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Long-term survival of haptophyte and prasinophyte resting stages in marine sediment

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Resting stages of marine phytoplankton have been shown to have potential for long-term survival and to remain viable in marine sediments for up to about a century. This study documents for the first time long-term survival in haptophytes and prasinophytes, by germination of resting stages of Isochrysis galbana and Mantoniella squamata from up to 40-year-old sediment layers. Germination was induced by setting up sediment slurries in L1 medium at 15°C. Cyst formation was induced in culture strains acquired from the germinations by keeping mixtures of strains in the light or dark at salinities of 20 or 30. The identity of the two species was confirmed by light and electron microscopy as well as LSU and SSU rDNA-based phylogenetic analyses.

Key words: cyst, Isochrysis, life-cycle, Mantoniella, phytoplankton, resting stage

INTRODUCTION

Resting stages of marine phytoplankton have been shown to have potential for long-term survival and to remain viable for up to about a century in sediments (Lundholm et al., 2011; Ribeiro et al., 2011; Ellegaard et al., 2013a). So far, such studies have mainly focused on resting stages of diatoms and dinoflagellates, two dominant groups in the marine phytoplankton. However, other groups play important roles in marine phytoplankton, and in this study, long-term survival is for the first time documented for species from two such groups, the haptophytes and prasinophytes.

Haptophytes (Haptophyta) are a group of mainly marine or brackish, mainly autotrophic or mixotrophic, microalgae comprising some hundreds of species (317 listed in the overview by Jordan et al., 2004). Some species produce toxins, which may cause mass mortalities of fish (e.g. Moestrup & Thomsen, 2003), whereas other species, notably Isochrysis galbana Parke 1949, are of commercial interest due to their utilization as feed in aquaculture (e.g. Bendif et al., 2013). Many species bear organic or calcified scales and some species alternate between stages with different types of scale-covering (e.g. Thomsen et al., 1991). Resting stages of haptophytes have been reported relatively rarely. The first report is from 1937, where cyst stages were reported from the genera Prymnesium Massart 1920 (Carter, 1937), confirmed by Conrad (1941), and Isochrysis (Parke, 1949). In all cases the cysts were found in cultured strains or by microscopical observation of cyst formation in plankton samples. Since then, cysts of Prymnesium have been reported sporadically, also from cultured strains (i.e. Pienaar, 1980; Wang & Wang, 1992; Beltrami et al., 2007). Cysts of haptophytes have not been reported from sediment samples or in situ in plankton, although Persson (2001) found the pavlovophycean species Pavlova cf. gryans seeded from a slurry of surface sediment. The role of these resting stages in the life cycle and survival of haptophytes is thus largely unexplored. Some of the cysts are very similar in shape and size to silicified chrysophyte stomatocysts, and there have been some reports indicating that haptophyte cysts might also contain silica (Parke, 1949; Pienaar, 1980).

Prasinophytes is a common term for several groups of green algae that branch out at the base of the green algal phylogenetic tree. In contrast to most groups of green algae, prasinophytes comprise mainly marine species and are typically characterized by a covering of scales (Sym & Pienaar, 1993). True resting stages have not to our knowledge been reported from marine prasinophytes. Some genera, Pterosperma Pouchet,
1893 and *Halosphaera* F.Schmitz, 1878, form a so-called phycoma stage (e.g. Sym & Pienaar, 1993), but this has not been shown to serve as a resting or survival stage. A palmellloid phase has been found in *Mantoniella squamata* (Manton & Parke) Desikachary 1973 (Manton & Parke, 1960). A cyst stage with scales of the Antarctic species *Pyraminonas gelidicola* McAfaden, Moestrup & Wetherbee 1982 was found in cultured strains, but it is not known whether these are temporary cysts or serve as resting stages (van den Hoff et al., 1989).

In this study we report germination of a haptophyte, *Isochrysis galbana*, and a prasinophyte, *Mantoniella squamata*, from marine sediments several decades old. We show induction of cyst formation in mixtures of culture strains kept for prolonged periods in dark or light conditions and discuss the implications of cyst formation and survival for the ecology and evolution of these organisms.

### MATERIALS AND METHODS

#### Sediment sampling

The sediment core from which the cultures were established was taken in 2005 at a water depth of c. 20 m in Mariager Fjord, a Danish sill fjord connected to the Kattegat. The fjord is characterized by bottom water anoxia and low sediment disturbance in the deep part where the core was sampled (Fallesen et al., 2000). The core was taken with a modified HAPS corer and X-rayed intact, but no structures were discernible in the X-ray image. It was stored in the dark and cold (4°C) with water intact over the sediment surface until sliced. Except for the top few cm, the outer 5–10 mm of each slice was scraped off to avoid smearing between the layers.

#### Sediment core dating

The age-depth model was based on $^{210}$Pb and $^{137}$Cs gamma spectrometry at the Gamma Dating Centre, Department of Geosciences and Natural Resource Management, University of Copenhagen. The measurements were carried out on a Canberra ultralow-background Ge-detector. $^{210}$Pb was measured via its gamma-peak at 46.5 keV, $^{226}$Ra via the granddaughter $^{214}$Pb (peaks at 295 and 352 keV) and $^{137}$Cs via its peak at 661 keV.

#### Cyst formation in cultures

**Germination from sediment samples.** To induce germination from the sediment, small subsamples of sediment layers down to 50 cm were incubated under 60–100 µmol photons m$^{-2}$ s$^{-1}$ light in microwells with L1 growth medium at 15°C in a light:dark cycle of 16:8 h. The wells were checked regularly and single cells were isolated from those wells in which growth occurred using a capillary pipette. Each isolated cell was rinsed in drops of culture medium and transferred to a new well containing fresh L1 medium. Furthermore, single cysts were isolated from the sediment, and incubated under the same conditions as the slurries (see above). One of these germinated into a *Mantoniella* strain. A total of nine strains (seven of *Isochrysis* and two of *Mantoniella*) were established (see Table 1) and kept under the same conditions as the microwells. Growth in the sediment slurries was detected in layers down to 39 cm.

**Microscopy**

Cysts formed in culture were studied using a Zeiss Axiophot light microscope fitted with an AxioCam HRc camera (Zeiss, Oberkochen, Germany). Material from a flask of mixed

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### Table 1. Information on the strains: sediment depth, sediment age, strain name, analyses performed, species identification and accession numbers.

<table>
<thead>
<tr>
<th>Depth</th>
<th>Age (approx.)</th>
<th>Isolate</th>
<th>Analyses</th>
<th>Identification</th>
<th>Accession numbers</th>
</tr>
</thead>
<tbody>
<tr>
<td>0–3 cm</td>
<td>0–5 years</td>
<td>A A3</td>
<td>Sequenced, TEM (whole mount &amp; sections), plus TEM on rinsed bulk sediment from 0–3 cm</td>
<td><em>Isochrysis galbana</em></td>
<td>KU600439 (LSU), KU600442 (SSU)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>B A4</td>
<td>TEM (whole mount)</td>
<td><em>Mantoniella</em> sp.</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>B B6</td>
<td></td>
<td><em>Isochrysis</em> sp.</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>B D3</td>
<td></td>
<td><em>Isochrysis</em> sp.</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>B3</td>
<td></td>
<td><em>Mantoniella</em> sp.</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>C2</td>
<td></td>
<td><em>Isochrysis</em> sp.</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>C5</td>
<td></td>
<td><em>Mantoniella</em> sp.</td>
<td></td>
</tr>
<tr>
<td>15–16 cm</td>
<td>25–30 years</td>
<td>B5</td>
<td>Sequenced, TEM (whole mount and sections), sequenced TEM (whole mount &amp; sections)</td>
<td><em>Isochrysis galbana</em></td>
<td>KU600441 (LSU), KU600444 (SSU)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C3</td>
<td></td>
<td><em>Mantoniella squamata</em></td>
<td>KU600438 (SSU)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cyst5</td>
<td>Sequenced</td>
<td><em>Mantoniella squamata</em></td>
<td>KU600446 (SSU)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CCMP 1323</td>
<td>Sequenced</td>
<td><em>Isochrysis galbana</em></td>
<td>KU600440 (LSU), KU600443 (SSU)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>K-0633</td>
<td>Sequenced</td>
<td><em>Isochrysis galbana</em></td>
<td>KU600445 (SSU)</td>
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<tr>
<td></td>
<td></td>
<td>K-0284</td>
<td>Sequenced</td>
<td><em>Mantoniella squamata</em></td>
<td>KU600447 (SSU)</td>
</tr>
</tbody>
</table>
strains (named Isomix6), which had been incubated at salinity 30 in the dark, was fixed with Lugol’s iodine, rinsed in ethanol, spun down by centrifugation and air-dried. The dried material was mounted on stubs and examined in a JEOL 6330F scanning electron microscope. To determine if resting stages could be identified directly in sediment layers, a slurry of raw sediment from sample 0–3 cm (surface sediment) was rinsed using standard diatom rinse methods as described above. The rinsed material was placed on a coated copper grid and air-dried. For studying ultrastructural details of motile cells (with a view to confirmation), motile cells of *Mantoniella* isolate C3 and *Isochrysis* isolates A A3 and B5 were transferred to coated copper grids and fixed in the vapour from osmium tetroxide before being shadow cast with chromium. For thin sections, cells of *Isochrysis galbana* were fixed by adding 1 ml of 25% glutaraldehyde to 25 ml of culture B5. Cells were then centrifuged to form a pellet and the liquid exchanged for 4% glutaraldehyde in 0.2 M cacodylate buffer containing 0.4 M sucrose. It was left here for 1.5 h before rinsing for 1.5 h in three changes of buffer with decreasing sucrose content. Post-osmication was in 1% osmium tetroxide in seawater overnight. The subsequent dehydration was through a graded ethanol series, followed by two changes of propylene oxide, 5 min in each change. The pellet was then embedded in Spurr’s resin and sectioned with a diamond knife on a LKB ULtratome V ultramicrotome. Sections were stained with uranyl acetate followed by lead citrate. The TEM was a JEOL JEM-100SX transmission electron microscope (Jeol, Tokyo, Japan).

**Sequencing and phylogeny**

Cells from cultures were harvested by centrifugation, frozen at −20°C, and DNA extracted following Lundholm *et al.* (2002). For both haptophytes and prasinophytes, the SSU rDNA region was amplified using the primers ND1 and ND6 and sequenced using also primers ND3, ND7, ND9 and ND2 (Ekulund *et al.*, 2004). For the haptophytes, the D1–D3 region of the LSU rDNA was amplified using the primers D1R-F, D3B-R for PCR plus D2C for sequencing (Lundholm *et al.* 2002). Similar sequences found in GenBank using the BLAST algorithm (Altschul *et al.*, 1997) were included in the alignments. The rDNA sequences were aligned using Clustal W (Thompson *et al.*, 1994) and adjusted manually. Initial alignments were analysed using neighbour joining (NJ) in PAUP* version 4.0b.8 (Swofford, 2003) and reduced to the final alignment (for accession numbers see Table 1). Bayesian analyses were performed using MrBayes 3.1.2 (Ronquist & Huelsenbeck, 2003), using four chains run for 2 000 000 generations. The temperature was set to 0.2, sample frequency was 100, and the number of burn-in generations was 5000. All distance, parsimony, and likelihood analyses were performed using PAUP* version 4.0b.8 (Swofford, 2003). Maximum-parsimony (MP) analyses were done with 1000 heuristic searches. All distance analyses used NJ (1000 replicates) with the GTR (general time reversible) model. The optimal model for maximum likelihood (ML) analyses was found with a 99% level of significance using Modeltest version 3.7 (Posada & Crandall, 1998); the proposed model used for the ML analyses, GTR+I+G (general time reversible model + proportion of invariable sites + gamma distributed rate variation among sites) of all dataset included 1000 random addition replicates. In all NJ, MP and ML analyses we made 1000 bootstrap replicates.

**Energy dispersive X-ray spectrometer (EDS; for chemical characterization)**

Samples from Isomix6 (see above) were taken with a plastic pipette and rinsed twice in plastic falcon tubes with distilled water. Afterwards, a drop of the rinsed material was air-dried onto a SEM stub and examined for the presence of silica by EDS on a FEI Helios EBS3duel beam microscope. As a positive control, stomatocysts formed in a cultured strain of the chrysophyte *Ochromonas glossopara* (CCMP strain 2060) were also analysed.

**RESULTS**

**Dating**

The core showed concentrations of unsupported $^{210}\text{Pb}$ of around 100 Bq kg$^{-1}$ in the upper part of the core and a rather irregular profile below 23 cm depth (Fig. 1). A peak in the concentration of $^{137}\text{Cs}$ was observed at a depth of around 12 cm. A $^{210}\text{Pb}$ based chronology was calculated using the CRS-model (Appleby & Oldfield, 1978). The inventory of unsupported $^{210}\text{Pb}$ in the bottom of the core (below 44 cm) was calculated on the basis of a regression of unsupported $^{210}\text{Pb}$ vs. accumulated mass depth above that level (Appleby, 2001; Andersen, *in press*). This chronology dates the peak in $^{137}\text{Cs}$ to 1989 ± 3 years, which fits the expected Chernobyl-origin (1986) of this material. The chronology (Fig. 2) is therefore considered to be reasonably robust, despite the rather irregular profile of unsupported $^{210}\text{Pb}$. The approximate ages of each depth from which we obtained strains are shown in Table 1.

**Germinations**

Haptophyte or prasinophyte cells were identified in three layers (0–3 cm, 15–16 cm and 24–25 cm) after germination occurred. In most wells, growth was detected after c. 1 month of incubation, but germination probably occurred well before this. They may have been present in other layers as they are small and may have been overlooked (or outcompeted) in wells where e.g. diatoms were prolific. The most common organisms growing in the slurries were different species of small *Chaetoceros*, which were found in most layers down to 27 cm. Seven individual, potential cysts were isolated from layer 0–3 cm. They were small, spherical or sub-spherical and yellowish in colour. One of these cysts germinated into a *Mantoniella* culture. Table 1 summarizes the layers and isolates of *Mantoniella* and *Isochrysis*, including which analyses were performed on each isolate. The species name is included for those isolates that were
included in the phylogeny. The strains from other isolates were very similar to those identified, and are most likely the same, but we did not confirm the species identity of these isolates by sequencing.

Description of cysts

Intact *Isochrysis galbana* cysts formed in culture (after c. 1 month) were small, almost spherical (Figs 3, 4) and yellowish in colour. They measured 3.4–5.3 µm in diameter (n = 20; measured on light micrographs of cysts formed in culture) and the empty cysts showed a small circular opening of c. 0.5 µm in diameter (Figs 5, 6, 8). The cysts were found in monoclonal cultures as well as in mixtures of culture strains that had been kept for months under either dark or light conditions, as well as both at salinities of 20 and 30, but not in medium without nitrogen. Similar cysts were also found in TEM analyses of raw sediment that was rinsed by standard methods to remove non-silica material (Fig. 7). TEM sections of cysts from cultured strain B5 (Fig. 10) showed a thick (c. 0.2 µm) wall with granular unevenly distributed protuberances (Fig. 10). The cell content included a chloroplast with a single pyrenoid, and vesicles of unknown function (Fig. 10).

*Mantoniella* cysts formed in culture (after c. 1 month) were more or less spherical (Fig. 9) and yellowish in colour. They were 4–5 µm in diameter (n = 7). Similar to *Isochrysis* cysts, they were found in monoclonal cultures as well as in mixtures of culture strains that had been kept for months under either dark or light conditions, as well as at salinities of 20 and 30, but not in medium without nitrogen. One of the single isolated cysts from the sediment (0–3 cm) germinated (= cyst 5).
Tests for silica in *Isochrysis* cysts

As the cysts remained intact after processing by standard methods for rinsing diatom frustules, indicating that they might contain silica, we analysed the elemental composition of the cyst wall using the EDS function of the scanning electron microscope. These tests for silica in cysts from Isomix6 indicated the presence of small amounts of silica (from 0–3%). Similar analyses showed c. 20% silica in cysts of *Ochromonas gloeopa* Skuja 1964, the chrysophyte we included as a positive control (data not shown).

Motile stages

The motile stage of *Isochrysis galbana* was, in our isolates, c. 3.6 × 2.7 μm in size (n = 2), with 2 identical flagella (c. 5–7 μm long) each with an acronema, and a short haptonema (Fig. 11). Several layers of rimless body scales were present, measuring 0.20–0.29 × 0.23–0.32 μm in size (Figs 12, 13). The haptonema was covered by scales 70–90 × 70–80 nm in size.

The motile stage of *Mantoniella squamata* had one long and one very short flagellum. It had three types of spiderweb scales, two types on the cell body (measuring c. 0.21 and 0.11–0.13 μm in width, respectively (Fig. 14), and one type on the flagella, 0.21–0.23 μm in length and 0.17–0.19 μm wide. In addition, two rows of flagellar hair scales were present (cf. Moestrup, 1990).

Phylogeny

Sequences of nuclear SSU rDNA of the two prasinophytes, strain C3 and cyst5 were identical to a strain K-284 isolated from the Sound, Denmark. Two base pairs separated these from another strain of *M. squamata* (strain CCAP 1965/1 with accession number X73999), whereas nine base pairs differed from *M. antarctica*. Phylogenetic analyses place our organism in a not well-supported clade with *Mantoniella squamata*, with *M. antarctica* Marchant 1989 as a very close sister group (Fig. 15).

In the phylogenetic analyses of both LSU and SSU nuclear rDNA, the two sequences of haptophyte strains from the sediment clustered in a clade of *Isochrysis galbana sensu stricto* (Fig. 16), as recently defined by Bendif et al. (2013).

**DISCUSSION**

For the first time we document resting stages of small haptophyte and prasinophyte flagellates from sediment samples and, by germinating them from dated layers of a sediment core, we show that they have the capability to remain viable for at least 40 years in situ.

*Isochrysis* cysts

The cysts of *Isochrysis galbana*, found in culture and in the sediment samples, are morphologically similar to haptophyte cysts previously described from cultured strains and observed forming under the microscope in fresh plankton samples. Parke (1949) illustrated and described cysts of *Isochrysis galbana* as spherical, 5–6 μm in diameter and with an opening of c. 1.5 μm, closed by a domed plug when intact. The newly formed cysts were depicted as smooth, while mature and germinated cysts were shown, and described, as covered by small protuberances. Carter (1937), in her classic study from the Isle of Wight, described cysts of *Prymnesium parvum* Carter 1937; apparently the first description of cysts in haptophytes. The cysts were ovate or elliptical in shape, 9–11 × 5–7 μm. The wall was thin, transparent and delicately rugose, and the single cell present within the cyst was motionless. It developed flagella at maturity and escaped through a narrow pore. A plug was not observed, and Carter speculated that these cysts might be temporary cysts. Wang & Wang (1992) depicted light micrographs of empty cysts of *Prymnesium saltans* Massart 1920. These cysts were spherical to subspherical, larger than the cysts found here (c. 10–15 μm) and with a c. 2–4 μm-diameter hole. Conrad (1941) showed similar illustrations of cysts of *P. saltans*. Pienaar (1980) described the *Prymnesium* cysts he observed as identical to those described by Conrad (1941), although they look more oval in shape in the illustrations shown.

The haptophyte cysts depicted in the literature and found here are strikingly similar to stomatocysts of some chrysophytes (e.g. Piête et al., 2009), which are known to be siliceous. Indeed, the presence of silica in haptophyte cysts has also been reported by previous authors. Parke (1949) wrote that the cellulose membrane of *Isochrysis galbana* cysts thickens and becomes impregnated with silica, but she did not describe how the presence of silica was determined. Pienaar (1980) described the *Prymnesium* cysts as having scales and did a preliminary investigation to determine the composition of an electron dense material seen in thick TEM sections of these scales. This study (by Kevex analysis) showed that one of the major components of this material was silica. According to him, *Prymnesium* is the only prymnesiophyte known to produce cysts or to deposit silica. More recently Yoshida et al. (2006) described a new haptophyte genus, *Hyalolithus* Yoshida, Noél, Nakayama, Naganuma & Inouye 2006 as characterized by bearing siliceous scales on the vegetative cells, which were shown to be produced in a different manner than usual for scales in haptophytes. Apart from this recent paper, reports of silica in scales or cysts of haptophytes are sporadic and not well documented. In the present study, we noted that the cysts were still
Long-term survival of haptophytes and prasinophytes
Fig. 15. Phylogenetic tree based on SSU rDNA maximum likelihood analyses of Mamiellophyceae and other prasinophytes. Bootstrap analyses shown refer to maximum parsimony (MP)/neighbour-joining (NJ)/maximum likelihood (ML).

Figs 3–14. Fig. 3. Light micrograph of intact cysts of *Isochrysis galbana* formed in culture strain 24–25 B5. Fig. 4. Light micrograph of a mixture of empty and intact cysts of *Isochrysis galbana* formed in Isomix. Fig. 5. Light micrograph (close-up) of a *Mantoniella* cyst formed in strain 24–25 C3. Fig. 6. Light micrograph an *Isochrysis galbana* cyst formed in Isomix, showing the pore/opening. Fig. 7. Transmission electron micrograph of a cyst found in rinsed, raw, sediment from layer 0–3 cm. Fig. 8. Scanning electron micrograph of a cyst of *Isochrysis galbana* produced in Isomix. Fig. 9. LM of a mixture of empty and intact *Mantoniella* cysts formed in strain 24–25 C3. Fig. 10. Transmission electron micrograph of a section of a cyst of *Isochrysis galbana* formed in culture strain B5, composite of four micrographs. Fig. 11. TEM whole mount of an *Isochrysis galbana* flagellate cell from strain 0-3A A3. Fig. 12. TEM tangential section of body scales of *Isochrysis galbana*. Isolate B5. Fig. 13. TEM transverse section of *Isochrysis galbana* showing the proximal part of the haptonema covered with haptonema scales and cross sections of body scales. Isolate B5. Fig. 14. Three different types of scales of *Mantoniella squamata*, detached from the cell. The three elongate scales are from the flagella, while the small on the right and the large square one on the left are from the cell body. Isolate C3. Scale bars = 10 µm (Figs 3, 4); 5 µm (Figs 5–7, 9); 1 µm (Fig. 8); 0.5 µm (Fig. 10); 2 µm (Fig. 11) and 0.2 µm (Figs 12–14).
intact after being subjected to standard diatom silica rinsing procedures. We therefore attempted to confirm the presence of silica in *Isochrysis galbana* cysts by EDS analysis of whole SEM preparations or single cysts. The analyses showed trace amounts of silica (a few per cent) while a similar analysis of chrysophyte stomatocysts indicated an order-of-magnitude higher silica content (c. 20%). Thus, although the resting stage of *Isochrysis galbana* has a striking morphological similarity to some types of stomatocysts and although we saw indications of silica contents in the cysts, we were not able to confirm that haptophyte cysts are, or can be, silicified. Rather, our data, though inconclusive, indicated that these cysts had very little, if any, silica in their walls.

**Mantoniella cysts**

We found evidence of resting stages of *Mantoniella* by the establishment of strains from slurries of sediment samples up to 40 years old. We were also able to isolate single cysts from surface sediment slurries, which germinated into *Mantoniella* vegetative stages.

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Fig. 16. Phylogenetic tree of Isochrysidales inferred from maximum likelihood analyses of SSU rDNA. Bootstrap analyses shown refer to neighbour-joining (NJ)/maximum parsimony (MP)/maximum likelihood (ML), and finally Bayesian inference shown as percentages.
and we were able to induce formation of cysts in mixed and monoclonal culture strains. This is the first report of a resting stage in a marine prasinophyte.

The identity of the organisms

The phylogeny and taxonomy of the Isochrysidaceae, the family of haptophytes to which *Isochrysis galbana* belongs, has recently been revised (Bendif et al., 2013). Data from DNA sequence-based phylogenies indicated that several species and genera in this family were polyphyletic and the authors made a number of taxonomic changes. Previously, *Isochrysis galbana* appeared to be polyphyletic; but according to the revisions proposed by Bendif et al. (2013), the strains found in the present study belong to *Isochrysis galbana sensu stricto*. The other clade associated with ‘I. galbana’ strains has been renamed *Tisochrysis lutea* (Bendif & Probert 2013). Strain K633 from SCCAP under the name *Isochrysis* sp. as well as CCMP1323 named *Isochrysis galbana* was also included in our phylogenies and clustered in the revised *Isochrysis galbana* clade. However other strains (from culture collections) previously known as *Isochrysis galbana* now belong to *Tisochrysis lutea* (Bendif et al., 2013). The species is of commercial interest due to its use as aquaculture feed and whether both species have actually been used for this and are equally suited, is an open question. However, apart from the clear separation in DNA-based phylogenetic position, the two taxa also appear to be different in chemical profile (Bendif et al., 2013), which could affect their function as feed-organisms. The strains found here are morphologically very similar to previous descriptions of *Isochrysis galbana*, both with regard to the flagellate and the cyst stage. One possible, minor difference is the lack of a clear central protuberance on the scales of the strains examined here, which was reported for *I. galbana* by Green & Pienaar (1997) as ‘a small central swelling’.

The prasinophytes have long been known to be paraphyletic (e.g. Sym & Pienaar, 1993) and *Mantoniella* belongs to the group Mamiellophyceae. Our strains appear in the same not well supported clade as other strains of *Mantoniella squamata*. However, a strain of *M. antarctica* seems to be very closely related. The two species differ mainly by the distinctive basket scales which are found on the flagella of *M. antarctica* (Marchant et al., 1989). They were never seen in our preparations.

Survival

This study constitutes the first report of a resting stage in a marine prasinophyte species, the first detailed report documenting haptophyte resting stages in situ as well as the first documentation of the potential for long-term survival of haptophytes and prasinophytes. Many phytoplankton organisms form resting stages as part of their life cycle. These often serve as survival stages for overwintering or survival under other conditions that are not conducive for vegetative growth and act as an anchor to the benthic environment as they are non-motile and sink to the sediment after being formed. Such stages play important roles in the population dynamics of planktic organisms (e.g. von Dassow & Montresor, 2011; Ellegaard et al., 2013b) and can also be important in long-term survival and evolutionary histories of these organisms (Ribeiro et al., 2011). This study shows that resting stages of the two groups of small flagellates can be preserved for 40 years in marine sediments, and remain viable. This has implications both for the potential for long-term survival of populations of these organisms and for their life-history dynamics, where the potential for a benthic seed-bed must be taken into account. If the empty or dead remains of these stages can be preserved, they have the potential to be used as palaeoenvironmental indicators, in the same manner as, for example, coccoliths from other haptophyte groups are currently used. The fact that the *Isochrysis galbana* cysts could apparently withstand the treatment used for diatom frustules in sediments illustrates that they are quite robust.

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DISCLOSURE STATEMENT

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AUTHOR CONTRIBUTIONS

M. Ellegaard: original concept, sampling, drafting and editing manuscript, microscopy; N. Lundholm: original concept, editing manuscript, culture experiments, sequencing and phylogeny, microscopy; T.J. Andersen: dating, editing manuscript; Ø. Moestrup: microscopy, editing manuscript.

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