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Phytoflagellate diversity in Roskilde Fjord (Denmark), including the description of *Pyramimonas octopora* sp. nov. (Pyramimonadales, Chlorophyta)

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**ABSTRACT**
Identification of planktonic flagellates is challenging, and multiple species are often lumped together, limiting our understanding of their diversity and ecology. Our aim was to investigate changes in the phytoplankton community composition over the annual cycle in Roskilde Fjord, Denmark, focusing on the identification of organisms smaller than 20 μm. Weekly microscope and pigment data indicated a low contribution of diatoms and dinoflagellates and the importance of cryptophytes, euglenophytes and green algae, with high contributions of alloxanthin and chlorophyll b. Community composition using Serial Dilution Cultures (SDC) confirmed the high contribution of cryptophytes, prasinophytes and euglenophytes in the phytoplankton community. In contrast, diatoms only played a minor role. Seasonal changes were observed within cryptophytes, with *Teleaulax* dominance during cold months shifting to *Hemiselmis* in summer. Small prasinophytes and pico-eukaryotes were always present but had a low relative contribution to the total phytoplankton biomass. Cultured organisms enabled the identification of *Hemiselmis cf. cryptochromatica*, recorded for the first time in European waters, and an undescribed *Pyramimonas* species. We describe the new species as *Pyramimonas octopora* sp. nov., placed within *Pyramimonas* subg. *Vestigiferae*, based on morphological and molecular (SSU rDNA) data. The distinctive features of the new species are the eight irregularly rounded perforations arranged in a square box with rounded corners around the centre of the base plate of the box scales, and the complex crown scales, not observed in previously described *Pyramimonas* species.

**INTRODUCTION**
Phytoplankton comprises different taxonomic groups, with organisms spanning over a broad size range, from less than one to a few hundred micrometres. The diversity of phytoplankton communities is related to the efficiency of resource use and ecosystem stability (Ptacnik et al. 2008). In many marine and estuarine ecosystems, small-sized phytoplankters are given less attention in comparison to larger phytoplankton such as diatoms and dinoflagellates, which often dominate the biomass. However, small phytoplankton is a pervasive part of the community and can dominate phytoplankton at specific periods of the year, especially when chlorophyll a concentrations are low (Smetacek 1981; Levasseur et al. 1984; Haraguchi et al. 2015). Thus, even though high biomass events are rarely associated with small-sized organisms (<20 μm), this size fraction is considered an important component of the planktonic communities due to their capability to cope with low nutrient conditions (Edwards et al. 2012), their high division potential (Banse 1982) and because they fit the optimal prey size for different predators (Hansen et al. 1994, 1997).

Despite the large diversity found among flagellates, they are often pooled together into coarse groups in which size is the only distinctive feature (Thronsden 1993; Zingone et al. 2015). This leads to loss of information on diversity, ecology and functionality of a significant portion of the phytoplankton community (Piwowz 2019). One of the main issues limiting a deeper characterization of small-sized phytoplankton is that many of them do not preserve their distinctive features with the fixatives commonly used in routine light microscopy analysis (Hasle 1978; Thronsden 1978a). Even among organisms that still preserve features such as shape and flagella, other key information like motility and colour is nevertheless lost, which in some cases can hamper proper identification (Thronsden 1993) and even distinction between autotrophic and heterotrophic organisms. Studies investigating the taxonomic composition of small flagellates usually increase the knowledge of local diversity, demonstrating that flagellate diversity is often overlooked (Thomsen 1992; Thomsen & Buck 1998; Bergesch et al. 2008; Percopo et al. 2011). In addition, species abundance and contribution change over seasons and years (Thomsen 1992; Cerino & Zingone 2006), demonstrating that the small flagellate assemblages are dynamic, with their composition responding to changes in the environment. Therefore, they are more than only a ‘background’ community.
At the mesohaline temperate estuary Roskilde Fjord (RF), Denmark, long-term data have shown that small organisms make up a large proportion of the phytoplankton community (Haraguchi 2018). Hence, the present study aims to investigate the phytoplankton community composition during the 2016 annual cycle in RF, focusing on the identification of small flagellates (<20 µm). Additionally, we include the description of a new species, *Pyramimonas octopora* sp. nov.

**MATERIAL AND METHODS**

**Area description**

Roskilde Fjord (RF) is a mesohaline, shallow (average depth is 4 m) and well-mixed estuary located in Denmark (Fig. S1). It has two large basins connected by a narrow channel, with the outer (northern) basin connected to the adjacent Kattegat by a narrow opening. Due to RF morphology, low river discharge and tidal influence, the freshwater residence time in the inner basin can be up to 8 months (Kamp-Nielsen 1992). The catchment area in the region is dominated by agriculture, resulting in high nutrient inputs that stimulate primary production (Staehr et al. 2017).

**Sampling**

Surface water (2 litres) was sampled approximately weekly from March 2016 to January 2017 at a fixed shallow (c. 2-m depth) station (55°41′N, 12°5′E; Fig. S1). Samples were brought back to the laboratory for processing of nutrients and phytoplankton within 10–20 min after sampling. Temperature (°C) and salinity (%) were measured immediately upon arrival in the laboratory with an YSI Professional Plus multi-parameter hand-held metre (YSI Inc., Yellow Springs, Ohio, USA).

**Nutrients**

Samples for inorganic nutrients (NO$_3^-$, NO$_2^-$, NH$_4^+$ and PO$_4^{3-}$) were collected from GF/F-filtered water and stored frozen in 30-ml acid-washed plastic bottles until analysis. The samples were analysed on a San ++ Continuous Flow Analyser (Skalar Analytical B.V., Breda, Netherlands) as previously described (Grasshof 1976; Kaas & Markager 1998). Detection limits were 0.04, 0.1, 0.3 and 0.06 µM for NO$_2^-$, NO$_3^-$, NH$_4^+$ and PO$_4^{3-}$, respectively. Dissolved inorganic nitrogen (DIN) concentrations were calculated as the sum of the concentrations of NO$_2^-$, NO$_3^-$ and NH$_4^+$. Dissolved inorganic phosphorus (DIP) is expressed as PO$_4^{3-}$ concentration.

**Pigments**

For the determination of pigment concentrations 300–500 ml of sample were collected on GF/F filters and stored at ~80°C until analysis. Prior to analysis, pigments were extracted overnight at ~20°C, in 10 ml acetone. Pigment extracts were analysed by an HPLC method (Wright et al. 1991) performed in a Shimazu LC 10A (Shimazu Corporation, Kyoto, Japan) system with a Supelcosil C18 column (250 × 4.6 mm, 5 µm). Pigments were identified by their retention times as compared to pure pigment standards (DHI Water & Environment, Denmark) at an absorption spectrum at 449 nm, following Wright et al. (1991), as modified by Schlüter et al. (2000). Chlorophyll a (Chl a) was used as a proxy of total phytoplankton biomass, whereas the following accessory pigments were assumed indicative of phytoplankton groups: alloxanthin (cryptophytes); chlorophyll b (green algae; euglenophytes), neoxanthin and violaxanthin (green algae); diadinoxanthin ('golden-brown algae', e.g. diatoms, dinoflagellates, haptophytes), fucoxanthin (diatoms); and peridinin (dinoflagellates) (Jeffrey et al. 1997).

Pigment data was analysed by the Bayesian compositional estimator (BCE) developed by Van Den Meersche et al. (2008). The method uses a series of input ratios selected based on knowledge of the water body studied. We used the pigment ratios of Henriksen et al. (2002) but replaced the diatom ratio by the ratio of Eutreptiella gymnastica obtained from Higgins et al. (2011). This was done because of the minimal role of diatoms in RF (low concentration of fucoxanthin and microscopical confirmation of scarcity) and the frequent occurrence of Eutreptiella spp. For each sample, the pigment ratios were multiplied by the Chl a concentration to obtain Chl a proportion for each group. Group-specific carbon biomass was estimated assuming a C to Chl a ratio of 30, which is suitable for the area (Jakobsen & Markager 2016).

**Community composition and cell enumeration**

Serial dilution cultures (SDC; Thronsden 1978b, 1993) were conducted once per month on 10 occasions during the study. Five replicates of five 1:10 dilution steps (1 ml to 0.1 µl of the original sample) were incubated in 20-ml glass tubes containing L-1 medium (Guillard & Hargraves 1993) with a salinity of 12, similar to values found in the inner basin of RF. Tubes were kept at 10°C in saturated PAR light at an irradiance of 100–120 µmol m$^{-2}$ s$^{-1}$ (12:12 h light:dark regime), until analysis with a Nikon Ti-U inverted light microscope. For each serial dilution, tubes were analysed after 2 and 4 weeks from sampling. Each tube was gently homogenized before observation and 5–10 drops were observed with a Nikon Ti-U inverted light microscope equipped with a camera. Each drop was screened under 100× and 400× magnification, using differential interference contrast, to assess the organisms in the whole subsample. For a more detailed view on organisms, especially the smaller ones, 1000× magnification was employed. When dinoflagellates were present, cells were stained with calcofluor and analysed using epifluorescence (Andersen & Thronsden 2003). All organisms, including the unidentified ones, were registered with pictures, drawings and descriptions, ensuring a unified catalogue for all dilution steps and replicates. Cell abundances of different taxonomical units were estimated from the most probable number (MPN) tables, considering the number of tubes showing growth (Andersen & Thronsden 2003). To account for cell size variations among different taxa, cell abundances were transformed into biovolumes by normalizing cell numbers by taxa volumes. Taxa volumes were calculated using a geometrical approach (Sun &
Liu 2003), with the average cell dimensions (as the average of measurements of 10–30 live individual cells of each taxon, depending on the abundance in the samples). Volumes were converted to carbon using the general protist formula from Menden-Deuer & Lessard (2000). Some organisms were isolated, and cultures established, allowing further investigation and species identification by electron microscopy (EM) and/or molecular phylogenetic analyses.

**Electron microscopy**

Whole mounts of different flagellate cultures were examined with a JEM-1010 transmission electron microscope (Jeol Ltd., Tokyo, Japan). Briefly, drops of cultures were placed on formvar/carbon-coated grids; then cells were fixed with 1% OsO₄ vapour. Grids were left to dry at c. 50°C and gently rinsed with distilled water to remove salts. Finally, grids were stained with 4% uranyl acetate for 5 min and rinsed very quickly in distilled water.

For thin sectioning of Pyramimonas octopora, two fixation methods were used. In the first method, the material was fixed 1:1 with 1% OsO₄ in 0.2 M cacodylate buffer for 40 min, dehydrated in an ethanol series followed by propylene oxide, and embedded in Epon 812. During the second fixation method, cells were fixed 1:1 with 1.5% glutaraldehyde in 0.2 M cacodylate buffer and 0.18-M sucrose for 80 min, rinsed in the buffer with decreasing sucrose content over 2.5 h, and post fixed in 1% OsO₄ in distilled water overnight. It was then dehydrated and embedded as above. Thin sections were collected on single-hole slot grids and stained in uranyl acetate and lead citrate. They were observed with a JEM-1010 transmission electron microscope.

Cryptophytes were examined with a JSM-6335F scanning electron microscope (Jeol Ltd., Tokyo, Japan) for investigation of cell periplast features. Cultures (800 µl) were fixed for 30 min in a mixture of 960 µl OsO₄ (4%), 960 µl L-1 medium (12 salinity) and 480 µl HgCl₂. Samples were concentrated onto 1- or 5-µm pore-sized filters, depending on the cell size. Filters were rinsed with Milli-Q water, dehydrated through an alcohol series and critical-point dried (Bal-Tec CPD 030, Balzers, Liechtenstein).

**Molecular phylogeny**

Cells of established cultures were concentrated by centrifugation and the pellets kept frozen (−20°C) until extraction. DNA was extracted using the CTAB method following Lundholm et al. (2002). For most species, the SSU rDNA region was amplified using the primers ND1 and ND6, and for sequencing additionally the primers ND2, ND3, ND9 and ND7 (Ekelund et al. 2004). For cryptophytes, SSU and LSU rDNA regions were amplified with a semi-nested PCR following Hoef-Emden (2008). For SSU, the first PCR was done with the primers CrN1F and ITS505R, followed by a second step with the primers CrN1F and BR. The LSU region was amplified first by the primers CrN1F and LSU1433R, followed by a second step using the primers CrN3F and LSU1433R.

PCR products were purified using a QIAquick PCR purification kit (Qiagen, Germany) and analysed on an automated sequencer (ABI3730XL, Applied Biosystems). Alignments for each major taxonomic group were established based on taxonomically relevant taxa from GenBank and similar sequences found using the BLAST algorithm available in GenBank (Altschul et al. 1997). For several taxa, basic sequence comparisons confirmed the morphological taxonomic identity and no further phylogenetic analyses were performed. Sequence alignments were done in BioEdit (Hall 1999). For Pyramimonas, the SSU rDNA alignment included 48 taxa and 1683 nucleotide positions, with four taxa belonging to the genera Mamiella, Micromonas and Mantoniella as outgroup. Distance, parsimony, and likelihood analyses were performed using PAUP* version 4.0b.8 (Swoford 2003). Maximum parsimony (MP) analyses were done with 1,000 heuristic searches and distance analyses used NJ with the GTR (general time-reversible) model. The optimal model for the maximum likelihood (ML) analyses was a general time-reversible model with the proportion of invariable sites and gamma distribution (GTR + I + G), according to the Akaike information criterion with a 99% level of significance in Modeltest version 3.7 (Posada & Crandall 1998). Heuristic searches performed ML analyses with 10 random-addition replicates and the tree bisection-reconnection (TBR) branch-swapping algorithm. In NJ, MP and ML analyses, 1,000 bootstrap replicates were performed. Bayesian analyses were performed using MrBayes 3.1.2 (Ronquist & Huelsenbeck 2003), using four chains run for 1,200,000 generations. The temperature was set to 0.2, the sample frequency was 100, and the number of burn-in generations was 3,000.

**Statistical analysis**

Carbon biomass estimated from pigments and SDC were compared using a simple linear regression, using estimates from the pigments as the independent variable. The linear model and plots were done in R (R Core Team 2015).

**RESULTS**

**Seasonal environment variability**

Salinity during the study period was relatively stable, ranging from 10 to 14, while temperature varied between 1.4°C in winter and 21.7°C in summer (Fig. 1). Variability in temperature and nutrients shows that the study captured distinct phases of the annual cycle in RF, with high DIN concentrations in winter compared to summer, while DIP concentrations were lower in winter and increased over summer (Figs 1, 2). DIN and DIP were quickly depleted between March and April (Fig. 2) coinciding with the spring bloom, as indicated by the sharp increase in Chl a (Fig. 3). Periods with increased Chl a concentrations (>10 µg l⁻¹) were associated with high biomass of cryptophytes (Figs 3, 4). All SDCs were established from communities with relative low phytoplankton biomass, indicated by Chl a < 5 µg l⁻¹ (Fig. 3), and the total C biomass estimated from pigments was less than 141 µg l⁻¹ (Fig. 4).

**Community composition**

Despite relatively similar Chl a concentrations (1.46–4.70 µg l⁻¹) among the samples used for flagellate analyses, both pigment
composition and SDC indicated distinct communities over the annual cycle (Figs 3, 4). Cryptophytes were found in all the samples, and overall were the group with highest biomass contribution during the year, with C biomass ranging from 3.7 to 451.8 µg C l⁻¹ in early August and late March, respectively (Fig. 4). Although cryptophyte biomass values larger than 100 µg C l⁻¹ were mostly observed in spring, isolated peaks were also observed in summer and autumn, but did not coincide with the SDC (Fig. 4). In summer, cryptophyte biomass was lower than 10 µg C l⁻¹ and increased to 20–70 µg C l⁻¹ over autumn (Fig. 4). Prasinophytes were the second most important group, with biomass in the range 0–174 µg C l⁻¹. Two biomass peaks of prasinophytes, with biomass above 100 µg C l⁻¹, were observed in late March and late May, followed by a period with biomass values of about 30–40 µg C l⁻¹ in July (Fig. 4). After September, prasinophyte biomass was consistently low (Fig. 4). Euglenophytes (0–94.8 µg C l⁻¹) and dinoflagellates (0–103.5 µg C l⁻¹), had similar contributions to the overall biomass, although they were less important than cryptophytes and prasinophytes. While euglenophytes contributed with low to moderate biomass (c. 20–40 µg C l⁻¹) over the year, dinoflagellate biomass had a marked occurrence in summer, with peaks in the range of 40–100 µg C l⁻¹ (Fig. 4). Pigments also indicated the presence of cyanophytes, pelagophytes, chlorophytes, and prymnesiophytes, although their contributions were smaller than those of the groups described before (Fig. 4).

Total carbon biomass estimated from SDC/MPN was 17–335 µg l⁻¹ and showed relatively small variation over the months in agreement with Chl a concentration (Figs 3, 5), except in December 2016, when the SDC/MPN estimated high abundances of cryptophytes and euglenophytes while Chl a concentrations were not notably different from the other samples (Figs 3, 5). Although the total biomass estimated from pigments ranged from 26.2 to 657.7 µg C l⁻¹ over the whole period (Fig. 4), the range for the coinciding SDC observations was much smaller (43.9–141.1 µg C l⁻¹), being comparable to most of the SDC estimates, except for December 2016. A linear regression comparing total biomass estimates from both methods yields a poor correlation (p = 0.461); however, it is strongly biased by the high biomass estimated by the SDC in December 2016. If the December sample is excluded, the linear model becomes significant (p = 0.022; R² = 0.436; slope = 0.818), indicating that there is agreement between C biomass estimated by the two methods and that the SDC tends to yield lower values than the pigments.

In accordance with the pigment data, the biomass estimated from SDC showed that cryptophytes were present throughout the year and constituted the most important group (>50% on five occasions), followed by heterotrophic nanoflagellates (10%–50% on five occasions) and euglenophytes (10%–50% on four occasions; S2). Although prasinophytes and pico-eukaryotes did not contribute much to the total C biomass, they were present in all observations (Figs 6, S2), and the latter reached high cell abundances (>1.5 × 10⁴ cells ml⁻¹) on three occasions (Fig. 6). Photosynthetic dinoflagellates were abundant in the SDC in May and August 2016 (Figs 5, S2), coinciding with pigment samples with a high contribution of dinoflagellates (Fig. 4). In those samples, Heterocapsa rotundata (Lohmann) Gert Hansen, plus an unidentified small species of the Suessiaceae and unidentified gymnodinioids were recorded. The SDC from September 2016 was the only case in which diatoms were abundant compared to autotrophic flagellates (Figs 5, S2). In that sample, Skeletonema marinoi Sarno & Zingone, Chaetoceros cf. tenuissimus, Cylindrotheca closterium (Ehrenberg) Reimann & J.C. Lewin and unidentified centric taxa smaller than 10 µm were recorded. Haptophytes were also recorded in most of the observations (except in April, July and December 2016), but at low concentrations, as shown by the pigment data (Figs 4, 5). Chrysochromulina was the most common haptophyte, recorded in all haptophyte observations, and organisms belonging to Pavlovothecaceae were also recorded on three occasions during summer (May, June and August 2016).

A closer look at the composition of the main autotrophic groups showed some seasonal variation. All the euglenophytes observed belonged to the genus Eutreptiella (Figs 11, 12). Although these could not be identified to species level,
morphological variations were observed at different times of the year, with smaller cells (c. 20 µm) observed in the cold months (April, December and January) and larger cells (>30 µm) in warmer months (May and July). Cryptophytes and prasinophytes showed seasonality in the generic composition. *Teleaulax* was the main contributor to cryptophyte biomass in March, April and October 2016 until January 2017 (Fig. 7). From June to November 2016, high abundances of small *Hemiselmis* were observed (Fig. 8), and they dominated the cryptophyte biomass between June and September 2016, when the cryptophyte biomass was generally low (Fig. 7). Small-sized organisms, mainly prasinophytes and pico-eukaryotes, were more abundant in spring and summer, respectively (Figs 6). *Mantoniella* was recorded in all samples, but the highest prasinophyte biomass and abundance, in May, was dominated by *Tetraselmis* (Figs 9, 10). Species of *Pyramimonas* were observed mainly from April to June (Figs 9, 10), in reasonable agreement with the pigments (Fig. 4).

Establishment of cultures supported the identification of organisms that could not be identified by light microscopy. The SDC allowed for observation of diversity among small flagellates and showed this assembly’s composition to change over the annual cycle of 2016. Examples of some of the organisms are shown in Figs 11–27. Although some organisms were recorded by the SDC (e.g. heterotrophic nanoflagellates, diatoms and dinoflagellates), they were not always identified. In total, 15 flagellate taxa were identified (Table 1), belonging to cryptophytes (7), prasinophytes (4), haptophytes (2) and pico-eukaryotes (2). Information on the strains, including methods used for identification and GenBank accession numbers, are summarized in Table 1. Of the identified species in RF, we highlight the occurrence of *Hemiselmis cryptochromatica* C.E. Lane & J.M. Archibald, recorded for the first time in European waters, and *Pyramimonas octopora* sp. nov. Some cultures could not be identified to species level due to problems with sequencing or lack of reference data: *Euteuptella* (April, May, November and January), *Tetraselmis* (May and August), *Hemiselmis* (July, October and November) and pico-eukaryotes (April and May).

Phylogenetic analyses of cryptophyte SSU rDNA (Fig. S3) revealed a clade comprising the genus *Teleaulax*, in which *T. amphioxeia* (W. Conrad) D.R.A. Hill strains from March and April clustered with *T. amphioxeia* strains from GenBank with high support, and the *T. acuta* from October clustered with *T. acuta* (Butcher) D.R.A. Hill from GenBank with high support. A separate *Rhodomonas* clade comprised *R. salina* (Wislouch) Butcher from June clustering with *R. salina* as well as other *Rhodomonas* taxa from GenBank. Within a *Chroococcus* clade, *C. venticiss N. Carter from January clustered with another strain of the same species. In the *Hemiselmis* clade, *H. virens* Droop from March clustered with high support with sequences of two *H. cf. virens* from GenBank, while another strain from GenBank was found outside that branch but within the *Hemiselmis* clade. *Hemiselmis cryptochromatica* formed a well-supported distinct branch within the *Hemiselmis* clade, in which our June isolate clustered with a sequence obtained from the culture from which the type material was derived.

**Formal description of new species**

*Pyramimonas octopora* L. Haraguchi, Moestrup, H.H. Jakobsen & Lundholm sp. nov.

Figs 28–42

**DIAGNOSIS:** Elongated cells with average cell length 9.5 ± 1.2 µm and width 7.2 ± 1.1 µm. The eyespot comprises two rows of carotenoid droplets, near the apical end of the cell in two adjacent chloroplast lobes. The four flagella are slightly longer than the cell. Each flagellum is covered by a layer of small pentagonal scales, in addition to limuloid scales and hair scales. A layer of box scales covers the cell body, each box scale with eight round irregular perforations in the base plate, arranged in a rounded square around the centre of the base plate. Crown scales have a squared base with pointed edges. A cross-shaped structure is formed by struts from the middle of each side of the outer rim and meeting in the centre. In each quadrant defined by the cross-shaped structure, the outer parts of the rim are connected by two smaller structures arranged at about 45 degrees in relation to the cross, forming a complex pattern. From each corner, a tubular structure rises upward, all four meeting at the top, from where a central tubular column connects to the base plate centre.

**HOLOTYPE:** A fixed and plastic-embedded sample of culture Poct_06 is deposited at C, the herbarium at the Natural History Museum of Denmark (museum number C-A-99692) (Fig. 35 illustrates the holotype). Accession number MW451603 for the SSU sequence of the holotype strain.

**TYPE LOCALITY:** Type material was collected at the inner portion of Roskilde Fjord, Denmark (55°41’N, 12°55’E) in June 2016. Salinity was 13.1 and temperature 19.8°C.

**ETYMOLOGY:** Latin adjective *octopus, -a, -um*, eight pores, referring to the box scale with eight round irregular perforations in the base plate.

**Morphology**

**LIGHT MICROSCOPY**

The cells have an average length of 9.5 ± 1.2 µm and a width of 7.2 ± 1.1 µm (n = 27, mean ± s). The apical portion of the cell is truncated, but some notches can occasionally be observed, corresponding to the four anterior lobes of the cell. Cell morphology varies from inverse pyramidal to U-shaped (Figs 28, 29, 31). The single chloroplast is bright light green, and cup-shaped with four anterior lobes (Figs 29, 31). The pyrenoid is eccentric and located in the antapical part of the cell (Figs 28, 29, 31). Four thick flagella emerge from the flagellar pit, which is about ½ of the cell length (Fig. 31). The flagella are slightly longer than the cell. The two eyespots are located near the cell apex, often at the edge of a cell corner (Figs 28–31).

**TRANSMISSION ELECTRON MICROSCOPY**

This species presents morphological characteristics common to species of *Pyramimonas* subg. *Vestigiferae* McFadden, D.R.A. Hill & Wetherbee. These include two bi-layered eyespots (Figs 32, 34), an eccentric pyrenoid with invading thylakoids (Figs 28, 29), a square syntosome (Fig. 37), and square under-layer scales ornamented with perforations (Fig. 42).
Figs 3–10. Seasonal variation in phytoplankton community composition in Roskilde Fjord.

Fig. 3. Chlorophyll a concentration. Large dots represent samples in which serial dilution cultures (SDC) were established.

Fig. 4. Biomass estimated from the proportion of accessory pigments using the Bayesian Compositional estimator (BCE). Phytoplankton group abbreviations: Chloro = chlorophytes, Crypto = cryptophytes, Cyanop = cyanophytes, Dino = autotrophic dinoflagellates, Eugl = euglenophytes, Pelago = pelagophytes, Prasino = prasinophytes, and Prymne = prymnesiophytes.

Figs 5, 6. Main groups enumerated by SDC and MPN as carbon biomass and cell abundances. Phytoplankton group abbreviations: Crypto = cryptophytes, Diat = diatoms, Dino = autotrophic dinoflagellates, Eugl = euglenophytes, Hapto = haptophytes, HNF = miscellaneous heterotrophic nanoflagellates, NID = non-identified organisms, Pico_euk = eukaryotes <2 µm, and Prasino = prasinophytes.

Fig. 5. Estimates based on carbon biomass.

Fig. 6. Estimates based on cell abundances.

Figs 7, 8. Cryptophyte composition at genus level as carbon biomass and cell abundances.

Fig. 7. Estimates based on carbon biomass.

Fig. 8. Estimates based on cell abundances.

Figs 9, 10. Prasinophyte composition at genus level as carbon biomass and cell abundances.

Fig. 9. Estimates based on carbon biomass.

Fig. 10. Estimates based on cell abundances.
The flagella are covered by an underlayer of pentagonal scales about 40 nm wide (usually 38–42 nm) overlain by nine rows of limuloid scales. Each limuloid scale is 260–300 nm long and 170–190 nm wide, with a longitudinal spine directed anteriorly (Figs 33, 42, 45). At least two ribs diverge from the centre of the scale towards the margins, one located close to the spine base and the other more posteriorly (Figs 33, 42, 45). Three perforations of irregular size are found in the anterior part of the scale, one very close to the base of the longitudinal spine and the other two near the anterior end (Figs 33, 42, 45). Hair scales are present (Fig. 33) but were not examined in detail.

The cell body is covered with a layer of square box scales (Figs 35, 41, 42), in which the only ornamentation are eight perforations on the base, arranged in a square with round corners around the centre. The perforations are irregular in shape and size and occasionally very small. Each box scale is about 200 nm wide and about 80 nm high (Figs 41, 35). The crown scales, situated on top of the box scales (Fig. 41), have a square base with pointed edges, with a cross structure from the middle portion of each side of the outer rim, meeting at the centre. In each quadrant defined by the cross, the two parts of the rim were connected by two smaller structures arranged at an angle of c. 45 degrees in relation to the central cross, forming an intricate pattern on the base of the crown scale (Fig. 39). From each corner, a tubular structure rises upwards, the four structures meeting at the top, from where a central tubular column connects to the centre of the base (Figs 35, 41, 44). A layer of small square scales with perforations (Fig. 38) is present only in the flagellar pit (Fig. 41). Footprint scales can be found between the box scales and measure approximately 60 nm in length when measured in thin sections, one end being slightly thicker than the rest (Fig. 40).

**Cell motion**

In culture, cells usually swim forward in a straight line, rotating along the cell axis. To turn to a new direction, the cells stop, turn and then resume swimming. Cells can also be found with their flagella attached to a surface and moving in circles.

**Phylogenetic analyses**

Phylogenetic trees were based on alignments of 1683 positions of the SSU rDNA (Fig. 46). The genus *Pyramimonas* made up a monophyletic clade, weakly supported (67/-/- as NJ/MP/ML), to which 'Prasinopapilla vacuolata' was the closest sister taxon (Fig. 46). The designation 'Prasinopapilla vacuolata' was not validly published (no description) and no further information on the organism was found, except what was mentioned in GenBank. *Pyramimonas octopora* clustered within the *Vestigiferae* clade, which was monophyletic and highly supported (98/88/83). The clade *Vestigiferae* appeared as sister to the clade *Punctatae*, with no
Within the Vestigiferae clade, three main clades were observed: the basal clade included *P. disomata* Butcher *ex* McFadden, D.R.A. Hill & Wetherbee and received high bootstrap values (98/88/93 as NJ/MP/ML), whereas the two other clades were supported by the moderate bootstrap values 96/66/- (NJ/MP/ML). The second clade comprised *P. octopora*, an unidentified *Pyramimonas* (strain RCC1987 from the Beaufort Sea; JN934670) and an uncultured chlorophyte (clone CCW80 from rRNA library-based sample from Cape Cod, US; AY180034). The third clade comprised the cold-water species *P. discoicola* Harðardóttir, Lundholm, Moestrup & T.G. Nielsen and *P. gelidicola* McFadden, Moestrup & Wetherbee, and a few unidentified organisms (Fig. 46).

**DISCUSSION**

Our results on flagellate diversity and composition in a well-studied and monitored environment demonstrate that part of the phytoplankton diversity has been overlooked by traditional monitoring. The findings highlight the importance of small phytoplankton biodiversity surveys.

**Methodological considerations**

In this study, phytoplankton community composition was assessed by two very distinct methods: pigments and SDC/MPN. Overall, there was a fair agreement between the methods, which showed similar trends for phytoplankton biomass and composition, except for one out of 11 samples. The methods have very different strengths and limitations, and therefore discrepancies were expected. On the other hand, the number of samples was too small to make a robust comparison between methods and more studies are required to make a proper comparison between the two methods. The main limitations of the methods are described below, indicating potential sources of bias for each method.
Marker pigments are well suited for analysing a large number of samples but suffer limitations for inferring the taxonomic composition because many pigment markers, such as diadinoxanthin and chlorophyll b, are shared by different algal groups (Jeffrey et al. 1997). When HPLC data use specific procedures to project the community composition, as the Bayesian estimator used in this study, the results can be used to estimate the biomass contribution of different phytoplankton groups. However, as the pigment ratios vary with the environment, those procedures require good knowledge of the local populations and their pigments (Higgins et al. 2011), as was the case for RF where pigment ratios exist from previous studies (Henriksen et al. 2002). Another problem might come from pigments derived from non-phytoplanktonic groups, as mixotrophic ciliates, which are common in RF and can be abundant at specific times of the year (Haraguchi et al. 2018).

The SDC allows for a much deeper taxonomic classification, but it is laborious and time-consuming, limiting its application (Thronsden 1978b; Cerino & Zingone 2006). In addition, estimates from SDC are somewhat biased as they favour species that grow well under culture conditions (Cerino & Zingone 2006), and cell abundances estimated by the SDC/MPN method often underestimate the number of cells. Small grazers might be present in the tubes, as the case of this study, and their presence might impact the occurrence of certain taxa. Additionally, as species with different growth rates are present in phytoplankton, the method might indicate the false absence of an organism that grows too fast or too slow even though it was present in the sample (Cullen & MacIntyre 2016). Likely because of these issues, the SDC/MPN application to assess the whole community has been very limited but it has been indicated as a potential method to assess cell viability in ballast waters (Cullen & MacIntyre 2016). The semi-quantitative nature of the method is also a disadvantage; however, it has been shown that precision and reproducibility for cell viability can also be obtained for the MPN method when validation protocols are used (MacIntyre et al. 2019).

Our SDC/MPN results were able to reproduce some patterns previously observed in RF, such as the dominance of small phytoplankton (Haraguchi et al. 2018) and the summer reduction of the cryptophyte Teleaulax (Altenburger et al. 2020). Given the many constraints associated with the method, which limits the quantification capability, it was a surprise to see that it reproduced total biomass trends from the pigments. In addition, the possibility to establish cultures allowed for a more detailed investigation of a few organisms, an effort that was already relevant for the knowledge on the local biodiversity. However, it is important to highlight that part of the phytoplankton could not be properly assessed in this study, and the efforts were too limited to link biodiversity and ecology.

Metagenomics features as one of the methods to assess biodiversity that are well established (Johnson & Martiny 2015). Massana et al. (2015) in a survey in different coastal environments in Europe showed that the diversity of larger protists is relatively well known, whereas small flagellate diversity remains much less explored. A comparison between SDC and next-generation sequencing for diatom resting spores demonstrated a good agreement between records obtained with both methods (Piredda et al. 2017). Piredda et al. (2017) also emphasized the importance of curated reference sequences, highlighting a potential synergy between SDC and metagenomics.

Cryptophyte diversity: ecological implications and challenges

Cryptophytes were the main taxonomic group among the nanoflagellates in RF according to pigment data and were found by SDC during all months. However, quantitative studies focusing on species composition and dynamics of cryptophytes in marine systems are scarce (Laza-Martínez et al. 2012). In the Kattegat basin, adjacent to RF, cryptophytes have been recorded at all sampling occasions, the most common species found by Hill et al. (1992) but Plagioselmis prolonga. The acuta Butcher ex Novarino, I.A.N. Lucas & S. Morrall, Hemiselmis virescens and Teleaulax acuta. Teleaulax amphioxidea is the most important cryptophyte in RF in terms of biomass (Haraguchi et al. 2018; Altenburger et al. 2020), and the species was recently described as having a dimorphic life cycle, in which the form known as T. amphioxidea is the diploid stage and the former Plagioselmis prolonga is the haploid stage (Altenburger et al. 2020). In our study, T. acuta and T. amphioxidea (the diploid form) were observed to occur together, especially during the winter months. The haploid form of T. amphioxidea (the former Plagioselmis prolonga – see Altenburger et al. 2020), was less often recorded, and its lower abundance might reflect a potential life-cycle change, from the haploid to the diploid form, as SDC was established using nitrogen-rich culture media (Altenburger et al. 2020). The dependence of T. amphioxidea on high DIN concentrations likely leads to
Figs 32–42. *Pyramimonas octopora* sp. nov., TEM. Figures 32–34, 36–41 are from thin sections; Figs 35, 42 are from whole mount preparations stained with uranyl acetate. All scale bars = 200 nm, except for Figs 32, 34 and 36 in which the scale bars = 1 µm.

Fig. 32. Transverse section through the flagellar pit, with four flagella (1–4), scale reservoir (S), nucleus (N) and eyespots (E) near the cell corner.

Fig. 33. Section through the flagella showing hair scales (HS), limuloid scales (LS) and under-layer of pentagonal scales (PS).

Fig. 34. Longitudinal section showing nucleus (N), bi-layered eyespot (E) and scale reservoir (S) close to the apical part of the cell.

Fig. 35. Body scales: box scales (BS), crown scales (CS) and limuloid scales (LS). Note detail on the ornamentation of the box scales, with eight perforation on the base.

Fig. 36. Longitudinal section showing scale reservoir (S), nucleus (N) and pyrenoid (Py) penetrated by thylakoids.

Fig. 37. Detail of a longitudinal section showing the square synistosome (Sy) and the four flagella (1–4).

Fig. 38. Details of the underlayer body scale.

Fig. 39. Details of the crown scale base.

Fig. 40. Detail showing the footprint scales (indicated by arrows) between the box scales.

Fig. 41. Cell surface detail showing the underlayer body scales (US), box scales (BS) and crown scales (CS) at the surface.

Fig. 42. Limuloid (LS) and box scales (BS).
the summer dominance of *Hemiselmis* among the cryptophytes. While the high abundances of *Hemiselmis* in RF during summer match the reports for the Gulf of Naples (Cerino & Zingone 2006) and for Kattegat (Hill et al. 1992), it was in the latter region also recorded in other seasons. Observations of cultured *Hemiselmis* spp indicate that *Hemiselmis* can endure nutrient stress, making it suitable to survive in the DIN-limited RF during summer. Findings of the cryptophyte assemblage in RF corroborate previous studies (Hill et al. 1992; Cerino & Zingone 2006), all showing that the composition within a taxonomic group changes over time, most likely reflecting distinct environmental conditions. However, studies focusing on *Hemiselmis* ecology in nature are usually limited in time, being restricted to cruises or short-lived surveys such as this study, limiting the comprehension of key aspects of their ecology. *Hemiselmis* records by traditional monitoring in RF are scarce and most likely these algae end up being added to non-identified flagellates. This is likely the case for many other locations, highlighting a bottleneck to assess *Hemiselmis* over longer time scales.

Additionally, the identification of *Hemiselmis* to species level is challenging because of the small cell size and the lack of distinct morphological features. For many years, distinction of the marine *Hemiselmis* species was based on colouration (Lane & Archibald 2008). Such limitations make *Hemiselmis* one of the most enigmatic cryptophyte genera. We established five strains in culture but were able to identify only two species level: *H. virescens* and *H. cf. cryptochromatica*, the latter recorded for the first time in Europe. Apart from the phylogenetic support, the *H. cf. cryptochromatica* culture exhibited the same pale-green colour as stated in the original description of the species (Lane & Archibald 2008). No information on environmental conditions of the type material of *H. cryptochromatica* has been obtained, and the only ecological information available is the maximum temperature conditions of cultures, up to 21°C (Lane & Archibald 2008), making it difficult to make ecological inferences on environmental preferences and distribution. Our material of *H. cf. cryptochromatica* was isolated in June, at a water temperature of c. 20°C, but the isolate also grew in the culture at 10°C, indicating potential plasticity in ecological requirements. The other three unidentified *Hemiselmis* strains have characteristics that resemble *H. cryptochromatica*, such as size and culture colouration (Lane & Archibald 2008), but one of the strains (Hemi_11) was isolated in November 2016 when the temperature was about 6°C. Whether those belong to same species or not, those observations highlight that marine *Hemiselmis* ecology and diversity remain largely unknown.

**Prasinophytes: hidden diversity in RF**

Prasinophytes were common in RF, but with a lower relative contribution to the total biomass when compared to the main groups, cryptophytes and euglenophytes. Prasinophytes were mainly important around spring, although they were also present during the following months, and this applied especially to *Mantoniella squamata* (Manton & Parke) Desikachary, a common species in Danish waters, which occurs all year round (Moestrup 1992). Picococci were present in all samples, and two strains, one collected in August and the other in December, were identified as two potentially distinct species of the prasinophyte genus *Ostreococcus*. Due to the small size and limited morphological characteristics, species identification based on morphology is not feasible, and organisms can be distinguished based on genome (Palenik et al. 2007), further supporting the ecotypes distinction proposed by Rodriguez et al. (2005). Considering the environmental differences (temperature, light, nutrient availability) found between August and December in RF, it is likely that the two *Ostreococcus* strains are distinct species. Small prasinophytes can be subjected to intense grazing pressure, with reported abundance decline coinciding with blue mussel larval development in Oslo Fjord (Backe-Hansen & Thordsen 2002). In RF, ciliate grazing is very intense at all seasons, controlling especially small-sized phytoplankton populations (Haraguchi et al. 2018). As most prasinophytes recorded in this study were smaller than 10 μm, their lack of abundance after May (Figs 9, 10) is probably related to grazing intensification towards summer.

Among the prasinophytes from RF, an undescribed species belonging to the genus *Pyramimonas* was found. This new species possessed morphological characteristics of *Pyramimonas* subg. *Vestigiferae*, such as small squared under-layer scales with perforations in the flagellar pit (corresponding to type 1 from McFadden et al. 1986), the excentric pyrenoid with invading thylakoids, the square synistosome, and two bi-layered eyespots (McFadden et al. 1986). Since the description of the subgenus *Vestigiferae* by McFadden et al. in 1986 (invalidly published for lack of type; validated in McFadden et al. 1987) these features have been consistently reported for all species within the group, therefore representing stable features (Daugbjerg et al. 2020). The swimming behavior in culture is similar to what has been described from other species of this subgenus, with cells swimming forward, rotating along the cell axis and stopping to change direction (Daugbjerg & Moestrup 1992; Daugbjerg et al. 2020; Harđardottir et al. 2014). In LM, live cells resembled *P. orientalis*, but the characteristics of the large body scales (observed in EM), which are determinant for species identification (Norris & Pienaar 1978), indicated a different identity. Eight round perforations ornamented the box scales at the base, arranged around the centre in a square with rounded corners. Furthermore, the crown scale had distinctive characteristics, such as the intricate pattern on its base.
**Pyramimonas cyclotreta** Daugbjerg & Moestrup is the only other *Pyramimonas* species with box scales possessing round holes in the base plate (Daugbjerg & Moestrup 1992). *Pyramimonas octopora* box scales differ from the arctic *P. cyclotreta* by 1) the way the holes are arranged around the centre (in a square with round corners in *P. octopora* and in circle in *P. cyclotreta*); 2) not having striations; and 3) the lack of a central spine. The crown scales of *P. cyclotreta* are simpler than those of *P. octopora*, especially regarding the crown scale base pattern. The crown scales most similar to *P. octopora* were recorded in an organism similar to *P. orientalis* (*P. aff. orientalis* CCAP 4, in fl. 12 of Pennick 1984). However, Pennick’s drawing did not show the two smaller structures arranged in a nearly 45° angle in relation to the mid cross, as in the RF material. In the past years, two new species of *P. subg. Vestigiferae* were described, the arctic *P. discoiocola* (Harðardóttir et al. 2014) and *P. tatianae* (Daugbjerg et al. 2020). Morphologically, *P. octopora* differs from those two species by the more anterior position of the eyespot and by the morphology of the large body scales (as

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**Fig. 46.** Phylogenetic analysis of *Pyramimonas* spp based on sequences of SSU rDNA. Bootstrap values supporting the branches are given at each node as estimated by different methods (Neighbour-Joining NJ/Maximum Parsimony MP/Maximum Likelihood ML).
discussed above). While the phylogenetic SSU analyses placed *P. octopora* within the *Vestigiferae* clade, the closest organism was an uncultured chlorophyte (AY180034) sampled in a tidal pool (at 10°C and salinity c. 30) at Cape Cod, USA (Stoeck & Epstein 2003). Our phylogenetic tree supports a distinction between *P. disomata* and *P. octopora* as belonging to separate clades within the subgenus *Vestigiferae*. The general SSU phylogeny confirmed previous studies (Suda et al. 2013; Harårdottir et al. 2014).

**Final considerations**

Our results exemplify a latent diversity in the small-sized phytoplankton in RF. Annual changes were observed between distinct taxonomic groups but also within the same taxonomic group, such as cryptophytes. Such changes might not be evident at coarser taxonomic levels, yet the hidden diversity probably reflects a range of ecological strategies related to seasonal changes in nutrient supply and grazing. The study allowed us to record *Hemiselmis* cf. *cryptochromatica* for the first time in Europe, and to describe a new *Pyramimonas* species in a well-studied site such as RF. These findings indicate that the known diversity on the smaller phytoplankton fraction is still expanding, impacting the knowledge of phytoplankton ecology and monitoring, and directly affecting diversity indices and reference databases used for metabar-coding. Current knowledge of diversity and functioning of phytoplankton communities is obviously lacking in detail. This applies not only to poorly studied areas but also to areas such as Roskilde Fjord, which have been studied and monitored for many years.

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**DISCLOSURE STATEMENT**

No potential conflict of interest was reported by the authors.

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