ON THE COVER: *Phaeocystis* colony stained with chromomycin A3, observed by epifluorescence microscopy, and treated in false color by image analysis. The dark areas correspond to cell nuclei.

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MORPHOLOGY, PLOIDY, PIGMENT COMPOSITION, AND GENOME SIZE OF CULTURED STRAINS OF *PHAEOCYSTIS* (PRYMNESIOPHYCEAE)[1]

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ABSTRACT

We examined cell morphology, ploidy level, cell size, pigment composition, and genome size in 16 cultured strains of Phaeocystis *Lagerheim. Two strains originated from the Antarctic, 3 from the tropical Western Atlantic, and 11 from temperate regions (Eastern Atlantic, English Channel, North Sea, and Mediterranean Sea). Thirteen strains made colonies morphologically similar to* P. globosa *Scherffel, whereas three never formed colonies under any circumstances. Five-rayed star-like structures with filaments were observed in 11 strains. In several strains, two ploidy levels were observed, one (haploid) linked to flagellates and one (diploid) linked to colonies. Cell size did not appear to be a very good criterion for distinguishing strains since size distributions overlapped. Pigment analysis by reversed-phase–high-performance liquid chromatography allowed the strains to be grouped into three clusters that differed from each other mainly by the relative proportions of three carotenoids: fucoxanthin, 19′-hexanoyloxyfucoxanthin, and diadinoxanthin. All strains contained low levels of 19′-butanoyloxyfucoxanthin. Differences in genome size measured by flow cytometry delimited at least five groups. On the basis of both pigment composition and genome size, six clusters were defined, one corresponding to an Antarctic species (possibly* P. antarctica*), one to* P. globosa*, and the rest probably to several yet-undescribed species or subspecies. Two main conclusions emerge from this study. First, the taxonomy of the genus* Phaeocystis *needs to be clarified through a combination of morphological, biochemical, and molecular studies. Second, sexuality is a prevalent phenomenon in* Phaeocystis*, but controls of the sexual cycle are most likely strain-dependent.*

Key index words: cell size; flow cytometry; genome size; HPLC; Phaeocystis; *pigments; ploidy; Prymnesiophyceae; taxonomy*

Phaeocystis Lagerheim is a prymnesiophyte genus that is distributed worldwide, extending from the Arctic to the Antarctic and through tropical and temperate areas (Baumann et al. 1994). It has attracted wide interest recently because of its significance to the global carbon cycle, especially in coastal and polar regions (Lancelot et al. 1987, Smith et al. 1991). After the establishment of the genus 100 years ago, several species were described in the early part of the century. Sournia (1988) tried to clarify the taxonomic status of the genus and determined that, until more detailed studies are done, only two bona fide species could be recognized: *P. pouchetii* (Hariot) Lagerheim and *P. scrobiculata* Moestrup. Since his work, at least two other species have been convincingly revived: *P. globosa* Scherffel (Jahnke and Baumann 1987, Baumann et al. 1994, Medlin et al. 1994) and an Antarctic species referred to as *P. antarctica* Karsten (Baumann et al. 1994, Medlin

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TABLE 1. *Characters of the four* Phaeocystis *species recognized to date (adapted and extended from Baumann et al. 1994 and Medlin et al. 1994).*

Species	Habitat	Colonies		Flagellates	
		Shape	Cell distribution	Production of star-like features	Body scales
P. pouchetii	Arctic North Atlantic	Globular	In packets	Five rays[a]	?
P. globosa	North Atlantic	Round	Even at the periphery	Five rays[b]	Circular, 0.18 × 0.19 μm, 48 rims[b] Oval, 0.10 × 0.13 μm, 30 rims
P. antarctica?	Antarctic	Round	Even and dense	Five rays[c]	Two types uncharacterized[c]
P. scorbiculata	South Pacific North Atlantic	?	?	Nine rays[d]	Circular-oval, 0.19 × 0.21 μm[d] Oval, 0.60 × 0.45 μm

[a] Baumann and Jahnke 1986.
[b] Parke et al. 1971. Although the authors referred the material they examined to *P. pouchetii*, it was most likely *P. globosa* (this paper and Medlin et al. 1994).
[c] Medlin et al. 1994.
[d] Moestrup 1979.

et al. 1994). The main taxonomic features that distinguish these species are colony shape, excretion by cells of star-like features, and habitat (Table 1). However, for some species, these features are characteristic of only the colonial form, while for others, only the flagellate form (Table 1). Very recently, Medlin et al. (1994) established a firm basis for the distinction of three species, *P. pouchetii*, *P. globosa*, and *P. antarctica*, through the analysis of 18S ribosomal RNA (rRNA) sequences. Characteristics of the genus *Phaeocystis* need to be examined in much more detail in order to establish firm criteria for its taxonomy.

For the present work, we selected 16 *Phaeocystis* strains and examined features that may be taxonomically relevant. We also addressed the issue of ploidy level. This has never been discussed for *Phaeocystis* and is of likely significance to its taxonomy.

MATERIAL AND METHODS

Sixteen *Phaeocystis* strains were used (Table 2). Rationale for strain selection is presented in the Results section.

For routine transfer, cultures were maintained in K medium (Keller et al. 1987) at their optimum growth temperature (either 4°, 15°, or 20° C) under a 12:12 h LD cycle of about 100 μmol photons·m^{-2}·s^{-1}. For pigment and cell size determinations, 800-mL batch cultures were grown using standard conditions with the exception that, when possible, we used constant illumination to avoid variability linked to synchronous growth. Some strains, however, did not grow well under continuous light and had to be exposed to light–dark cycles. Cultures were sampled once per day for cell size determination and twice, during exponential and stationary phases, respectively, for pigment composition.

To verify the presence/absence of filaments and star-like structures in the cultures, cells were fixed with glutaraldehyde (final concentration 1%) in cacodylate buffer (0.1 mol·L^{-1}). Two milliliters of culture were gently filtered on a Nuclepore filter (pore size = 0.8 μm) and rinsed with distilled water to remove salt and fixatives before being air-dried. The filters were then mounted onto a stub and coated with gold-palladium before examination under a Hitachi S 520 (Tokyo, Japan) scanning electron microscope. To determine the morphology of flagellated cells in four of the strains (Rosko A, PCC 64, PCC 147, Naples), this procedure was completed by a dehydration step through a graded series of ethanol concentrations and by critical-point drying.

To identify five-rayed stars, cultures were stained with Alcian blue (0.05% final concentration). A drop was left to dry on the slide, which was examined using an Olympus (Tokyo) BH2 microscope with a 40× phase-contrast objective.

For cell measurements by microscopic image analysis, cells were fixed using Lugol's glutaraldehyde fixative (Rousseau et al. 1990). Three milliliters of culture were filtered on a 13-mm-diameter, 0.2-μm-pore size, Millipore Isopore filter. The filter was inverted onto a 0.01% polylysine–coated slide, and cells were mounted into a PBS/glycerol mixture and examined using a BH2 microscope (40× phase-contrast objective). The image analysis system consisted of a black and white CCD camera (Cohu 4710, San Diego, California), a video monitor (Trinitron KX14CP1, Sony, Japan), a personal computer, a digitizing board MATROX MVP (Dorval, Québec), and the software package Biocom 200 (Biocom, Les Ulis, France). Measurements were performed on about 100 cells from randomly selected fields.

Cell volume in μm^3 was determined using a Coulter counter model ZM (Coulter Electronics, Hialeah, Florida) equipped with a 76-μm orifice and coupled with a C1000 Channel Analyzer. Volume distribution data were transferred to a personal computer for analysis and analyzed with a custom software CYTOPC (Vaulot 1989). Beads of diameter 9.57 μm (DNA Check, Coulter) were used for calibration.

Genome size was determined by flow cytometry on isolated nuclei, which were released by diluting dense cell cultures 1:5 or 1:10 into a buffer consisting of 30 mmol·L^{-1} $MgCl_2$, 20 mmol·L^{-1} Na-citrate, 55 mmol·L^{-1} HEPES, 125 mmol·L^{-1} sorbitol, 5 mmol·L^{-1} EDTA, 0.1% w/v Triton X100, and 5 μL·mL^{-1} β-mercaptoethanol (all from Sigma). Chicken red blood cells (2.33 pg DNA·$cell^{-1}$) diluted into Alsever buffer with 1% Triton X-100 were added as DNA internal standards (Brown et al. 1991). Nuclei were stained with either Hoechst 33342 (HO, 5 μg·mL^{-1} final concentration), chromomycin A3 (CA3, 40 μg·mL^{-1}; 1 mg·mL^{-1} CA3 stock is made in 1 mmol·L^{-1} $MgCl_2$), or propidium iodide (PI, 30 μg·mL^{-1}). For PI, 10 μL·mL^{-1} of a stock RNAse solution (10 mg·mL^{-1}; 1:1 RNAse A and B, Sigma R-4875 and R-5750) was added. HO samples were analyzed with ultraviolet excitation after a 10–15-min incubation and CA3 with 457 nm and PI with 488 nm after 20 min. Genome sizes and GC% values were estimated as described in Godelle et al. (1993).

To determine ploidy, cells were fixed with 0.5% paraformaldehyde, frozen in liquid nitrogen, and then stored at −80° C (Vaulot et al. 1989). Prior to flow cytometric analysis, thawed samples were stained directly with CA3 at a final concentration of 40 μg·mL^{-1}. Chicken red blood cells and 1.98-μm Polysciences YG beads were used as internal standards.

For chlorophylls and carotenoids, 100-mL samples were prefiltered through a 10-μm mesh to eliminate colonies, collected on GF/F filters, immediately frozen in liquid nitrogen, and kept at −80° C. Analysis was performed as described in Kraay et al. (1992). Filters were extracted in 100% methanol and buffered with 2% ammonium acetate in a CO_2-cooled Braun homogenizer

TABLE 2. *List of* Phaeocystis *strains used in this study. NA = information not available.*

Strain	Origin	Isolation date	Latitude	Longitude	Depth (m)	Temperature (°C)	Isolator	Method of isolation	Deposited
SK 22	Wedell Sea	Nov–Dec 90	54°20'S	3°20'W	0–30	1.5	Alfred Wegener Institute	One (?) colony	
SK 32	Wedell Sea	Nov–Dec 90	60°40'S	15°42'W	0–30	−1.7	Alfred Wegener Institute	One (?) colony	
JNorth	North Sea	28 May 86	54°24'N	4°17'E	NA	8.6	J. Jahnke	NA	
NIOZ 1	North Sea	1 Apr 89	52°5'N	4°45'E	NA	8	W. van Boekel	One colony	
NIOZ 2	North Sea	1 Oct 90	52°5'N	4°45'E	NA	NA	W. van Boekel	One colony	
Rosko A	English Channel/Roscoff	28 May 91	48°43'N	3°59'W	0	12	V. Cariou	One colony	
Rosko C	English Channel/Roscoff	3 Jun 91	48°43'N	3°59'W	0	12	R. Casotti	One colony	
Rosko D	English Channel/Roscoff	3 Jun 91	48°43'N	3°59'W	0	12	R. Casotti	One colony	
Rosko E	English Channel/Roscoff	3 Jun 91	48°43'N	3°59'W	0	12	R. Casotti	One colony	
PCC 64	English Channel/St E1	23 Sep 52	50°9'N	4°22'W	0	NA	M. Parke	One flagellate	Plymouth Culture Collection
PCC 147	English Channel/St E1	13 Jun 55	50°9'N	4°22'W	70	NA	I. Adams	One flagellate	Plymouth Culture Collection
PCC 540	E. Atlantic	17 Jul 82	47°37'N	8°53'W	NA	NA	P. Course	One colony	Plymouth Culture Collection
Naples	Mediterranean Sea/Gulf of Naples	15 Mar 91	40°48'N	14°15'E	20	22	Stazione Zoologica	200 single cells	
Big IVM1	Gulf Stream	25 Feb 81	NA	NA	NA	NA	L. Provasoli	NA	Bigelow CCMP 629
Big 677-3	W. Atlantic/Surinam	6 Jun 65	6°45'N	53°19'W	NA	26	R. Guillard	One colony	Bigelow CCMP 628
Big 1209	Gulf of Mexico	1 Feb 80	29°15'N	85°54'W	NA	NA	L. Brand	NA	Bigelow CCMP 627

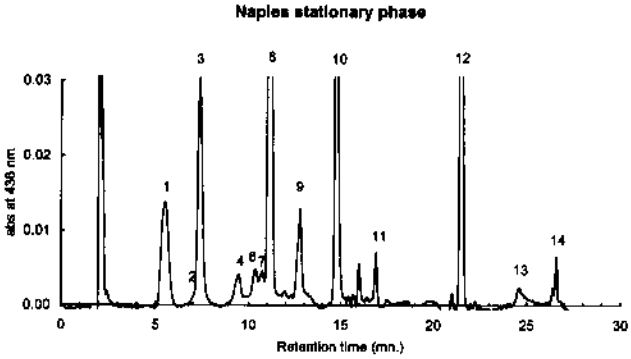

FIG. 1. Pigment chromatogram (HPLC) of *Phaeocystis* (strain Naples, stationary phase). See Table 3 for peak identification.

for 20 s. The methanol extract was filtered over a GF/F glass-fiber filter (10 mm diameter). A volume of 100 μL was injected into the liquid chromatograph. The high-performance liquid chromatography (HPLC) instrument was a Waters Associates liquid chromatograph consisting of a 600E gradient module with a system controller and a model 991 photodiode array detector. The column was an Rsil C18 (Biosil, Biorad RSL). Three solvents (HPLC-grade Baker) were used as described previously (Kraay et al. 1992): A) 0.5 mol·L^{-1} ammonium acetate in methanol and water, 85:15, v:v; B) acetonitrile and water, 90:10, v:v; and C) 100% ethyl acetate. Flow rate was 0.8 mL·mn^{-1}. Pigments were identified (Fig. 1, Table 3), and concentrations were calculated as in Kraay et al. (1992). Chlorophyll (Chl) c_3 concentration was estimated using an extinction coefficient similar to that for Chl c_1, after transposition of the absorption spectra in the blue-green region (446 nm for Chl c_2 to 440 nm for Chl c_3). Cluster analysis was performed with SYSTAT software (Evanston, Illinois).

RESULTS

Strain selection. We used 16 strains from either cold, temperate, or tropical waters (Table 2). Strains PCC 64 and 147 were of particular interest because they were subjected to a very detailed study by Parke et al. (1971) that provided some of the taxonomic basis for the identification of flagellated cells of *Phaeocystis*. Strain Big 677-3 was also the object of previous studies (Guillard and Hellebust 1971, Bjornland et al. 1988). All the strains studied by us could be assigned to either *P. globosa* or *P. antarctica*. We were unable to obtain strains with characters of *P. pouchetii* or *P. scrobiculata*; the latter, to our knowledge, has never been cultivated.

Cell morphology. Only three strains (PCC 64 and 147 and Naples) did not form colonies (Table 4). Whereas the Antarctic strain formed small colonies, with cells well separated and densely packed, all other strains formed large colonies with cells distributed at the periphery that are typical of the *P. globosa* morphotype (Baumann et al. 1994). In routine cultures in K medium (N/P ratio about 90), some colony-forming strains stopped producing colonies and remained flagellated (Table 4). We have not been able to find a reliable and reproducible way to manipulate culture conditions to switch back and forth between colonial and flagellate forms for any of the strains.

TABLE 3. *List of pigments identified by HPLC.*

Peak no.	Pigment	Abbreviation	λ_{max} (nm)
1	Chlorophyll c_3	Chl c_3	452
2	Chlorophyll c_1-like	Chl c_2-like	440
3	Chlorophyll c_2	Chl c_2	449
4	19'Butanoyloxyfucoxanthin	19'BFuco	446
5	Fucoxanthin derivative	Fuco derivative	449
6	Fucoxanthin	Fuco	449
7	19'Hexanoyloxyfucoxanthin derivative	19'HFuco derivative	449
8	19'Hexanoyloxyfucoxanthin	19'HFuco	447
9	*Cis* of 19'HF or of Fuco	*Cis*-Fuco	449
10	Diadinoxanthin	Diadino	446
11	Diatoxanthin	Diato	449
12	Chlorophyll *a*	Chl *a*	664
13	Phytol chlorophyll *c*-like	Phytol Chl *c*-like	449
14	Carotenes (α, β, and *cis*-)	Caro	453

We observed, at one time or the other, flagellated cells in all strains, i.e. no strain displayed only colonial cells. Scanning electron microscopy of flagellated cells was performed on four strains (Fig. 2). The Naples strain had a rougher cell surface, a shorter haptonema, and longer flagella than Rosko A, PCC 64, and PCC 147, which all looked very similar.

Filaments that are excreted by cells and arranged in a five- or nine-rayed star pattern represent an important taxonomic peculiarity of the genus *Phaeocystis* (Parke et al. 1971, Moestrup 1979, Pienaar 1991, Moestrup and Larsen 1992). Using either scanning electron microscopy (Fig. 2) or phase-contrast microscopy after Alcian blue staining (Fig. 3), we observed five-rayed stars in all European strains but not in the Antarctic or tropical strains (Table 4). The latter, however, produced filaments, except for Big IVM-1. The European strains always exhibited stars when flagellates were the dominant life form; stars were frequently, but not always, absent when colonies were dominant.

Ploidy. Ploidy appears to be taxonomically important since it is linked to the cell type: Rousseau et al. (1994) recently suggested that while colonies and so-called swarmers (i.e. flagellated cells that coexist with colonies) are diploid, microzoospores are haploid. We detected difference in ploidy level only in European strains that had the potential to form colonies (Table 4). For example, the Rosko A strain displayed different DNA cytograms for cultures with and without colonies (Fig. 4). When comparing cell DNA levels to that of chicken erythrocytes, it is evident that the majority of cells in the culture with colonies had twice as much DNA as those in the culture devoid of colonies. In the absence of detailed information on chromosome numbers, it is logical to identify the cells with the lower DNA level (1C) as haploid and those with the higher DNA level (2C) as diploid.

In a culture devoid of colonies (Fig. 5), 80% of the cells were 1C and 20% were 2C. These 2C cells could be either haploid in G_2 or diploid in G_1. However, the former hypothesis is unlikely because the proportion of 2C cells remained high after the culture had stopped dividing. This proportion even increased during the senescence period whereas the proportion of cells in G_2 usually decreased when

TABLE 4. *List of strain characters. Plus (+) sign means that character has been observed at least once for the strain.*

Strain	Optimum growth temperature (° C)	Ability to form colonies	Flagellated cells observed	Five-rayed stars observed	Two ploidy levels observed	Dominant form in routine transfer (K medium N/P ≈ 90)
SK 22	4	+	+	Filaments		Colony
SK 32	4	+	+			Colony
JNorth	15	+	+	+	+	Flagellate haploid
NIOZ 1	15	+	+	+	+	Flagellate haploid
NIOZ 2	15	+	+	+	+	Flagellate haploid
Rosko A	15	+	+	+	+	Flagellate haploid/diploid
Rosko C	15	+	+	+	+	Flagellate haploid
Rosko D	15	+	+	+	+	Flagellate haploid
Rosko E	15	+	+	+	+	Flagellate haploid
PCC 64	15		+	+		Flagellate
PCC 147	15		+	+		Flagellate
PCC 540	20	+	+	+	+	Colony diploid
Naples	15–20		+	+		Flagellate
Big IVM1	22	+	+			Colony
Big 677-3	22	+	+	Filaments		Colony
Big 1209	22	+	+	Filaments		Colony

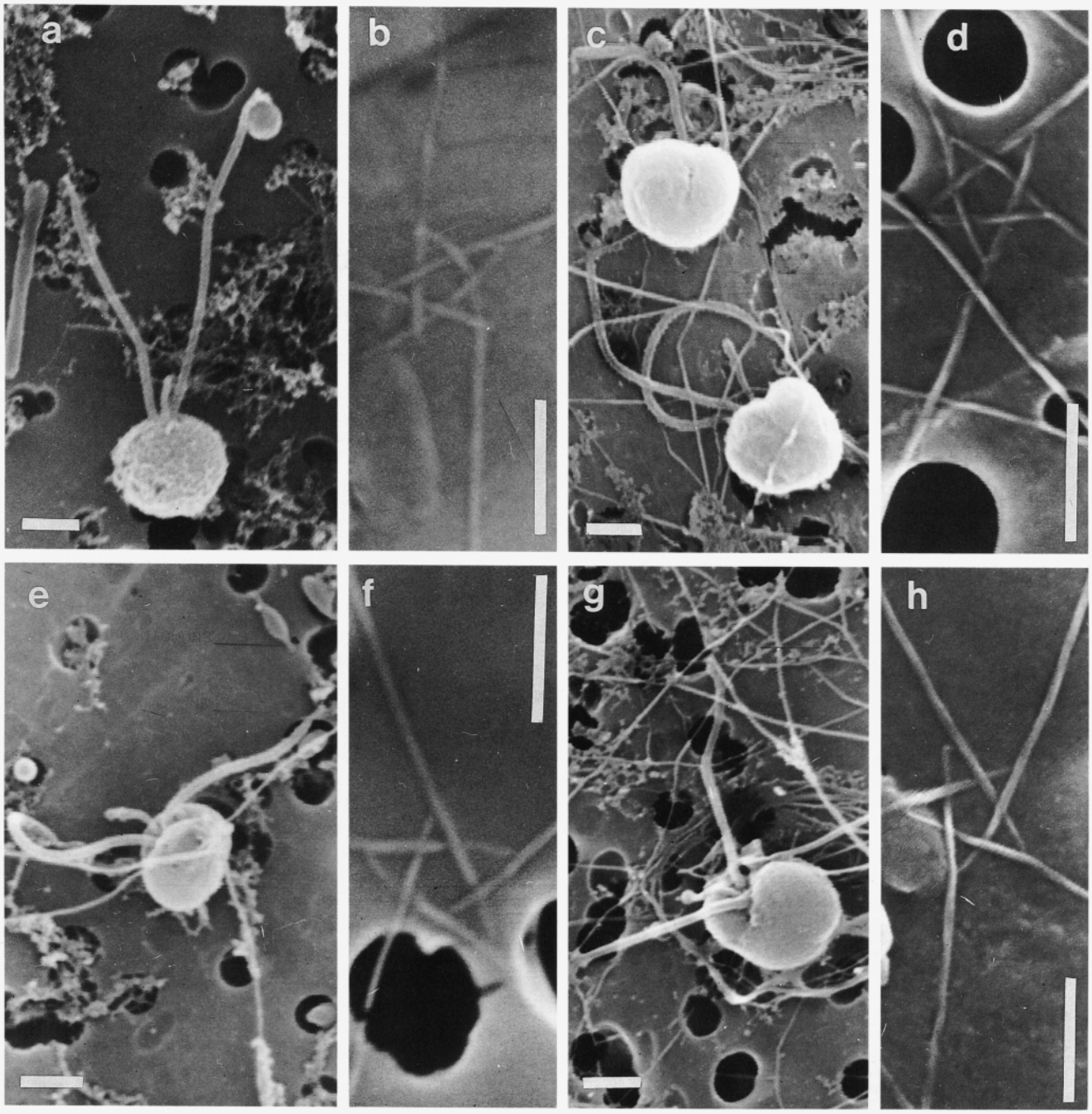

FIG. 2. Scanning electron micrographs of *Phaeocystis* flagellated cells and pentagonal star-like structures after critical point drying. a, b) Strain Naples. c, d) Strain PCC 147. e, f) Strain PCC 64. g, h) Strain Rosko A. Scale bars = 1 μm.

division stopped. Indeed, strains PCC 64 and 147, which are haploid (see later), always had very few cells in G_2, even when actively growing (data not shown). Therefore, in this mainly haploid culture, it is likely that diploid cells were present, resulting either from fusion of haploid cells or from division of an initial stock of diploid cells.

In a culture with colonies (Fig. 6), 2C cells dominated. The small number of 4C cells observed were probably diploid in G_2 because their proportion decreased sharply at the end of the exponential phase. In this culture, there was always a small, albeit significant, proportion of 1C cells, suggesting that haploid cells may be continuously produced either by meiosis in culture with colonies or, alternatively, by division of the small fraction of haploid cells present in the inoculum. We also have examples of cultures of this strain completely devoid of haploid cells (data not shown).

These observations extended to all European col-

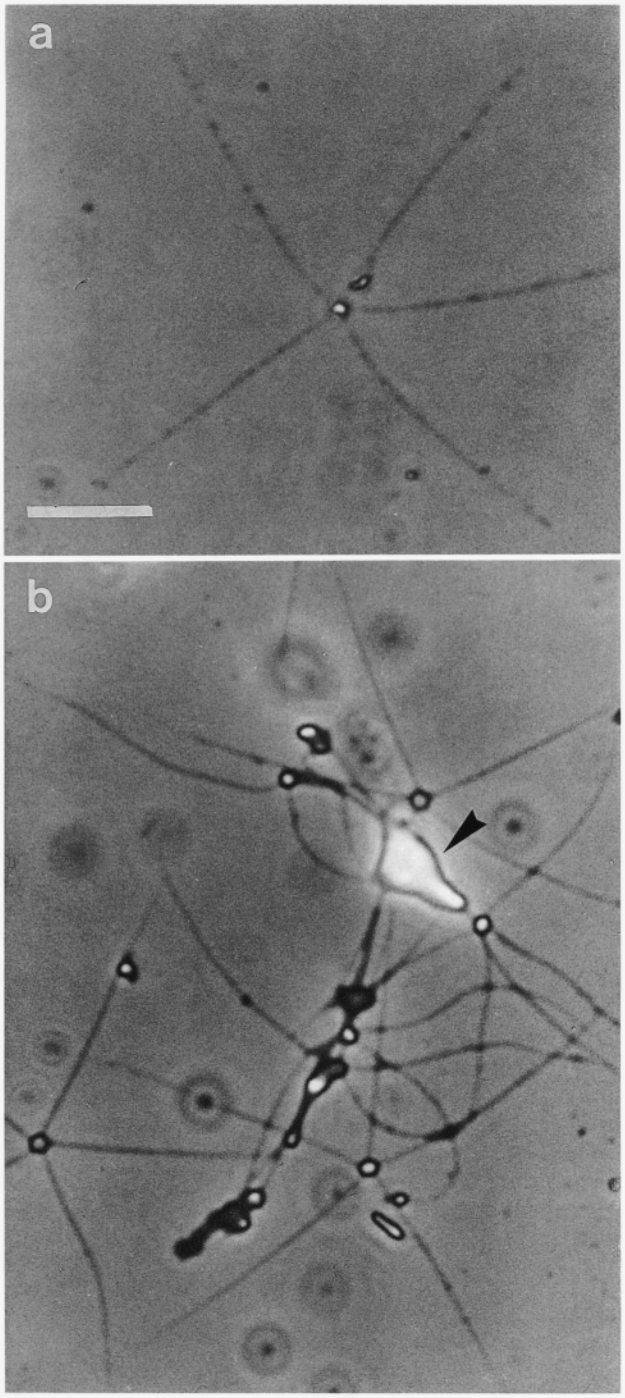

FIG. 3. Five-rayed star and filaments visualized by Alcian blue staining in optical microscopy. Strain Rosko A. Scale bar = 10 μm. a) Single star. b) Filaments and stars entangled around a dried-up cell (arrowhead).

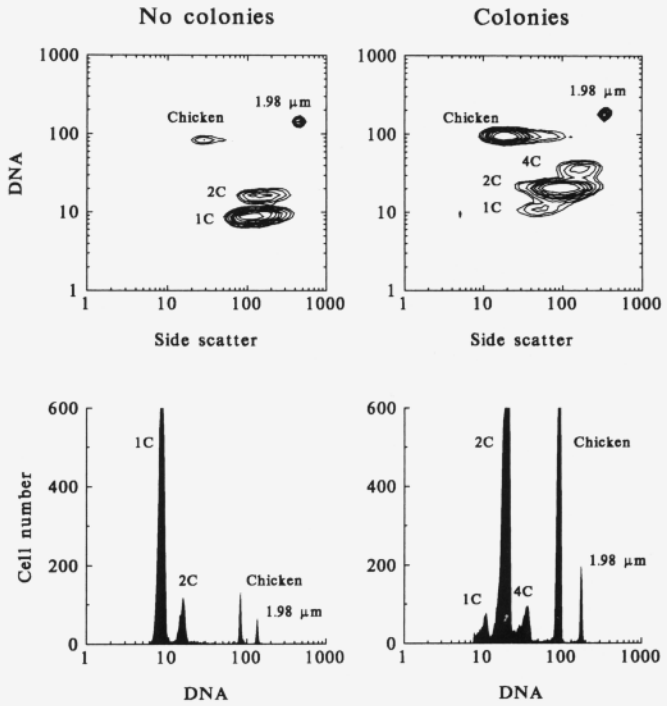

FIG. 4. Strain Rosko A. Top: Flow cytograms of cell DNA vs. side scatter (a function of cell size) for a culture without colonies (left) and a culture containing colonies (right). Contours correspond to constant cell number. Chicken erythrocytes and 1.98-μm beads were used as internal standards. Bottom: DNA distributions.

ony-forming strains, for which the following rule seemed to apply. When colonies were present, 2C cells dominated; when colonies were absent, 1C cells dominated. The only exception was a Rosko A culture that contained only 2C cells and never produced colonies for at least 1 year. The two European strains PCC 64 and 147 that never formed colonies (Parke et al. 1971) represented the 1C haploid level (see below).

In the Arctic and tropical strains, we never observed two ploidy levels (Table 4). Because cultures of these strains always produced colonies, it is not clear whether they were really unable to produce haploid cells or that our culture conditions prevented formation of haploid cells.

Cell size. Mean cell diameter (population averaged, Table 5) varied 1.5-fold between 3.8 μm (Naples) and 5.7 μm (NIOZ 1), while extreme values spanned a 2-fold difference, corresponding to an 8-fold cell volume range. Although some of this variability was strain-specific, most of it could be attributed to growth phase and ploidy (see later). The noncolonial strains Naples and PCC 64 and 147 had the smallest average sizes while the other strains were indistinguishable (Table 5). Actual size distributions overlapped considerably (Fig. 7).

The average cell volume could vary 2-fold during growth in batch culture. In general, cells were significantly larger during the exponential than the phosphorus-limited stationary phase (not shown). Haploid cells were, on average, smaller than diploid cells, as demonstrated for strain Rosko A both for cultures containing a majority of one cell type (Figs. 5, 6; compare average volumes) and within a culture containing both cell types (Fig. 4; 1C cells have smaller side scatter); however, cell size distributions over-

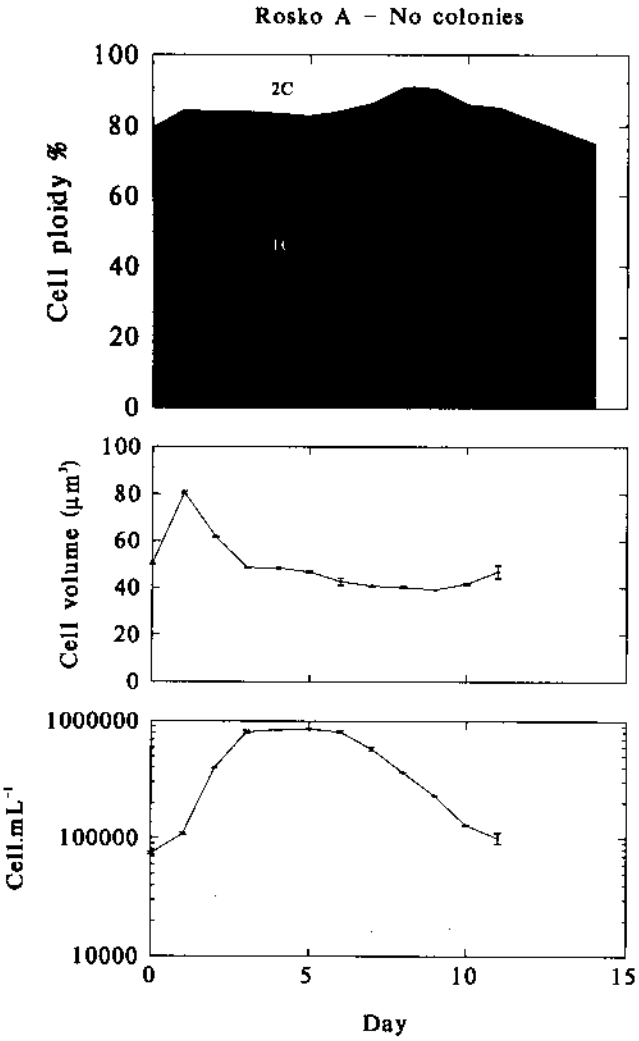

FIG. 5. Strain Rosko A. Culture devoid of colonies. Time course of cell numbers (bottom), cell volume (middle), and percentages of haploid (1C) and diploid (2C) cells (top). On bottom and middle figures, error bars correspond to standard deviation.

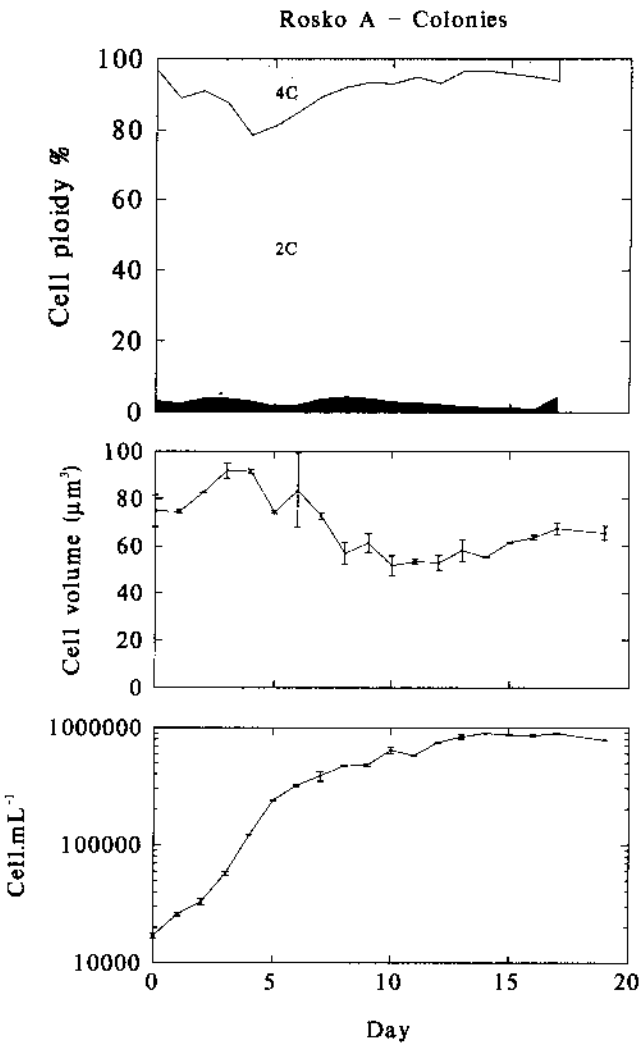

FIG. 6. Same as Figure 5, but for a culture containing colonies.

lapped. In cultures with both single and colonial cells (both diploid), measurement by image analysis established that colonial cells were always smaller and more elongated than single cells (ANOVA, significant at the $P < 0.001$ level, data not shown), with the exception of the NIOZ strain for which the difference in volume was not significant.

Pigment composition. For 12 strains, reverse-phase HPLC (Fig. 1) was used to establish pigment com-

TABLE 5. *Population averages of equivalent spherical cell diameter (µm) determined with a Coulter counter for* Phaeocystis *strains during batch culture (see temperature and light conditions in Table 6).*

Strain	Mean	Minimum	Maximum
Naples	3.8	3.4	4.6
SK 22	5.1	4.7	5.6
Big 1209	4.8	4.0	6.8
Big IVM1	4.7	4.3	5.2
Big 677-3	4.7	4.3	5.0
PCC 540	4.7	4.5	4.9
JNorth	5.2	4.6	5.5
NIOZ 1	5.7	4.9	6.2
NIOZ 2	5.0	4.6	5.4
PCC 147	4.4	3.7	5.2
PCC 64	4.4	4.0	4.8
Rosko A	4.7	4.5	5.0

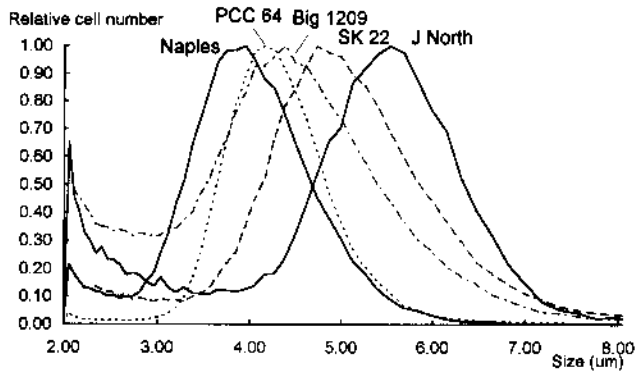

FIG. 7. Population distributions of equivalent spherical diameter for single cells as determined with the Coulter counter for five strains. Cultures of Big 1209, SK 22, and JNorth contained colonies.

TABLE 6. *Accessory pigments of different* Phaeocystis *strains expressed either as ratios to Chl* a *or as % of total carotenoids. Averages are for clusters as indicated in Figure 8. Exp = exponential phase; Sta = stationary phase.*

Cluster	Strain	Colonies present in culture	Phase	Temperature (° C)	Light (LD)	Sampling date	Ratio to Chl *a*					% of total carotenoids										
							Chl c_1	Chl c_2	Chl c_3	Phytol Chl *c*	Carotenoids	19'BFuco	Fuco	Fuco derivative	19'H-Fuco	19'HFuco derivative	Cis-Fuco	Diadino	Diato	alpha-Caro	beta-Caro	cis-Caro
1	Naples	No	Exp	15	24:0	02/07/92	0.005	0.13	0.21	0.027	1.02	2.1	6.3	0.0	65.7	0.0	3.6	18.2	1.9	0.5	1.7	0.0
1	Naples	No	Sta	15	24:0	02/13/92	0.004	0.14	0.19	0.013	1.03	3.0	2.7	0.0	55.7	2.2	7.8	24.4	1.7	1.0	1.2	0.2
1	SK 22	Yes	Exp	4	24:0	03/13/92	0.000	0.19	0.10	0.000	0.45	traces	6.3	0.0	79.7	0.0	0.0	5.7	7.1	0.0	1.2	0.0
1	SK 22	Yes	Sta	4	24:0	03/17/92	0.000	0.22	0.12	0.000	0.71	traces	0.0	0.0	74.2	0.0	0.0	9.3	15.0	0.0	1.5	0.0
1	Average						0.002	0.17	0.15	0.010	0.80	1.3	3.8	0.0	68.8	0.5	2.8	14.4	6.5	0.4	1.4	0.1
2	Big 1209	Yes	Sta	20	24:0	10/03/91	0.000	0.16	0.28	0.000	0.70	1.2	45.0	0.0	44.1	2.2	0.0	4.6	1.8	1.1	0.0	0.0
2	Big 1209	Yes	Exp	20	24:0	01/24/92	0.000	0.20	0.31	0.000	0.90	0.9	26.7	0.0	54.9	5.0	5.6	3.7	2.5	0.0	0.7	0.0
2	Big 1209	Yes	Sta	20	24:0	01/28/92	0.002	0.20	0.29	0.000	0.91	0.8	28.3	0.0	54.1	6.9	2.2	5.2	1.8	0.1	0.7	0.0
2	Big 677/3	Yes	Exp	20	24:0	10/17/91	0.004	0.18	0.28	0.000	0.88	1.4	39.3	0.0	43.4	3.2	4.1	5.5	2.1	0.1	0.9	0.0
2	Big 677/3	Yes	Sta	20	24:0	10/22/91	0.005	0.18	0.39	0.000	1.15	3.8	22.6	0.0	57.1	4.2	2.5	6.0	2.9	0.0	0.8	0.0
2	Big IVM1	Yes	Exp	20	24:0	09/25/91	0.010	0.17	0.17	0.000	0.77	0.9	45.0	0.0	34.3	3.2	2.7	9.4	1.6	0.9	1.5	0.4
2	Big IVM1	Yes	Exp	20	24:0	10/01/91	0.004	0.19	0.37	0.000	0.91	0.7	31.0	0.0	49.9	4.8	4.7	6.6	1.5	0.1	0.6	0.1
2	PCC 540	Yes	Exp	15	24:0	02/03/92	0.005	0.14	0.22	0.000	0.53	7.7	41.1	0.0	28.3	8.1	5.7	5.1	2.3	0.1	1.3	0.3
2	PCC 540	Yes	Sta	15	24:0	02/05/92	0.005	0.13	0.25	0.000	0.54	6.3	39.4	0.0	33.2	7.3	4.2	4.6	3.7	1.3	0.0	0.0
2	Average						0.004	0.17	0.28	0.000	0.81	2.6	35.4	0.0	44.4	5.0	3.5	5.6	2.2	0.4	0.7	0.1
3	JNorth	Yes	Exp	15	24:0	08/05/91	0.004	0.19	0.21	0.000	0.97	0.0	76.4	2.1	3.0	0.7	2.5	11.1	2.4	0.2	1.4	0.0
3	JNorth	Yes	Sta	15	24:0	08/10/91	0.004	0.20	0.30	0.000	0.96	0.4	67.3	6.0	2.7	4.2	3.5	9.2	5.7	0.1	0.8	0.1
3	JNorth	Yes	Exp	15	12:12	02/11/92	0.010	0.16	0.20	0.000	0.76	0.2	71.7	9.1	1.3	1.3	2.3	9.5	1.2	0.8	2.1	0.4
3	JNorth	Yes	Sta	15	12:12	02/14/92	0.005	0.13	0.19	0.000	0.92	0.4	53.7	4.4	2.1	3.9	7.9	22.5	2.6	0.5	1.7	0.4
3	NIOZ 1	Yes	Exp	15	24:0	08/05/91	0.007	0.17	0.22	0.000	0.47	0.0	81.9	2.8	2.7	1.3	1.8	4.7	3.1	0.0	1.8	0.0
3	NIOZ 1	Yes	Sta	15	24:0	08/10/91	0.007	0.21	0.38	0.000	0.57	0.0	63.3	3.5	6.3	2.8	9.6	5.1	8.5	0.0	1.0	0.0
3	NIOZ 2	Yes	Exp	15	24:0	08/01/91	0.010	0.19	0.20	0.000	0.80	0.4	78.8	5.4	1.3	1.4	1.4	7.3	2.1	0.2	1.5	0.1
3	NIOZ 2	Yes	Sta	15	24:0	08/08/91	0.007	0.19	0.30	0.000	0.85	1.1	60.9	3.6	4.6	5.7	8.0	11.3	3.0	0.2	1.3	0.3
3	PCC 147	No	Exp	15	12:12	12/03/91	0.007	0.21	0.28	0.000	1.16	0.0	83.0	0.0	4.1	0.8	3.0	6.6	0.6	0.3	1.3	0.3
3	PCC 147	No	Sta	15	12:12	12/07/91	0.004	0.15	0.29	0.000	0.97	0.9	70.0	0.0	10.6	3.3	0.5	10.9	1.9	0.2	1.4	0.3
3	PCC 64	No	Exp	15	24:0	12/04/91	0.009	0.21	0.25	0.000	1.09	traces	77.6	0.0	2.6	1.5	7.1	9.2	0.5	0.1	1.4	0.0
3	PCC 64	No	Sta	15	24:0	12/09/91	0.003	0.13	0.25	0.000	1.08	0.5	60.5	0.7	6.1	1.0	0.5	23.7	5.2	0.3	1.2	0.2
3	Rosko A	Yes	Exp	15	24:0	09/20/91	0.006	0.20	0.22	0.000	0.78	0.2	75.2	8.1	0.6	0.0	6.1	6.2	1.2	0.5	1.6	0.3
3	Rosko A	Yes	Sta	15	24:0	09/25/91	0.005	0.18	0.25	0.006	1.04	1.5	51.2	11.3	4.3	3.1	8.8	16.0	3.1	0.1	0.6	0.1
3	Rosko A flag	No	Exp	20	12:12	02/24/92	0.007	0.16	0.22	0.000	0.82	0.3	74.8	1.1	4.7	1.3	4.0	9.5	2.1	0.4	1.5	0.2
3	Average						0.006	0.18	0.25	0.000	0.88	0.4	69.8	3.9	3.8	2.1	4.5	10.9	2.9	0.3	1.4	0.2

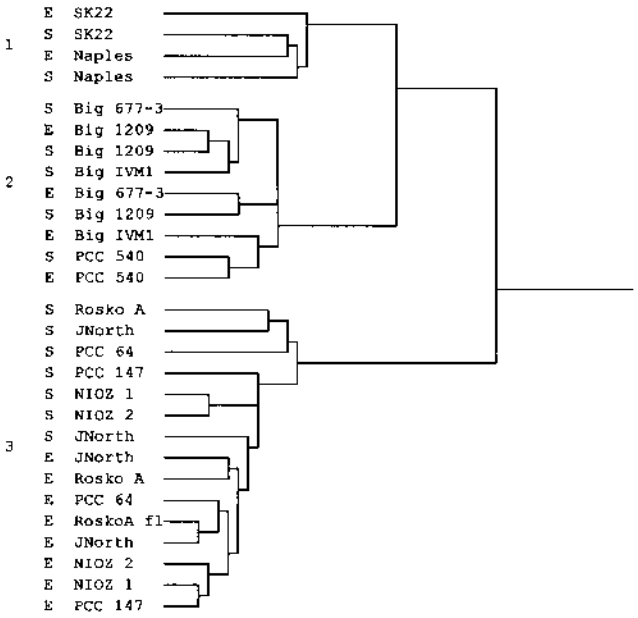

FIG. 8. Linkage tree between *Phaeocystis* strains, based on the relative contribution of the different carotenoids (see Table 6). The single linkage method (nearest neighbor) was used in conjunction with euclidean distances. Numbers on the left refer to clusters.

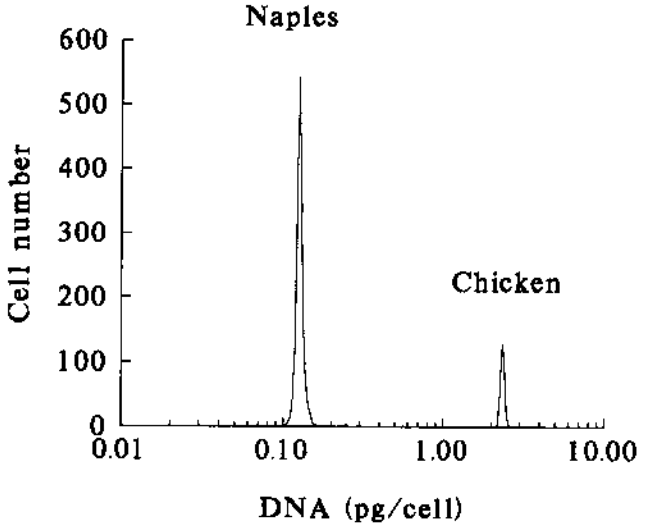

FIG. 9. DNA distribution (log scale) of nuclei of strain Naples and chicken erythrocytes stained with PI and measured by flow cytometry.

position of single cells after colony removal (Table 6). All strains contained similar major accessory pigments (see Table 3 for abbreviations): Chl c_2 and c_3, fucoxanthin (Fuco), 19′-hexanoyloxyfucoxanthin (19′HFuco), and diadinoxanthin (Diadino). Minor pigments such as Chl c_1 or 19′-butanoyloxyfucoxanthin (19′BFuco) were only present in certain strains. Certain accessory pigments varied very little between strains. For example, the ratio Chl c_2/Chl *a* remained between 0.1 and 0.2 for all strains. In contrast others varied over a much wider range, in particular Fuco and 19′HFuco. To classify the different strains objectively, a cluster analysis was performed on the basis of the ratios of 10 carotenoids (diatoxanthin [Diato] and Diadino were pooled together because they form part of a cycle, e.g. Olaizola et al. 1992) to the total carotenoid content (Table 6). Three clusters were clearly distinct (Fig. 8), corresponding to geographic origins. The first cluster was formed by Naples (Mediterranean Sea) and SK 22 (Antarctic), the second by all tropical Western Atlantic strains and PCC 540, and the third by all other North European strains. The changing proportions of the two principal carotenoids (Fuco and 19′HFuco) explained most of the clustering (Table 6). With respect to 19′HFuco, Naples clearly had the highest relative content, followed by SK 22, the Western Atlantic strains, and then PCC 540. 19′HFuco was much less abundant in North European strains (<10% of total carotenoids). For Fuco, the opposite trend was observed. The thirdmost abundant carotenoid (Diadino) was markedly higher for the Naples and the North European strains than for the other strains. Other pigments that played only a minor role in the clustering process provided additional discrimination between strains. For example, Naples and SK 22 could be differentiated within cluster 1 by the presence of Phytol Chl *c*, 19′BFuco, and *cis*-Fuco in the former strain and the abundance of Diato in the latter. Within cluster 2, PCC 540 was clearly characterized by a much higher contribution of 19′BFuco (>6%). Finally, strains from cluster 3 contained the Fuco derivative.

Ploidy apparently does not affect pigment composition. For example, the haploid strains PCC 64 and 147 as well as the haploid culture of Rosko A clustered tightly with the diploid North European strains (Fig. 8). The growth phase (exponential vs. stationary) appeared to be of some importance in the North European strains because exponential and stationary cultures clustered independently. This was apparently related to a decrease in the ratio Fuco/19′HFuco from exponential to stationary growth phase.

Genome size. Flow cytometric analysis of nuclei stained with PI, a DNA fluorochrome that has no basepair specificity and standardized to chicken red blood cells produced information on genome size (Fig. 9). Values for G_1 cells ranged between 0.12 pg DNA·cell^{-1} for Naples and 0.44 pg for PCC 540 (Table 7). The combined use of HO, an AT-specific stain, and PI (Godelle et al. 1993) provided an estimate of the average GC% around 51%, while the combined use of CA3, a GC-specific stain, and PI provided a GC% value of 57%. This discrepancy is most likely linked to the use of chicken as a standard, because its GC% value (42.7%) is too far from that of *Phaeocystis*. One may assume that *Phaeocystis* GC% is close to the average of these two values, i.e. about 54%. Within the *Phaeocystis* strains, no significant

TABLE 7. *DNA content of* Phaeocystis *strains determined by flow cytometry. Strains were assigned to the same cluster if only one DNA peak was observed when strains were mixed together (see Fig. 10).*

Strain	DNA content (pg·cell^{-1}) (n = 2) Mean	SD	Ploidy (assumed)	DNA content 1C (pg·cell^{-1})	DNA content 1C /Naples	DNA cluster
Naples	0.12	0.00	1	0.12	1.00	1
SK 32	0.35	0.02	2	0.18	1.27	2
SK 22	0.37	0.00	2	0.18	1.30	2
Big 1209	0.34	0.02	2	0.17	1.38	3
Big IVM1	0.38	0.00	2	0.19	1.46	4
Big 677-3	0.38	0.02	2	0.19	1.47	4
JNorth	0.21	0.00	1	0.21	1.65	5
NIOZ 1	0.21	0.01	1	0.21	1.64	5
NIOZ 2	0.20	0.00	1	0.20	1.60	5
Rosko A haploid	0.22	0.01	1	0.22	1.67	5
Rosko A diploid	0.41	0.02	2	0.21	1.73	5
Rosko C	0.21	0.02	1	0.21	1.69	5
Rosko D	0.21	0.00	1	0.21	1.70	5
Rosko E	0.21	0.01	1	0.21	1.67	5
PCC 64	0.21	0.00	1	0.21	1.64	5
PCC 147	0.20	0.00	1	0.20	1.63	5
PCC 540	0.44	0.03	2	0.22	1.71	5

differences in GC% strains could be reliably determined with this method.

For most strains (except Rosko A), only one ploidy level (either haploid or diploid) could be analyzed. Therefore, the assumption was made (see earlier) that cultures containing colonies were diploid whereas those without colonies were haploid (Rosko A being an exception). The inferred haploid (1C) genome size ranged from 0.12 to 0.22 pg·cell^{-1} (Table 7). Three groups seemed to emerge: Naples at 0.12 pg·cell^{-1}; Antarctic and tropical strains at 0.17–0.19 pg·cell^{-1}; and North European strains at 0.20–0.22 pg·cell^{-1}.

The use of chicken nuclei as an internal standard (Fig. 9) is not very satisfactory because its genome size is 10–20 times higher than that of *Phaeocystis*. As an alternative, the Naples strain was used as an internal standard for the other strains. The data obtained this way (Table 7) were of better quality and allowed us to split the middle group into three: Antarctic strains, Big 1209, and Big 677-3 + Big IVM1. However, cells may release substances that interfere with the staining of nuclear DNA. Therefore, the only way to be sure that two strains have equal or different genome sizes is to mix them together before nuclei are released. If the DNA histogram of the mixture displays two peaks (Fig. 10), the two strains have different genome sizes and should be placed in two different clusters. In contrast, the observation of a single peak allows the strains to be grouped together. These mixing experiments were performed using the GC-specific stain CA3 that provided a better discrimination than PI. They confirmed that the Antarctic strains were distinct from Big 1209 by about 8% (Fig. 10, SK 32 shows as a shoulder) and that Big 1209 and Big 677-3/IVM1 were different by about 9%. In contrast,

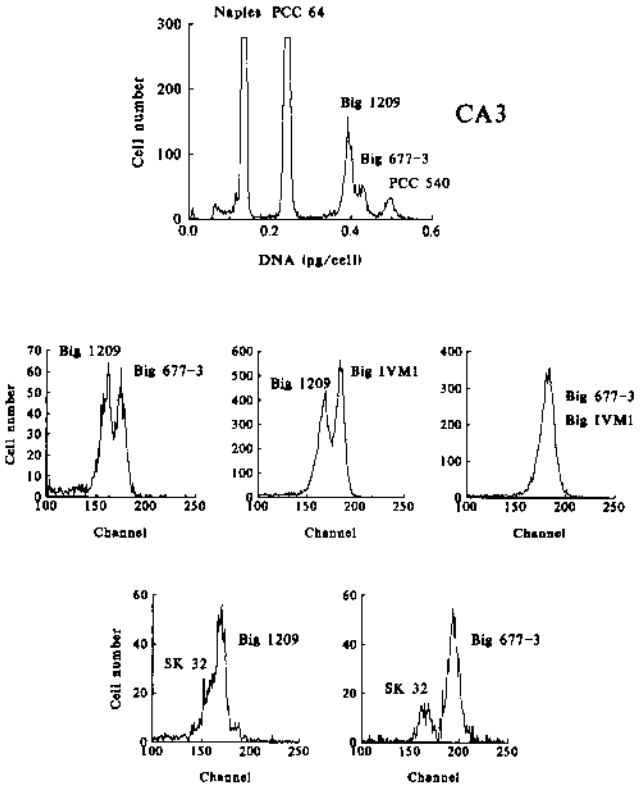

FIG. 10. DNA distribution of mixtures of nuclei from different *Phaeocystis* strains stained with CA3 and measured by flow cytometry.

no difference could be detected within the Antarctic strains SK 22 and SK 32, within the North European species, or between Big 677-3 and Big IVM-1, leading finally to five clusters (Table 7). Because the differences observed in mixtures appeared more marked with the GC-specific CA3 than with the non-base-specific PI, they could be due to a slight difference in GC% as well as a difference in DNA content.

DISCUSSION

Until now, the main criteria used for *Phaeocystis* taxonomy have been colony morphology (shape, distribution of cells), habitat, star-like features, and body scales (Table 1). Some of these parameters, such as colony shape or habitat are, however, not fully reliable, i.e. colonies with the *P. pouchetii* and *P. globosa* morphotypes may occur in the same habitat (Rick and Aletsee 1989). Other features such as scales are difficult to determine because they can only be observed on flagellated cells using transmission electron microscopy. In the present paper, we instead emphasized pigment composition and genome size as taxonomic markers.

Colony morphology did not appear to be very useful in distinguishing between strains because it was quite similar for all colony-forming strains with the possible exception of the two Antarctic strains SK 22 and SK 32 for which cells appeared more

densely packed. However, colony packing, or, quantitatively, the ratio of colony volume to cell number, is very dependent on environmental conditions. For example, Cariou's (1991) data indicate that for a typical colony with 100 cells, colony volume is 77 times larger at low (about 1 $\mu mol \cdot L^{-1}$) than at high phosphate (about 5 $\mu mol \cdot L^{-1}$) concentrations. In contrast, colony morphology is probably useful to distinguish *P. pouchetii* from other species (Baumann and Jahnke 1986), although strain PCC 540 that usually displayed *P. globosa*-like colonies sometimes exhibited *P. pouchetii*-like colonies (not shown).

Presence or absence of colonies cannot be used as a taxonomic character. We established that strains PCC 64 and 147, which have never formed colonies and were classified as *P. pouchetii* by Parke et al. (1971), are very similar in other respects to North European strains with *P. globosa*-like colonies. We also demonstrated with strain Rosko A that ploidy and colony formation are interconnected, with flagellates being mostly haploid and colonial cells diploid. One notable and unexplainable exception to this rule was observed for Rosko A: one flagellate culture stayed diploid.

Morphological differences between haploid and diploid phases have been observed in other Prymnesiophyceae, e.g. *Pleurochrysis pseudoroscoffensis* (Gayral and Fresnel 1983) and *Emiliana huxleyi* (Course et al. 1994). In the former species, however, the colonial and free-living phases are haploid and diploid, respectively, opposite to *Phaeocystis*.

Why do some strains (PCC 64 and 147 and Naples) fail to give colonies, i.e. to produce diploid cells? One possible explanation is that *Phaeocystis* possesses multiple mating types, as is common in mushrooms (Kües and Casselton 1993). These strains, which have been isolated initially as flagellates, may contain only a single mating type and therefore cannot fuse with themselves to produce diploid cells.

In light of these results, conditions for colony formation are therefore better thought of in terms of conditions necessary for ploidy change. These conditions are still very much unknown. Both nutrients and the necessity of a solid substrate have been invoked (reviewed in Rousseau et al. 1994). Recently, calcium concentration has been convincingly shown to be critical for the gelling of the polysaccharide matrix (van Boekel 1992), while low light may drastically reduce colony yield (Peperzak 1993). With respect to nutrients, Veldhuis and Admiraal (1987) found that, in batch cultures, low orthophosphate levels increased colony yield with a critical threshold around 1 $\mu mol \cdot L^{-1}$. However, Riegmann et al. (1992) found that in semicontinuous cultures, low P/N favored flagellated forms while high P/N favored colonies. Indeed, in agreement with the latter, routine transfer to K medium (P/N about 1/90) yielded the flagellate form in all North European strains with the exception of PCC 540 (Table 4). However, PCC 540 as well as all tropical and Antarctic strains still formed abundant colonies for P/N about 1/90. Our data suggest that nutrient effects on colony formation are strain-dependent; this could explain the contradiction between the two studies cited earlier, which used different strains. Also, studies of colony formation should be very carefully conducted, if, as suggested earlier, mating types are present in *Phaeocystis*. Both switches from colonies to flagellates and back from flagellates to colonies should be demonstrated because flagellates may well lose the ability to make colonies after a few generations if only noncompatible mating types remain in the culture.

Pentagonal star formation may also be linked to sexuality because it is always associated with flagellates, i.e. haploid cells (except in the odd diploid culture of Rosko A flagellates). This criterion must therefore be used very cautiously for taxonomic definition. In particular, we cannot tell whether the Antarctic and tropical strains are incapable of forming stars or whether this reflects our inability to obtain haploid flagellates; in fact, Medlin et al. (1994) reported stars in the Antarctic SK strains. Finally, we observed stars in both PCC 64 and 147 strains, whereas Parke et al. (1971) did so only in PCC 147. We do not know whether PCC 64 has evolved or has been contaminated since their study.

Flagellate morphology and scales that are important for taxonomy (Table 1) were not investigated by transmission electron microscopy in the present work. From scanning electron microscopy, however, Naples appears to have a slightly different morphology than the other strains, i.e. a rougher cell surface and longer flagella (Fig. 2).

Our data for cell size (Table 5) clearly falls within values that have been previously reported (Parke et al. 1971, Rousseau et al. 1990, Weisse and Scheffel-Möser 1990). PCC 147 and 64 cells are in the range given by Parke et al. (1971). However, cell size does not discriminate very clearly between strains. Only Naples cells are significantly smaller. In fact, the larger part of size variability is linked to growth conditions and ploidy. The 8-fold variability among all strains makes it very difficult to use a single conversion factor between cell number and carbon content (e.g. Rousseau et al. 1990).

In contrast to cell size, pigment composition appears very useful in discriminating among strains. Objective clustering based on the relative percentage of the different carotenoids led to three well-defined groups (Fig. 8) that were most clearly discriminated by their relative proportions of Fuco and 19'HFuco (Table 6). Culture conditions and ploidy levels have little effect on pigment signature.

Nichols et al. (1991) and Buma et al. (1991) found higher contributions of Fuco to carotenoids (4–25% and 83–93%, respectively) than we did for Antarctic strains, suggesting that there might be a large diversity of strains in the Antarctic. The contributions of 19'HFuco and Fuco to carotenoids reported earlier for strain Big 677-3 (34 and 40%, respectively,

TABLE 8. *Final clustering of* Phaeocystis *strains into six groups.*

Cluster	DNA cluster	Pigment cluster	Other distinguishing character (see text)	Strain	Origin
I	1	1	Rough cell surface, long flagellum	Naples	Mediterranean Sea
II	2	1	No stars?	SK 22, SK 32	Wedell Sea
III	3	2	No stars?	Big 1209	Gulf of Mexico
IV	4	2	No stars?	Big IVM1, Big 677-3	W. Atlantic
V	5	2	Condition for colonies, optimum temperature	PCC 540	E. Atlantic
VI	5	3		JNorth, NIOZ 1 and 2, Rosko A to F, PCC 64 et 147	North Sea and English Channel

Bjornland et al. 1988) fell into the range found here (Table 6). Values for another North Sea strain (Buma et al. 1991) place it in cluster 3 (North European strains). Finally, it is clear that there must be additional pigment clusters besides the three we found; in a strain from the Australian current (CS-165, Wright and Jeffrey 1987), 19′BFuco accounted for more than 40% of the carotenoids, against at most 8% in our strains.

Phaeocystis haploid genome size ranged between 0.12 and 0.22 pg (i.e. 1.09–2.00 10^8 basepairs) and separated the strains into five groups (Table 7). Although genome size variations have been observed within plant species (Bachmann 1993), it is useful to distinguish morphologically similar species of microalgae (e.g. Partensky et al. 1988). In the case of *Phaeocystis*, the groups observed fit tightly to the geographical origin. For example, all the North European strains displayed exactly the same genome size, as confirmed by mixing them 2 by 2. It is well known that genome and cell size correlate well (Shuter et al. 1983) and, in this respect, it is noteworthy that the smallest *Phaeocystis* strain (Naples) had also the smallest cell size.

CONCLUSION

If all parameters measured in this study are considered together, clusters obtained from pigment data are coherent with those obtained from genome size. Interestingly clusters were ordered similarly. Moreover, these clusters coincided with geographical origin. Therefore, we propose a clustering of the strains into six groups (Table 8).

I. Naples strain. This strain is clearly distinguished from the others by its geographical origin (the Mediterranean Sea), its lower genome size, and its pigment composition (very little Fuco). Moreover, cells are smaller and have a rougher surface and possibly longer flagella than the other strains. A question arises whether natural populations of *Phaeocystis* from the Mediterranean Sea (e.g. Delgado and Fortuño 1991) share similar characteristics. Indeed, two *Phaeocystis* strains recently isolated from the middle of the northwestern Mediterranean Sea display the same genome size as the Naples strain (N. Simon, pers. commun.).

II. Antarctic strains. Their main distinguishing features are colony morphology, pigment composition, and genome size.

III–IV. Tropical strains. Although the three tropical strains are indistinguishable for most of the measured parameters, strain Big 1209 clearly has a smaller genome size than the other two. Interestingly, it originates from the Gulf of Mexico, while the other two were isolated from the Atlantic Ocean proper.

V–VI. North European strains. All North European strains are quite similar, in particular with respect to genome size. Only PCC 540 seems different enough to justify a cluster of its own. First, it is related to tropical strains in terms of pigment composition; second, it forms colonies under conditions for which none other do; third, it has a slightly higher optimum temperature for growth. Finally, it was isolated from the Northeast Atlantic, while all other North European strains were from the English Channel or the North Sea.

It is evident from Medlin et al. (1994) that cluster II corresponds to what these authors call *P. antarctica* (the status of this species is still dubious, Baumann et al. 1994). Despite their differences, all the other clusters could be included in *P. globosa* according to the present state of the taxonomy. It seems logical, however, to consider only the North European cluster VI as the true *P. globosa*. It includes in particular PCC 64 and 147 that were initially classified as *P. pouchetii* (Parke et al. 1971). The other clusters (I, III, IV, and V) correspond probably to several yet-undescribed species or subspecies.

Our study investigated only the tip of the iceberg, because we were not able to include strains from many oceanic areas, in particular the Arctic, the Pacific and Indian Oceans, and the South Atlantic. From these areas, *P. pouchetii* (Baumann et al. 1994), *P. scrobiculata* (Moestrup 1979), strain CS-165 (Wright and Jeffrey 1987), and the flagellates described by Pienaar (1991) all may constitute separate species, increasing the *Phaeocystis* species count to almost eight, the number Sournia (1988) tallied in his review.

Our hypotheses should be confirmed by more detailed morphological studies, in particular at the ul-

trastructural level, and by sequencing genes such as that of 18S rRNA. Our work suggests that some criteria for *Phaeocystis* taxonomic determination, such as the shape of colonies or the presence of five-rayed stars, should not be used alone but in combination with other characteristics, because these criteria may be linked to the sexual cycle, for which conditions of induction are still largely unknown and possibly strain-dependent. Laboratory studies should use reference strains that have been well identified and preferably deposited in culture collections. The establishment of cultured strains from areas other than those investigated in this study is highly desirable. Finally, a detailed study of the type species of the genus *P. pouchetii* is urgently needed, as the strains examined by Parke et al. (1971) are most likely referable to *P. globosa*.

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