. 2007). The then-named *Chattonella verruculosa* has become a dominant causative organism forming recurrent ichthyotoxic blooms in summer and winter in Japanese waters (Yamaguchi et al. 1997). *Chattonella* aff. *verruculosa* was initially classified as a Raphidophyceae (Hara et al. 1994), but genetic analysis of the 18S region revealed a closer relationship with the class Dictyochophyceae (Bowers et al. 2004). Further work by Hosoi-Tanabe et al. (2007) and Edvardsen et al. (2007) confirmed this systematic treatment. Based on these studies, a new genus was proposed for this species and it was named *Pseudochattonella verruculosa* (Hosoi-Tanabe et al. 2007).

Since they were first recorded in European waters, *Pseudochattonella* spp. have been observed on an almost annual basis and have been held responsible for a number of fish mortality events causing substantial economic losses in the North Sea, Skagerrak and Kattegat (Edvardsen et al. 2007, Riisberg and Edvardsen 2008). Strains isolated from a massive bloom in the Skagerrak in 2001 and in the Kattegat in 2006, which killed farmed salmon amounting to 1,100 tons and 18 tons, respectively were found to belong to a new species *P. farcimen*. This new species differed both morphologically and physically to *P. verruculosa* (Edvardsen et al. 2007). Both *P. farcimen* and *P. verruculosa* now appear to have become well established in Scandinavian waters. *P. farcimen* seems to be an endemic species and a common constituent of the late winter to early spring phytoplankton community (Riisberg and Edvardsen 2008). It has now been observed along the German coast from the North Sea coast of Schleswig to Holstein, the Danish Coast in the North Sea and Skagerrak, the Swedish west coast from Hvaler to

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Bergen (Naustvoll 2010) and the Gulf of Gdansk, Poland (Łotocka 2009) and also potentially in French and Dutch waters (Naustvoll 2010).

The blooms of *Pseudochattonella* spp. that developed in 1998, 2000, 2002 and 2004 occurred in April and May, when water temperatures reached a maximum of 18°C (Riisberg and Edvardsen 2008). However, the blooms that formed in 2001, 2006, 2007 and 2011 developed from January to March and often overlapped with the spring diatom bloom, when water temperatures were much lower, at 2°C–5°C (Edvardsen et al. 2007, Riisberg and Edvardsen 2008). The causative species of each bloom was not determined at the time. However, it was suspected that due to the distinct temperature preferences determined by Yamaguchi et al. (1997) on *P. verruculosa* and *P. farcimen* by Jakobsen et al. (2012) the blooms that developed earlier in the season were formed by *P. farcimen* and the later blooms more exclusively *P. verruculosa*.

Cell counts are the usual measure to quantify harmful algae and it is these count estimates along with toxicity measurements that make up the basis for all stakeholder decisions regarding fisheries production and human health. However, it is almost impossible to identify *Pseudochattonella* down to species level, as they appear very similar under the light microscope, particularly if fixed in Lugol's iodine (LES, personal observation). Both species are variable in size (5–50 µm), possess two flagella of unequal length and have numerous golden-brown chloroplasts. Under favorable conditions cells become elongated, whereas under less favorable conditions they can become oval or spherical in shape. Cells can also be either smooth or have a warty appearance due to the presence of protruding oval mucocysts (Edvardsen et al. 2007). However, marked differences can be observed when comparing the shape of the nucleus in thin-sectioned material examined under a transmission electron microscope (TEM). The nucleus of *P. verruculosa* is rounded, whereas it is branched in *P. farcimen*. Subtle differences also exist in the morphology of their flagella hairs (Edvardsen et al. 2007). However, TEM observations are impractical as a means of routine monitoring. Due to the morphological similarities in gross cell outline as described above, the task of identifying and separating the two species of *Pseudochattonella* using light microscopy of Lugol fixed samples is not a trivial task. This is problematic as the most commonly used fixative in HAB monitoring programs is Lugol's iodine (Eckford-Soper and Daugbjerg 2015^a). The problem is also compounded by fixative induced changes in form, for example, loss of mucocysts (Jakobsen et al. 2012). To add to the uncertainty, morphology in terms of size and shape changes in response to the growth phase (LES, ND, personal observation). For more detailed studies of the geographical distribution, bloom dynamics, life cycle, succession, and

toxic effects of *Pseudochattonella*, correct delineation between the two species is necessary (Riisberg and Edvardsen 2008).

Numerous molecular detection methods have been developed and tested for a range of HAB species, which quantify either RNA or DNA. Molecular tools may overcome the problems associated with identifying these morphologically similar species as well as having the potential to be applied to automated high-throughput systems. Some of the molecular methods include whole cell approaches such as hybridization assays, for example, fluorescent in situ hybridization (FISH; Touzet et al. 2010), sandwich hybridization assays (Zhen et al. 2009) and FISH-Flow cytometry (Eckford-Soper et al. 2013). All these methods use species-specific rRNA targeting probes. Enumeration can also be based on cellular homogenates treated with molecular probes. These methods include: microarray for the detection of toxic algae (Medlin 2013, Dittami et al. 2013a) and PCR and real-time qPCR (Penna and Galluzzi 2013), as well as the enzyme-linked immunosorbent assays based kits for toxicity testing (Trainer and Hardy 2015).

qPCR has been comprehensively tested and extensively used to identify and quantify a number of HAB species in natural marine samples including *Alexandrium* spp. (Erdner et al. 2010, Toebe et al. 2013, Eckford-Soper and Daugbjerg 2015b), *Pseudonitzschia* spp. (Fitzpatrick et al. 2010), *Prymnesium parvum* (Galluzzi et al. 2008, Manning and Claire 2010, Eckford-Soper and Daugbjerg 2015b), *Pfiesteria* spp. (Bowers et al. 2000, Lin et al. 2006), *Dinophysis* spp. (Kavanagh et al. 2010), *Gambierdiscus* spp. (Vandersea et al. 2012), and *Karenia* spp. (Yuan et al. 2012), for example.

Molecular studies of *Pseudochattonella* spp. have shown that they differ from each other in the nucleotide sequences of SSU, LSU, and ITS rDNA as well as the protein encoding genes *Cox1*, *psbA* and *rbcl* (Edvardsen et al. 2007, Riisberg and Edvardsen 2008). PCR, qPCR, dotplot hybridization and microarray probes have all been developed with varying degrees of success for *Pseudochattonella* spp. (Dittami and Edvardsen 2012). However, the species-specific primers previously developed by Dittami et al. (2013b), which were based on the nuclear-encoded LSU rDNA region, could not quantitatively distinguish between the two species in a mixed assemblage due to crossover between species.

Hence, the aim of this study was to develop and test molecular primers and hydrolysis probe pairs for the identification and enumeration of the two currently recognized *Pseudochattonella* species. As gene copy number variation is a common form of genome diversity and calculated cell densities are based on the assumption that the DNA content remains stable during the different growth cycles, we also aimed to test their number and variation over time (i.e., from stationary to death phase). Two

primer sets were designed: one that was specific for *P. verruculosa* and one set that was revealed to amplify both *P. farcimen* from *P. verruculosa*. Thus, by subtracting the cell estimates for *P. verruculosa* from the combined results it should be possible to calculate the cell numbers for *P. farcimen* (i.e., *P. farcimen* cell number = *Pseudochattonella* spp. cell number – *P. verruculosa* cell number).

MATERIALS AND METHODS

Experimental conditions. The following microalgal cultures were used: *P. farcimen* was obtained from the Scandinavian Culture Collection of Algae and Protozoa, Copenhagen, Denmark (strain K-1809) and *P. verruculosa* was kindly provided by Bente Edvardsen (strain JG8). Both strains were grown in L1 media at 30‰. Growth conditions were chosen to represent optimum temperatures for *P. verruculosa* and *P. farcimen*. *P. verruculosa* was grown at 15°C and *P. farcimen* at 4°C, both under a light intensity of 110 $\mu\text{mol photons} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ and a light:dark cycle of 16:8 h. Prior to experimentation cultures had been maintained in exponential growth at their respective temperatures by frequent sub-culturing. Experiments were conducted in triplicate. Inoculums for the experiments were taken from exponential phase of stock cultures. The inoculum cell density was $\approx 2,000 \text{ cells} \cdot \text{mL}^{-1}$ and cells were allowed to grow in batch mode until they reached post stationary cell death phase when the experiments were terminated.

Enumeration of total cell abundances. Daily pH of the culture media was measured using a Jenway 3510 pH meter before sub-samples (3.6 mL) were removed from each flask. Cells were preserved in acidified Lugol's iodine solution (660 $\mu\text{g I}_2$). From this sub-sample 1 mL was removed for DNA analysis by qPCR, 1.2 mL was removed for cell enumeration and counted in a Sedgewick Rafter counting chamber (LeGresley and McDermott 2010) at $\times 100$ magnification using an Olympus CH-2 microscope. A further 1.2 mL was removed and mixed with the corresponding sample taken from the other *Pseudochattonella* species. From this 2.4 mL "mixed" sample, 1 mL was removed for DNA analysis and 1.2 mL for cell enumeration as described above. Figure S1 in the Supporting Information illustrates the sampling strategy. DNA samples were centrifuged (6,000g, 10 min) and the supernatant removed. Cell pellets were then washed in 1 \times PBS buffer, centrifuged (6,000g, 10 min) and the supernatant removed. Finally 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.

The empirical growth rate μ was defined as number of cell divisions $\cdot \text{d}^{-1}$ and defined as (Levasseur et al. 1993):

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

where N_1 and N_2 are the abundances at time₁ (t_1) and time₂ (t_2), respectively. The Minitab software package was used for statistical comparison of growth rates over the duration of the exponential phase.

Field validation. Field samples were taken near Horsens fjord in the vicinity of Snaptun on the east coast of Jutland, Denmark. Water samples (100 mL) were taken over a 10-d

period in March-April 2015 during a *Pseudochattonella* bloom at a depth of 3 m. Samples were then preserved in acidified Lugol's iodine solution (660 $\mu\text{g I}_2$), from this 20 mL was removed for enumeration by light microscopy (Uthermöhler method) and 1 mL was removed for enumeration by qPCR. Samples for DNA analysis were centrifuged (4,000g, 20 min) and the supernatant removed. Cell pellets were then washed in 1 \times PBS buffer, centrifuged (6,000g, 10 min) and the supernatant removed. The material was stored at -20°C until extraction of total genomic DNA. Samples for cell enumeration by light microscopy were settled for 24 h before being examined under an inverted microscope (Olympus IX81 Olympus, Tokyo, Japan). Micrographs were taken using a DP72 digital camera (Olympus).

Standard curves. Standard curves were obtained by amplifying the DNA extracted from the 10-fold serial dilutions (10^1 – 10^7 total cell number) of known cell concentrations in triplicate measurements ($n = 3$). For the qPCR assay a template of each target species was added in equal concentrations to each reaction. Reactions were carried out with a no-template control containing ddH₂O.

Extraction of total genomic DNA. Samples for DNA extraction were chosen so that they represented different stages of the entire growth cycle for both single species and mixed samples. Hence, a total of 72 samples from eight days were chosen and selected: two in the lag phase, one at the start of the exponential growth phase, two during the exponential growth phase, two in the stationary phase and one in the death phase (Fig. S2 in the Supporting Information). These data points corresponded to days: 0, 2, 9, 16, 21, 30, 33, and 37.

The selected samples were thawed and the cell pellets were re-suspended in 10 μL of ddH₂O before being transferred to the reaction tube supplied with the DNA extraction kit. The storage tube was then given a further 2–3 washes using 10 μL of ddH₂O to ensure all material had been transferred. *Pseudochattonella* cells are notoriously sticky (LES, personal observation) and care should be taken to ensure that all cells are transferred. For all experiments, extraction was carried out using the Powerplant Pro DNA isolation Kit (MO BIO, Carlsbad, CA, USA) according to manufacturers recommendations with one exception; an aliquot (4 μL) of Internal DNA extraction control (Primer Design, Southampton, UK) was added to the lysis buffer prior to extraction. Loss in percent was calculated from absolute qPCR values generated from the control before and after extraction. Cell numbers used to generate calibration curves were reduced by the same percentage. Total elution volume of DNA was 50 μL and samples were stored at -20°C until analysis.

Primers and hydrolysis probe for qPCR. The primers and hydrolysis probe designed for this study were based on previously published nuclear-encoded LSU rDNA sequences of six strains of *P. verruculosa* (with GenBank accession numbers given in parenthesis): JG8 (AM850226), CAWR 21 (AM850224) and CAWR 22 (AM850225), NIES-670, ver-P (AM040504), CAWDC03 (JF701986) and four strains of *P. farcimen*: UIO 110 (AM040502), UIO 114 (AM850222) and UIO 115 (AM850223) from Norway and one from Danish waters. The LSU rDNA sequence of the Danish strain is not available in GenBank. The qPCR amplicon (97 base pairs) was identical for all strains of *P. farcimen* and *P. verruculosa*, respectively. We used the online service provided by Integrated DNA technologies (<https://eu.idtdna.com>) for primers and probe design and followed their recommendations. The LSU rDNA sequences available for *P. verruculosa* from the North Sea (JG8) is 497 base pairs long, setting some limitations to the possibilities for species-specific primer design when compared to *P. farcimen* for which 1,200 base pairs were available. Additionally we wanted to have an annealing tem-

perature for our *Pseudochattonella* assay similar to that in other assays of ichthyotoxic microalgae in multiplex qPCR (Eckford-Soper and Daugbjerg 2015b). This added further constraints to the assay design for *Pseudochattonella* spp. *P. farcimen* and *P. verruculosa* were amplified using the primer and probe sequences described in Table 1. The alignment of 97 base pair long fragment used for qPCR with position of forward and reverse primers and the hydrolysis probe is illustrated in Figure S3 in the Supporting Information. Primers and probe concentrations were optimized for the CFX96 Touch Real-time PCR detection system (Bio-Rad Hercules, CA, USA). Optimized conditions for qPCR were with 20 μ L reactions with 4 μ L of HOT FIREPolI Probe qPCR Mix Plus (no ROX; 1 \times final concentration; Solis BioDyne Tartu, Estonia.), 2 μ L of template, 0.5 μ L of each appropriate primer, 1 μ L of probe, and 12 μ L ddH₂O. 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.

Inhibition assay. Due to potential inhibition, the amplification performance was analyzed using a dilution series consisting of 10²–10⁶ cells (counted by light microscopy [LM]) in triplicate during the late exponential and stationary phases. Samples were centrifuged at 4,000g for 20 min and the supernatant removed. Cell pellets were then washed in 1 \times PBS buffer, centrifuged at 6,000g for 10 min and the supernatant removed. The material was stored at –20°C until extraction of total genomic DNA and amplification as described above.

Determination of copy number. To estimate the number of LSU rDNA gene copies a dilution series of known concentrations of LSU rDNA gene products was analyzed together with the extractions of known cell number from days: 0, 2, 9, 16, 21, 30, 33, and 37. Replicate samples consisting of 10⁵ cells were centrifuged (6,000g, 10 min), the supernatant removed and the pellet frozen over night (–20°C) before extraction and amplification as described above and then purified by ethanol precipitation. rDNA concentrations were measured with a Qubit 2.0 fluorometer (Invitrogen, Carlsbad, CA, USA.) following the manufacturer's instructions. Copy numbers were calculated using the following equation (Perini et al. 2011):

$$\text{Copy number} = (A \times 6.022 \times 10^{23}) \times (B \times 1 \times 10^9 \times 660)^{-1} \quad (2)$$

where A is the template concentration in ng, B is the length in base pairs of the PCR product, 6.022 \times 10²³ is Avogadro's number and 660 is the average molecular weight of one base pair. The standard curve for both species was obtained by amplifying the specific internal fragment of 97 base pairs from 10-fold serial dilutions with copy number ranging from 10¹ to 10⁶ in triplicates (data not shown). The number of copies was determined using the qPCR standard curve to obtain the number of copies per cell.

RESULTS

Specificity and sensitivity. Cross-reactivity between the two species was tested on laboratory cultures of known cell numbers in both single and mixed samples. Primer set 1 (Table 1) proved to be specific to *P. verruculosa* and did not amplify *P. farcimen* while the primer set 2 amplified both species. When the results obtained from the *P. verruculosa* specific primer set were subtracted from the genus specific results we were able to obtain accurate cell number

estimates for *P. farcimen*. The specificity of each primer set was further tested by screening them against non-target organisms known to co-occur in Danish waters including *Alexandrium tamarense*, *A. pseudogonyaulax*, *A. ostensfeldii*, *A. minutum*, *Dictyocha speculum*, *P. parvum*, *Karenia mikimotoi*, *Chrysochromulina polylepsis*, and *Karlodinium veneficum*. Only the two target species were detected with no cross-reactivity between the other species (data not shown).

To determine overall cell numbers, 10-fold serial dilutions of known cell concentrations from cultured material were prepared for the standard curves in triplicate. Concentrations ranged from 10¹ to 10⁵ cells for both species. The two primer sets exhibited an amplification efficiency (E) of between 95% and 100% and the R²-values exceeded 0.99 in all cases (Fig. S4 in the Supporting Information). For both species the reproducible detection limit for cell numbers was 10¹ for both species. An internal extraction control was used to assess extraction efficiency of the DNA extraction kit. Percentage loss varied between 7% and 11% and did not vary between species (*P* > 0.05).

Maximum specific growth rates and cell densities. *Pseudochattonella verruculosa* and *P. farcimen* were studied in batch culture experiments carried out in triplicate. The linear phase of logarithmic plots of cell abundances were examined and the mean maximum cell specific growth rates were calculated. The length of the exponential growth was observed between days 2 and 21 for *P. verruculosa* and days 9 and 30 for *P. farcimen* (Fig. 1). Maximum specific growth rates were found to be significantly different for *P. farcimen* (0.28 div · d⁻¹) and for *P. verruculosa* (0.38 div · d⁻¹; Mann–Whitney, *P* < 0.05).

Mean peak cell densities differed significantly between the two species varying from 1.8 \times 10⁶ cells · mL⁻¹ for *P. farcimen* and 2.8 \times 10⁶ cells · mL⁻¹ for *P. verruculosa* (Mann–Whitney *P* < 0.05).

Count comparisons of single species and mixed samples. A comparison of cell counts obtained by qPCR and light microscopy using a Sedgwick-Rafter chamber was performed on the individual species of *Pseudochattonella*. qPCR samples were selected to represent the entire growth cycle and corresponded to days: 0, 2, 9, 16, 21, 30, 33, and 37. During the lag phase and exponential growth phase cell numbers closely matched. However, when cultures reached the stationary and death phases the qPCR method appeared to underestimate the cell densities compared to the LM counts. Despite this when a Mann–Whitney test was used to evaluate the differences between the mean cell densities obtained from both species using the different counting methods, the cell densities were not significantly different (*P* < 0.05; Fig. 1).

The mixed samples were assessed twice by qPCR. Firstly, they were run with the *Pseudochattonella* genus specific primers (primer set 2, Table 1) to

TABLE 1. Primers and hydrolysis probes for identification and enumeration of the species of *Pseudochattonella* used in this study. Total amplicon length = 97 bp.

Species	Strain number	Primer set	Forward primer (5'-3')	Reverse primer (5'-3')	Hydrolysis probe (5'-3')
<i>Pseudochattonella verruculosa</i>	JB08	1	GGGAGAAGTC CTTTGGAACAAGG	GCTAATGGA GTCGAGTTGC	TexasRed-TCAGA GAGGGTGACAAT CCCGTCT-BHQ2
<i>P. farcimen</i>	K-1809	2	GGGAGAAATT CTTTGGAACAAGG	CCTAGTGGA GTCGAGTTGC	Same as above

obtain the total cell counts and then with the *P. verruculosa* species-specific primers. *Pseudochattonella verruculosa* cell densities were then subtracted from the total to obtain the cell numbers for *P. farcimen*. We then compared the qPCR cell counts for each species with the expected cell densities from the mixed samples. As the mixed samples were generated by creating a 50:50 mix of each species, the expected cell densities were calculated by dividing the total cell counts obtained for each species LM by two (Fig. 2). For *P. farcimen* cell densities were similar throughout the entire growth cycle and there was no significant difference between the results obtained from LM and enumeration by qPCR (Mann-Whitney $P < 0.05$). However, for *P. verruculosa* cell densities were similar during the lag and exponential phases (Mann-Whitney $P < 0.05$), but the qPCR method provided an underestimation of cell numbers during the stationary and death phases.

Inhibition assay. Inhibition tests showed no inhibition for either *P. farcimen* in the late exponential (amplification efficiency = 95%, $R^2 = 0.99$) or stationary phase (amplification efficiency = 95%, $R^2 = 0.99$), or for *P. verruculosa* during the late exponential phase (amplification efficiency = 96%,

$R^2 = 0.99$) or stationary phase (amplification efficiency = 94.9%, $R^2 = 0.98$).

Determination of copy number. The average number of LSU rDNA copies in each growth phase for *P. farcimen* was $12,933 \pm 124$ in the lag phase, $12,522 \pm 297$ during the exponential growth phase, $7,141 \pm 131$ during the stationary phase and $3,655 \pm 150$ during the death phase. The average number of copies for *P. verruculosa* was $9,775 \pm 118$ in the lag phase, $9,796 \pm 75$ during the exponential growth phase, $8,526 \pm 65$ during the stationary phase and $5,387 \pm 119$ during the death phase. For both species there was with no significant reduction in LSU rDNA copy number per cell until the late exponential growth, stationary and death phases ($P < 0.5$; Fig. 3).

Field validation. As part of a monitoring program, field samples were taken from waters off the east coast of Jutland, Denmark in March–April 2015 when water temperatures were between 4.3°C and 8.8°C and salinities between 20 and 25. As with most monitoring programs only one sample was taken at each time point. It was impossible to distinguish between *P. farcimen* and *P. verruculosa* by light microscopy of fixed material but qPCR results suggested that only *P. farcimen* was present until water temperatures had reached 8.8°C when low numbers of 163 and 153 cells \cdot L⁻¹ of *P. verruculosa* were picked up using the qPCR assay. Figure 4 shows a comparison of cell number estimates by LM and qPCR and cell counts closely matched between methods until the post stationary death phase of the bloom on the 24th March 2015. Light micrographs of what was counted as *Pseudochattonella* cells are included as Figure 5.

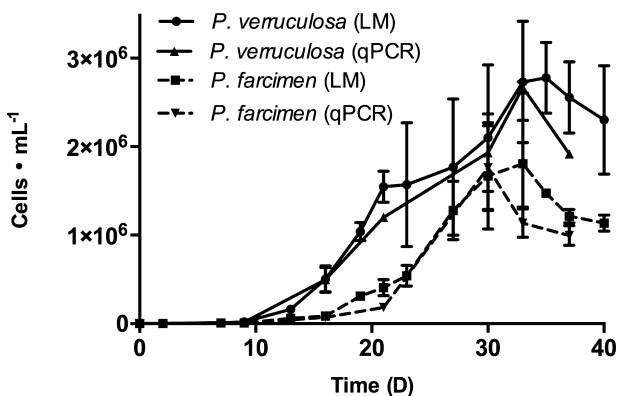


FIG. 1. Comparison of enumeration methods. Number of cells \cdot mL⁻¹ for *Pseudochattonella verruculosa* calculated by light microscopy (●) and by qPCR (▲) and the number of cells \cdot mL⁻¹ for *P. farcimen* calculated by light microscopy (■) and by qPCR (▼). All results are means of triplicate flasks and error bars are standard deviations ($n = 3$).

DISCUSSION

Pseudochattonella in Scandinavian waters. The aim of this study was to design, develop and test species-specific primers and probe set for the two only known species of *Pseudochattonella*, viz *P. verruculosa* and *P. farcimen*. Here we used a hydrolysis probe for additional specificity and constrained the primers and probe design to allow for multiplexing qPCR by having the same annealing temperature (60°C). We propose that this approach permits greater efficiency

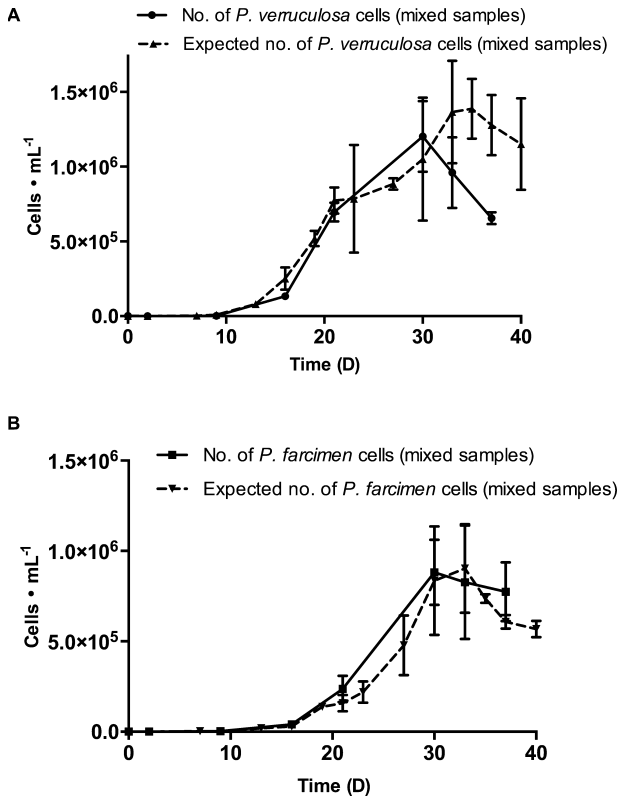


FIG. 2. Comparison of *Pseudochattonella* spp. cell counts using qPCR and their expected number in mixed cultures using LM. Number of cells · mL⁻¹ for *Pseudochattonella verruculosa* estimated by LM (●) and calculated by qPCR (▲) (A) and the cell densities · mL⁻¹ for *P. farcimen* estimated by LM (■) and calculated by qPCR (▼) (B). The expected cell densities were calculated by dividing the total cell counts obtained in LM by two.

in larger ecological studies when up to 4 species can be detected simultaneously (Eckford-Soper and Daugbjerg 2015b). However, our tests revealed that the primers-probe set for *P. verruculosa* was species specific, whereas the primers-probe set for *P. farcimen* gave positive results for both species. We therefore tested thoroughly whether subtracting cell numbers obtained using the *P. verruculosa* primers-probe from the cell numbers obtained using the primers-probe set for *P. farcimen* provided cell numbers of the latter species. Our results revealed that this approach worked very well when compared to cell numbers enumerated from light microscopy and as our tests covered the entire growth cycle. However, it should be noted that when cells entered the stationary and death phase the gene copy number underestimated the cell numbers. This so-called subtracting approach will be confounded when a third co-occurring species of *Pseudochattonella* is described, but until then the method can act as a work around the limited sequence data currently available for *P. verruculosa*. We therefore suggest it as an early warning detection

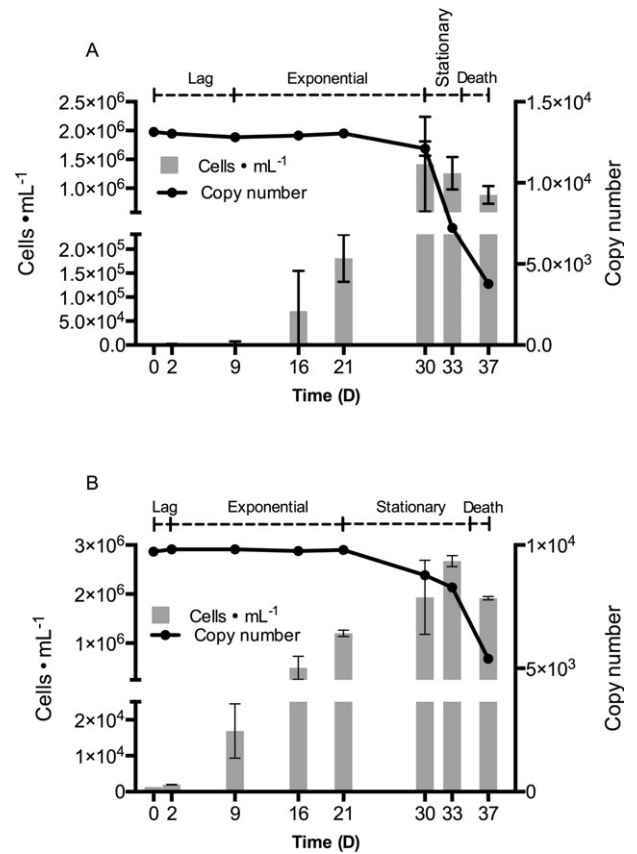


FIG. 3. Comparison of cell density and LSU rDNA copy number. Cell densities (left Y-axis) and LSU rDNA gene copy numbers (right Y-axis) as a function of time (day 0–37) during the growth experiments of (A) *Pseudochattonella farcimen* and (B) *Pseudochattonella verruculosa*. Broken lines above the calculated parameters indicate the different phases in the growth experiments. The left Y-axis is divided for reasons of scaling. Error bars are standard deviations ($n = 3$).

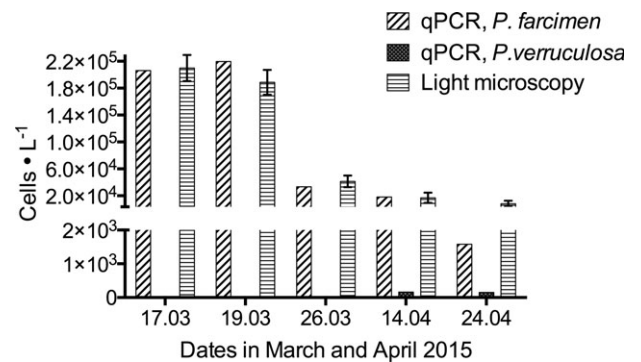


FIG. 4. Comparison of *Pseudochattonella* spp. cell counts in five field samples collected during a small bloom in March–April 2015, Denmark. Data were derived from qPCR of *Pseudochattonella farcimen* and *Pseudochattonella verruculosa* and *Pseudochattonella* spp. using light microscopy (Utermöhl method). Error bars are from precision percentage calculation of light microscopy cell counts.

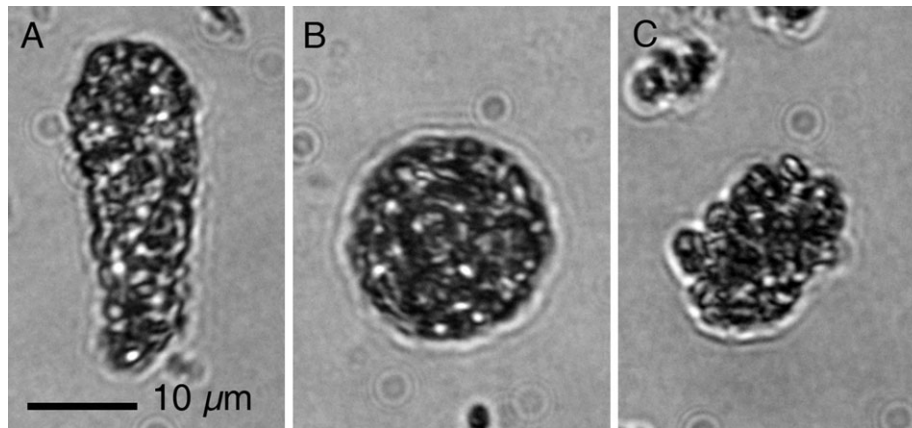


FIG. 5. Light microscopy (differential interference contrast) of *Pseudochattonella* cells as revealed in field samples and observed in an inverted microscope. (A) Elongated cell, (B) rounded cell, and (C) intermediate cell.

tool also allowing for studies of natural bloom dynamics during early and exponential growth.

Estimation of cell numbers using the qPCR assay. The observed underestimation of cell numbers in stationary and death phases of the growth cycle when based on the qPCR assay could be due to different reasons. As due care was taken to remove all the cells loss of material during processing and DNA extraction probably cannot explain the variation in cell number between the two methods used (LM vs. qPCR). Total qPCR results could also have been affected by inhibiting substances such as excreted extracellular substances. As the most common method to overcome inhibition is to dilute the inhibitors below their effective concentration we studied the amplification performance of the assay using a dilution series to remove any potential inhibitors. We did not observe a reduction in amplification efficiency and so direct inhibition did not cause the perceived reduction in cell numbers.

Rather we advocate that the underestimation was due to a reduction in LSU rDNA copy number as the cultures reached stationary and death phases. rDNA copy numbers have previously been shown to vary between and within species and strains and is thought to be one of the main reasons for contrasting results (Elser et al. 2008). Other species including *Alexandrium* spp. (Erdner et al. 2010), *Emiliana huleyi* (Nejstgaard et al. 2008) and *Ostreopsis* cf. *ovata* (Perini et al. 2011) have been observed to modify their copy number with physiological conditions and growth stage, with copy numbers declining as the cultures age. In *Alexandrium* spp. copy numbers have been shown to vary with varying conditions including: light:dark cycle, temperature and medium make up (Galluzzi et al. 2010). Previously Dittami and Edvardsen (2012) tried to correlate RNA content to cell numbers of *Pseudochattonella* but this proved to be unsuccessful.

These results show that variations in copy number are an important factor to consider when using DNA based detection methods especially when examining microalgae in the stationary and death phases. Distinct populations of closely related clonal lineages can form within the same species, each displaying different ranges of physiological capabilities. Each population may respond to and bloom under different environmental conditions meaning that a bloom could comprise of a mix of different growth phases (Rynearson et al. 2006). So, without determining the genetic composition of bloom-forming species it is recommended that a more appropriate quantification standard should take into account variations in both copy number and species diversity. This could be by creating standards using in situ target cells or to group a number of strains over a range of growth stages in order to calculate an average. Otherwise, the results obtained during the later stages of a bloom may only be semi-quantitative (Nejstgaard et al. 2008, Erdner et al. 2010, Dittami and Edvardsen 2012).

Cross-reactivity and sensitivity testing. The primers and probe sets used in this study were tested against the target and non-target species, which are found to co-occur in Danish waters. These included the closely related *D. speculum*, the dinoflagellates *A. tamarense*, *A. pseudogonyaulax*, *A. ostensfeldii*, *A. minutum*, *K. veneficum*, and *K. mikimotoi* and finally the haptophytes, *P. parvum*, and *C. polylepis*. Both primer and probe sets proved to be specific for *Pseudochattonella*, with a high amplification efficiency of between 95% and 100%. The reproducible limit of detection for cell numbers was below that of LM at 10^1 cells for both species, again highlighting the assays use under pre- and early bloom conditions.

Growth rates and bloom densities of Pseudochattonella spp. Maximum specific growth rates differed between the two species ranging from $0.28 \text{ div} \cdot \text{d}^{-1}$

for *P. farcimen* and $0.38 \text{ div} \cdot \text{d}^{-1}$ for *P. verruculosa*. This disparity in growth rates appears to be consistent with other studies. Skjelbred et al. (2013) described growth rates of between $0.41\text{--}0.52 \text{ div} \cdot \text{d}^{-1}$ for *P. farcimen* and $0.51\text{--}0.61 \text{ div} \cdot \text{d}^{-1}$ for *P. verruculosa* depending on the strain studied, whereas a study by Yamaguchi et al. (1997) observed an even greater growth rate for *P. verruculosa* of $1.74 \text{ div} \cdot \text{d}^{-1}$ at 15°C . However, Jakobsen et al. (2012) recorded much lower growth rates for *P. farcimen* of $0.25\text{--}0.26 \text{ div} \cdot \text{d}^{-1}$ which were much more consistent with those observed in this study. The contrast between growth rates of the same species most likely highlights the large differences in strain growth dynamics that exist in *Pseudochattonella* spp.

In the environment, cell densities of $\approx 10,000 \text{ cells} \cdot \text{mL}^{-1}$ have been associated with fish mortalities (Bourdelaïs et al. 2002). The mean peak cell densities differed significantly between the two species varying from $1.8 \times 10^6 \text{ cells} \cdot \text{mL}^{-1}$ for *P. farcimen* and $2.8 \times 10^6 \text{ cells} \cdot \text{mL}^{-1}$ for *P. verruculosa*. The maximum cell densities for *P. farcimen* was two orders of magnitude higher than those described by Jakobsen et al. (2012) as they only observed densities of $4.3 \times 10^4 \text{ cells} \cdot \text{mL}^{-1}$. Others have also described densities of $5 \times 10^5 \text{ cells} \cdot \text{mL}^{-1}$ for *P. verruculosa* (Imai et al. 1996).

Field results. In the field sample validation qPCR results suggested that only *P. farcimen* was present in March, as the water temperature ranged between 4.3°C and 5.1°C . However, in April, *P. verruculosa* was picked up in low concentrations by the qPCR assay when the water temperature reached 8.8°C . Both species have never before been observed simultaneously in the environment mostly due to the lack of specific detection methods. The temperature was below the optima for *P. verruculosa* of $12^\circ\text{C}\text{--}20^\circ\text{C}$ described by Yamaguchi et al. (1997) and Skjelbred et al. (2013). However, some cold-water species including *Pseudochattonella* species have been observed to have a higher temperature optima in culture compared to when they are found in the environment (Skjelbred et al. 2013). Total cell counts closely matched between the methods until the bloom reached the late post stationary death phase, when on the 24th of March 2015 the qPCR assay underestimated the total cell numbers. This underestimation is most likely due to a decline in the copy number caused by the diminishing environmental conditions. As with all phytoplankton-monitoring practices, the results were based on one count but the precision percentage error can be observed on the LM counts. The samples were taken during a period with a diverse community of nanoflagellates: six *Chrysochromulina* species, *Corymbellus aureus*, *Dichtyocha speculum*, as well as numerous unidentified species of cryptophytes and prasinophytes. This made identification of any individual species exceedingly difficult.

CONCLUSIONS

To the best of our knowledge, this is the first time that a molecular method has been developed where the cell number for species A is subtracted from the total cell number of species A+B to provide the cell number for species B. In terms of cell abundance estimation with qPCR, results were found to compare well with light microscopy in the lag and exponential phases for both *Pseudochattonella* species. These results underline the power of molecular tools in distinguishing between two closely related species that would have been difficult if not impossible to separate morphologically. Molecular approaches can give us a greater understanding of the distribution dynamics of these two closely related species through time and space and improve our understanding of their individual ecological niches. We propose that this method can be used in cases where two species are closely related and it is therefore difficult to design species-specific primers and probe sets or where nucleotide sequence data are still limited. This assay gives regulators a method, which allows for the discrimination and enumeration of these two closely related species, in turn allowing for its potential application in multi-species studies as well as monitoring programs.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web site:

Figure S1. Workflow diagram showing the design of sample processing. For each sampling date, we collected nine samples for enumeration by light microscopy and 9 samples for enumeration by qPCR. These included 3 × *Pseudochattonella farcimen* ($n = 3$), 3 × *P. verruculosa* ($n = 3$), and 3 × mixed ($n = 3$).

Figure S2. Time (days) in the growth curve where samples were selected for extraction of total genomic DNA.

Figure S3. Fragment of nuclear-encoded LSU rDNA highlighting the position of the forward primer, hydrolysis probe and reverse primer for *Pseudochattonella verruculosa* and *P. farcimen*. The designed primers for *P. verruculosa* showed to be species specific, whereas pairs for *P. farcimen* amplified both species.

Figure S4. Standard curves for *Pseudochattonella farcimen* (A) and *P. verruculosa* (B) showing efficiency (E) and R^2 -values which in all cases exceeded 95% and 0.99, respectively.