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Review

The ichthyotoxic genus *Pseudochattonella* (Dictyochophyceae): Distribution, toxicity, enumeration, ecological impact, succession and life history – A review

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ABSTRACT

The marine genus *Pseudochattonella* is a recent addition to the list of fish killing microalgae. Currently two species are recognised (viz. *P. verruculosa* and *P. farcimen*) which both form recurrent coastal blooms sometimes overlapping in space and time. These events and their ecological and economic consequences have resulted in great interest and concern from marine biologists and the aquaculture industry. Since the first recorded blooms in Japanese (late 1980s), Scandinavian (1993) and Chilean (2004) waters numerous studies have focused on understanding the causative means of the fish killing. Mortality is probably due to *Pseudochattonella* discharging mucocysts that cause gill irritation and damage to the fish fills. Here, a review is provided of the literature on *Pseudochattonella* that covers the last ca. 25 years and focus on a number of topics relevant to understanding the general biology of the genus including ways to distinguish the two species. The literature addressing biogeography and known harmful events is evaluated and based on these findings an updated distribution map is proposed. *P. farcimen* is presently restricted to North European waters. Despite being very difficult to delineate based on morphology alone the two *Pseudochattonella* species seem to have separate growth optima. In laboratory experiments *P. verruculosa* consistently has higher temperature growth optima compared to *P. farcimen* though periods of overlap have been noted in the field. The review ends by proposing five areas with knowledge gaps and each of these could form the basis of future studies.

Contents

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1. Introduction

The ichthyotoxic genus *Pseudochattonella* (Dictyochophyceae) is a marine heterokont phytoflagellate currently comprising two species (viz. *P. farcimen* and *P. verruculosa*). *Pseudochattonella* forms recurrent extensive blooms in coastal waters in Japan, New Zealand and Northern Europe (Baba et al., 1995; MacKenzie et al., 2011; Naustvoll et al., 2002). Recently the causative microalga for fish kill in Chile was identified to be *P.* cf. *verruculosa* (Alejandra Aguilera, personal communication). Blooms of *Pseudochattonella* spp. have proved be extremely damaging to fish not only those in intensive aquaculture systems but also wild stocks (Andersen et al., 2015). Fish mortalities of caged fish such as Atlantic salmon (*Salmo salar*) resulted in substantial financial losses to the aquaculture industry as well as impacting the marine ecosystem as a whole (Naustvoll, 2010; Clement et al., 2016). The mechanism of toxicity is yet unknown but the most common mode of action is by acute gill irritation and damage to the fish gills, reducing gas exchange efficiency (MacKenzie et al., 2011; Andersen et al., 2015). Here the literature surrounding the current biology of *Pseudochattonella*, one of the most recently added phytoflagellates to the list of harmful algal bloom (HAB) species is reviewed. The review is based on studies conducted over a period of ca. 25 years. The review ends with a proposal for the direction of future studies of *Pseudochattonenlla* to address some of the knowledge gaps uncovered.

2. Morphological characteristics and species identity

Both species of *Pseudochattonella* posses a variable morphology with size and form changing in response to growth phase and growth conditions (personal observations) making them virtually impossible to delineate to species level. Cell shape ranges from long allantoid to conical shaped and oval or small spherical cells (Fig. 1). These can occur with or without numerous mucocysts that when present are often evenly distributed giving the cell surface a warty-like appearance. Smaller spherical cells are often smooth. Elongated cells vary between 12–34 μm in length and 4–9 μm in width. Oval cells are approximately 14 μm long and 10 μm wide whereas smaller spherical cells are ca. 5 μm in diameter (Edvardsen et al., 2007). Spherical cells were observed to aggregate at the bottom of cell culture flasks, suggesting that they are less motile. Elongated cells contain 30–35 golden-brown chloroplasts (Edvardsen et al., 2007). Two heterokont flagella are present; a long anteriorly directed flagellum, which can range from 9 to 20 μm in length is used to pull the cell forward, while a shorter less obvious flagellum faces backwards. The mucocysts when present protrude outwards and vary in size and shape from oval to pointed, oblong and elongated. The peripheral mucocysts often discharge on fixation leaving an empty pocket and making identification using light microscopy difficult (Edvardsen et al., 2007; Jakobsen et al., 2012). Mucus secretion can be discharged laterally or posteriorly from the mucocysts forming tails. Some mucocysts have been observed to form chains (Chang et al., 2014). Small but 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 species-specific differences also exist in the morphology of their flagella hairs (Edvardsen et al., 2007).

3. Taxonomy

The taxonomic history of both species of *Pseudochattonella* (Y. Hara & Chihara) Hosoi-Tanabe, Honda, Fukaya, Inagaki & Sako has been in a state of flux. Originally the type species (viz. *P. verruculosa*) was described as *Chattonella verruculosa* Y. Hara et M. Chihara but it possessed certain features not present in the genus *Chattonella* B. Biecheler including the protruding mucocysts, the discoid chloroplast with embedded pyrenoids invaded by 1 or 2 canals and the absence of osmiophilic particles in the peripheral cytoplasm (Hosoi-Tanabe et al., 2007). Additionally a phylogenetic analysis based on nuclear-encoded SSU rDNA revealed that it clustered with members of the order Dictyochales within the class Dictyochophyceae. Hence, the genus *Pseudochattonella* was proposed for *P. verruculosa* (Y. Hara & M. Chihara) S. Tanabe-Hosoi, D. Honda, S. Fukaya, Y. Inagaki & Y. Sako making *Chattonella verruculosa* a synonym. One month later Edvardsen et al. (2007) suggested the new genus *Verrucophora* Eikrem, Edvardsen & Throndsen for *V. farcimen* Eikrem, Edvardsen & Throndsen and showed it to form a sister taxon to *P. verruculosa* in a phylogenetic analysis based on nuclear-encoded SSU rDNA. Due to reasons of priority (ICBN, Article 31) *V. farcimen* is now considered a synonym of *Pseudochattonella farcimen* (W. Eikrem, B. Edvardsen,

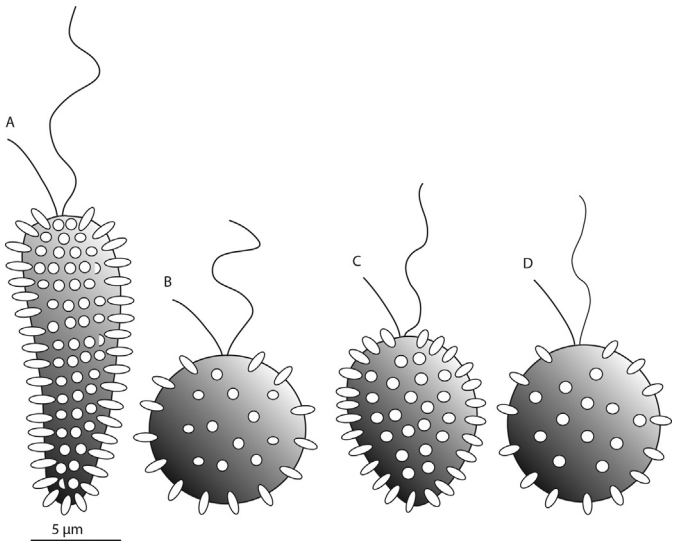

Fig. 1. Schematic drawings of *Pseudochattonella farcimen* (A, B) and *Pseudochattonella verruculosa* (C, D). (A, B) Two of the cell morphologies (elongate and small round shape). (C, D) Two of the cell morphologies (droplet and round shape). Numerous mucocysts are distributed on the surface of the cell.

& J. Throndsen) W. Eichrem (Eikrem et al., 2009). Hence, two species are currently assigned to the genus *Pseudochattonella*.

4. Molecular identification

As the indistinct morphological differences described above can only be observed under the transmission electron microscope (TEM), which is impractical for routine monitoring, molecular based methods have been applied for species identification. Despite the fact that the molecular sequence data available for species separation is still limited for *Pseudochattonella* spp. a handful of studies have attempted to use molecular tools for species identification and separation (Dittami et al., 2013a,b; Eckford-Soper and Daugbjerg, 2016; Riisberg and Edvardsen, 2008). The study by Riisberg and Edvardsen (2008) analysed a number of molecular markers including the nuclear-encoded small and large subunit rDNA, internal transcribed spacers ITS1 and 2 and 5.8S, chloroplast-encoded *psb*A, *rbc*L, and the RuBisCO spacer region, as well as the mitochondrial-encoded *cox*1 region. The sequence determination included a number of strains and SSU and LSU rDNA sequences were useful for delineating the two species.

Dittami et al. (2013b) attempted to develop a number of specific molecular methods for detection and separation of both *Pseudochattonella* species from environmental samples with varying degrees of success. The molecular technologies included dot blot hybridisation, PCR, qPCR and microarray. The genus specific SSU rDNA probes developed for the dot blot hybridisation assay proved to be specific for *Pseudochattonella* whereas a genus specific SSU-ITS1 rDNA primer set proved to be specific but with a too high intraspecific variability for distinguishing between species. A qPCR assay using LSU rDNA primers was able to distinguish the two species but was not able to accurately quantify cell numbers of either species. The microarray assay developed by the MIDTAL project with probes based on the dot blot SSU rDNA probes and LSU rDNA qPCR primers generated an indication of which species was dominant and had a detection limit of between 1000 and 5000 cells depending on the species (Dittami et al., 2013a).

To address the issue of limited sequence availability as well as low gene variability Eckford-Soper and Daugbjerg (2016) designed a qPCR assay for the detection and enumeration of *Pseudochattonella* spp. Two primers were designed, one genus specific for *Pseudochattonella* and one specific for *P. verruculosa* and a genus specific hydrolysis probe. Cell numbers for each species were then estimated by subtracting cell numbers for *P. verruculosa* from total cell numbers. Comparisons of cell abundance estimates obtained by qPCR and light microscopy (LM) showed no statistical difference until the stationary and death phases, when an under estimation in cell number occurred (Fig. 2). This reduction in assay efficiency was due to the reduction in gene copy number, which occurred alongside deteriorating culture conditions, making cell number estimated during this time only semi-quantitative. Until more sequence data becomes available this assay is perhaps the most accurate method available. Despite its shortcomings (e.g. when a third co-occurring species of *Pseudochattonella* is described and resides in the same biogeographic area as one or both of the currently known species) the assay will act as a suitable early warning detection tool, allowing for studies of natural bloom dynamics during early and exponential growth.

Molecular methods are also confounded by species, strain and growth phase differences in DNA and RNA content. Eckford-Soper and Daugbjerg (2016) showed a difference in copy number of the gene of interest between species with *P. verruculosa* having a significantly higher copy number compared to *P. farcimen* (Fig. 2). Mean DNA content has also been shown by Skjelbred (2012) to vary between the two species. Mean DNA content of *P. farcimen*

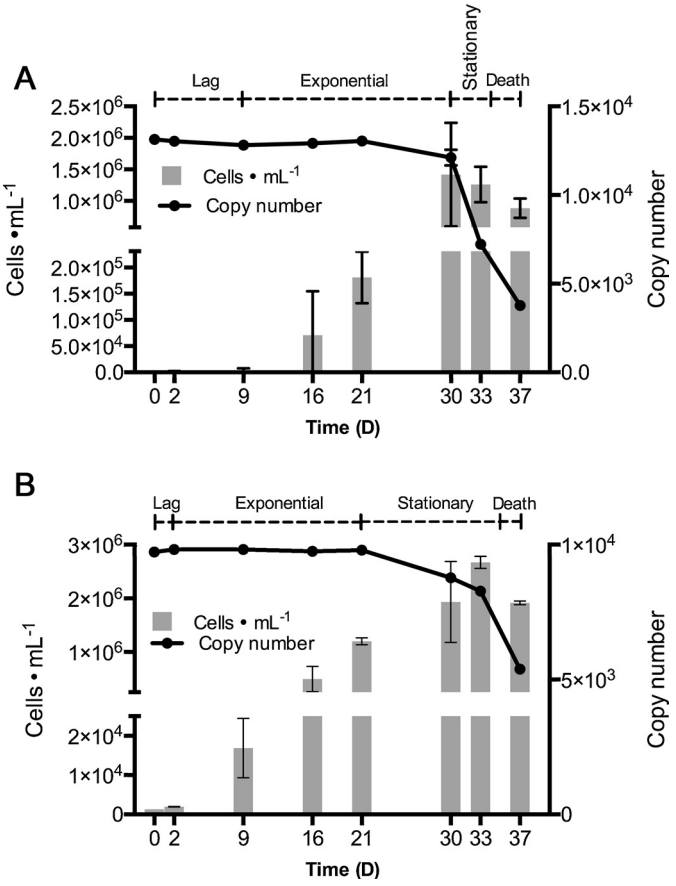

Fig. 2. Cell density and LSU rDNA copy number. Cell densities (left Y-axis) and LSU rDNA gene copy numbers (right Y-axis) as a function of time (day 0–37) during the growth experiments of *Pseudochattonella farcimen* (A) and *Pseudochattonella verruculosa* (B). The different phases in the growth experiments are indicated above the broken line. The left Y-axis is divided for reasons of scaling. Error bars are standard deviations ($n = 3$) (Eckford-Soper and Daugbjerg, 2016). Copyright Journal of Phycology, John Wiley and Sons.

was estimated to be 0.46 ± 0.11 pg cell^{-1} (453 Mbp) for nuclei stained with promidium iodide (PI) and 0.60 ± 0.11 pg cell^{-1} (590 Mbp) for SYBR green I. DNA content was higher by a ratio of 1.7 in *P. verruculosa*, where mean DNA content was estimated to be 0.77 pg cell^{-1} (750 Mbp) for PI and 1.42 ± 0.11 pg cell^{-1} (1393 Mbp) for SYBR green I (Skjelbred, 2012).

Molecular RNA based methods are also affected in a similar manner. Dittami and Edvardsen (2012) showed a reduction of per-cell RNA content in *Pseudochatonella* species by almost one order of magnitude depending on culture conditions. RNA yields ranged from 0.89 to 7.7 pg cell^{-1} in *P. farcimen* and 0.25 to 1.9 pg cell^{-1} in *P. verruculosa*, which makes it hard to estimate per-cell RNA yields. This affects the use of RNA based detection methods in *Pseudochattonella* species. Despite this, RNA content was well correlated with cell volume and therefore would act as a good proxy for total biovolume (Dittami and Edvardsen, 2012).

5. Life cycle

Chang et al. (2014) described a tentative life cycle for a strain of *P. verruculosa* isolated from New Zealand waters. They observed that under favourable conditions up to 90% of the cells were elongated with the rest varying between oval and pyroform and small and round. As cultures aged and conditions became suboptimal large numbers of small rounded or oval (7–12 μm) uninucleate cells formed. These cells contained fewer chloroplasts

compared to the larger elongated cells. It was thought that these small cells acted as gametes which fused to form zygotes. After this, some form of nuclear division occurred resulting in large multinucleated cells, which had four or more nuclei. The cytoplasm of each multinucleate cell then quickly divided with the chloroplasts amassing into four or more separate accumulations, each with their own nucleus. These accumulations were then enclosed to form numerous daughter cells within the original parent cell. In some circumstances the multinucleate cells fused with each other to produce massive aggregations (200 μm). Tomas et al. (2002) also observed these multicellular aggregations at the bottom of older cultures of *P. verruculosa* isolated from Japan and the North Sea. These massive aggregations can potentially contain huge numbers of daughter cells that eventually push their way out of the membrane of the parent cell. Once all daughter cells were released the entire membrane of the parent cell disintegrated (Chang et al., 2014). The function of these large aggregations has been debated. One popular hypothesis is that they act as resting stages when conditions become unfavourable (Chang et al., 2014; Jakobsen et al., 2012). A multinucleate stage very similar to *Pseudochattonella* has also been observed in another dictyochophyte *Dictyocha speculum* by Henriksen et al. (1993).

6. Chemical markers

6.1. Lipid markers

Differences are evident in the chemical composition of each species. *Pseudochattonella* spp. have also been shown to contain a high proportion of polyunsaturated fatty acids (PUFAs). PUFAs are thought to act as toxins, precursors for toxins and grazer deterrents (Dittami and Edvardsen, 2012). The high ratios of docosahexaenoic acid (DHA) to eicosapentaenoic acid (EPA) and the presence of high concentrations of the rare PUFAs C1s8:5n-3 and C16:1n5 are unusual features (Dittami and Edvardsen, 2012; Giner et al., 2008). *Pseudochattonella farcimen* has been shown to have several PLPA2 genes, which may be connected to the release of certain free PUFAs and polyunsaturated aldehyde (PUAs) compounds. These compounds have previously been shown to have toxic effects on several taxonomic groups including fungi and algae (Dittami et al., 2012; Leflaive and Ten-Hage, 2009).

Both species produce the rare 27-nor sterol called occelasterol that may substitute for cholesterol and could act as a chemotaxic marker. As occelasterol is unlikely to act as a substitute for cholesterol for grazing zooplankton it may be a mechanism for the reduction in grazing pressure of zooplankton, contributing to bloom formation (Giner et al., 2008).

6.2. Photosynthetic pigments

HPLC pigment analysis of the Wellington isolate revealed that chlorophyll *a* was the major pigment, but it also contained chlorophylls c_2 and c_3. Fucoxanthin, was the dominant carotenoid, and 19′-butanoyl-oxyfucoxanthin, diadinoxanthin, zeaxanthin and ditoxanthion were the minor pigments. Other pigments included β-carotene and ε-carotene (Chang et al., 2014). This pigment profile was in slight contrast to an analysis by Tomas et al. (2002) on a North Sea isolate of *P. verruculosa*. Like the Wellington isolate, the North Sea isolate contained chlorophylls *a*, c_2 and c_3, 19′-butanoyl-oxyfucoxanthin, fucoxanthin, diadinoxanthin, zeaxanthin but also chlorophyll c_1, violaxanthin and lutein. A comparison of a Japanese and North Sea isolate of *P. verruculosa* revealed the absence of antheraxanthin in the former. A pigment analysis of *P. farcimen* by Edvardsen et al. (2007) reported a composition of chlorophylls *a*, c_1, c_2 and c_3, 19′-butanoyl-oxyfucoxanthin, fucoxanthin, diadinoxanthin, and β-carotene, but unlike *P. verruculosa* it lacked zeaxanthin and violaxanthin. The slight variations in pigment profiles in strains of *P. verruculosa* seem to indicate population specific differences.

7. Biogeographical distribution and known fish-killing events

The currently recognised world-wide distribution of *P. verruculosa* and *P. farcimen* is illustrated in Fig. 3.

7.1. Japan

Pseudochattonella verruculosa (syn. *Chattonella verruculosa*) forms recurrent blooms in Japan, which have been associated with fish mortality such as amberjack (*Seriola quinqueradiata*), red (*Pagrus major*) and black (*Acanthopagrus schlegelii*) sea breams in early summer and winter since the 1980s (Baba et al., 1995; Imai et al., 1998, 1996; Yamamoto and Tanaka, 1990).

7.2. New Zealand

Pseudochattonella verruculosa is the only species of *Pseudochattonella* described in New Zealand waters (MacKenzie et al., 2011). The first fish-killing event attributed to *P. verruculosa* in New Zealand occurred in Queen Charlotte Sound, South Island in 2010 (MacKenzie et al., 2011). A phylogenetic analysis that included three different gene sequences (LSU rDNA, *rbc*L and COI) of a *Pseudochattonella* isolate termed the 'Wellington Isolate' by Chang et al. (2014) revealed conflicting results. The LSU rDNA and *rbc*L analyses resolved the Wellington isolate with other but geographically separated strains of *P. verruculosa* whereas the COI analysis resolved it with cultures of *P. farcimen*. It was suggested that this isolate was a product of a hybridisation event between *P. farcimen* and *P. verruculosa* resulting in the inclusion of the *P. farcimen* mitochondrial genome into a *P. verruculosa* with a nuclear and plastid background. Then either a gene conversion or backcrossing into *P. verruculosa* has reduced or erased all *P. farcimen* LSU sequences. As *P. farcimen* has not yet been observed outside European waters this scenario needs to be confirmed using a genomics approach. Only then can we infer the true phylogeny of this isolate.

7.3. Scandinavia

The first registered bloom of *Pseudochattonella* in European waters was recorded between April and May 1998. Further, re-examination of preserved water samples may have found *Pseudochattonella* in samples collected in Kattegat in as early as 1993 (Riisberg and Edvardsen, 2008). The 1998 bloom formed off the west coast of Germany, Denmark, Sweden and Norway killing 350 tonnes of farmed fish as well as some wild stocks (Riisberg and Edvardsen, 2008). As the organism responsible for this event resembled that of *Chattonella verruculosa* from Japanese waters (Hosoi-Tanabe et al., 2007) it was initially named *Chattonella* aff. *verruculosa*.

A second bloom in 2000 occurred between April-May in the German Bight and off the Coast of Jutland, Denmark with cells reaching 11×10^6 cells L^{-1} at its maximum (Lu and Goebel, 2000). This was followed by another extensive bloom between March and April in 2001, reaching from Kattegat, Skagerrak up to the southern coast of Norway (Naustvoll et al., 2002). Two smaller blooms occurred in 2004 along the Swedish west coast and another in the Danish North Sea (Naustvoll et al., 2002). Since then *Pseudochattonella* spp. have been recorded almost annually in North European waters causing large scale fish mortality events in the Kattegat or waters along the Danish west coast leading to substantial economic losses (Edvardsen et al., 2007; Riisberg and Edvardsen,

Fig. 3. Distribution map of *Pseudochattonella farcimen* (○) and *Pseudochattonella verruculosa* (■).

2008). The biogeographic range is now thought to include the German coast from the North Sea coast of Schleswig to Holstein, the Danish North sea coast and the Skagerrak, the Swedish west coast from Hvaler to Bergen (Naustvoll, 2010) and the Gulf of Gdansk, Poland (Łotocka, 2009) and also potentially in French and Dutch waters (Naustvoll, 2010).

The *Pseudochattonella* blooms which developed in along the coasts of Germany, Denmark, Sweden and Norway in 1998, 2000, 2002, and 2004 occurred between April and May, when water temperatures were relatively warm, peaking at 18 °C (Backe-Hansen et al., 2001; Lu and Goebel, 2000; Riisberg and Edvardsen, 2008). The blooms that formed in 2001, 2006, 2007, 2009, 2011 and 2015 developed from January to March and often overlapped with the spring bloom of diatoms. During these periods the water temperatures were much lower ranging from 2 to 5 °C (Andersen et al., 2015; Edvardsen et al., 2007; Riisberg and Edvardsen, 2008; Eckford-Soper and Daugbjerg, unpublished). Until recently it has not been possible to actively delineate and quantify the two species and therefore the causative species of each bloom was not determined at the time. It is now proposed that due to the distinct temperature preferences determined by Yamaguchi et al. (1997) and on *P. verruculosa* and *P. farcimen* by Jakobsen et al. (2012) and Skjelbred et al. (2013) the blooms that developed earlier in the season were exclusively formed by *P. farcimen* and the later blooms more exclusively *P. verruculosa*. Further evaluation of nuclear, chloroplast and mitochondrial encoded DNA sequences later identified that the strains isolated from the German North Sea in 2000 were *P. verruculosa*, whilst strains isolated from the Skagerrak in 2001 and 2006 were *P. farcimen* (Riisberg and Edvardsen, 2008). This isolate was also shown to be almost identical in five different genes (LSU, SSU rDNA, *cox1*, *psb*A and *rbc*L) to *P. verrucuolsa* strains isolated from blooms in Japan and New Zealand. This certainly indicates that *P. verruculosa* could be an introduced species in Scandinavian waters (Hopkins, 2000). Despite the apparent separation in temperature ranges a period of overlap does occur between the species as Eckford-Soper and Daugbjerg (2016) observed the two species co-occurring in environmental samples from Danish waters in 2016 (Fig. 4). In March 2016 *Pseudochattonella verruculosa* appeared in low densities (~160 cell ml^{-1}) alongside of *P. farcimen* (~18,000 cells ml^{-1}) when water temperatures were 8–9.5 °C.

7.4. USA

On the eastern coast of the United States, extensive monitoring programmes have recorded a *P. verruculosa*-like species in Chesapeake Bay and the brackish lagoons in South Carolina (Hayes and Lewitus, 2003). Despite this, a later study using SSU rDNA sequence data and pigment analysis grouped the American isolate together with the raphidophytes (Tomas et al., 2002). Hence, *Pseudochattonella* species have not yet been documented in the USA.

7.5. Chile

Pseudochattonella cf. *verruculosa* was first recorded in January of 2004 in the vicinity of the Cholgo area (Mardones et al., 2012). It has reoccurred in January and February 2005, 2009, 2011 and most recently in March 2016 (Clement et al., 2016; Mardones et al., 2012). During the first recorded bloom *P.* cf. *verruculosa* cell densities reached a maximum of 40 cells ml^{-1} at a water temperature of 15 °C and a salinity of 32.5 (Clement et al., 2016). More recently peak abundances have reached approx. 400 cell ml^{-1} when water temperatures ranged between 10 and 18.5 °C and salinities between 11 and 33. In March 2016 a massive fish kill was recorded in the northern Chiloé Archipelago, Calbuco

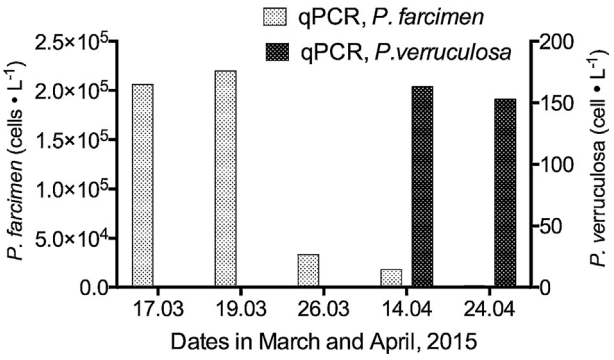

Fig. 4. Occurrence of *Pseudochattonella farcimen* and *Pseudochattonella verruculosa* in Danish waters (March–April, 2015). Identity confirmed by qPCR assay.

and Reloncaví Sound and Fjord with an estimated loss of 100,000 tonnes of Atlantic and Coho salmon and trout. This equals as much as 15% of Chile's yearly production and an estimated export loss of $800 million. During that bloom peak cell densities reached 7700 cells ml^{-1} (Clement et al., 2016).

8. Optimal growth conditions

8.1. Environmental parameters

In situ observations of previous *Pseudochattonella* blooms indicate that these commonly occur in coastal water masses characterised by shallow water or a shallow pycnocline, a salinity of 20–30 and a relatively high concentrations of nitrate (Chang et al., 2014). Populations take 2–3 weeks of growth to turn into a major bloom. During pre-bloom conditions nutrient concentrations are high with ample but not too high solar illumination (Backe-Hansen et al., 2001). Studies of vertical profiles have indicated that cells commonly accumulate in the upper few metres, with the highest abundances occurring in the top 5–10 m, but they have also been observed down to 30 m in the Skagerrak. *Pseudochattonella* species can dominate in late winter/early spring conditions when light due to the low solar angle and short day length can limit the compensation value as photosynthetic photon flux density has been shown by Skjelbred et al. (2013) to range between 4.2 and 14.9 μmol photons m^{-2} s^{-1} and saturation irradiances between 18.3 and 51 μmol photons m^{-2} s^{-1}. Jakobsen et al. (2012) observed a lower compensation irradiance of around 1.3 μmol photons m^{-2} s^{-1} making *P. farcimen* an ideal competitor at low light levels.

A number of laboratory studies have been carried out to assess the optimal conditions for growth in both species. These studies have assessed maximum specific growth rates under changing light, temperature and salinity regimes. Jakobsen et al. (2012) observed positive growth for *P. farcimen* over a range of temperatures with positive growth of 0.09 d^{-1} at as low as 1 °C. Some authors suggest that 10 °C is close to the upper temperature limit of *P. farcimen* (Edvardsen et al., 2007; Jakobsen et al., 2012). Contrary to this, Eckford-Soper and Daugbjerg (unpublished) demonstrated positive growth up to 15 °C and Skjelbred et al. (2013) observed positive growth as high as 20 °C with optimal growth occurring between 9 and 14 °C. In the study by Skjelbred et al. (2013) maximum specific growth rates ranged from 0.41 ± 0.04 and 0.52 ± 0.05 depending on the strain. The study also calculated the preferred salinity being in the range of 19 and 25. This preference for intermediate salinities might be an adaptation in order to reduce the energy required for osmotic adjustment (Skjelbred et al., 2013).

Despite having a temperature optima at higher temperatures the 'lowest minimum rates of loss' were observed at temperatures and salinities lower than their apparent optima between temperatures of 5 and 8 °C and salinities of 26 and 31. The temperatures associated with the smaller rates of loss are more consistent with *P. farcimen* blooms in nature (Lu and Goebel, 2000; Backe-Hansen et al., 2001; Edvardsen et al., 2007; Riisberg and Edvardsen, 2008). As there are energetic costs in the production of a light harvesting apparatus and as temperatures increase so will enzymatic activities and respiration. This could give cold water species, which are adapted to low light conditions, a competitive advantage in late winter-early spring. Thus, as maintenance costs are reduced at lower temperatures it may not be necessary to have such high division rates, as long as irradiance is above the compensation point. The ability to maintain positive net growth at lower temperatures may give a number of competitive advantages, such as the ability to monopolise available nutrient sources before the appearance of competing taxa and by removing grazing pressure by blooming before herbivores.

Temperature optima for *P. verruculosa* in all studies is consistently higher compared to *P. farcimen* ranging from 16 to 27 °C. Growth ceased at lower temperatures with no positive growth occurring below 4 °C (Skjelbred et al., 2013). The failure of *P. verruculosa* to grow at the lower temperatures show that temperature represents a strong ecophysiological barrier for survival of *P. verruculosa* in colder waters. *P. verruculosa* has a broader salinity optima range from 20 to 32. These ranges are in accordance with field observations in Japan as well as the optimal ranges described in the laboratory using Japanese isolates by Yamaguchi et al. (1997) of 11–20 °C and 15–53. Growth rates of *P. verruculosa* are typically higher compared to *P. farcimen.* Yamaguchi et al. (1997) recorded growth rates as high as 1.74 d^{-1}, while others have recorded more modest growth rates ranging from 0.51 ± 0.15 to 0.61 ± 0.07 d^{-1}, Eckford-Soper and Daugbjerg (unpublished) and Skjelbred et al. (2013), respectively.

8.2. pH

pH tolerances vary between the species with *P. farcimen* being more sensitive to high pH. Eckford-Soper and Daugbjerg (unpublished) observed no growth above pH 8.4. Yet, Jakobsen et al. (2012), reported a higher pH tolerance of 8.9–9. In comparison, *Pseudochattonella verruculosa* had a higher tolerance to pH with growth ceasing at pH 9.1 (Eckford-Soper & Daugbjerg, unpublished). The reason for the observed difference in pH tolerance between the two species in the Eckford-Soper and Daugbjerg study is unknown. It may be speculated that photosynthetic activity due to the spring bloom of diatoms in temperate waters in combination with the growth of *P. verruculosa* results in higher pH levels. Thus, over time *P. verruculosa* has become adapted to tolerate higher pH levels. In contrast, *P. farcimen* blooms early in the season and prior to the diatoms. pH levels are therefore impacted less by photosynthetic activity with hardly any contribution from other microalgae.

9. Toxicity

The ichthyotoxic mechanism in *Pseudochattonella* is elusive with the mode of action yet to be fully determined. Both *P. farcimen* and *P. verruculosa* have been reported to be ichthyotoxic with fish mortalities being attributed to *P. verruculosa* as early as 1989. Despite this, the active compound (if any) responsible has to be characterised.

Research on toxicity has proven to be difficult. Most examinations of affected fish have shown damage to the gills, which could have either been caused by toxins, direct gill irritation of *Pseudochattonella* cells or mucus on the gills (Andersen et al., 2015). The threshold needed to induce fish death is species and most likely also strain specific. For *P. farcimen* the number of cells needed is thought to be approx. 500 cells ml^{-1} (Andersen et al., 2015). Contrary MacKenzie et al. (2011) demonstrated that only around 10 cells ml^{-1} of *P. verruculosa* were required to cause mortality of salmon in New Zealand.

A number of studies on the toxic effects of *Pseudochattonella* have been carried out on whole fish, gill cells, embryos and model organisms. This has led to different opinions regarding toxicity. Andersen et al. (2015) considered that live cells are required to induce a toxic effect on fish, whereas Skjelbred et al. (2011) observed toxic effects using algal extracts only. Despite this, most studies relate toxicity to some effect on the gill tissue (Andersen et al., 2015). Caged salmon exposed to *P. farcimen* during a bloom in the Skagerrak in 2001 showed necrosis in the gill epithelia, bleeding in the orbital cavity and peritonitis. Skjelbred et al. (2011) demonstrated an impaired functioning and performance of the gills in cod (*Gadus morhua*) and salmon smolts (*Salmo salar*) that

had been exposed to very high cell densities of 1–3 $\times$ 10^7 cells L^{-1} of *P. verruculosa*. Symptoms included hypertrophy, oedema, detachment of the epithelial cells, thickening of the secondary lamellae as well as necrotic cells and cells with swollen nuclei. Although no fish deaths occurred during this study it was suspected due to the impaired gill performance the effects would have eventually proved to be fatal. MacKenzie et al. (2011) only observed signs of gill irritation during histological examinations in the form of mucous cell hypertrophy (swelling) on cultured stocks of salmon (*Oncorhynchus tshawytscha*) exposed to *P. verruculosa* in New Zealand. It was however, suspected that this might have been an artefact of their method of fish euthanasia. On examination of rainbow trout (*Oncorhynchus mykiss*) exposed to *P. farcimen* Andersen et al. (2015) detected epithelial lifting as well as nuclear disintegration in the gill tissue, which had increased the distance between the water and the pillar cells causing a reduction in gas exchange efficiency. MacKenzie et al. (2011) have been one of the only authors to document effects on organs other than the gills when they detected shrinkage of the glomeruli within the kidneys and extensive diffuse hydropic changes in the livers of moribund salmon. Liver problems have also been reported after exposure to *P. verruculosa* (Morten B.S. Svendsen, personal communication).

Size and species related susceptibility differences have been highlighted in a number of studies. Larger fish appear to be more susceptible to the effects of *Pseudochattonella* compared to smaller fish. In May 1998 a dense bloom of *Pseudochattonella* spp. occurred off the south-west coast of Norway killing 350 tonnes of Atlantic Salmon (*Salmo salar*). This was followed by the deaths of wild stocks of garfish (*Belone belone*), herring (*Copea harengus*), sand eel (*Amnocytes* spp.) and mackerel (*Scromber scrombrus*). A large percentage of the fish that were affected were over 2 kg with a much lower mortality rate in smaller fish. Andersen et al. (2015) observed species related susceptibility differences in the toxic effects of *P. farcimen* on different species. No effect was seen on whitefish (*Coregonus lavaretus*) or cod (*Gadus* spp) in either the laboratory or field. Larger rainbow trout (*Oncorhynchus mykiss*) ($<$500 g) and salmon 1–2 kg, exhibited behavioural changes when exposed to *P. farcimen*. The behavioural responses included irregular and upside down swimming and no escape reaction. It was hypothesised that size related differences in the susceptibility are due to larger fish being more readily affected by a reduction in gill efficiency in terms of gas exchange.

Skjelbred et al. (2011) undertook a number of in vitro cytotoxicity assays using algal extracts of *P. farcimen* and *P. verruculosa* including an assay with chinook salmon embryo cells and rainbow trout primary hepatocytes and strain and species specific differences were evident. The cytotoxic effects on the embryo cells included decreased mitochondrial activity, metabolism inhibition and loss of membrane integrity in the primary hepatocytes. The toxicological effects of the extracts were greatest within the first 24 hours and declined thereafter. In this instance the toxic effects were attributed to the free fatty acids STA (stearidonic acid) and EPA (eicosapentaenoic acid).

Chang et al. (2014) tested the cytotoxicity of whole cells as well as a lipophylic extract of the Wellington isolate on rotifers. In the feeding experiments using whole cells, rotifers began to discharge undigested food and cellular material through their oral cavities. Within 24 h 30% of the rotifers had perished whereas after 90 h 90% had perished. Again the dead animals had discharged undigested food and other cellular material through their oral cavities.

Skjelbred et al. (2011) performed toxicity tests on the nauplii of brine shrimp (*Artemia leach*) and no acute toxicity was detected after 24 h of exposure to either species of *Pseudochattonella*. During this period *Artemia* actively fed on the algal cells resulting in sticky faecal pellets, which caused individuals to become stuck to each other through their faeces. This mode of action on grazers could reduce the grazing pressure.

10. Knowledge gaps

We still have knowledge gaps in the basic understanding of the biology and harmful effect of *Pseudochattonella* spp. Some of these gaps are identified below and could form the basis of future studies.

10.1. Allelopathic ability

Environmental conditions in late winter-early spring are characterised by low light levels but plentiful nutrients. In order to bloom under such conditions *Pseudochattonella* must be able to successfully compete against more competitive groups such as the diatoms when like other flagellates they have low growth rates (Jakobsen et al., 2012). The actual mechanism allowing *Pseudochattonella* to actively compete against other phytoplankton species is yet unknown. Jakobsen et al. (2012) failed to observe any chemically mediated allelopathic interactions between *P. farcimen* and the dinoflagellate *Heterocapsa triquetra* and the diatoms *Chaetoceros decipiens* and *C. diadema* in mixed growth experiments. Nevertheless, species-specific differences in sensitivity are common and some species can influence the allelopathic efficiency of other species (Prince et al., 2008). It would therefore be interesting to further assess the allelopathic ability of both species of *Pseudochattonella* on a number of co-occurring phytoplankton species across a number of microalgal groups.

10.2. Nutrient utilisation

Nutrient supply is an important factor influencing the overall structure of the microbial food web. A mechanism for successful competition is to have high uptake efficiencies and nutritional modes as many competition studies suggest that high nutrient uptake and high nutrient affinities are advantageous to primary producers. It would be interesting to assess the nutrient uptake rates of *Pseudochattonella* species and how this compares to competing phytoplankton species in order to assess niche differentiation. Data could be used in resource competition models to simulate competition between phytoplankton species in late winter-early spring.

10.3. Molecular characterisation

In order to better characterise and further develop molecular based assays for distinguishing between the two species more sequences need to be determined. This will also allow a more detailed phylogenetic analysis of both recognised *Pseudochattonella* species with the as yet undetermined Wellington isolate and any potential hybrids. A connection needs to be made between traditional biological, ecological and system modelling approaches and newer molecular genomic-based techniques. 'Omics' studies have revolutionised the understanding of how species have evolved and adapted to their changing environment through mechanisms such as gene or protein regulation. It creates a greater understanding of how organisms react and respond to changing conditions. Combining omics data with ecophysiological measurements and environmental studies will provide a fully integrated approach to understanding the structure and function of ecosystems.

10.4. Biogeography

The understanding of the biogeographical distribution pattern of the two *Pseudochattonella* species is incomplete. It is speculated

also to exist in French and Dutch waters but this has not been confirmed. The identity of the Chilean species of *Pseudochattonella* was only recently confirmed by molecular methods but the extent of their distribution range is still unknown. A more comprehensive understanding of the species distribution along with environmental parameters would allow us to examine functional relationships and to better predict and model the dynamics of future blooms.

10.5. Harmful effects

The mechanism of harm, which leads to mortality in fish and predatory invertebrates, is yet to be established. If the effect is caused by direct intoxication (i.e. toxic compound(s)) or by gill irritation leading to mucus secretion and ultimately suffocation needs to be determined. Isolation and characterisation of intracellular and extracellular material including determining the chemical composition of the discharged mucus from the mucocysts would give us a greater understanding of the processes. This would provide a greater understanding as to the harmful modes of action of *Pseudochattonella* spp.

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