J. Phycol. **42,** 859–871 (2006)

DOI: 10.1111/j.1529-8817.2006.00240.x

LIFE CYLE AND SEXUALITY OF THE FRESHWATER RAPHIDOPHYTE *GONYOSTOMUM SEMEN* (RAPHIDOPHYCEAE)[1]

Rosa Isabel Figueroa

Instituto Oceanográfico Vigo, Cabo Estai-Canido, 36200 Vigo, Spain

and

Karin Rengefors[2]

Limnology, Department of Ecology, Lund University, Ecology Building, 22362 Lund, Sweden

Previously unknown aspects in the life cycle of the freshwater flagellate *Gonyostomum semen* (Ehrenb.) (Raphidophyceae) are described here. This species forms intense blooms in many northern temperate lakes, and has increased in abundance and frequency in northern Europe during the past decades. The proposed life cycle is based on observations of life cycle stages and transitions in cultures. Viable stages of the life cycle were individually isolated and monitored by time-lapse photography. The most common processes undertaken by the isolated cells were: division, fusion followed by division, asexual cyst formation, and sexual cyst formation. Motile cells divided by two different processes. One lasted between 6 and 24 h and formed two cells with vegetative cell size and with or without the same shape. The second division process lasted between 10 and 20 min and formed two identical cells, half the size of the mother cell. Planozygotes formed by the fusion of hologametes subsequently underwent division into two cells. Asexual cyst-like stages were spherical, devoid of a thick wall and red spot, and germinated in 24–48 h. Heterogamete pairs were isogamous, and formed an angle of 0–90° between each other. Planozygote and sexual cyst formation were identified within strains established from one vegetative cell. The identity of these strains, which was studied by an amplified fragment length polymorphism analysis, was correlated with the viability of the planozygote. Resting cyst germination was described using cysts collected in the field. The size and morphology of these cysts were comparable with those formed sexually in culture. The excystment rate was higher at 24° C than at 19 or 16° C, although the cell liberated during germination (germling) was only viable at 16° C. The placement of *G. semen* within the Raphidophyceae family was confirmed by sequence analysis of a segment of the 18S ribosomal DNA.

Key index words: **AFLP; cysts; encystment; *Gonyostomum semen* life cycle; raphydophyceans; sexuality; SSU.**

Abbreviation: **AFLP, amplified fragment length polymorphism**

Gonyostomum semen is the most common freshwater member of the raphidophyceans, and like several of its marine counterparts it is considered a nuisance alga. *G. semen* often forms intensive blooms (Pithart et al. 1997, Willén 2003) and may dominate the phytoplankton community by as much as 98% for extended periods (Le Cohu et al. 1989). This alga adversely affects lakes used for recreation, as it discharges mucilaginous strands upon contact, thereby covering bathers with a slimy layer causing itching and other allergic reactions (Cronberg et al. 1988). The distribution of *G. semen* is widespread, and has been described in various locations in Europe, Asia, Africa, North America, and South America (Eloranta and Räike 1995). In the last decades, there has been an increase in the abundance and occurrence of this flagellate in Nordic countries including Sweden, Finland, and Norway (Hongve et al. 1987, Cronberg et al. 1988, Lepistö et al. 1994), while before the 1980s, the occurrence of these blooms was rare.

G. semen is most common in brown-water lakes, i.e. lakes with moderate to high humic content (50–60 mg $Pt \cdot L^{-1}$ or approximately 10 mg $DOC \cdot L^{-1}$) and slight acidity, which are typically found in forested areas (Cronberg et al. 1988). However, principal component analyses show no direct connection with pH, but rather with humic content and nutrients, especially phosphorus (Cronberg et al. 1988, Eloranta and Räike 1995). Although peat processing and general eutrophication of forest lakes have been suggested as a probable cause (Eloranta and Räike 1995), the recent spreading of this species remains largely unexplained.

G. semen can form extremely dense blooms, reaching a biomass of over 1800 µg chl $a \cdot L^{-1}$ (Pithart et al. 1997). However, an explanation behind the bloom formation and competitive advantage of *G. semen* over other algae is lacking. Vertical migration has been suggested as one important adaptation allowing

[1]Received 30 August 2005. Accepted 26 April 2006.
[2]Author for correspondence: e-mail karin.rengefors@limnol.lu.se.

G. semen to maximize both nutrient uptake and photosynthesis (Eloranta and Räike 1995). Another important characteristic of *G. semen* is that it has an overwintering benthic stage or resting cyst (Drouet and Cohen 1935). The resting cyst is considered as an adaptation to survive during unfavorable environmental conditions (Fryxell 1983), including predation (Hansson 1996, Hansson 2000). A life cycle with an alternation between a benthic and a planktonic stage is a trait that *G. semen* has in common with a number of other freshwater and marine harmful algal bloom formers, including other raphidophytes, dinoflagellates, and cyanobacteria. In these species, the cyst stage has been shown to play an important role in bloom initiation (Anderson and Wall 1978) and seasonal succession (Anderson and Rengefors 2006). Moreover, in the dinoflagellates, resting cysts are known to form following sexual reproduction, making the cyst stage important in connection to gene recombination and genetic diversity of populations (Pfiester and Anderson 1987).

Despite the importance of the life cycle in the ecology of phytoplankton, it has to date been poorly investigated in *G. semen*. Previous works have reported aspects of asexual reproduction (Drouet and Cohen 1935) and of isolated steps of the sexual cycle (Cronberg 2005). Nevertheless, a detailed description has never been given of sexual reproduction, in which single cells are followed through the process, nor has there been a study of mating type in cultures, two aspects of great importance in phytoplankton life cycles.

Marine raphidiophytes, a small taxon whose members are almost all classified as "harmful" species, have in contrast been studied in more detail. Despite previous reports of sexual cyst formation (Nakamura et al. 1990) in these marine relatives, the cyst stage was later claimed to be haploid (Yamaguchi and Imai 1994, Itakura et al. 1996). *Chattonella antiqua* (Hada) and *Chattonella marina* (Subrahmanyan) Hara et Chihara have diplontic life histories, in which vegetative cells are diploid and cysts are haploid (Yamaguchi and Imai 1994). A similar life cycle is also suggested for *Heterosigma akasiwo* (Hada) Hada (Imai et al. 1993a), which, like *G. semen* (Salonen and Rosenberg 2000), exhibits diel vertical migrations (Watanabe et al. 1983, Yamochi and Abe 1984, Nagasaki et al. 1996).

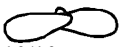
Here, we investigated the life cycle of *G. semen* by following the development and fate of the morphological types that were commonly observed in culture. The individual monitoring of these cells allowed us to report undescribed aspects in the life cycle of raphidiophytes, such as two kinds of vegetative division, fusion of holo- and heterogametes, and both asexual and sexual cyst formation. Part of the small subunit (SSU) of the ribosomal DNA was sequenced in order to provide a molecular identification of *G. semen*, and to determine its phylogenetic relationship with other raphidophytes.

MATERIAL AND METHODS

Cultures. In these experiments, cultures of *G. semen* were established through the isolation of several individual motile cells from a water sample of Lake Dagstorpsjön in August 2002. The cultures were grown in modified WC culture medium (MWC) (Guillard and Lorenzen 1972) and maintained at 15° C at 30 µmol photons $\cdot m^{-2} \cdot s^{-1}$ on a 12:12 light:dark (L:D) photo-cycle. Two strains from single-cell isolations, named G2 and G3, were established by the isolation of individual cells from this culture.

Life cycle study. Observations and size determination were carried out on vegetative cells, cysts formed in laboratory, and natural cysts. For monitoring of individual cells, different life cycle stages of *G. semen* (Table 1) were isolated from six different treatments: (i) MWC-replete medium, (ii) medium without nitrate (N) added, (iii) medium without phosphate (P) added, (iv) medium without N nor P added, (v) medium without N and placed 72 h in darkness, and (vi) medium without P and placed 72 h in darkness. Both cultures were exposed to each of the treatments. Duplicate sterile polystyrene petri dishes (Iwaki, Tokyo, Japan, 35 mm diameter) were inoculated with exponentially growing cells (4000–6000 cells $\cdot mL^{-1}$) to a final concentration of 700 cells $\cdot mL^{-1}$ in a total volume of 10 mL of each treatment medium. Fifty cells or pairs of cells with different morphology were individually isolated, photographed, and separately transferred to wells of tissue culture plates (Iwaki, 6.4 mm diameter) filled with MWC medium. Culture plates were incubated under the same conditions as those previously described for culture maintenance. The isolated cells were checked at least once daily and were photographed with a CANON EOS digital camera. They were also measured at × 630 magnification

TABLE 1. Morphologies most commonly observed in clonal cultures of *Gonyostomum semen* after 5 days of incubation in MWC medium.

Morphology and numbers of cells isolated from each clone (G2/G3)	Process[a]
 10/10	Division type 1
 7/6	Division type 2
 10/10	Fusing pairs of hologametes
 7/3	Fusing pairs of heterogametes
 5/5	Temporary cysts
 4/5	Cytoplasmic reduction

[a]Classification based on results from this study.
MWC, modified WC culture medium.

using an inverted microscope (Axiovert Zeiss 135, Oberkochen, Germany) and an Image IPplus analyzer (Media cybernetics, Berkshire, UK).

Cyst germination study. For natural cyst observations, sediment samples were collected from Lake Bokesjön, Southern Sweden, in May 2004, using a gravity core sampler. Small particles were removed with a 20 µm sieve and the remaining sediment was stored in airtight plastic jars in darkness at 4° C. Twenty to 25 cysts were individually isolated to sterile polystyrene petri dishes (Iwaki, 6.4 mm diameter), and subsequently incubated at three different temperatures (16, 19, and 24° C), and checked for excystment every 24 h for 15 days. The excystment was defined as the complete emergence of the protoplast from the cyst even if the germling remained non-motile (Anderson and Wall 1978). The number of germinated cysts divided by the total number of cysts was used to determine germination success. The development of the germinated cells was checked at least daily, and the cells were photographed and measured at × 630 as described above.

Nuclear staining. Individual cells were fixed for 10–15 min in 2% glutaraldehyde in 0.01 M PBS buffer, pH 7.4 (Sigma, St. Louis, MO, USA). Subsequently, they were washed with several drops of PBS buffer, stained with 10 mM Hoechst 33342 (Sigma) or 1:100 Sybr green (Molecular Probes, New Brunswick, NJ, USA) in 0.01 M PBS pH 7.4 for 30 min, washed again, and observed with an epifluorescence microscope (LEICA DMLA, Wetzlar, Germany) at 497 nm. Photographs were taken using a digital camera (CANON EOS, Tokyo, Japan).

Genetic analysis. DNA fingerprint analysis was performed on the G2 and G3 strains in order to check whether phenotypic differences were reflected in the respective genotypic profiles. Amplified fragment length polymorphism (AFLP) was utilized to distinguish the strains, as this technique has been successfully implemented to detect clonal differences in dinoflagellates (Figueroa et al. 2006).

DNA extraction. Cells in the exponential growth phase from the G2 and G3 strains were harvested by centrifugation (3000g for 10 min at 4° C) and the total DNA was extracted, according to Bolch et al. (1999), and stored at − 80° C.

AFLP analysis. Modified protocols were followed for AFLP analyses based on Vos et al. (1995). Ten microliters of the extracted DNA (10 ng/µL) from each sample was first digested with 2.5 units of *Eco*RI (Amersham Pharmacia Biotech, Piscataway, NJ, USA) and TruI (Fermentas, Vilnius, Lithuania) in a total volume of 20 µL for 1 h at 37° C. Ligation to the ends of the DNA fragments was made by adding T-4 ligase (USB corporation, Cleveland, OH, USA) (0.5 U per sample) and adaptors, at a concentration of 0.01 µM for the E-adaptor and at 0.1 µM for the M-adaptor and incubating 3 h at 37° C. Duplicate DNA templates were made for all cultures. The preamplification reaction was performed using a DNA Thermal Cycler (Perkin Elmer Applied Biosystems 9600, Foster City, CA, USA), and carried out using 20 cycles (94° C, 30 s; 56° C, 30 s; 72° C, 60 s). Following the preamplification step, the product was diluted (10×) with water and 2.5 µL was used for selective amplification. Six primer combinations were used: 1: *Eco*RI-TCG and MseI-CGC; 2: *Eco*RI-TCT and MseI-CGA; 3: *Eco*RI-CGG and MseI-CGG; 4: *Eco*RI-TGA and MseI:CGG; 5: *Eco*RI-TCG and MseI-CGA; and 6: *Eco*RI-TGA and MseI-CGA. The reaction mix contained 10 µL preamplified product, 1.8 µL of water, 0.4 U of Taq DNA polymerase, 4 µL 1 mM dNTPs, 0.06 µL E-primer (100 µM), 0.06 µL M-primer (100 µM), 2 µL $MgCl_2$ (25 mM), and 2 µL PCR buffer (10×). Amplification by touchdown PCR was performed with an initial denaturation at 94° C for 2 min and a first cycle at 94° C for 30 s, 65° C for 30 s, and 72° C for 60 s. During the next 12 cycles, the annealing temperature was reduced by 0.7° C per cycle down to 56° C, whereas the last 23 cycles were the same as described for preamplification.

The selective amplification was stopped by adding 10 µL of formamide dye (100% formamide, 10 mM EDTA, 0.1% xylene cyanol ff, 0.1% bromophenol blue) to the samples that were stored at + 4° C overnight before running on the gel. After 3 min denaturation at 95° C, 3.5 µL was loaded onto a 6% polyacrylamide gel. The fragments were separated at 30 W for 1–2 h, and detected by fluorescein-labeled E-primers in a FlourImager (Vistra Fluorescens, Molecular Dynamics Inc., Sunnyvale, CA, USA). The results were stored as TIFF files for further processing.

Phylogenetic analyses. Strain GSBO2 isolated from a single cell collected in Lake Bokesjön, Southern Sweden, was used. DNA was extracted as for AFLP. The SSU of ribosomal DNA was amplified and sequenced using two different universal primers: 4616 (forward) 5′-AACCTGGTTGATCCTGCCAG-3′ and 4618 (reverse) 5′-TGATCCTTCTGCAGGTTCACCTAC-3′. The reactions were set up in 25 µL volumes, with 25 ng DNA template, 0.5 U of Taq DNA polymerase (AmpliTaq, Applied® Biosystems, Stockholm, Sweden), 3 µL $MgCl_2$ (25 mM), 1.25 µL dNTP (10 mM, Applied Biosystems), and 1 µL of each primer. The following amplification protocol was followed: 5 min at 94° C, 35 cycles at 94° C for 1 min, 55° C for 5 min, 72° C for 2 min, and a final extension step at 72° C for 7 min. The PCR product was cleaned with the PCR-M Clean Up System (Viogene, Sunnyvale, CA, USA) and sequenced using the BigDye Terminatior v1.1 Cycle Sequencing Ready Reaction Kit (Applied Biosystems) using the primers 4616F, 4618R, Euk29F, and Euk517R (Coyne et al. 2005). The partial SSU sequence was submitted to GenBank (accession no. DQ408616).

For the phylogenetic analyses, the sequences were edited with BioEdit (v7.04.1, Hall 1999) and aligned using MEGA3 (Kumar et al. 2004). Additional raphidophyte and outgroup sequences were downloaded from GenBank for phylogeny constructions. The phylogeny was estimated using Neighbor-Joining as implemented in MEGA3 under the Tajima–Nei substitution model with a γ-distributed rate of variation across sites. Branch support for the phylogram was estimated by 1000 bootstrap pseudoreplicates.

RESULTS

Based on our results, we have proposed a life cycle for *G. semen*, which is summarized in Figure 1. The physiological development of each depicted process is based on the individual isolation and monitoring of a total of more than 50 cells.

Vegetative cycle. Vegetative cells of *G. semen* with normal morphology and size (Table 2) have two flagella attached at an apical pit, with one directed forward and one backward when swimming (Fig. 2a). These cells had a central nucleus that occupied approximately 1/7 of the cell volume (Fig. 2b).

Vegetative cells in culture underwent three main processes: division, fusion, or asexual cyst formation. Division occurred by two different mechanisms. Division type 1 (Figs. 1 and 3) lasted between 6 and 24 h, and involved the elongation of the dividing cell proceeding until two cells with vegetative size were formed. The newly formed cells had the same or different lengths. This process took place in two planes, with the formation of a lobe on one side of the caudus

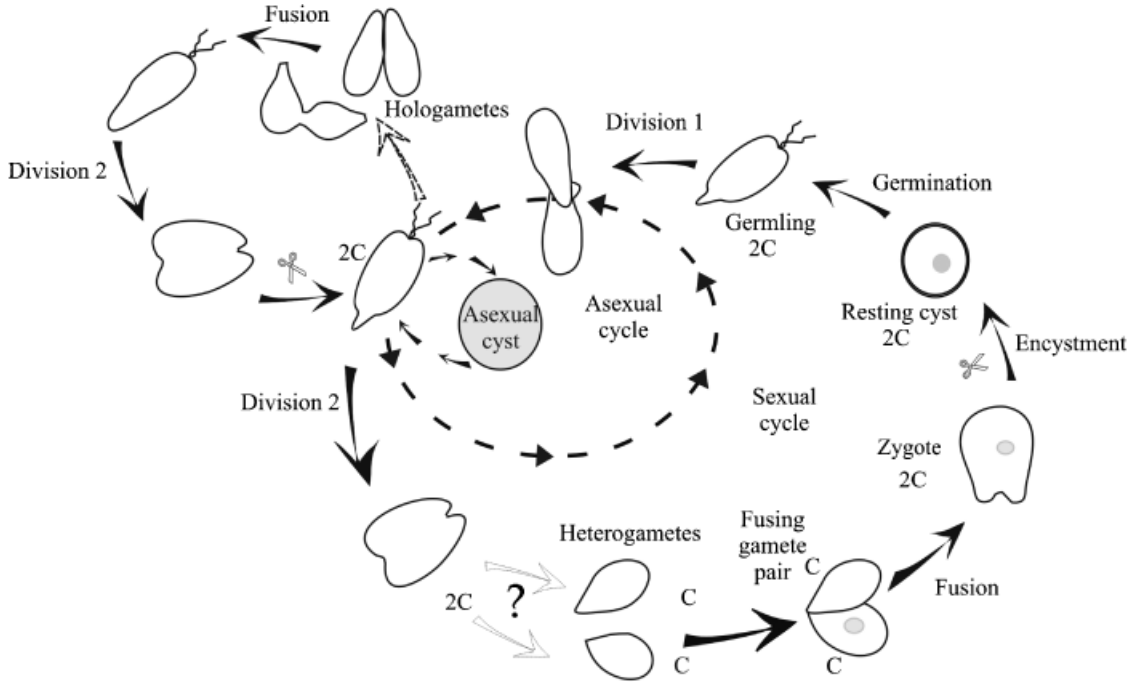


FIG. 1. Life cycle of *Gonyostomum semen* in culture and DNA contents assuming that vegetative cells are 2C in the G1 phase (see text for explanation). The scissors indicate the steps not allowed in the clonal culture G3.

at an early stage of the division (Fig. 3, a and b). This lobe later formed the two "tails" of the cells. In this example, the cells formed had a different size and remained attached by a thin cytoplasmic bridge during several hours before splitting (Fig. 3c). At other times, the process proceeded in the same plane (Fig. 3, d–f). In these cases, cell enlargement (without or with symmetry, Fig. 3, d and e, respectively) was not followed by the formation of the two cell tails, which only appeared at the end of the division process (Fig. 3f). The division of the nucleus may have occurred in very early stages of division, since cells in initial stages of division (Fig. 3g) already had two distinct nuclei (Fig. 3h). In addition, some large (63–78 μm in length) vegetative cells in culture contained two nuclei (Fig. 3i).

In division type 2 (Figs. 1 and 4, a–f) cell constriction was evident at the posterior and anterior part of the cell (Fig. 4a). Cells with a morphology similar to Figure 4a had one large nucleus that occupied most of the cell, and in which short and thick chromosomes (30–50) were observed (Fig. 4b). The mid-furrow (Fig. 4c) and the nuclear division (Fig. 4d) progressed until the formation of two identical cells half the size of the mother cell (Fig. 4, e–f). The main characteristic

TABLE 2. Cell dimensions and distinctive features of the different *Gonyostomum semen* life cycle stages in culture.

Stage	Dimensions	Distinctive features	Duration
Vegetative cell	36–92 μm long 23–69 μm wide	No fixed shape, yellowish-green in color, with two flagella in an apical pit	—
Fusing pairs	23–25 μm long 18–24 μm wide	Isogamy. 0–90° angle of coupling	10 min to 3 h
Planozygote	Young (<24 h): 35–39 μm long 23–26 μm wide. Old (1–3 days): 40–45 μm long 33–37 μm wide	Flat apex. Could present a red spot	48–72 h
Hypnozygote (resting cyst)	27–39 μm diameter	Double wall, reservoir granules, one or two reddish-brown spots.	>3 months
Germling from resting cyst	55–66 μm long 31–43 μm wide	Vegetative morphology. The red spot is left in the cyst shell.	48–96 h
Temporary cyst	28–38 μm	Almost spherical, dark color, and no granules or double wall	24–48 h

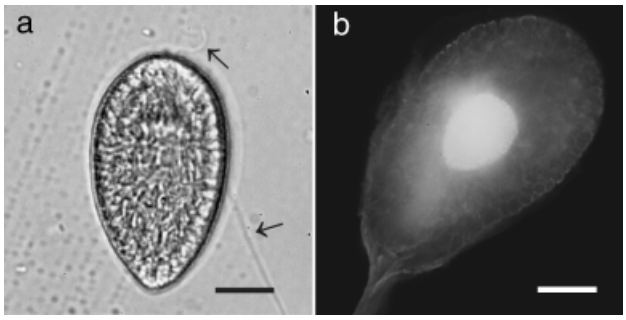


FIG. 2. Vegetative cell of *Gonyostomum semen*. Scale bars, 10 µm. (a) Vegetative cell with the two flagella pointing in opposite direction. (b) Nucleus of a vegetative cell after staining with Hoescht.

that differentiated this process from the division type 1 was that there was no cell enlargement, and consequently, the cells formed were always identical to each other and half the size of the mother cell. Another important difference was the nuclear development, where nuclear division occurred before cell enlargement in type 1, whereas nuclear and cytoplasmic divisions were simultaneous processes in type 2. Division type 1 was commonly observed in the morning. Type 2 division, on the other hand, was more frequent before the onset of the dark period, and was also enhanced by stressful conditions, such as nutrient limitation (mainly N reduction), or long periods of darkness.

We also observed that some cells lost cytoplasmic volume through a cytoplasmic furrow (Fig. 4g). Cells in the early stages of this process had one nucleus located in the constriction area (Fig. 4h), although it ended with the formation of only one nucleated cell and a cytoplasmic residue.

Hologamete fusion. Cell fusion was a rather frequent process in exponentially growing cultures. Fusions occurred between isogamous or anisogamous cell pairs and followed two pathways: at an angle or longitudinal. Fusions at an angle (Fig. 5) were more common and began with the attachment between one cell's apical area and the upper lateral area of the other cell, forming an angle of 0°–90° between the cells (Fig. 5a). At this stage, nuclei were approaching each other (Fig. 5b). The fusion progressed as shown in Figure 5, c–e, and a larger and longer cell than the originals was formed (Fig. 5f). After the complete fusion, only two flagella were observed in these zygotes. This cell contained one nucleus of 22–26 µm

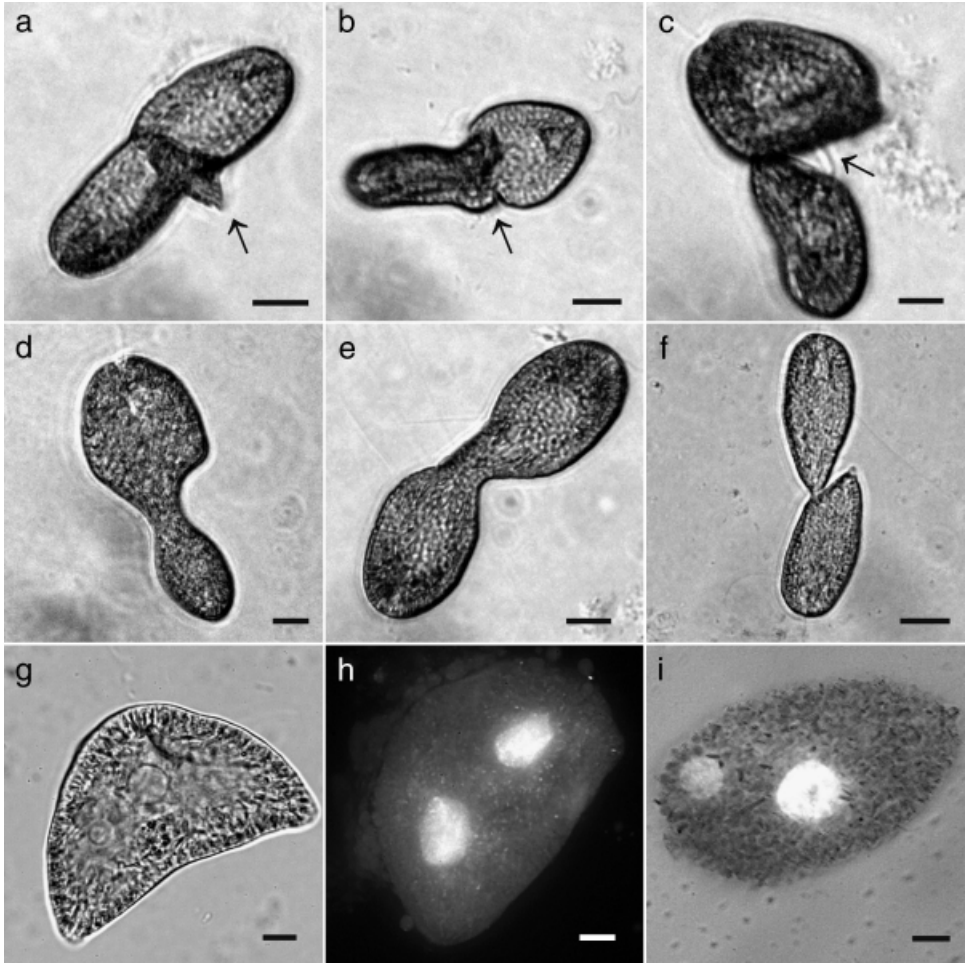


FIG. 3. Time-lapse photography of cells in vegetative division type 1. Scale bars, 10 µm. (a) First division stage showing the formation of a lobe (arrow) between the emerging cells. (b) Division stage in (a) after 24 h development. (c) Final stage of the division and formation of two cells of different sizes. The arrow indicates the cytoplasmic bridge that is the last nexus between both cells. (d) Asymmetric division type 1 without lobe formation. (e) Symmetric division type 1 without lobe formation. (f) Final stage of the division (e) and formation of two cells with the same size. (g) Culture cell in division type 1. (h) Nuclear staining of the cell in (g). (i) Culture vegetative cell showing the presence of two nuclei.

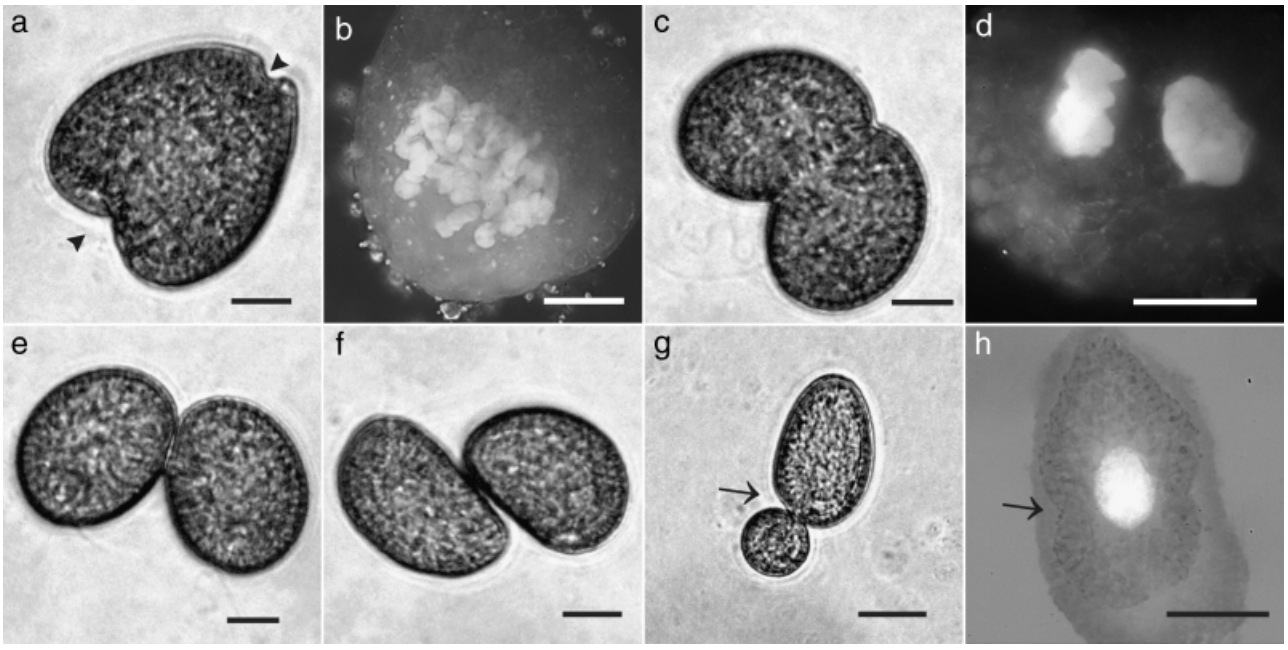


FIG. 4. Time-lapse photography of a vegetative divisions type 2. Scale bars, 10 μm. (a) Initial stage of division and formation of cytoplasmic constrictions on both sides of the cell (arrow heads). (b) Nuclear staining of a cell in similar stage of division than (a). (c) Progression of the division (a) and formation of a mid-cytoplasmic furrow. (d) Nuclear division in a cell with a morphology similar to (c). (e–f) Final stages of division (a). (g–h) Culture cell undergoing cystoplasmic reduction by the formation of a cytoplasmic furrow (arrow) and the presence of one nucleus in the constricted area (arrows) in a early reduction stage (h).

diameter (Fig. 5g), and the subsequent division resulted in two cells with similar morphology (Fig. 5h).

Longitudinal fusion began by the attachment of both lateral sides of the cells, which progressively fused (Fig. 6, a–c). After 24 h, the cell formed by this fusion process (Fig. 6d) divided (Fig. 6e), and formed two similar cells with smaller size and a marked constriction between the main body and the tail area (Fig. 6f). These cells were viable and progressively acquired a normal vegetative morphology. Fusing stages were

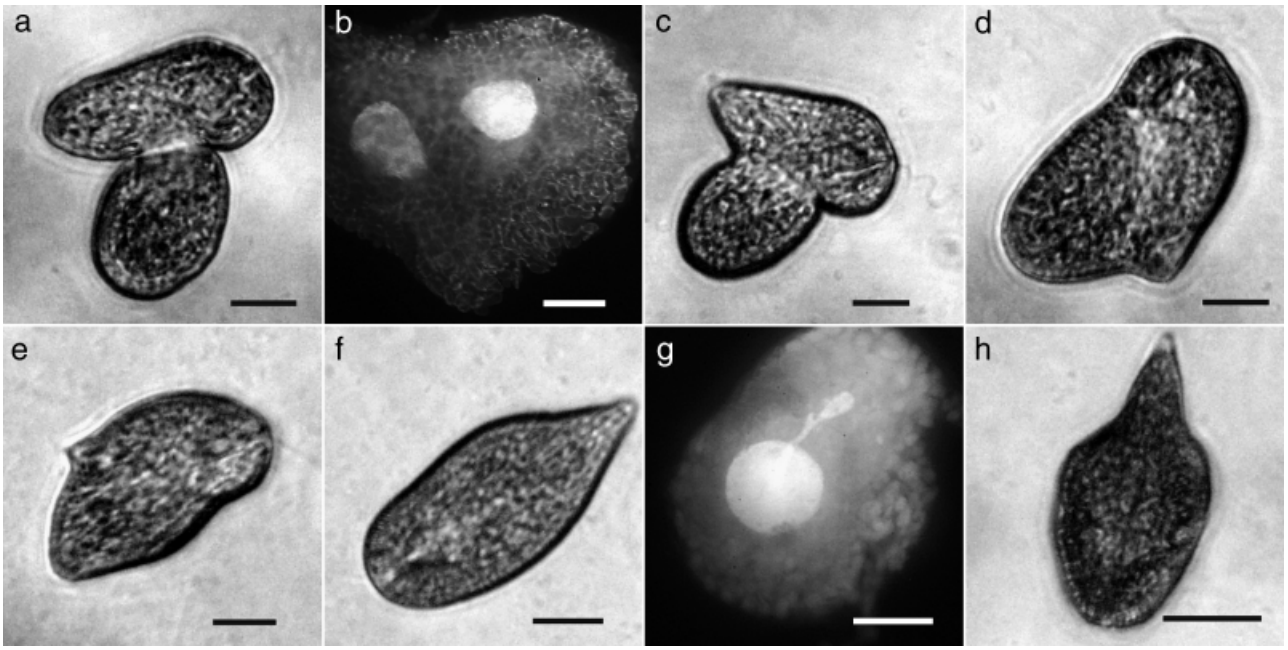


FIG. 5. Time-lapse photography of fusion with angle of hologametes. Scale bars, 10 μm. (a) Initial stage of fusion. (b) Nuclear arrangement in a cell with similar morphology than (Fig. 6a). (c–f) Progression of the fusion (a) and new cell formation. (g) Nuclear staining of a cell formed by a fusion with angle. (h) One of the two cells formed by the division of the cell (f).

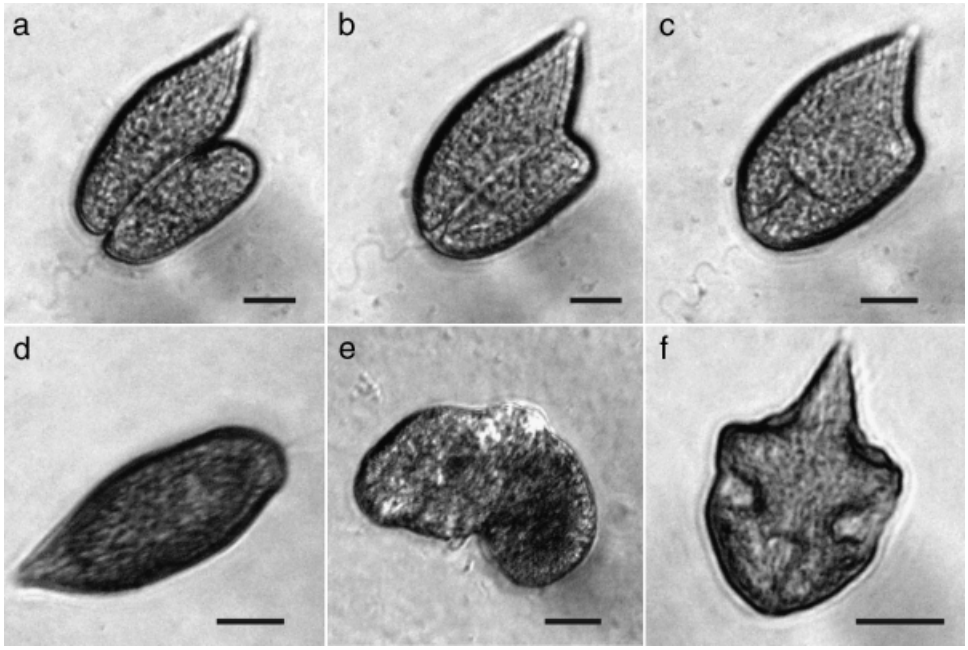


FIG. 6. Time-lapse photography of an equatorial fusion of hologametes. Scale bars, 10 μm. (a) Initial stage of fusion. (b–d) Progression of the fusion and new cell formation. (e) Cell in division type 2. (f) One of the two cells formed by the division (e).

always viable in the G2 strain but never in the G3 strain. This pattern of viability was observed in all studied cases, which involved the monitoring of at least 10 cell pairs in the process of fusion from each strain. Intermediate morphologies of the fusing process as shown in these time-lapse photographs were routinely observed in cultures.

Asexual cyst formation. In stressed cultures, the formation of asexual cyst-like stages was observed. Under long periods of darkness and/or N and/or P depletion, vegetative cells acquired a circular shape and settled on the bottom of the culture plates (Fig. 7a). These cysts were distinct from sexual cysts (see paragraph below), because they were larger, and lacked a double wall and red spot. Furthermore, an empty wall was not left after excystment (Fig. 7b), which occured 24–72 h after their isolation in fresh MWC medium. Figure 7c depicts a massive temporary cyst formation at the bottom of a culture previously exposed to N-limited medium and 72 h of darkness.

Heterogamete fusion, planozygote, and resting cyst formation. Fusing gamete pairs were observed in old cultures and in medium with N^- or P^- depletion. As seen with the fusion of vegetative cells, the zygotes were unviable in clone G3, but encysted successfully in strain G2. Heterogametes were isogamous (both gametes had the same size), and paler and smaller than vegetative cells (Table 2), and sometimes contained a reddish brown vacuole. Fusing gamete pairs formed an angle of 0°–90° between each other. Figure 8a shows a fusing pair with a 0° angle between the gametes. During the fusing process (6–12 h), the cells first attached the apices, which led to the formation of typical two-tailed cells with a flat apex (Fig. 8b). Two-tailed cells can be considered syncytiums, as they contained two nuclei (Fig. 8c) and four flagella arising from the apical pit (Fig. 8d). In Figure 8e, a fusing pair with a 90° angle between the gametes is shown. Although the fusing process was different from that in the previous case (compare Fig. 8f with 8, a and b), it eventually led to the formation of a two-tailed cell (Fig. 8g). Later, the tails fused (Fig. 8h) and zygote formation was completed (Fig. 8i). One-tailed zygotes contained one elongated nucleus in the central position (Fig. 8j) and only two flagella. Old planozygotes usually contained one brown-reddish spot (Fig. 8k), and the nucleus was positioned at the

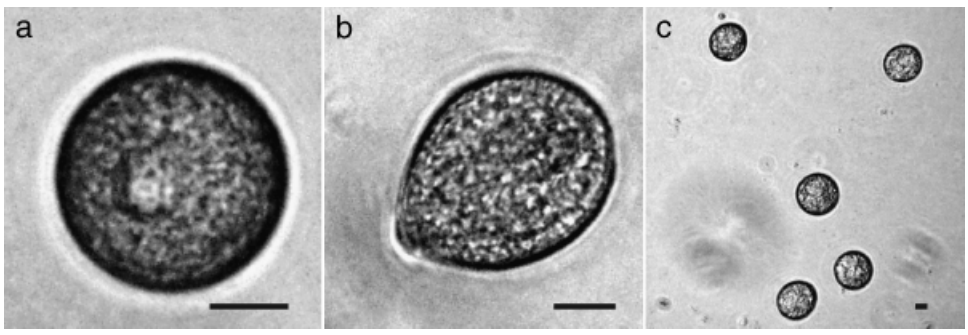


FIG. 7. Formation and excystment of temporary cysts. Scale bars, 10 μm. (a) Temporary cyst. (b) Germling from temporary cyst. (c) Temporary cysts in the bottom of a culture.

tail of the cell (Fig. 8l). Between 2 and 4 days from the isolation of fusing pairs, double walls and round cysts (29–39 μm diameter) were formed in clone G2 (Fig. 8m). However, these cysts degraded 6–8 days after their formation in the culture plates of gamete pair isolation. No indications existed that planozygotes could omit the resting stage and undergo division directly. Sexual cysts contained one nucleus (9.5–13 μm in diameter, Fig. 8, n and o) located toward one end of the cyst. In some cysts, the nucleus was elongated and located in a more central position (Fig. 8p). Some culture cysts had two red spots (Fig. 8q). This feature was explained by the existence of red spots in both fusing gametes and often, although not in all cases, co-occurred with the presence of two nuclei (Fig. 8r). In these cysts, the distance between the two nuclei was variable. Some of them were adjacent and almost fused (Fig. 8s) and were localized at a more central position (Fig. 8t). In the followed cases, fusing gametes with none or only one red spot formed one-spot cysts.

Resting cyst germination. To describe the morphology of the germling from resting cysts, we studied the germination of natural cysts. The size and morphology of those cysts were comparable with those obtained in our cultures (Table 3). Cysts were round, green, and with a thick wall. They typically had a reddish-brown spot, although two spots were also observed (Fig. 9, a and b) as in the culture cysts. Cysts germinated in greater proportions at high temperatures, but the germlings were only highly viable at 16° C. At 24° C, the germination percentage reached 61%, but no single viable germling was observed. At 19° C, 43% of the cysts germinated, although germling viability remained low (11%). Only 36% of the cysts germinated at 16° C, but the post-excystment viability was as high as 60%.

Excystment began by the appearance of a small hole in the double wall of the cyst (Fig. 9c). Later, the germling exited through this opening, always leaving behind the red spot in the empty cyst (Fig. 9d). Zero to 24 h after excystment, the germling had attained vegetative cell morphology (Table 2 and Fig. 9e). On one occasion, we observed the germination of two cells. Newly excysted cells had one nucleus with the same characteristics as observed in vegetative stages (Fig. 9f). The germling divided into two cells after 72–120 h.

Genetic analyses. To determine whether *G. semen* strain identity impacted the efficiency of the life-cycle processes studied, an AFLP analysis was carried out on the cultures G2 and G3. In this analysis, 3 of the 6 primers combinations used showed clear differences between the strains (primer combinations 2, 3, and 6, Fig. 10). As several differences were observed with the selective amplification 6, only the most obvious ones are indicated by arrows in Figure 10.

The partial SSU (436 bp) phylogeny clustered *G. semen* with the other freshwater and marine raphidophytes (Fig. 11). This clade had high bootstrap support (94%). *G. semen* was most closely related to *Vacuolaria virescens*, the only other freshwater species in the analyses (100% bootstrap).

DISCUSSION

Knowledge of the life cycle stages and processes is essential to understanding the ecology and environmental role of a species in a certain area (Litaker et al. 2002). Here, we present detailed photographic evidence of cell morphology and transitions between life stages of the full life cycle of the nuisance alga *G. semen*.

Our main results can be summarized as follows: *G. semen* ordinarily multiplies with a longitudinal cell division (division type 1). Close to onset of darkness, and mainly under stressful conditions, a depauperating division (division type 2) also takes place. *G. semen* has a sexual cycle, which is induced by N^- or P^- depletion. Under these conditions, small, pale gametes are formed, which fuse in pairs and form a two-tailed cell that becomes rounded, acquires one to two brownreddish pigmented spots inside, and transforms into a round resting cyst. Vegetative stages can undergo asexual cyst formation and fusion, but the zygote formed by this process divides but never encysts. Our data are the first to our knowledge that reported step by step the formation of resting sexual cysts in a raphidophycean flagellate, from the fusion of gametes to the zygote encystment. These results show interesting similarities to and differences from marine members of this group.

Asexual reproduction. Asexual reproduction of *G. semen* vegetative stages occurred through two types of pathways that we have termed as division type 1 and 2. Division type 1 was the main way of division in exponentially growing cultures, and resulted in cells with vegetative size and morphology. In contrast, division type 2 was a depauperating kind of division, as two cells half the size of the mother cell were formed. Presumably, type 2 division occurred as a response to stress, as it was more frequent near the dark period, after placing the cultures in darkness for 72 h, or in N^--limited medium. These results agree with previous work on marine raphidophytes showing that irradiance was important for nuclear DNA replication. For example, *C. antiqua* and *C. marina* generally divide once during one period of darkness (Ono 1988), and several divisions occurred at night in cultures of *Heterosigma akashiwo* (Honjo and Tabata 1985). Previously, only division type 2 has been described for the asexual division of this alga (Cronberg 2005). This fact may reflect stressful conditions, given that no acclimated cultures but rather lakes samples were used.

The two types of divisions described for *G. semen* differed in their nuclear development. Nuclear division occurred before cell division in division type 1, whereas both processes were simultaneous in division type 2. In the latter process, the chromosomic structure was clearer (Fig. 4b), which might be explained by a smaller DNA amount. The characteristics of type 1

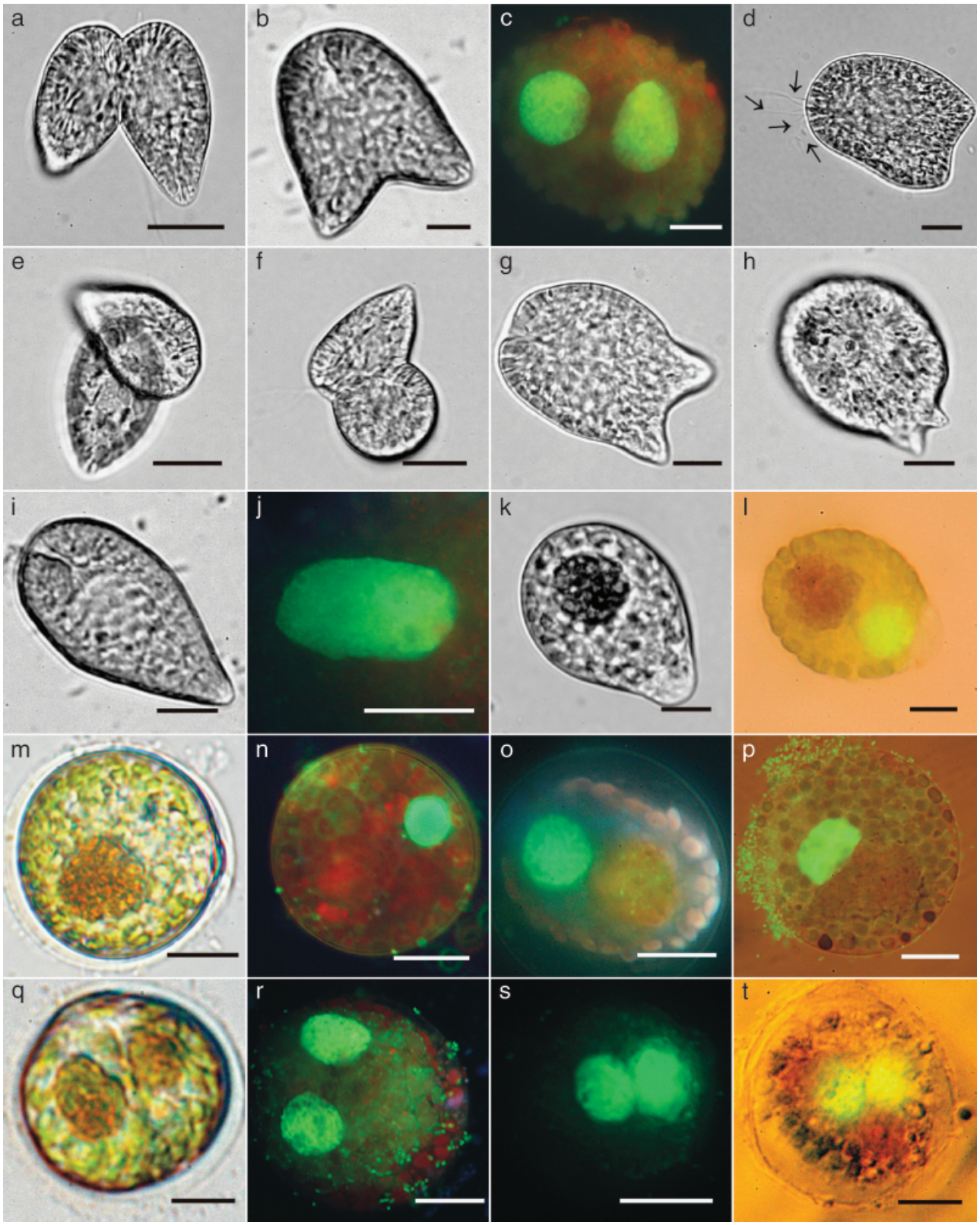


FIG. 8. Time-lapse photography of heterogamete fusion and zygote formation in the strain G3. Scale bars, 10 μm. (a) Isolated fusing gamete pair forming a 0° angle between the gametes. (b and c) Planozygote formation and nuclear staining. (d) Different planozygote showing four flagella arising from the apical pit (arrows). (e) Isolated fusing gamete pair forming 90° angle between the gametes. (f–i) Planozygote formation. (j) Nuclear staining of the planozygote (i). (k and l) Planozygote with a red spot showing the nucleus near the tail area. (m–p) One-spot cysts and nuclear staining showing the nucleus usually opposite the red spot and in peripheral position (n and o), though on occasion almost central (p). (q) Two-spot resting cyst. (r-t). Nuclear staining of two-spot resting cysts showing different arrangement of the nuclei.

TABLE 3. Size comparison between natural and culture cysts of *Gonyostomum semen*.

Origin of cysts	Median ± standard deviation (μm)	Range (μm)
Field ($N = 63$)	31.7 ± 1.89	27–37
Culture ($N = 25$)	33.6 ± 3.41	28–40

division suggest that this is the mitotic and normal type of vegetative cell division. Type 2 division, on the other hand, may be a haploidization process, based on cell size, morphology, nuclear development (Fig. 1), and in the fact that the resulting cells are smaller than vegetative ones and probably contain less DNA.

Sexual reproduction. In this study, we were able to prove sexual reproduction in *G. semen*. We provided the step-by-step evidence that small and pale cells fused and that the resulting two-tailed planozygote eventually lost motility and formed resting cysts. Nutrient stress has been reported as a trigger for sexuality and resting stage formation in many dinoflagellate species (von Stosch 1973, Chapman et al. 1982, Sako et al. 1987, Kelley and Pfiester 1990). Accordingly, we observed the formation of fusing gamete pairs under nutrient-deficient conditions, mainly in medium without N. In contrast to results for many dinoflagellate species, in *G. semen,* we observed that gametes capable of resting cyst formation were distinctly different from vegetative cells. Heterogametes were characteristically smaller, paler, and differently shaped than vegetative cells (heterogamy) (Fig. 8, a and e). "Small cells" were also identified as gametes in *C. antiqua* (Nakamura et al. 1990). In *H. akashiwo*, pre-encystment cells also differed in shape, size, and color from vegetative cells (Imai 1989, 1990, Imai et al. 1993b). In the present study, we also verified that cells of *G. semen* with vegetative, rather than gamete morphology (hologametes) underwent processes of fusion in culture. The fusion of hologametes, is a novel finding in the study for raphidophytes. This process differed from the fusion of heterogametes in both the morphology of the cells involved and the fate followed by the fused cell. This kind of fusion did not form two-tailed zygotes, and the resulting cell underwent division. This fact suggests that although vegetative cells may have surface recognition molecules as those in gametes, the fusion cannot produce resting cysts. Interestingly, we never observed fusion of holo- with heterogametes.

We documented an important phenotypic difference in the life cycle processes between the strains of *G. semen* used. Fusion of vegetative-like cells and sexual cyst formation were unviable processes in one of the strains, while zygote division and encystment were successfully achieved in the other one. The AFLP analysis on the cultures G2 and G3 showed different fingerprint patterns, suggesting that there were genotypic differences between the two strains. In *Heterosigma* strains, RAPD variability was related to phenotypic variation between natural and cultured samples (Han et al. 2002). Based on this evidence, we inferred that genetic factors likely affected the sexual behavior of the *G. semen* strains.

Another result is that sexuality might be under the control of at least two loci, as cell mating and cytoplasmic fusion were processes achieved independently of the viability of the zygote. Consequently, only asexual reproduction was possible in the strain G3, albeit that cell mating and fusion processes were also observed. In this strain, outcrossing would be necessary to complete the cycle proposed in Fig. 1. The existence of a sterile

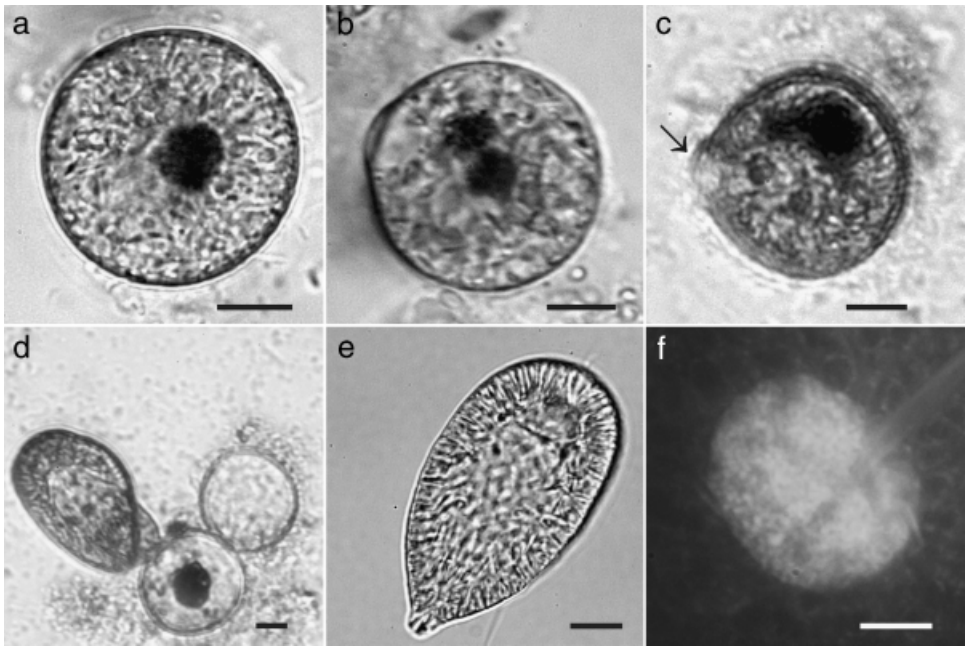


FIG. 9. Resting cyst germination. Scale bars, 10 μm except in (f), which is 5 μm. (a–b) Resting cysts from natural samples with one and two red spots, respectively. (c) In an initial stage of germination, an opening (arrow) is formed in the double wall of the resting cyst. (d) Resting cyst germination. (e) Newly excysted cell (0–24 h). (f) Nucleus from the germling in Fig. 10e after staining with Hoescht.

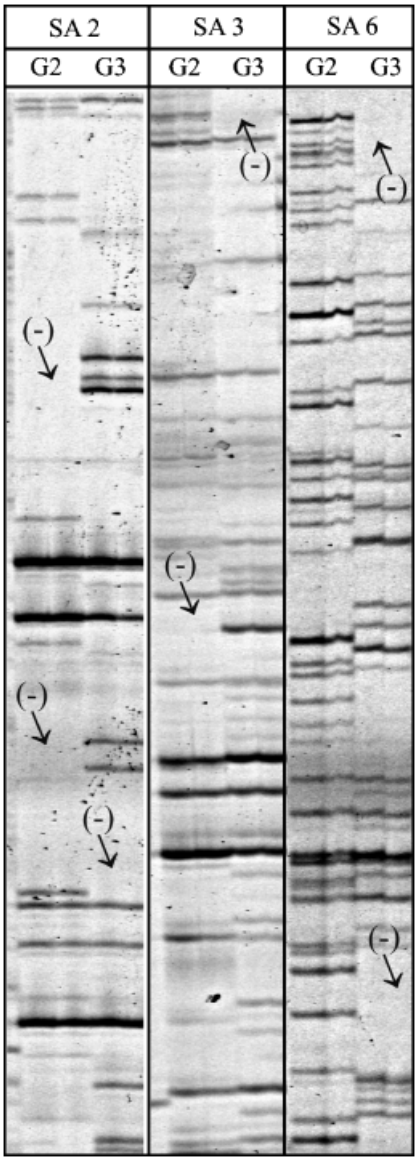


FIG. 10. Amplified fragment length polymorphism analysis showing the pattern of the clonal strains G2 and G3. SA 2, SA3, and SA6 are the primer combinations used. Each two lines correspond to one duplicate analysis. Arrows indicate when bands are missing in one of the strains.

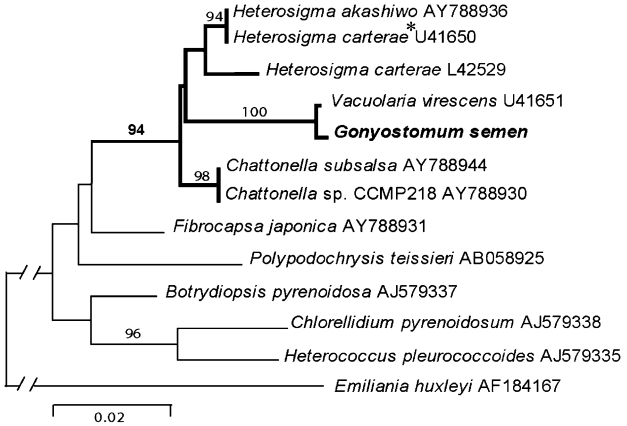


FIG. 11. Neighbor-joining tree based on a partial 18S rDNA sequences of raphidophyte and xanthophytes. Note the well-supported clade including *Gonyostomum semen* with other marine rapahidophytes and the freshwater species *Vacuolaria virecens*. *Emiliania huxley* was included as an outgroup. Bootstrap values are based upon 100 replicates. Scale shows substitutions per sites. **Heterosigma carterae* is synonymous to *Heterosigma akashiwo* (see text for explanation).

strain is also plausible. The same sexual pattern was observed in four other new clonal strains tested (unpublished data), which in all cases were capable of gamete mating and cyst formation. In conclusion, our results suggest that in most strains of *G. semen*, sexual fusion and cyst formation can be completed within strains established from a diploid, vegetative stage.

Nonetheless, the sexual cysts formed during our experiment degraded with time. This phenomenon was also observed by Imai (1989), who reported that some unknown factors affected the formation of viable cysts of *C. marina* in culture. As seen in our results, encystment was successfully achieved in *C. marina* but the cysts had a very low incidence of germination. One explanation for this result might be that culture conditions were not adequate for cyst maturation. *C. antiqua* and *C. marina* complete cyst formation after sinking to the sea bottom (Imai and Itoh 1988). For these species, the combination of factors such as nutrient depletion, adherence to solid surfaces, and low light intensity was assumed to be essential for cyst formation and maturation (Imai 1989). In fact, for maturation and then germination to occur, *Chattonella* cysts need a low storage temperature (below 11° C) for more than 4 months (Imai et al. 1991).

We observed that uninucleate sexual cysts are produced in *G. semen* cultures, and that binucleate cysts might be produced by a premature encystment of the fused cell (syncytium), as is suggested by the existence of two nuclei in different steps of fusion (Fig. 8, r–t) that are located centrally as in the young planozygote stage (Fig. 8i). Imai (1989) also observed that most cysts formed in *C. marina* cultures were uninucleate, although some cysts were binucleate. Imai proposed that, as described for some chrysophycean flagellates (Sandgren 1983), three types of cysts might be produced in cultures: uninucleate asexual, binucleate asexual (autogamic), and binucleate sexual (zygotic). In our experiments, however, we found no evidence of asexual resting cyst formation.

Resting cysts of *H. akashiwo*, *C. antiqua*, *C. marina*, and *G. semen* have been suggested to be haploid, based on the small size of both the cyst diameter and nucleus. This fact has been attributed to their presumed haploid DNA content, which was similar to that observed in small cells formed in N-limited medium and more or less half in content of that observed in normal vegetative cells (Imai et al. 1993a). The sexually formed cysts of *G. semen* in our cultures were also smaller than the vegetative cells. However, the cell size was not related to an asexual encystment, but was due to the

small size of the gametes along with the contraction of planozygotes during encystment. For example, in Fig. 8m, we show a cyst of 28.4 µm of diameter, even though it was formed by a planozygote of 37 µm in length (5 h after gamete fusion). Sexual cyst size fitted the normal range found for natural cysts, which ranged from 27 to 37 µm.

Among the marine raphidiophytes studied, a dormancy period is not always required before cyst activation can occur. For example, *Heterosigma* resting cells do not require a mandatory period of dormancy (Han et al. 2002). In contrast, *Chattonella* cysts undergo a mandatory dormancy that in nature extends from summer to the following spring (Imai and Itoh 1987, Imai et al. 1991). *G. semen* cysts may also have overwintering benthic stages that require a mandatory period of dormancy, because in preliminary studies, natural cysts stored in darkness at 4° C failed to excyst during a 3-month period (unpublished data). The highest viability was found at 16° C, which corresponds with late spring/early summer temperatures in Swedish lakes. Temperatures between 20 and 25° C promoted the germination of cysts out of dormancy, although they have a negative effect on the viability of germlings. Temperature is also a crucial factor in the germination of natural cysts of *Chattonella* and *Heterosigma*. Cysts from *H. akashiwo* did not germinate below 10° C, and reached maximum germination values between 15 and 25° C (Imai and Itakura 1999). *Chattonella* cysts also have an optimum temperature range for germination and viability between 20 and 25° C, which corresponds with Japanese seawater temperatures during summer (Imai et al. 1991).

Sexual versus asexual reproduction. Previous studies on the life cycles of raphidophytes (Imai 2003) showed interesting and contradictory results regarding sexuality. Initial studies on marine species pointed to some evidence of sexuality. For example, Nakamura et al. (1990) observed sexual fusion and zygote encystment in *C. antiqua* under culture conditions, which suggested that cysts were diploid and products of haploid fusion. Similarly, Imai (1989) reported that both sexual and asexual processes could be responsible for cyst formation in *C. marina*. However, Imai (1989) also suggested that asexual cyst formation seemed to be most important in nature, as natural cysts contained a nucleus with a size similar to the one observed in vegetative stages cultured in N-limited medium. Based on fluorometric analysis of nuclear DNA, Yamaguchi and Imai (1994) later concluded that *C. antiqua* and *C. marina* cysts had a haploid DNA content, and this suggested the same life cycle pattern as observed for the encystment of *H. akashiwo* (Itakura et al. 1996). In these species, it was proposed that DNA diploidization occurred after excystment without fertilization (autodiploidy), as the DNA content of cells of *C. antiqua* and *C. marina* established from germinated cysts was 2C and 4C (2 and 4 DNA contents, assuming that vegetative cells are 2C in G1 phase). In contrast, we have verified that the cell and nucleus size of *G. semen* planozygotes are attained before encystment, and that the germling acquires vegetative morphology within 24 h after germination. In contrast to depictions by Cronberg (2005), only one diploid cell germinated in most cases, although germination of two cells was also observed on one occasion.

Apart from resting cyst formation, we have observed that *G. semen* cells formed asexual, short-lived cysts when they were exposed for 72 h to dark conditions. These temporary resting states allow the cell to withstand short-term environmental fluctuations, as has been reported for the marine raphidophycean *H. akashiwo* (Imai and Itakura 1999). In *G. semen*, this round non-motile form can re-establish a vegetative, motile existence in 24–72 h when conditions become favorable again (replete medium and light).

Phylogenetic analyses. Additionally, we confirmed the placement of *G. semen* among the raphidophycean family by nucleotide sequence analyses of part of the 18S (SSU) rDNA. The phylogenetic tree showed that *G. semen* clustered in a well-supported clade with the marine genera *Heterosigma* and *Chattonella*. *G. semen* also formed a distinct group with the other freshwater taxon, *V. virescens* Cienkowski. Potter et al. (1997) have previously shown that *V. virescens* is a sister taxon to *Chattonella subsalsa* Biecheler and *Heterosigma carterae* (Hulburt) Taylor (synonymous of *H. akashiwo* (Throndsen 1996)). We also noted that the marine species *Fibrocapsa japonica* Toriumi et Takano was not part of the *G. semen*, *Heterosigma*, and *Chattonella* clade. Its position received low bootstrap support and thus remains unclear.

We thank Polina Polykarpou, M. Svensson, I. Ramilo, and A. Fernández-Villamarín for technical assistance. We also thank Ramiro Logares for help with the phylogenetic analyses. The research was supported by a CSIC grant for training of research staff to R. I. F., by the Swedish Research Council (VR) to K. R., and by the SEED project (GOCE-CT-2005-003875).

Anderson, D. M. & Rengefors, K. 2006. Community assembly and seasonal succession of marine dinoflagellates in a temperate estuary—the importance of life cycle events. *Limnol. Oceanogr.* 51:860–73.

Anderson, D. M. & Wall, D. 1978. Potential importance of benthic cysts of *Gonyaulax tamarensis* and *G. excavata* in initiating toxing dinoflagellate blooms. *J. Phycol.* 14:224–34.

Bolch, C. J. S., Blackburn, S. I., Hallegraeff, G. M. & Vaillancourt, R. E. 1999. Genetic variation among strains of the toxic dinoflagellate *Gymnodinium catenatum* (Dinophyceae). *J. Phycol.* 35:356–67.

Chapman, D. V., Dodge, J. D. & Heaney, S. I. 1982. Cyst formation in the freshwater dinoflagellate *Ceratium hirundinella* (Dinophyceae). *J. Phycol.* 18:121–9.

Coyne, K. J., Handy, S. M., Demir, E., Whereat, E. B., Hutchins, D. A., Doblin, M. A. & Cary, S. C. 2005. Improved quantitative real-time PCR assays for enumeration of harmful algal species in field samples using an exogenous DNA reference standard. *Limnol. Oceanogr. Methods* 3:381–91.

Cronberg, G. 2005. The life cycle of *Gonyostomum semen* (Raphidophyceae). *Phycologia* 44:285–93.

Cronberg, G., Lindmark, G. & Björ, S. 1988. Mass development of the flagellate *Gonyostomum semen* (Raphidophyta) in Swedish forest lakes—an effect of acidification? *Hydrobiologia* 161: 217–36.

Drouet, F. & Cohen, A. 1935. The morphology of *Gonyostomum semen* from Woods Hole, Massachusetts. *Biol. Bull. Mar. Biol Lab.*, Woods Hole 68:422–39.

Eloranta, P. & Räike, A. 1995. Light as a factor affecting the vertical distribution of *Gonyostomum semen* (Ehr.) Diesing (Raphidophyceae) in lakes. *Aqua Fennica* 25:15–22.

Figueroa, R. I., Rengefors, K. & Bravo, I. 2006. Effects of parental factors and meiosis on sexual offspring of *Gymnodinium nolleri* (Dinophyceae). *J. Phycol.* 42:350–62.

Fryxell, G. A. 1983. *Survival Strategies of the Algae*. Cambridge University Press, New York, 154 pp.

Guillard, R. R. L. & Lorenzen, C. J. 1972. Yellow-green algae with chlorophyllide c. *J. Phycol.* 8:10–24.

Hall, T. A. 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symp. Ser.* 41:95–8.

Hansson, L.-A. 1996. Behavioural reponse in plants: adjustment in algal recruitment induced by herbivores. *Proc. Roy. Soc. London* 263:1241–4.

Hansson, L.-A. 2000. Synergistic effects of food chain dynamics and induced behavioral responses in aquatic ecosystems. *Ecology* 81:842–51.

Han, M.-S., Kim, Y.-P. & Cattolico, R. A. 2002. *Heterosigma akashiwo* (Raphidophyceae) resting cell formation in batch culture: strain identity versus physiological response. *J. Phycol.* 38:304–17.

Honjo, T. & Tabata, K. 1985. Growth and dynamics of *Olisthodiscus luteus* in outdoor tanks with flowing coastal water and in small vessels. *Limnol. Oceanogr.* 30:653–64.

Hongve, D., Løvstad, O. E. & Bjørndalen, K. 1987. *Gonyostomum semen*—a new nuisance to bathers in Norwegian lakes. *Verh. Internat. Verein. Limnol.* 23:430–34.

Imai, I. 1989. Cyst formation of the noxious red tide flagellate *Chattonella marina* (Raphidophyceae) in culture. *Mar. Biol.* 103:235–9.

Imai, I. 1990. Physiology, morphology, and ecology of cysts of *Chattonella* (Raphidophyceae), causative flagellates of noxious red tides in the Inland Sea of Japan. *Bull. Nansei Natl. Fish. Res. Inst.* 23:63–166 (in Japanese with English abstract).

Imai, I. 2003. Life history of raphidophycean flagellates. *In* Okaichi, T. [Ed.] *Red Tides*. Terra Scientific Publishing Company, Tokyo, pp. 111–24.

Imai, I. & Itakura, S. 1999. Importance of cysts in the population dynamics of the red tide flagellate *Heterosigma akashiwo* (Raphidophyceae). *Mar. Biol.* 133:755–62.

Imai, I., Itakura, S. & Itoh, K. 1991. Life cycle strategies of the red tide causing flagellates *Chattonella* (Raphidophyceae) in the Seto Inland sea. *Mar. Pollut. Bull.* 23:165–70.

Imai, I., Itakura, S. & Itoh, K. 1993a. Cysts of the red tide flagellate *Heterosigma akashiwo*, raphidophyceae, found in bottom sediments of northern Hiroshima Bay, Japan. 1993. *Nippon Suisan Gakk.* 59:1669–73.

Imai, I., Itakura, S. & Ouchi, A. 1993b. Occurrence of *Chattonella* red tide and cyst dynamics in sediments in northern Hiroshima Bay, the Seto Inland Sea, Japan. *Nippon Suisan Gakk.* 59: 1–6 (in Japanese with English abstract).

Imai, I. & Itoh, K. 1987. Annual life cycle of *Chattonella* spp., causative flagellates of noxious red tides in the Inland Sea of Japan. *Mar. Biol.* 94:287–92.

Imai, I. & Itoh, K. 1988. Cysts of *Chattonella antiqua* and *C. marina* (Raphidophyceae) in sediments of the Inland Sea of Japan. *Bull. Plankton Soc. Japan* 35:35–44.

Itakura, S., Nagasaki, K., Yamaguchi, M. & Imai, I. 1996. Cyst formation in the red tide flagellate *Heterosigma akashiwo* (Raphidophyceae). *J. Plankton Res.* 18:1975–9.

Kelley, I. & Pfiester, L. A. 1990. Sexual reproduction in the freshwater dinoflagellate *Gloeodinium montanum*. *J. Phycol.* 26: 167–73.

Kumar, S., Tamura, K. & Nei, M. 2004. MEGA 3: Integrated software for Molecular Evolutionary Genetics Análysis and sequence alignment. *Briefings Bioinform.* 5:150–63.

Le Cohu, P. R., Guitard, J., Comoy, N. & Brabet, J. 1989. *Gonyostomum semen* a potential nuisance in large French reservoirs? The case of the Pareloup lake. *Arch. Hydrobiol.* 117:225–36.

Lepistö, L., Antikainen, S. & Kivinen, J. 1994. The occurrence of *Gonyostomum semen* (Ehr.) Diesing in Finnish lakes. *Hydrobiologia* 273:1–8.

Litaker, R. W., Vandersea, M. W., Kliber, S. R., Noga, E. J. & Tester, P. A. 2002. Reply to comment on the life cycle and toxicity of *Pfiesteria piscicida* revised. *J. Phycol.* 38:1268–72.

Nagasaki, K., Itakura, S., Imai, I., Nakagiri, S. & Yamaguchi, M. 1996. The disintegration process of a *Heterosigma akashiwo* (Raphidophyceae) red tide in northern Hiroshima Bay, Japan, during the summer of 1994. *In* Ysumoto, T., Oshima, Y. & Fukuyo, Y. [Eds.] *Harmful and Toxic Algal Blooms*. Intergovernmental Oceanographic Commission of UNESCO, Paris, pp. 251–54.

Nakamura, Y., Umemori, T., Watanabe, M., Kulis, D. M. & Anderson, D. M. 1990. Encystment of *Chattonella antiqua* in laboratory cultures. *J. Oceanogr. Soc. Japan* 46:35–43.

Ono, C. 1988. Cell cycles and growth rates of red tide organisms in Harima Nada aea, eastern part of Seto Inland Sea. *Japan. Bull. Akashiwo Res. Inst. Kagawa Pref.* 3:1–67 (in Japanese with English abstract).

Pfiester, L. & Anderson, D. M. 1987. Dinoflagellate reproduction. *In* Taylor, F. J. R. [Ed.] *Biology of Dinoflagellates*. Blackwell Scientific Publications, Oxford, pp. 611–48.

Pithart, D., Pechar, L. & Mattsson, G. 1997. Summer blooms of raphidophyte *Gonyostomum semen* and its diurnal vertical migration in a floodplain pool. *Arch. Hydrobiol. Suppl. Algolog. Stud.* 85:119–33.

Potter, D., Saunders, G. W. & Andersen, R. A. 1997. Phylogenetic relationships of the Raphidophyceae and Xanthophyceae as inferred from nucleotide sequences of the 18S ribosomal RNA gene. *Am. J. Bot.* 84:966–72.

Sandgren, C. D. 1983. Survival strategies of chrysophycean flagellates: reproduction and the formation of resistant resting cysts. *In* Fryxell, G. A. [Ed.] *Survival Strategies of Algae*. Cambridge University Press, Cambridge, pp. 23–48.

Sako, Y., Ishida, Y., Nishijima, T. & Hata, Y. 1987. Sexual reproduction and cyst formation in the freshwater dinoflagellate *Peridinium penardii*. *Nippon Suisan Gakk.* 53:473–8.

Salonen, K. & Rosenberg, M. 2000. Advantages from diel vertical migration can explain the dominance of *Gonyostomum semen* (Raphidophyceae) in a small, steeply-stratified humic lake. *J. Plankton Res.* 22:1481–853.

Throndsen, J. 1996. Note on the taxonomy of *Heterosigma akashiwo* (Rapidophyceae). *Phycologia* 35:367.

von Stosch, H. A. 1973. Observations on vegetative reproduction and sexual life cycles of two freshwater dinoflagellates, *Gymnodinium pseudopalustre* Schiller and *Woloszynskia apiculata* sp. nov. *Br. Phycol. J.* 8:105–34.

Vos, P., Hogers, R., Bleeker, M., Reijans, M., van de Lee, T., Hornes, M., Frijters, A., Pot, J., Peleman, J. & Kuiper, M. 1995. AFLP: a new technique for DNA fingerprinting. *Nucleic Acids Res.* 23:4407–14.

Watanabe, M. M., Nakamura, Y. & Kohata, K. 1983. Diurnal vertical migration and dark uptake of nitrate and phosphate of the red tide flagellates, *Heterosigma akashiwo* HADA and *Chattonella antiqua* (Hada) Ono (Raphidophyceae). *Jpn. J. Phycol.* 31:161–6.

Willén, E. 2003. Dominance patterns of planktonic algae in Swedish forest lakes. *Hydrobiologia* 502:315–24.

Yamaguchi, M. & Imai, I. 1994. A microfluorometric analysis of nuclear DNA at different stages in the life history of *Chattonella antiqua* and *Chattonella marina* (Raphidophyceae). *Phycologia* 33:163–70.

Yamochi, S. & Abe, T. 1984. Mechanisms to initiate a *Heterosigma akashiwo* red tide in Osaka Bay. II. Diel vertical migration. *Mar. Biol.* 83:255–61.