Journal of Sea Research 76 (2013) 105–113

Contents lists available at SciVerse ScienceDirect

Journal of Sea Research

journal homepage: www.elsevier.com/locate/seares

Characterization of *Phaeocystis globosa* (Prymnesiophyceae), the blooming species in the Southern North Sea

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ARTICLE INFO

Article history:
Received 5 October 2011
Received in revised form 11 July 2012
Accepted 24 July 2012
Available online 31 July 2012

Keywords:
Phaeocystis
P. globosa
Phylogeny
Prymnesiophyceae
Haploid-diploid
Life cycle

ABSTRACT

Despite significant research dedicated to the marine genus *Phaeocystis*, which forms large blooms in the coastal waters of the Southern North Sea, some aspects of the taxonomy and biology of this species still suffer from a sketchy knowledge. It is currently admitted that *P. globosa* is the species that blooms in the Southern North Sea. This has however, never been confirmed by SSU rDNA sequencing which constitutes nowadays, together with the morphology of the haploid flagellate, a reliable taxonomic criterion to distinguish between *Phaeocystis* species. Also, although the fine morphology of the haploid scaly flagellate is well known, there is a lack of comparable and harmonized description of the other cell types, i.e. colonial cells and diploid flagellates, previously identified within the *Phaeocystis* life cycle.

In this study, we used SSU rDNA sequencing, light and electron scanning microscopy and flow cytometry to identify and characterize three cell types produced in controlled and reproducible manner from two strains of *Phaeocystis* isolated from the Belgian coastal zone. Our morphometry and sequencing data confirm unambiguously that *P. globosa* is the species that blooms in the Southern North Sea, but suggest in addition that both strains are representative of the original *P. globosa* Scherffel. Our study compares, for the first time since the species description, the fine morphometry and ploidy features of diploid colonial cells, diploid and haploid flagellates originating from same strains, providing therefore unambiguous identification criteria for distinguishing them from each other. The diploid stage, colonial or flagellated cell, is thus characterized by a naked surface, has a size range nearly twice that of the haploid flagellate and do not produce the chitinous filaments specific of the haploid stage. Colonial cells lack flagella and haptonema but possess on their apical side two appendages, which elongate to form the flagella of the diploid flagellate.

1. Introduction

Blooms of the haptophyte *Phaeocystis* occur every spring in the coastal waters of the Eastern Channel and the Southern Bight of the North Sea (*e.g.*, Breton et al., 2006; Cadée and Hegeman, 1991; Seuront et al., 2006). This haptophyte has a complex life cycle involving different types of nanoplanktonic free-living and colonial cells enclosed in an exopolysaccharidic matrix. It is under this latter form that *Phaeocystis* blooms massively during spring (*e.g.* Lancelot et al., 1998).

Although it has long been referred to as *P. pouchetii* in the previous literature (e.g. Baumann et al., 1994), it is currently admitted that the species blooming in the North Sea is *P. globosa* Scherffel (type locality: Helgoland) based on colony morphology, *i.e.*, a globular shape (Antajan et al., 2004), and some physiological and biochemical properties (Baumann et al., 1994; Jahnke, 1989; Jahnke and Baumann, 1987; Vaulot et al., 1994). Despite significant *Phaeocystis*-based research in this coastal area (*e.g.* Lancelot et al., 1994; Veldhuis and Wassmann, 2005), the identity of the blooming species has never been determined based on the reliable taxonomic criteria nowadays used to distinguish between *Phaeocystis* species: the SSU rDNA sequence coupled to the detailed morphometric features of the scaly flagellates, *i.e.* size, structure and arrangement of body scales and star-forming threads (*e.g.*, Lange et al., 2002; Medlin and Zingone, 2007; Medlin et al., 1994; Zingone et al., 1999). The other cell types reported in the life cycle of *Phaeocystis* have never been used to identify the species due to the lack of fine morphological description of these cells.

Up to now, four cell types have been described from strains isolated in the Channel and the Southern North Sea and identified as *P. globosa*. These are the diploid colonial cell, the diploid flagellate and two types of haploid flagellates (Cariou et al., 1994; Kornmann, 1955; Parke et al., 1971; Peperzak et al., 2000; Rousseau et al., 1994, 2007; Vaulot et al.,

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http://dx.doi.org/10.1016/j.seares.2012.07.011

1994). These different cells are involved in a haploid–diploid life cycle where diploid colonial cells are formed after syngamy of haploid flagellates and where the diploid flagellate is able to reinitialize the colonial stage (Peperzak et al., 2000; Rousseau et al., 2007).

These different cell types are of quite different ecological significance. The success of colonial cells has been attributed to specific ecological properties, *i.e.* higher growth rates (Veldhuis et al., 2005) in nitrate-rich areas (Lancelot et al., 1998) and resistance to losses (*e.g.* Hamm et al., 1999; Lancelot et al., 1998, 2002; Verity and Medlin, 2003) conferred by the colonial structure. On the other hand, the flagellates have been shown better adapted to compete under conditions of ammonium and phosphate limitation (Riegman et al., 1992; Veldhuis et al., 1991), are controlled by protozoan grazing pressure (Admiraal and Venekamp, 1986) and are more vulnerable to virus infection than colonies (Brussaard et al., 2007). The type of flagellated cells, haploid or diploid, investigated in these experimental studies is very often unclear and is generally reported as *Phaeocystis* flagellates or free-living cells, making uncertain to which cell type the ecological properties refer. This is due to the lack of clear and complete morphological characterization of the different flagellates existing with the *Phaeocystis* life cycle (Rousseau et al., 2007), as only the morphology of the haploid flagellates has been resolved with electron microscopy (Antajan et al., 2004; Parke et al., 1971; Vaulot et al., 1994).

Verifying the identity of the *Phaeocystis* species blooming in the Southern North Sea and providing a complete description of the different cell types are therefore required for improving fundamental knowledge on *Phaeocystis* taxonomy, biology and ecology. With this objective we combined molecular (SSU rDNA sequence analysis), microscopy (light and scanning electron microscopy) and flow cytometry on three cell types, originating from two *Phaeocystis* strains isolated from the Belgian coastal zone (BCZ).

2. Methods

2.1. Phaeocystis cultures

Strains BCZ99 and BCZ05 were isolated as colonies from station 330 (N 51° 26.05; E 2° 48.50) in the BCZ during the spring bloom in 1999 and 2005 respectively. Three cell types, one colonial and two free-living flagellates of different sizes, were obtained from both strains. Colonial (C) cells were generated through the vegetative pathway for colony formation, *i.e.* when new colonies are produced from a suspension of cells freshly released from mature colonies (Rousseau et al., 2007). The larger flagellates (Mfl) were also observed during this vegetative pathway when part of the cells released from colonies subsequently developed flagella. In the cultures used in this study, Mfl represented 13% of the total cells (C + Mfl) some 28 h after release from colonies in BCZ99 while they amounted to 30% of the population after 36 h in BCZ05. For both strains, Mfl appearance was monitored by sampling cells every 3–4 h. Pure cultures of these large flagellates could never be obtained. The smaller flagellates (μfl) were produced within colonies and were maintained as pure cultures of free-living cells. All isolates were grown in F20 medium as described in Rousseau et al. (1990) at 8 ± 2 °C under a 12 h:12 h light:dark cycle at 100 μmol photons·m^{-2}·s^{-1} (Philips TLD 30/86). The different analyses performed on both BCZ99 and BCZ05 cell types are summarized in Table 1.

2.2. DNA extraction, SSU rDNA amplification and sequencing

Molecular analysis was performed on BCZ99 and BCZ05 C cells only. For this, 50 mL of colony cultures of both strains was centrifuged at 7500 rpm to concentrate cellular material. Slightly different procedures were used for both strains. For BCZ99, the CTAB protocol of Ishida et al. (1999) was used for extracting DNA and the entire SSU rDNA gene was amplified using the eukaryotic primers Euk 328f and Euk 329r as described in Moon-van der Staay et al. (2000). The PCR products were then cloned using the TOPO TA cloning kit (Invitrogen). For strain BCZ05, DNA was extracted using the Doyle and Doyle (1990)'s protocol, the SSU rDNA was amplified using the primers Euk 528f (Elwood et al., 1985), Euk 328f and Euk 329r (Moon-van der Staay et al., 2000) and the PCR products were purified with the Qiaquick PCR purification kit (Qiagen). Full-length 18S rRNA gene sequences of both strains were determined using an ABI prism 3100 sequencer (Applied Biosystems, France).

Table 1
Set of analyses performed on the different cell types of BCZ99 and BCZ05. C: colonial cells; Mfl: larger and μfl: smaller flagellates.

Strains	SSU rDNA	LM		SEM	Flow cytometry
		Living	Preserved		
BCZ99	C		C Mfl μfl	C Mfl μfl	–
BCZ05	C	C Mfl μfl	C Mfl μfl	C Mfl μfl	C Mfl μfl

2.3. Phylogenetic analyses

The full-length SSU rDNA sequences of BCZ99 and BCZ05 colonial cells were deposited in GenBank database under accession number AY851301 and EU127475, respectively. They were compared to *Phaeocystis* spp. full-length SSU rDNA sequences available in GenBank (Table 2). Three different methods, *i.e.* maximum parsimony (MP), neighbour-joining (NJ) and maximum likelihood (ML), were used for phylogenetic analyses. *Emiliania huxleyi*, another Prymnesiophyceae, was used to root the trees. Sequences were aligned using CLUSTALX and the alignment refined by

Table 2
Geographic origins and SSU rDNA sequence GenBank Acc. No. of algal strains used in the phylogenetic analysis.

Species and strain	Origin	GenBank ID
Phaeocystis antarctica		
CCMP1374	Antarctica, McMurdo Sound, 77°50′S, 163°E	X77477
SK20	Antarctica, Weddell Sea, 67°50′S, 20°51′W	X77478
SK21	Antarctica, Weddell Sea, 67°50′S, 20°51′W	X77479
SK22	Antarctica, Weddell Sea, 54°20′S, 3°20′W	X77480
SK23	Antarctica, Weddell Sea, 63°15′S, 58°20′W	X77481
Phaeocystis cordata		
Naples C2A1	Mediterranean Sea, 40°49′N, 14°15′E	AF163147
Phaeocystis globosa		
BCZ05	Southern North Sea, 51°26′N, 2°48′E	EU127475
BCZ99	Southern North Sea, 51°26′N, 2°48′E	AY851301
CCMP627	Gulf of Mexico, 29°15′N, 85°54′W	AF182111
CCMP628	Caribbean Sea, Surinam, 6°45′N, 53°19′W	AF182112
CCMP1524	North Pacific, Thailand, 15°N, 100°E	AF182109
CCMP1528	Galapagos, 1°23′S, 89°39′W	AF182110
PCC540	East Atlantic, 47°37′N, 8°53′W	AF182115
P162	South Africa	AF182113
SK35	North Sea, German Bight	X77476
Phaeocystis jahnii		
B5	Mediterranean Sea, Gulf of Naples	AF163148
Phaeocystis pouchetii		
P360	Raunefjorden, Norway	AF182114
SK34	Greenland Sea, East Greenland Current	X77475
Phaeocystis sp.		
OLI 51004*	Equatorial Pacific, 0°S, 150°W	AF107086
PCC559	North Atlantic	AF180940
Emiliana huxleyi (Texel B)	North Sea	AF184167

* Environmental sequence, no culture available.

hand using BIOEDIT. Poorly aligned positions and divergent regions were eliminated using Gblocks (Castresana, 2000). Different nested models of DNA substitution and associated parameters were estimated using Modeltest 3.06 (Posada and Crandall, 1998). The Akaike Information Criterion (AIC) in Modeltest selected a TrN + I (Tamura-Nei including the proportion of invariable sites; Tamura and Nei, 1993) model of DNA substitution with proportion of invariable sites = 0.7897) and substitution rates of R(b) [A-G] = 1.5847, R(e) [C-T] = 3.4601 and 1.0 for all other substitution rates (−lnL = 3481.9707). NJ, MP and ML were done using the PAUP*4.0b10 version (Swofford, 2002). Bootstrap values for NJ and MP were estimated from 1000 replicates.

2.4. Light microscopy

C, Mfl and μfl cells of both strains were observed at a magnification 1000× using a phase contrast microscope (LEITZ FLUOVERT, Germany). Size of both living (BCZ05) and preserved (BCZ99 and BCZ05) cells was measured with a graduated reticule and was reported as the average of linear measurements performed in parietal or apical/antapical view. Cells were preserved with 1% (final concentration) of the lugol-glutaraldehyde solution (Verity et al., 2007) and stored at 4 °C in the dark.

2.5. Scanning electron microscopy

SEM observation was performed on preserved C, Mfl and μfl cells after application of the critical point drying procedure. Samples were preserved with a lugol-glutaraldehyde solution (10% final concentration) and were centrifuged on a plastic coverslip coated with Poly-L-Lysine, dehydrated in a gradient of ethanol concentrations and dried with a Balzer's equipment before being placed on a stub. Samples were then gold coated and observed with a field emission SEM (HITACHI 4500, Japan).

2.6. Flow cytometry

Relative DNA content of the three cell types of strain BCZ05 was estimated on a FACScan (Becton Dickinson, USA) flow cytometer. Prior to analysis, glutaraldehyde preserved cells (1% final concentration) were left to settle for at least 48 h. Relative DNA content and cell cycle were estimated by measuring DNA fluorescence after staining cells with SYBER Green I (at 3.10^{-4} final concentration of the commercial solution) for 15 min. The DNA fluorescence was measured every 3–4 h during 36 h on pure cultures of C and μfl cells under a 12 h–12 h light–dark cycle. Such measurements were however not feasible on Mfl due to their short life span, low and unpredictable abundance and permanent mixing with colonial cells. In this study, the ploidy level of Mfl was therefore assessed by detecting relative DNA content changes during the kinetics of Mfl production from C cells in strain BCZ05. For this purpose, the DNA relative fluorescence of only C cells was compared to the one of a mixed population C + Mfl cells where Mfl represented 30% of total cell number. The relative DNA content was first estimated by comparison of the individual histograms obtained for μfl, C and C + Mfl cultures. In a later step, μfl cells in G1 phase were added to C and C + Mfl samples as internal standard to allow direct comparison of the respective DNA content. The cell cycle phases were retrieved from DNA histogram fitting using a cell cycle analysis software (ModFit LT Verity Software House Inc.).

3. Results

3.1. Species identification

When compared to the SSU rDNA of *Phaeocystis* strains listed in Table 2, BCZ99 and BCZ05 SSU rDNA sequences yield similarity ranges of 95.1–99.3% and 95.4–99.7%, respectively (Table 3). Both BCZ99 and BCZ05 show the highest similarity with *P. globosa* strains CCMP627, CCMP628, CCMP1524, CCMP1528, PCC540, P162 and SK35 (Table 3).

Similar SSU rDNA phylogenies were obtained from the MP, NJ and ML methods. The phylogenetic tree inferred by the ML method is shown in Fig. 1 with bootstrap values indicated for NJ/MP. This phylogeny demonstrates that BCZ99 and BCZ05 strains belong to the *P. globosa* clade which includes now 9 SSU rDNA sequences from cultured strains (Fig. 1). Interestingly, strains BCZ99, BCZ05 and SK35, all originating from the North Sea, belong to a same sub-group within the *P. globosa* clade (Fig. 1). This sub-group differs by 3–10 nucleotides (nt) from the other *P. globosa* strains, this difference being higher for BCZ99 (7–10 nt) and BCZ05 (4–7 nt) than for SK35 (3–4 nt; Table 3). This genotypic difference is in the same order of magnitude as that existing within the *P. antarctica* clade (1–7 nt) but also between closely related species such as *P. pouchetii* and *P. antarctica* (7–11 nt). The environmental sequence OLI51004 is very close but does not belong properly to the *P. globosa* clade from which it differs by 9–17 nt (Fig. 1; Table 3). This difference would exceed the lower limit between *P. antarctica*/*P. pouchetii* and the rest of the *P. globosa* sequences (14–26 nt).

3.2. Morphometric characteristics of C, Mfl and μfl

The three cell types (C, Mfl and μfl) obtained for strains BCZ99 and BCZ05 have been characterized with LM (Fig. 2) and SEM (Figs. 3–5). These observations show that the three cell types have the same morphological features for both strains.

3.2.1. Colonial cells (C)

C cells are randomly distributed at the periphery of spherical colonies (Fig. 2a). Colonies were artificially broken to release C cells prior to LM and SEM observations. In both strains, C cells are generally round-shaped with two, often bi-lobed, or four plastids providing them a typical square or rectangular shape in apical view (Figs. 2b; 3a, b; 4d). The average size of C cells is about 8 μm when alive but decreases by about 25% (~6 μm) when cells are preserved (Table 4) and even much more (53%) after critical point drying procedure used for SEM observation (Figs. 3a, b; 4d). SEM observation shows that C cells have a smooth surface without scales. It also reveals the presence of two short appendages, 0.2–0.4 μm in length, inserted between the two plastids at the center of the apical pole (Figs. 3a, b; 4d). These appendages are not visible from LM observations. C cells are often found paired suggesting they were undergoing active cellular division (Figs. 3a, b; 5a).

3.2.2. Large flagellates (Mfl)

The Mfl originating from C cells transformation generally have a rounded shape (Figs. 2c; 3c, 4d) but some can keep the angular typical shape of C cells (Fig. 4c). They have a size comparable to that of C cells (Table 4; Fig. 4d) and, like these cells, have a smooth naked cell surface (Figs. 3d; 4d). Time-course experiment of C cells transformation into Mfl shows that the Mfl flagella develop from the apical appendages (Fig. 4a–c) which elongate progressively, reaching a maximum length of 6.4 μm (Table 4). At this stage, flagella tips are tapered (Figs. 3c; 4d). Mfl possess a short haptonema, 1.74 μm of maximum length (Table 4) which becomes apparent after flagella growth has started (Fig. 4c). Some of Mfl have vesicles at their posterior end (Fig. 2c).

3.2.3. Small flagellates (μfl)

The μfl observed in this study were produced inside colonies at the end of their growth. Interestingly the formation of these small flagellates occurred only within the healthy spherical colonies and was never observed in damaged or aggregated colonies. The μfl are slightly flattened on their posterior end, possess two chloroplasts (Fig. 2d), and have an average size of 4.65 μm when alive and 3.9 μm when preserved (Table 4). They possess two flagella equal in length (5.8 μm; Table 3; Fig. 2d) with a tapered tip and a haptonema (Fig. 3d, e). This latter has an average length of 1.74 μm (Table 4) and is characterized by a

Table 3
Similarity (%) between SSU rDNA of *Phaeocystis* sequences and *Emiliania huxleyi* (upper triangle) and absolute number of nucleotide differences between these sequences excluding gaps (lower triangle in bold).

	BCZ99	BCZ05	CCMP1374	SK20	SK21	SK22	SK23	Napl C2A1	CCMP627	CCMP628	CCMP1524	CCMP1528	PCC540	P162	SK35	OLI51004	PCC559	B5	P360	SK34	*E. huxleyi*
BCZ99		99,5	98,1	98,2	98,4	98,4	98,4	97	99,2	99,2	99,3	99,3	99,1	99,1	99,3	98,9	95,1	95,3	98,2	98,1	93,2
BCZ05	**7**		98,5	98,7	98,8	98,8	98,8	97,4	99,7	99,6	99,7	99,7	99,5	99,5	99,7	99,3	95,4	95,6	98,7	98,5	93,6
CCMP1374	**23**	**21**		99,2	99,4	99,4	99,4	97,8	98,5	98,5	98,6	98,6	98,4	98,4	98,3	98,4	95,6	95,8	99,2	99,1	93,7
SK20	**25**	**22**	**7**		99,5	99,4	99,5	97,9	98,7	98,6	98,7	98,7	98,5	98,5	98,4	98,5	95,7	95,8	99,2	99,1	93,8
SK21	**22**	**19**	**3**	**4**		99,6	99,7	98,1	98,8	98,8	98,9	98,9	98,7	98,7	98,6	98,7	95,9	96	99,4	99,3	94
SK22	**22**	**19**	**4**	**5**	**1**		99,7	98,1	98,8	98,7	98,8	98,8	98,7	98,6	98,5	98,6	96	96,1	99,4	99,3	94
SK23	**21**	**18**	**4**	**5**	**1**	**2**		98,1	98,8	98,8	98,9	98,9	98,7	98,7	98,6	98,7	95,9	96,1	99,5	99,4	94
NaplC2A1	**44**	**41**	**26**	**28**	**24**	**23**	**24**		97,5	97,4	97,5	97,5	97,4	97,3	97,2	97,2	96	96,1	97,9	97,8	93,9
CCMP627	**9**	**5**	**17**	**20**	**16**	**16**	**17**	**36**		99,8	99,8	99,9	99,6	99,7	99,4	99,2	95,5	95,7	98,7	98,5	93,5
CCMP628	**9**	**6**	**17**	**20**	**16**	**16**	**17**	**36**	**0**		99,7	99,8	99,5	99,6	99,4	99,2	95,4	95,6	98,6	98,5	93,5
CCMP1524	**10**	**4**	**17**	**19**	**15**	**14**	**16**	**39**	**1**	**1**		99,8	99,7	99,6	99,5	99,3	95,5	95,8	98,7	98,6	93,6
CCMP1528	**9**	**4**	**17**	**20**	**16**	**16**	**17**	**36**	**0**	**0**	**1**		99,7	99,7	99,5	99,3	95,5	95,8	98,7	98,6	93,6
PCC540	**10**	**7**	**17**	**19**	**15**	**14**	**16**	**39**	**1**	**1**	**0**	**1**		99,4	99,3	99,1	95,3	95,6	98,5	98,4	93,5
P162	**9**	**7**	**17**	**20**	**16**	**16**	**17**	**36**	**0**	**0**	**1**	**0**	**1**		99,3	99,1	95,3	95,5	98,5	98,4	93,4
SK35	**7**	**4**	**18**	**21**	**17**	**16**	**18**	**38**	**4**	**4**	**3**	**4**	**3**	**4**		99,1	95,2	95,4	98,4	98,3	93,4
OLI51004	**17**	**10**	**19**	**23**	**19**	**20**	**18**	**41**	**10**	**10**	**9**	**10**	**9**	**10**	**10**		95,2	95,5	98,5	98,4	93,5
PCC559	**78**	**74**	**65**	**70**	**65**	**64**	**63**	**61**	**74**	**74**	**71**	**74**	**71**	**74**	**76**	**79**		98,8	95,8	95,6	94
B5	**72**	**69**	**61**	**65**	**61**	**59**	**58**	**59**	**67**	**67**	**66**	**67**	**66**	**67**	**69**	**74**	**15**		95,9	95,8	94,1
P360	**26**	**21**	**8**	**11**	**6**	**7**	**7**	**29**	**21**	**21**	**19**	**21**	**19**	**21**	**21**	**26**	**70**	**70**		99,8	93,8
SK34	**26**	**23**	**9**	**11**	**7**	**7**	**8**	**35**	**21**	**21**	**21**	**21**	**20**	**21**	**22**	**27**	**71**	**70**	**1**		93,7
E. huxleyi	**104**	**100**	**99**	**96**	**97**	**98**	**98**	**98**	**106**	**106**	**102**	**106**	**102**	**106**	**106**	**100**	**96**	**96**	**102**	**103**	

distal swelling (Fig. 3e). The μfl cell surface is, contrarily to that of Mfl, covered with an external layer of flat scales (Fig. 3e). These latter are oval and circular, show a raised rim, and are of two sizes, i.e., ~ 0.18–0.22 μm for the larger, and ~0.15–0.18 μm for the smaller (Fig. 3f). Scaly μfl are invariably associated to fully extended filaments reaching up to 20 μm long and forming pentagonal star-like structures at their proximal ends (Fig. 3d). The μfl are also able of active division (Fig. 5b).

3.3. Flow cytometry analysis

The analysis of relative DNA fluorescence histograms of the μfl culture shows a well marked diel cell cycle with most of cells (>90%) in G1 phase during the light period and increasing amount of cells in S and G2 (45%) during the dark period (not shown). This suggests that cell division of μfl was synchronous and occurred at the end of the dark period. On the contrary, the DNA fluorescence histograms of C cells were broad, with one unique peak where no cell cycle phase could be discriminated by the ModFit software along the light–dark cycle (not shown). The comparison between the DNA histograms of μfl in G1 phase and that of C (Fig. 6a) and C + Mfl (Fig. 6b), shows that C and C + Mfl cells have four times as much DNA as μfl. Furthermore, no change in the relative DNA content occurs after the transformation of a part of colonial cells into flagellates (Fig. 6) suggesting that the C and Mfl cells have the same ploidy level.

4. Discussion

4.1. P. globosa is the blooming species in the Southern North Sea

Since the first description of the genus *Phaeocystis*, criteria to distinguish between species have considerably evolved depending on methodological development. Species distinction has been at first based on the description of the colonial stage and different cell types using light microscope (*e.g.*, Kornmann, 1955; Lagerheim, 1893; Scherffel, 1900) and, later, of the scaly flagellate using electron microscope (*e.g.*, Moestrup, 1979; Parke et al., 1971). Nowadays, identification of species within the *Phaeocystis* genus mainly relies on both the genotypic signature and the morphological features of the scaly flagellate, *i.e.* size, structure and arrangement of body scales and star-forming threads (*e.g.*, Lange et al., 2002; Zingone et al., 1999).

All the morphological and molecular features reported in our study converge to ascertain that both strains BCZ99 and BCZ05 isolated from the Southern North Sea belong to the species *P. globosa*. In both strains, growing colonies were perfectly spherical with colonial cells homogenously distributed at their periphery in agreement with the original LM description of the species by Scherffel (1899) more than one century ago and extended later by Kornmann (1955) even if erroneously named *P. pouchetii* at that time. Our observations agree with the description of colonial cells and the existence of two flagellates of significantly different size provided by Scherffel (1900) and Kornmann (1955) for *P. globosa*.

The morphometric features of the scaly haploid flagellates (μfl) from BCZ99 and BCZ05, as observed from LM and SEM, are in agreement with those reported for strains identified as *P. globosa*, *i.e.*, PCC147 (Antajan et al., 2004; Parke et al., 1971 who refer to *P. pouchetii*; Vaulot et al., 1994), Rosko A (Fig. 2 in Vaulot et al., 1994) and an isolate from BCZ (Fig. 3 in Antajan et al., 2004). The features specific to *P. globosa* can be summarized as follows: a cell size in the range 3–6 μm, the presence of two equal flagella 10–15 μm long, of a non-coiling short haptonema with a distal swelling, of long filaments reaching up to 20 μm and forming a pentagonal figure at their proximal end and covered by types of discoid scales with radiating ridges (Medlin and Zingone, 2007; Medlin et al., 1994).

The size range of living scaly flagellates of BCZ05 (3.6–5.8 μm; Table 3) corresponds to that measured for Scherffel (1900)'s "zoospore" (5 μm), Kornmann (1955)'s microzoospore (3–4.5 μm), Parke et al.

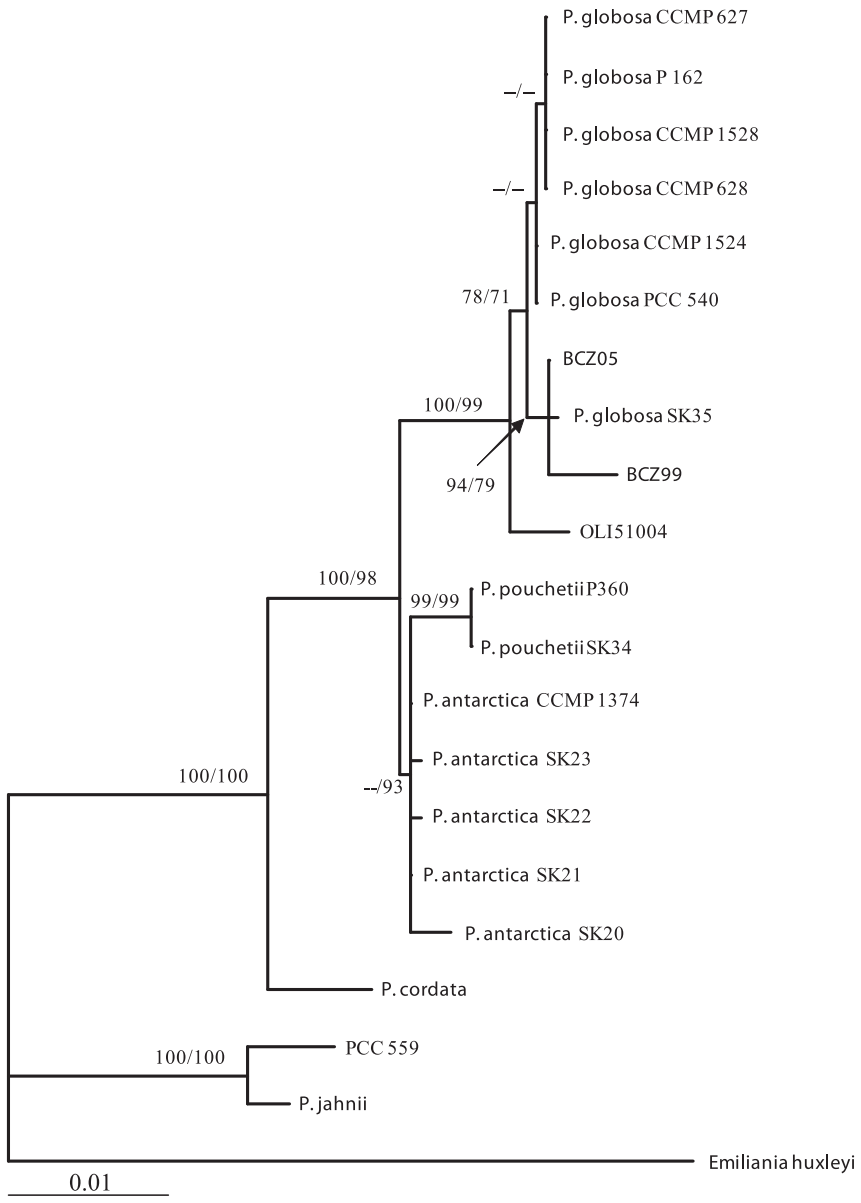

Fig. 1. Phylogeny of *Phaeocystis* based on SSU rDNA full-length sequences (1677 characters after data processing). The phylogenetic tree was inferred by the maximum likelihood (ML) method, using a TrN + I (Tamura-Nei including the proportion of invariable sites; Tamura and Nei, 1993) model of DNA substitution. Bootstrap values for NJ and MP, respectively, are indicated above internodes and were estimated from 1000 replicates (bootstrap values <70% are indicated by hyphens). The scale bar indicates 0.01% sequence divergence.

(1971)'s zoid (3–6 μm), all attributed to *P. globosa* haploid flagellates (Peperzak et al., 2000). The maximum length of the flagella (7 μm) and haptonema (1.74 μm) are close to those reported by Parke et al. (1971), *i.e.*, 1.5 times the cell length and a quarter to a third the length of the flagella respectively. The flagella length is however less than 10–15 μm as reported for cells from New Zealand waters by Moestrup (1979) and considered as a taxonomic criterion by Medlin et al. (1994). The pentagonal figure formed by the 20 μm filaments, made of alpha-chitin (Chrétiennot-Dinet et al., 1997), and observed under SEM in the haploid flagellate cultures constitutes another feature typical of the species. In both strains, a coating of oval and circular scales showing a raised rim is clearly visible from SEM observations. These scales are very similar in shape and size to the scales, depicted by Parke et al. (1971) for PCC147.

Finally, the genotypic identification of strains BCZ99 and BCZ05 also concludes that *P. globosa* is the blooming species in the Southern North Sea. This is supported by our phylogenetic reconstruction, which retrieves the five described *Phaeocystis* species, *i.e. P. globosa*, *P. pouchetii*, *P. antarctica*, *P. cordata and P. jahnii* in agreement with previous analyses (Edvardsen et al., 2000; Lange et al., 2002; Medlin and Zingone, 2007; Zingone et al., 1999). The two strains BCZ99 and BCZ05 unambiguously belong to the *P. globosa* clade that regroups strains from cold-temperate and tropical waters (Table 1). Within this clade, BCZ99 and BCZ05 appear together with SK35 in a sub-group of isolates originating from the Southern North Sea (Table 1; Fig. 1). Strain SK35 was indeed isolated from the German Bight, close to Helgoland (Medlin et al., 1994), the type locality given by Scherffel (1899) for *P. globosa*.

The Southern North Sea sub-group differs by 3–10 nt from the other *P. globosa* strains, *i.e.* a high genotypic differentiation for a conservative region which suggests a distinct species which would be the closest to the type material. That *P. globosa* is a complex of three or four cryptic species has already been suggested by Medlin and Zingone (2007) based on the high genotypic differentiation within the clade but also on the intra-specific sequence divergence of the internal transcribed

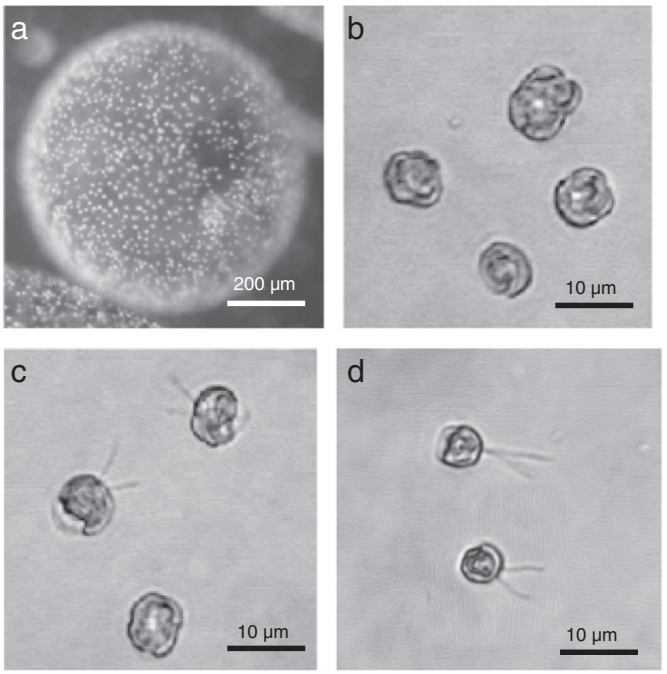

Fig. 2. LM photographs of preserved cells and colonies: a 600 µm-large spherical colony (BCZ05) containing some 1500 cells, colonial cells (BCZ05; b), two large flagellates and one colonial cell of similar size (BCZ05; c), and two small flagellates (BCZ99; d).

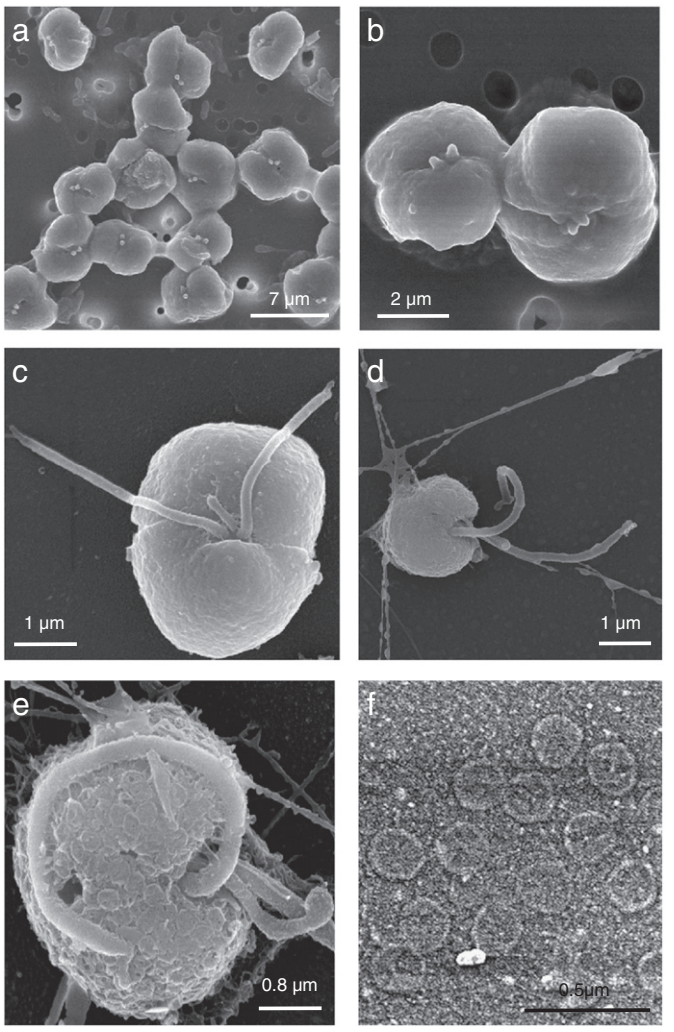

Fig. 3. SEM micrographs of the three cell types observed in both strains BCZ99 and BCZ05. Colonial cells C (BCZ99) released from the colony showing the two short appendages on their apical side and cell surface deprived of scale coverage (a, b); Large flagellate Mfl (BCZ99) showing the two flagella with a tapered end, the haptonema and the cell surface without scales (c); Scaly flagellate µfl (BCZ05) with the typical pentagonal star-forming filaments, the two flagella and the haptonema (d); Detail of a µfl showing the distal swelling of the haptonema and the cell surface covered by flat scales (e); Oval and circular scales of two sizes presenting a raised rim (BCZ99, f).

spacer (ITS). The *P. globosa* species diversity is also supported by pigment composition and genome size differences between North European and tropical strains (Vaulot et al., 1994). The difference in flagellar length between New Zealand and Belgian strains evidenced in this study constitutes another feature supporting species differentiation.

A careful consideration of shape and size of the scales, length of the flagella and other morphological details is further required to re-arrange the *P. globosa* taxon. In the case of a re-typification of the species *P. globosa*, it is likely that only one of the two genotypes will retain the name *P. globosa* and the other taxa from other areas would be described under new species names.

4.2. The three cell types of P. globosa

Besides the scaly flagellate used as species identifier, two other cell types were detailed in our study: the colonial non-motile cells and their derived flagellates. Although already reported in the *P. globosa* life cycle, these cells have, up to now, only been observed and described under LM (*e.g.* Kornmann, 1955; Peperzak et al., 2000; Rousseau et al., 1994). We did not observe non motile free living cells as such reported by Peperzak et al. (2000) and Rousseau et al. (2007).

Colonial cells of strains BCZ99 and BCZ05 lack scales, flagella and haptonema, possess 2 to 4 chloroplasts, show a longitudinal groove and do not release the filaments forming pentagonal figures. This is consistent with previous morphological observations of colonial cells of *P. globosa* (Kornmann, 1955; Peperzak et al., 2000; Rousseau et al., 2007; Scherffel, 1900; Vaulot et al., 1994) but also of *P. antarctica* (Zingone et al., 2011). SEM observations reveals on the apical pole of colonial cells two short appendages reported for the first time by Rousseau et al. (2007). These appendages were visible though not detailed on the *P. globosa* colonial cells described in Antajan et al. (2004; Fig. 3E). They were also present on a *P. jahnii* cell picture in Zingone et al. (1999; Fig. 30) and on *P. antarctica* (Rousseau et al., 2007; Zingone et al., 2011) suggesting that they might be a generic feature of *Phaeocystis* colonial cells. Although their composition and role remain to be elucidated, it seems that these appendages are at the origin of the two flagella which appear when colonial cells released from the colony transform into flagellates, as also observed for *P. antarctica* by Zingone et al. (2011). The mechanism and factors controlling flagella and haptonema growth when colonial cells are liberated from the polysaccharidic matrix are however not known yet.

The so-formed flagellates share most of morphometric features of colonial cells, *i.e.*, a naked surface devoid of scales and surface vesicles producing the star-forming filaments. They also have a similar size range, being sensibly larger than that of scaly flagellates (Table 4). Such a difference agrees with Kornmann's (1955) living cell size measurements, *i.e.* 4.5–8 µm for his colonial cells and larger flagellates (called swarmers) and 3–4.5 µm for his microzoospores. The lower size ranges notified by Kornmann (1955) for the three cell types could possibly be due to difference in cell cycle phases and growth rates of cultured cells at the time of measurement. Besides growth conditions, cell size depends on preservation and fixation procedures. When preserved with lugol-glutaraldehyde, all cells shrink by some 20%. As such, our colonial cell size range (4.8–7.8 µm for both strains) agrees well with that measured by Peperzak et al. (2000; 5.6–8.3 µm), Rousseau et al. (1990; 5.7–7.5 µm) for either lugol or lugol-glutaraldehyde fixed cells.

These flagellates also have the same ploidy level as colonial cells, as already suggested by Cariou et al. (1994) during a similar experiment of flagellate production from colonial cells. However these cells contain

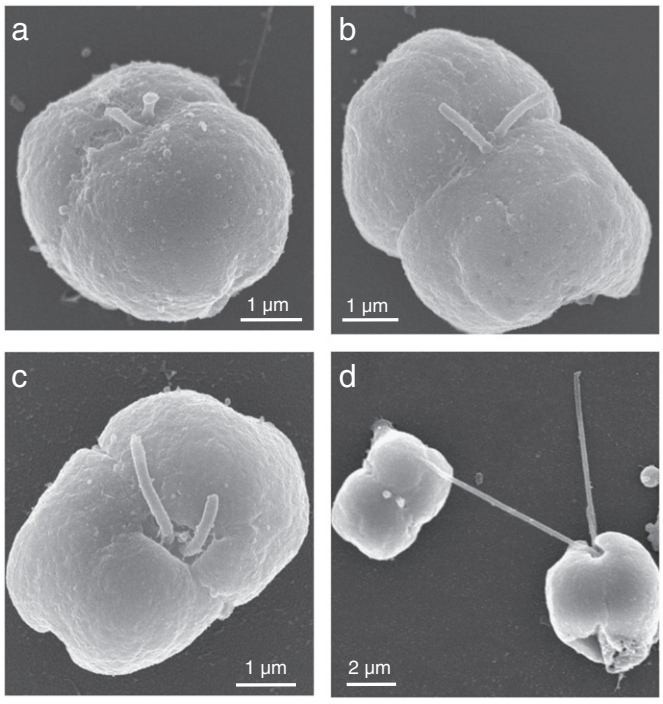

Fig. 4. SEM micrographs showing the progressive transformation of a colonial cell (a) into a flagellate of the same size deprived of scales and filaments (d). The two flagella originate from the apical appendages of colonial cells (a–c) and the haptonema becomes apparent once flagella growth has started (c).

four times more DNA than the haploid scaly flagellates, suggesting they would be tetraploid and not diploid as previously suggested by Cariou et al. (1994) and Vaulot et al. (1994). We suggest however that the tetraploidy observed in our experiment result from the presence of a very high proportion of diploid cells in S (2 + N) and G2 (4 N) phases. This assumption is confirmed by the complete absence of 8 N cells, which would result from tetraploid cell division (Fig. 6b). Such tetraploid cells have already been observed in colony culture but in much lower proportion (Vaulot et al., 1994). The absence of a diel cell cycle, as observed by Veldhuis et al. (2005) for field *P. globosa* populations, would suggest that colonial cells and derived flagellates undergo asynchronous division in our cultures.

Both haploid and diploid flagellates possess two flagella of similar length with a tapered end and a short non-coiling haptonema. The two flagellates differ in size and by the presence or absence of cell scale coverage and pentagonal filaments. The haploid flagellate is smaller in size (~5 μm), possess the scale and thread-like material specific to *Phaeocystis* while the diploid flagellate has the same size (7–8 μm) than colonial cells and is deprived of scales and chitinous filaments as already suggested by Peperzak et al. (2000) and Rousseau et al. (2007). We did not observe in our cultures the third flagellate type reported by Peperzak et al. (2000),

Table 4
Size range (μm) and measured cell number (n) performed on the three cell types observed in strains BCZ99 (only preserved cells) and BCZ05 (alive and preserved cells) at 1000× under LM. Maximum length of the flagella and haptonema are also reported. C: colonial cells; Mfl: larger and μfl: smaller flagellates.

Strain		BCZ99	BCZ05	
Cell type		Preserved	Alive	Preserved
C	Cell	4.6–7.8 (100)	5.8–10.4 (50)	4.8–7.1 (50)
Mfl	Cell	4.5–7.3 (100)	6.1–9.3 (35)	4.6–7.3 (85)
	Flagellum	7.5		
	Haptonema	1.7		
μfl	Cell	2.9–5.0 (35)	3.6–5.8 (50)	2.9–4.9 (50)
	Flagellum		7	6.4
	Haptonema		1.7	1.7

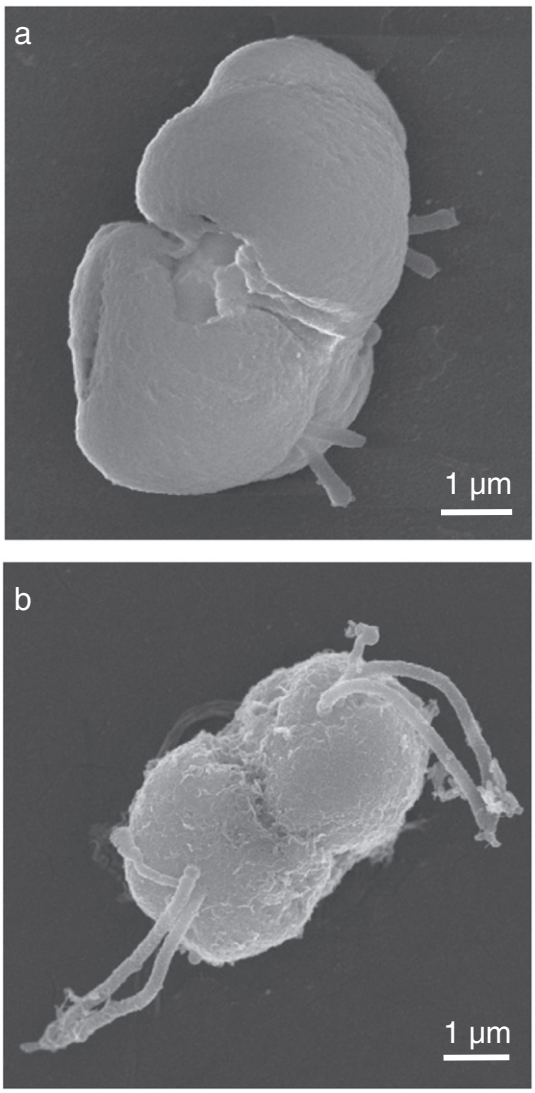

Fig. 5. SEM micrographs of mitotic division of a colonial cell with elongating appendages (a) and a haploid flagellate (b) both from strain BCZ05.

the so-called "microflagellate" slightly smaller than scaly haploid flagellate and lacking the filaments forming pentagonal stars. Interestingly, the two kinds of flagellate, one covered by scales and deploying five-rayed filaments, and another deprived of such features were also observed in *P. antarctica* colonial cultures. The *P. antarctica* flagella were, however, of unequal length (<12 μm and <18 μm, respectively) and the haptonema was relatively long (4.5 μm; Zingone et al., 2011).

4.3. Role of the three morphotypes in the P. globosa life cycle

The observation of three cell types characterized by distinct morphology and ploidy levels, *i.e.*, diploid colonial cells and flagellates, and haploid flagellates, gives support to the existence of a haplo-diploid life cycle within *P. globosa* as suggested by Peperzak et al. (2000) and Rousseau et al. (2007). In this cycle, haploid flagellates would be gametes undergoing syngamy to form diploid colonial cells and being formed through meiosis of colonial cells. In our cultures, the formation of scaly haploid flagellates was observed within spherical healthy colonies only. This observation has similarly been reported from the natural environment where "zoospores" (Scherffel, 1900) and "microflagellates" (Peperzak et al., 2000) were found in spherical colonies of various sizes at the end of the bloom. In any cases, scaly flagellate formation has never been depicted within *Phaeocystis*-derived aggregates. This suggests that intact healthy

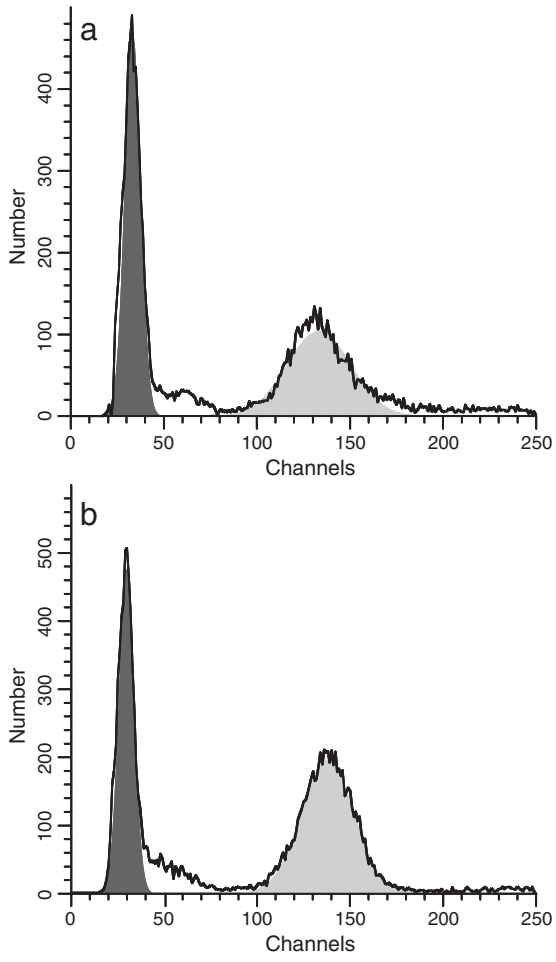

Fig. 6. DNA fluorescence frequency histograms (lines) and ModFit software fits (shaded areas) for a culture where μfl mainly in G1 phase (dark grey areas) were mixed with (a) only C cells and (b) C + Mfl cells (light grey areas) of strain BCZ05.

colonies would offer favorable conditions for scaly flagellate formation. Nutrient limitation has, among other factors, been suggested to play a role in flagellate formation at the end of a bloom (Verity et al., 1988; Escaravage et al., 1995). It is possible that the intact colonial matrix acts as a diffusion barrier (Ploug et al., 1999), inducing then the nutrient deprivation required for cell transformation. This would not be the case in aggregates where bacteria colonization ensures nutrient remineralization. In the field, this process results in the massive production of haploid flagellates often reported at the end of a *P. globosa* bloom, before disappearance of the colonial stage (*e.g.* Peperzak et al., 2000; Rousseau et al., 2007). The haploid scaly flagellate is the *P. globosa* form persisting in the water column between two blooms of colonial cells (Rousseau et al., 2007). It is also generally found in oligotrophic waters worldwide (Lancelot et al., 1998; Moestrup, 1979).

Besides sexual processes, the vegetative reproduction of the species occurs through two distinct pathways, *i.e.* colony growth when diploid colonial cells divide by mitosis within colonies and, colony generation when diploid flagellates reinitialize new colony forming after liberation into the ambient water (see the review of Rousseau et al., 2007). There are also few reports of colony division or budding (Verity et al., 1988; Rousseau et al., 1990). The production of colonies from aggregates containing densely packed cells, adhering to surface as observed recently in *P. antarctica* (Gaebler-Schwarz et al., 2010) was, at this stage, not observed in *P. globosa*.

The formation of diploid flagellates from colonial cells has been largely reported from culture observations under light microscopy (Cariou et al., 1994; Kornmann, 1955; Rousseau et al., 2007). In our experiments, the proportion of colonial cells undergoing transformation into flagellates varied between 1 and 30%. Different tests monitoring flagellate appearance after colony disruption show that this proportion is independent from cell growth rate, colonial size and/or stage (not shown). As they have a short life span, *i.e.* 24 h (Cariou et al., 1994), it is not sure that diploid flagellates are able of division. They probably pursue the cell cycle initiated by colonial cells, as suggested on Fig. 5b which shows the early stage of a diploid flagellate formation from a dividing colonial cell. The diploid phase in P. *globosa*, as colonial or flagellated cell, mainly occurs during the colonial blooms (Lancelot et al., 1998; Rousseau et al., 2007). It has been suggested that the diploid flagellate was not frequent in the natural environment, being produced after colony disruption under stormy or turbulent conditions (Rousseau et al., 2007).

5. Conclusions

Our morphometry and sequencing data confirm unambiguously that *P. globosa* is blooming in the Southern North Sea, but they also suggest that both strains BCZ99 and BCZ05 isolated from this coastal area could be representative of the original *P. globosa* described by Scherffel (1899). For the first time since the species description, this study compares the fine morphometry and ploidy features of three *P. globosa* cell types originating from a unique isolate, providing therefore unambiguous identification criteria for P. *globosa* diploid colonial cells, diploid and haploid flagellates. The diploid stage, colonial or flagellated cell, is characterized by a naked surface, has a size range nearly twice that of the haploid flagellate and do not produced the chitinous filaments specific of the haploid stage.

During our experiments, the three cell types alternating within the *P. globosa* life cycle were produced in a controlled and reproducible manner, so that a specific role can be attributed to each cell within the life cycle. Despite this, both sexual and vegetative reproduction processes and inherent cell transformation remain to be described in details. By identifying functional cell types, our study nevertheless constitutes a useful contribution towards a better knowledge of the taxonomy and biology of the species *P. globosa*.

Acknowledgments

This work is part of the projects “Advanced MOdelling and Research on Eutrophication” AMORE II (programme “Global Change, Ecosystems and Biodiversity” under contract EV/11/19) and AMORE III (programme “Science for a Sustainable Development” under contract SD/NS/03A) funded by the Belgian Science Policy. F. Rodriguez has been supported by Fundacion Caixanova. The authors wish to thank J.-Y. Parent and F. Jalabert for culturing *Phaeocystis* strains, B. Rivière for flow cytometry technical help, C. Courties for advice about the Modfit software, D. Gorand for assistance in using the scanning electronic microscope. This work was supported by the ASSEMBLE EU FP7 research infrastructure initiative and by the Contrat de Projet Etat-Région “Souchothèque de Bretagne”. The two anonymous referees are acknowledged for their constructive comments.

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