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The initiation of *Phaeocystis* colonies

Valérie Cariou[1], Raffaella Casotti, Jean-Louis Birrien and Daniel Vaulot[2]

CNRS et Université de Paris 6, Station Biologique BP 74, F-29682 Roscoff cedex, France

[1]*Present address: CREMA BP 5, F-17137 L'Houmeau, France*
[2]*To whom correspondence should be addressed*

Abstract. This study was designed to elucidate the sequence of events that leads to the formation of new colonies of *Phaeocystis* sp. (strain PCC 540) starting from single cells released from mature colonies. Colonies were first isolated by filtration onto a 10 μm mesh. Colonial cells were then liberated by shaking and inoculated into individual culture wells containing medium with a PO_4^{2-} concentration of ~1 μM. Cell size and shape were determined daily by image analysis, while chlorophyll and DNA distributions were estimated by flow cytometry. Released cells were non-flagellated and mostly located in the G_1 phase of the cell cycle. They developed flagella and up to 90% became motile within 24 h. Swarmers lost motility rapidly, became elongated, began to cycle again, excreted a mucilaginous compound and divided leading to new colonies within a few days. During this reproducible process, no change of ploidy could be observed. Colonies initially adhered to the bottom of culture wells. Frequent mixing drastically reduced the fraction of colonies produced and their volume. High initial PO_4^{2-} concentrations (5 μM) delayed colony appearance, whereas low concentrations (0.3 μM) prevented colony formation. The two main conclusions of this study are: (i) under favorable conditions (~1 μM PO_4^{2-}, no mixing), a large percentage of released colonial cells give back colonies after going through a flagellated stage; (ii) sexuality does not appear to be involved in this process.

Introduction

The Prymnesiophyte *Phaeocystis* has a world-wide distribution and is responsible for regular proliferations, in particular along coastal areas of the North Sea (Lancelot *et al.*, 1987). A thorough understanding of the *Phaeocystis* life cycle is a key element to explain the dynamics of this genus. Kornmann's (1955) classical study established the alternation between two morphologically distinct forms: single cells and colonies. Single cells may be either motile or non-motile and range between 3 and 10 μm in diameter. Colonies consist of an aggregation of non-motile cells that are located at the periphery of a multilayered polysaccharidic structure (Guillard and Hellebust, 1971; Chang, 1984; van Boekel, 1992). The origin of the colonial stage is still greatly discussed [see Rousseau *et al.* (1994) for a recent review]. Kornmann (1955) observed in culture that colonies may originate through three different pathways. First, colonial cells may multiply inside the colony without an increase of the colonial volume (macrozoospores). They subsequently develop flagella, extrude the matrix and produce new colonies. This pathway of colony formation has been very seldom observed. Second, smaller flagellated cells of 3–5 μm in diameter (microzoospores) may arise in cultures when colonies disappear. These cells form new colonies under specific conditions of culture, e.g. when cultures are illuminated with a fluorescent daylight lamp (Kornmann, 1955). Third, colonial cells released from colonies, and inoculated into fresh medium, may become

flagellated, attach to the surface of the culture vessels and develop into new colonies. This pathway is the most common and the most reproducible (Kayser, 1970; Lancelot and Mathot, 1985; Veldhuis and Admiraal, 1987).

Despite numerous studies, little is known about the sequence of events and the factors associated with the appearance of the colonial stage. In this study, we focus on the very first steps of *Phaeocystis* colony formation, starting from mechanically released colonial cells. Cell types, size, chlorophyll and DNA content were followed using a combination of image analysis and flow cytometry.

Method

Strain and culture conditions

Phaeocystis sp. strain PCC 540 (Plymouth Marine Laboratory, UK), isolated from the East Atlantic in July 1982, was used because of its very reproducible capacity to produce large numbers of colonies under laboratory conditions. Cultures were grown routinely in K medium (Keller *et al.*, 1987) at 12°C in an illuminated cabinet supplied with 100 $\mu E\ m^{-2}\ s^{-1}$ of continuous light. For colony-formation experiments, medium was prepared with sterile, filtered natural seawater enriched as in Veldhuis and Admiraal (1987), but without ammonia and with 1.24 μM phosphates.

Experimental design

The separation of colonies from single cells was achieved on exponentially growing cultures in K medium using gentle filtration (to avoid colony disruption) onto a 10 μm sterile mesh (Figure 1). The mesh was then washed with K medium, colonies were resuspended in this medium and broken by vigorous shaking by hand. The suspension was filtered again through a 10 μm sterile mesh to get rid of colonial debris and small unbroken colonies. Released colonial cells were inoculated at a final concentration of $\sim 10^4$ cells ml^{-1} into 24 well plates (Nunclon, Roskilde, Denmark). Each well contained 2 ml of the experimental medium. Plate cultures were sampled at $t = 0$ and $t = 6$ h, and subsequently every 24 h during 7 days. At each sampling time, two wells were sacrificed for cellular measurements (see Figure 1), leaving the others untouched to minimize mechanical disturbances.

Cell type analysis

Cellular observations and measurements were performed with an inverted microscope equipped with phase contrast (Olympus CK2, Tokyo, Japan). The mucilaginous envelope of the colonial stage was stained with Alcian blue and the flagellar system was colored with methylene blue. Different cell categories were visually discriminated: flagellated and non-flagellated single cells, spherical and elongated single cells, cell couples (groups of two cells), cell tetrads (groups of four cells) and colonies. Cell concentration in the supernatant (N_s, cells ml^{-1}) was measured with a Malassez counting chamber. The total number of cells on

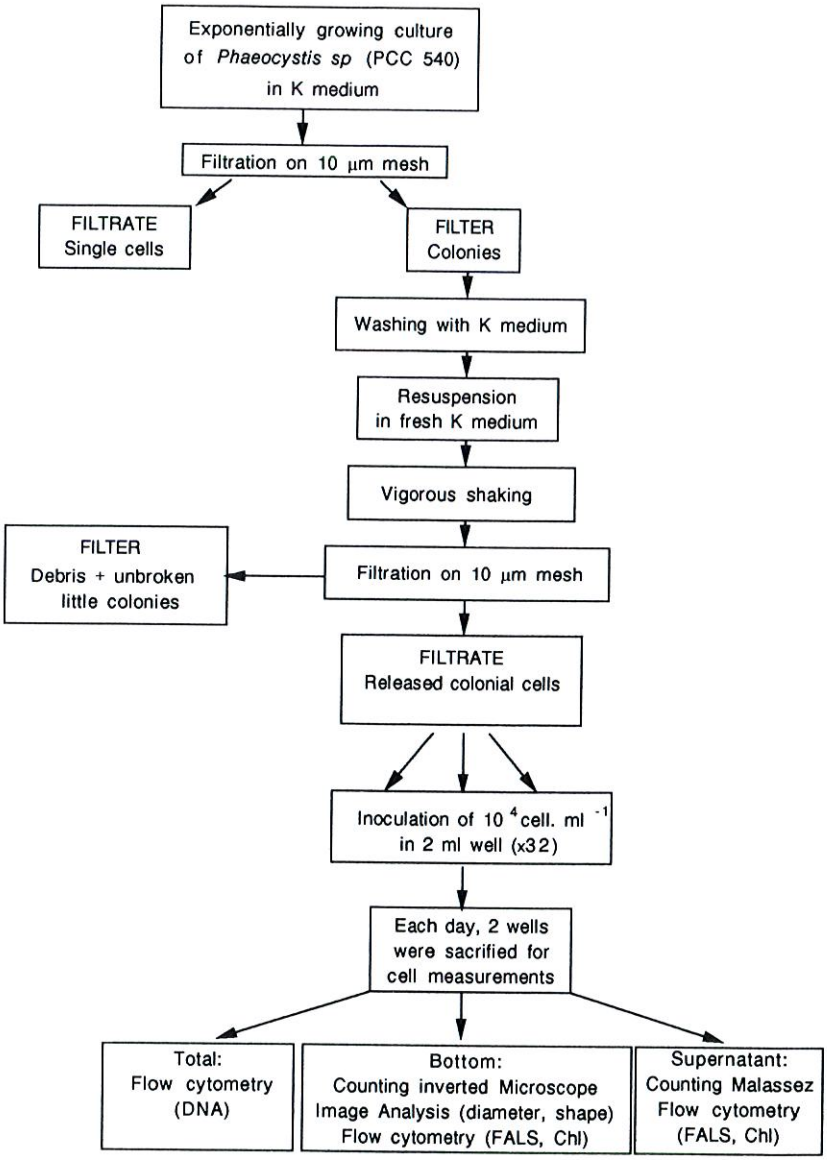

Fig. 1. Experimental design (see Method section).

the bottom (T_b, cells) was determined by counting 10 random fields under the inverted microscope and extrapolating to the total surface of the well bottom. The total cell concentration (cells ml^{-1}) in each category was then obtained as ($N_s + T_b/2$), since each well contained 2 ml. Each cell couple, cell tetrad and colony was counted as one element in its category. Percentages of the different categories were then calculated. Single cells represent the sum of elongated and spherical cells.

Image analysis

Morphometric measurements were performed directly in the wells under the inverted microscope (objective ×20; ocular ×2.5; epifluorescence block ×1.25) using an image-analysis system consisting of a black and white CCD camera (Cohu 4710, San Diego, CA), a video monitor (Trinitron KX14CP1, Sony, Japan), a personal computer, a digitizing board MATROX MVP (Dorval, Québec) and a software package Biocom 200 (Biocom, Les Ulis, France). Measurements were performed on ~100 cells from random bottom fields. The shape index (f) was computed as $f = 4.\pi.S/P^2$, where S is the cell surface and P the cell perimeter ($f = 1$ for a circle and $f = 0$ for a straight line). Morphometric measurements were not performed on single cells in the supernatant.

Flow cytometry

One milliliter of the supernatant was sampled gently using a pipette. The remaining volume was homogenized by stirring in order to resuspend the cells that had settled on the bottom of the well. Both subsamples were analyzed live for forward angle light scatter (FALS), a function of cell size, and chlorophyll fluorescence (CHL). For DNA analyses, which require a large number of cells, the whole volume of a well was homogenized by stirring, fixed with paraformaldehyde at a final concentration of 0.5%, frozen in liquid nitrogen and stored at −80°C. Prior to analysis, samples were thawed at room temperature, stained with 25 μg ml^{-1} of Chromomycin A3 (Sigma, St Louis, MO) for 90 min in the dark on ice (slightly modified from Boucher *et al.*, 1991). All analyses (FALS, CHL, DNA) were performed with an EPICS 541 flow cytometer (Coulter Electronics, Hialeah, FL) with excitation set at 457 nm and 100 mW. DNA-induced fluorescence was collected at 485 ± 22 nm and natural chlorophyll fluorescence was collected above 690 nm. FALS and CHL data are expressed in arbitrary units relative to 2.07 μm fluorescent beads (Pandex, Mundelein, IL). DNA distributions (see Figure 5 below) exhibited two peaks corresponding, respectively, to cells in the initial (G_1) and final (G_2 + M, where M is mitosis) phases of the cell cycle. These peaks were separated by a region containing cells in the process of replicating DNA (S phase). Since we did not measure a large number of cells (typically between 100 and 300), a full analysis of the cell cycle was not possible and we pooled together the S, G_2 and M phases.

Effects of mixing

A specific experiment was designed to test the effect of mixing on the development of the colonial form. Cell preparation and inoculation were as outlined in Figure 1. Each day following inoculation, a different well was mixed by stirring and then maintained untouched until the end. Two wells were mixed every day from day 1 to day 7. Eight others were maintained untouched during the entire experiment. Eight days after inoculation, the total number of colonies in each well was determined, summing colonies in the supernatant and on the

bottom (see above). The number of colonies produced was normalized to the initial concentration of inoculated cells. Colonial volume and cell number were also determined. Measurements were performed directly in the wells on 20 buoyant or settled colonies, using the image-analysis system.

Influence of phosphate concentrations

Released colonial cells, obtained as described in Figure 1, were inoculated at a final concentration of $\sim 3 \times 10^3$ cells ml^{-1} into 50 ml flasks (Nunclon, Roskilde, Denmark) containing 20 ml of experimental media with increasing concentrations of PO_4^{2-}; 0.35, 1.2, 2.5 or 5.4 μM. Daily observations were performed to detect the appearance of colonies.

Results

The process of colony formation

When colonies were broken and colonial cells resuspended in fresh medium with a PO_4^{2-} concentration around 1 μM (Figure 1), a reproducible series of events was observed. Cells, that were non-flagellated just after release from colonies, settled onto the bottom of the wells, became flagellated and then rapidly lost their flagella, reinitiated their cell cycle, elongated, divided and finally new colonies were formed within 3 days (Figures 2, 3, 4 and 5).

The released colonial cells had a mean diameter of 6.5 μm, 90% of them were spherical and 95% non-flagellated (Figures 3 and 4). Six hours after the inoculation, most of the cells had settled onto the bottom of the wells and began to develop flagella (Figures 2 and 3). Most of the cells were arrested in the G_1 phase at the beginning of the cell cycle, as reflected by the very low percentage of cells in the S, G_2 and M phases (Figures 4 and 5). Twenty-four hours after the beginning of the experiment, the number of cells on the bottom and in the supernatant had decreased (Figure 2). By that time, a large fraction of cells had become flagellated and motile (Figure 3).

The majority of cells remained motile during almost 24 h and then lost motility quasi-synchronously. As a consequence, no flagellated cells could be observed on the second and third day of the experiment (Figure 3). After losing motility, cells settled onto the bottom of the wells, became elongated (as shown by the decrease in shape index) and began to cycle again into the S and G_2 + M phases (Figure 4).

Cell division resulted in the appearance of cell couples, followed by cell tetrads a day later (Figure 3). Alcian blue staining revealed the presence of an extracellular envelope around elongated cells, cell couples and cell tetrads (Figure 6). When the number of cells inside a matrix increased beyond four (around day 3), the matrix boundary became visible under phase-contrast microscopy without coloration, giving what is called here a colony. Within colonies, cell division occurred about once a day. At first, colonies were found on the bottom of the wells (Figure 2): when moving the plates gently, it was

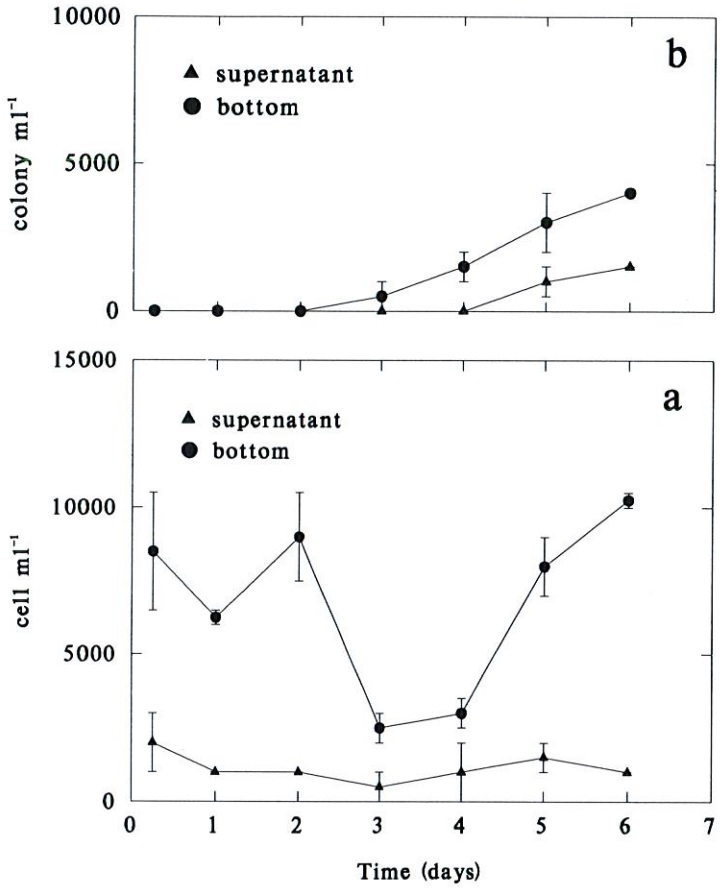

Fig. 2. Time course of the mean ±SD concentration of (**a**) single cells and (**b**) colonies in the supernatant and on the bottom of the wells during the experiment depicted in Figure 1.

observed that stages from elongated cells to small colonies had a tendency to stick to the bottom. On day 4, the largest colonies began to float (Figure 2).

Four days after inoculation, a fraction of single cells had failed to give colonies. These cells became flagellated again and divided within the formation of new couples and tetrads (Figures 2 and 3). The fraction of cells in the S + G_2 + M phases stabilized at ~15% on day 6 (Figure 4). At the end of the experiment, cells had regained a spherical shape, but were smaller than the cells inoculated initially (Figure 4). Cell size and shape were not determined for supernatant cells, but their forward light scatter (a function of cell size) and chlorophyll fluorescence appeared markedly different from those of bottom cells (Figure 4). The location of the G_1 peak did not change during the whole course of the experiment (Figure 5), suggesting that the ploidy level of the cell population was not modified.

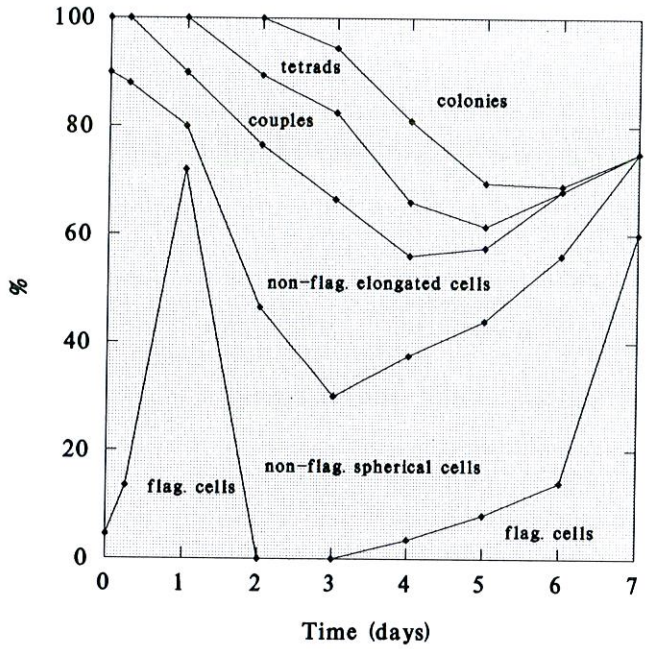

Fig. 3. Time course of cumulative percentages of six cell categories during the experiment depicted in Figure 1. Each cell couple, cell tetrad and colony was considered as one element in its category.

Influence of mixing on colony formation

The observation that colonies initially stick to the bottom induced us to test how mixing could affect colony formation (Table I). In cultures that were never mixed or mixed only once during the whole course of an experiment, ~40% of the inoculated cells gave colonies after 7 days. Cultures that were mixed every day during 7 days exhibited a drastic reduction in colony yield. Colonies originating from cultures mixed at least once were smaller and contained less cells than those from undisturbed cultures (Table I), with the sharpest reduction observed for cultures mixed every day.

Influence of PO_4^{2-} concentration on colony formation

When inoculated into media containing varying PO_4^{2-} concentrations (Table II), cells developed flagella as depicted above. However, the loss of motility and colony appearance were delayed with increasing PO_4^{2-} concentrations above 1 μM. At the lowest PO_4^{2-} concentration, only flagellated cells were observed and no colony appeared.

Discussion

These experiments confirm the existence of a well-ordered series of synchronous events leading from the release of colonial cells to the formation of new colonies,

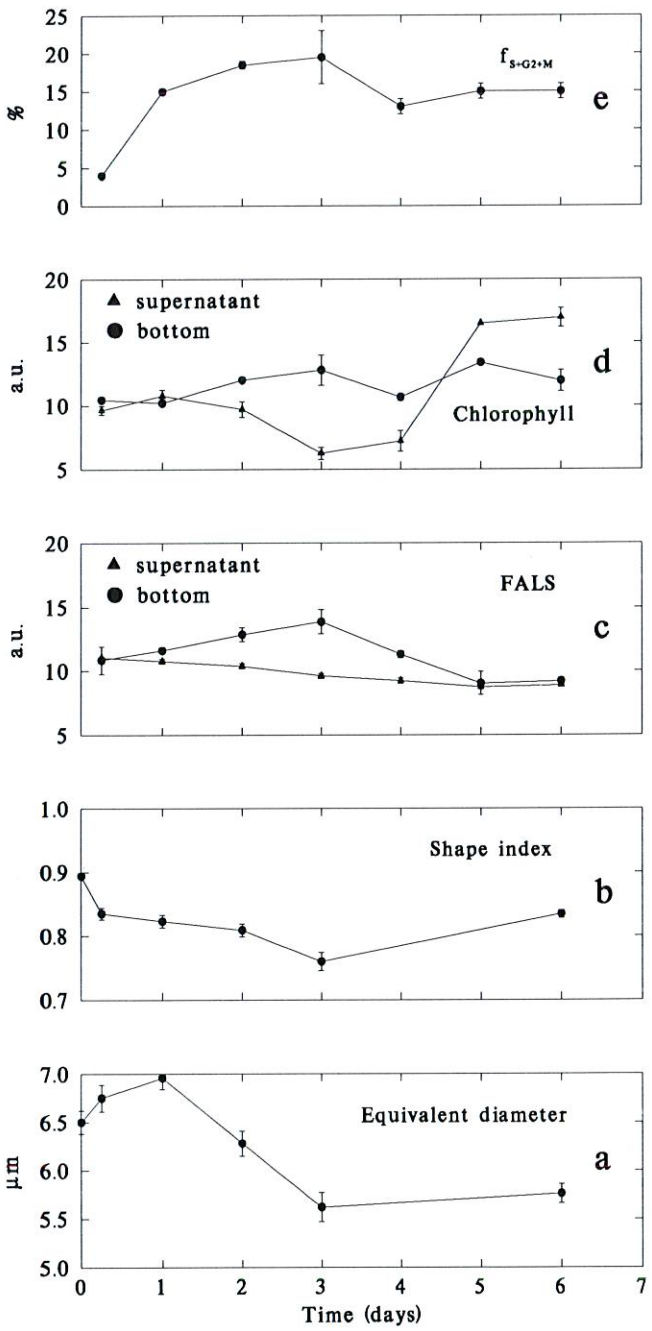

Fig. 4. Time course of the mean ±SD of cellular parameters for single cells in the experiment depicted in Figure 1. Cell equivalent diameter (**a**) and shape index (**b**) were determined by image analysis. Cell forward angle light scatter (**c**), cell chlorophyll fluorescence (**d**) and percentage of cells in the S + G_2 + M phase of the cell cycle (**e**) were determined by flow cytometry. Forward scatter and chlorophyll fluorescence are given in arbitrary units (a.u.) relative to 2.07 µm beads.

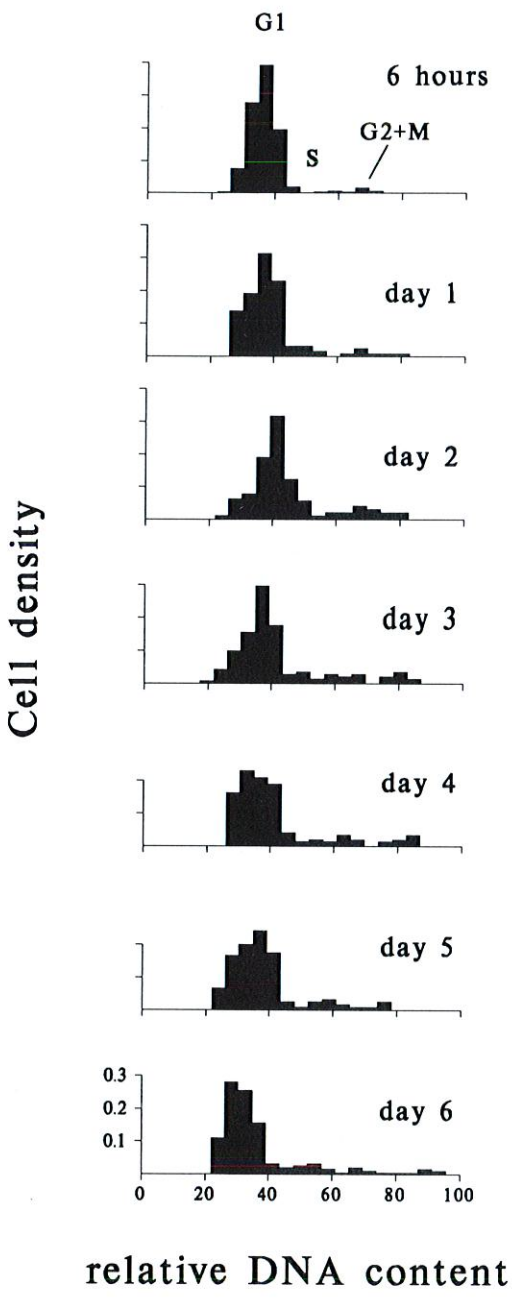

Fig. 5. Evolution of DNA distributions measured by flow cytometry during the course of the experiment depicted in Figure 1. The *x*-axis represents cell DNA content (in arbitrary units) and the *y*-axis, the fraction of cells with a given DNA content.

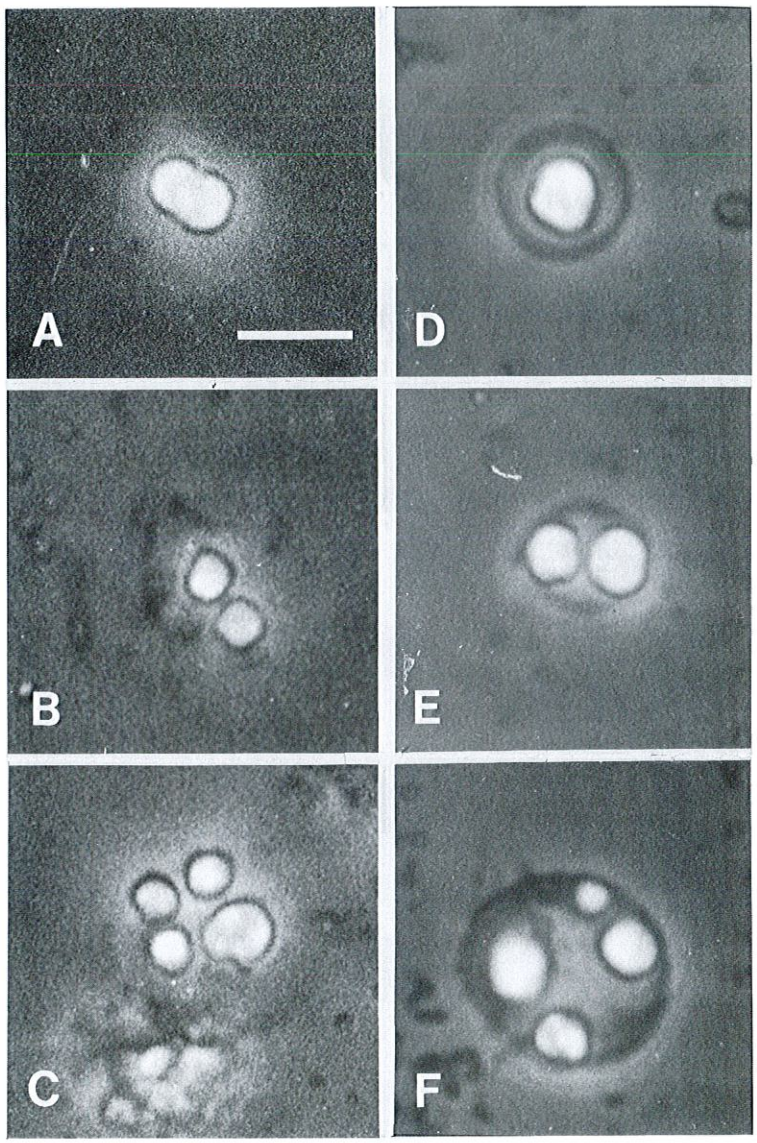

Fig. 6. First stages of *Phaeocystis* sp. colony formation. (**a**, **d**) Single cells (**b**, **e**) Cell couples. (**c**, **f**) Cell tetrads. (a, b, c) are unstained; (d, e, f) are Alcian blue stained. Scale bar = 10 μm.

after passage through a brief flagellated stage, as first observed by Kornmann (1955). Aggregation of free-living cells, another potential pathway for colony formation (Rousseau *et al.*, 1994), was not observed in our experiments.

Just after release from their matrix, cells are non-flagellated, in agreement with Chang (1984), who showed by electron microscopy that cells are devoid of any flagellar system when trapped within the colonial envelope. These liberated

Table I. Influence of mixing on colony formation. Cultures were prepared as depicted in Figure 1. The experiment lasted for 7 days. Wells were either left undisturbed, mixed once or mixed every day. Colony yield (number of colonies/number of inoculated cells), colony volume and number of cells per colony were determined at the end of the 7 day period. Mean ±SD (minimum–maximum)

Mixing	Colony yield (%)	Colonial volume (10^3 μm^3)	Colonial cell number	Replicates
None	39 ± 3	213 (92–310)	13.8 (2–50)	8
Once	38 ± 4	117 (51–179)	8.7 (1–48)	7
Every day	9 ± 0.4	14 (13–15)	9.0 (9–9)	2

Table II. Influence of phosphates on colony formation. Colonial cells were inoculated at ~3 × 10^3 ml^{-1} into fresh medium with varying phosphate concentrations

Initial $P\text{-}PO_4^{2-}$ (μM)	Day of colony appearance
0.35	Never
1.24	3
2.46	6
5.37	10

cells are able to develop flagella rapidly (Figure 3; Kornmann, 1955), but only for a short duration of the order of 24 h. The non-flagellated stage that follows settles onto the bottom and is the precursor of colonies. Since frequent mixing of cultures drastically reduces the proportion of produced colonies (Table I), cells may require a solid substrate during colony formation. Kayser (1970) had already observed the tendency of cells to adhere to culture vessels and put forward the hypothesis of a 'benthic' stage during the *Phaeocystis* life cycle. Although no benthic stage sensu stricto has been observed *in situ*, most field observations have revealed that young spherical colonies are in general attached to a substrate, such as *Chaetoceros* cells (Boalch, 1984; Rousseau *et al.*, 1994).

Settled cells, which are initially spherical and in the G_1 phase of the cell cycle, subsequently elongate, resume cycling and divide (Figure 4). Resumption of cell cycling is probably linked to the addition of fresh medium (Vaulot *et al.*, 1987). While dividing, some cells produce a mucilage, constituting the precursors of colonies. Chang (1984) established that cells excrete vesicles of chrysolaminarin (a polysaccharide formerly named leucosine) that spill around them, resulting in a multilayered structure. We were not able to determine on the basis of the measured parameters, i.e. size, chlorophyll content or DNA level, whether single cells that excrete mucilaginous compounds are different from those that do not. After the division of the initial cell, its non-motile daughters remain trapped inside the matrix which keeps increasing as cells continue to excrete. This leads to colonies, where division takes place about once a day (Kornmann, 1955). However, the generation time and synchronization of division inside colonies are not clearly established (Rousseau *et al.*, 1994). Initially, the matrix is visible only with Alcian blue staining (Figure 6). This envelope probably

becomes visible without staining when the amount of secreted polysaccharide is large enough, in our case when colonies contain more than four cells. Indeed, mucilage volume and colonial cell number are positively correlated (Rousseau *et al.*, 1990; Weisse and Scherffel-Möser, 1990). Alternatively, the visibility of the matrix may depend on its structure. Van Boekel (1992) suggested that the appearance of the membrane encircling the colony is linked to the optical properties of the colonial mucilage.

The observation that high PO_4^{2-} concentrations delayed colony appearance (Table II) is consistent with previous reports that nutrient depletion [either phosphate (Veldhuis *et al.*, 1986; Veldhuis and Admiraal, 1987) or nitrate (Lancelot and Mathot, 1985; Riegman *et al.*, 1992)] is a trigger for colony formation. More surprisingly, very low PO_4^{2-} concentrations (~0.3 μM) prevented totally colony appearance. Indeed, Riegman *et al.* (1992) observed colonies for phosphate concentrations of 1.2 μM, but none at or below 0.23 μM, when the medium contained nitrates as the single N-source (similar to what was used here). Colony formation may, therefore, occur only within a certain range of nutrient concentrations.

Other groups of microalgae, such as diatoms, are able to produce crysolaminarans, especially in culture when nitrogen or phosphorus is in limited supply, but do not display the formation of colonial structures (Myklestad, 1989). The gelling of the polysaccharide around the cells is an essential step in the colonial process. In the case of *Phaeocystis*, the precise structure of the excreted polysaccharide has not been established yet (van Boekel, 1992), but is likely a key to understand its gelling capacity. Moreover, gel formation is under the influence of specific ions (Rochas and Rinaudo, 1984; Hermansson, 1989). Indeed, van Boekel (1992) recently established that gelling of *Phaeocystis* mucilage depends on bivalent cations, e.g. below 2.5 mM Ca^{2+}, mucilage is formed but colony yield is drastically reduced.

During the time course experiment, cell ploidy did not change (Figure 5), but the data did not allow us to assert what was the ploidy level (haploid versus diploid). However, in a separate study (Vaulot *et al.*, 1994), we clearly established that colonial cells are always diploid. Therefore, since the experiment described here was started with colonial cells, it is highly probable that cells always remained diploid. If this is the case, both the short-lived flagellates that appeared just after colony disruption and the flagellates observed towards the end were diploid. These cells were similar in size and to *Phaeocystis* vegetative single cells (Weisse and Scherffel-Möser, 1990), in contrast to the smaller microzoospores that arise in old cultures and are haploid (Vaulot *et al.*, 1994). This strongly suggests that the process of colony regeneration described in this paper is not a sexual one.

This study raises many questions. The key steps of *Phaeocystis* colony formation, i.e. brief flagellum development, loss of motility, polymer excretion and gelling, should be examined in much finer detail and with a better temporal resolution. In particular, the factors (nutrient concentration, cation presence, mixing, etc.) triggering each transition should be determined. Moreover, other pathways of colony formation must exist, especially when no precursor cells are

present, as is the case for pure haploid microzoospore cultures devoid of colonies. In such cases, sexual conjugation must precede colony formation: this phenomenon has yet to be observed. Finally, both processes and controlling factors of colony formation may be strain dependent, as are many other cell properties (Vaulot *et al.*, 1994).

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