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CELL SIZE DIFFERENTIATION IN THE BLOOM-FORMING DINOFLAGELLATE *GYMNODINIUM* CF. *NAGASAKIENSE*[1]

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ABSTRACT

Two subpopulations differing essentially by their mean cell size were observed regularly in cultures and natural samples of the naked dinoflagellate Gymnodinium *cf.* nagasakiense *Takayama et Adachi (currently known as* Gyrodinium aureolum *Hulburt), a species which frequently forms red tides in North European seas. "Large" cells represented the typical form; they were morphologically similar to cells of the closely related Japanese species* G. nagasakiense, *which did not form any subpopulation of reduced size. "Small" and "large" cells of* G. *cf.* nagasakiense *had the same DNA content, but the nucleus of the former appeared to be much more condensed during interphase. Each cell type was able to divide and had its own growth dynamics; therefore, any intermediary between pure populations of "small" and of "large" cells were observed in culture. The "large" form generated a "small" cell by an atypical budding-like division, whereas the "small" form gave back a "large" form, once it ceased to divide, by simple enlargement of its cell body. Factors inducing cell size differentiation are yet unclear. Neither nitrogen nor phosphorus starvation induced a significant increase in the relative proportion of "small" and budding cells. Although cell size differentiation is associated with the formation of gametes in a variety of dinoflagellates, we demonstrated that "small" cells of* G. *cf.* nagasakiense *are able to divide asexually, in contrast to gametes of most other species. The high proliferative power of "small" cells as compared with normal cells suggests that they could play a significant role during red tides of* G. *cf.* nagasakiense; *in contrast, cells of the Japanese species* G. nagasakiense *could sustain high growth rates with larger cell size because this species generally blooms in waters much warmer than those found in northern Europe.*

Key index words: cell budding; cell size differentiation; dinoflagellate; flow cytometry; Gymnodinium *cf.* nagasakiense; Gyrodinium aureolum

In several species of cultured or wild dinoflagellates, cells with all the specific features but a reduced size (so-called "small" cells, Silva 1971) have sometimes been observed in addition to typical vegetative cells (Braarud 1957, Silva 1965, 1967, 1969, Partensky et al. 1988). The earliest observations of "small" cells were made on species of *Ceratium*; they were interpreted as either juvenile forms (Hensen 1887, quoted in von Stosch 1964) or resulting from seasonal variations (Lohmann 1908) or mutations (Kofoid 1909). Von Stosch (1964) observed the fusion of a "small" and a typical cell in *Ceratium tripos* (Müller) Nitzsch and concluded that the "small" form was a male gamete (anisogamy). Later, von Stosch (1973) described the sexual life cycles of *Gymnodinium pseudopalustre* Schiller and *Woloszynskia apiculata* von Stosch and found that fusing gametes in these species were of similar size (isogamy) but, again, smaller than the typical vegetative cells. More recently, several other dinoflagellate species were found to possess one or both gametes with a reduced size (Pfiester 1984, Pfiester and Anderson 1987). The formation of gametes is generally triggered by unfavorable conditions, such as a shortening of the light period and a low temperature (von Stosch 1973) or a deprivation of nitrogen (Pfiester 1975, Turpin et al. 1978) and/or phosphorus (Anderson et al. 1984). However, Silva (1965, 1967, 1969) observed the occurrence of "small" cells in a number of species under seemingly optimal conditions. The rapid increase of the relative proportion of these forms in culture suggested that they could divide (Silva 1971). Nevertheless, Silva was never able to conclude whether the pairs of "small" cells she observed in culture were fusing or dividing cells, i.e. whether "small" cells were gametes or vegetative cells.

Hypotheses on the mode of formation of "small" cells also differ among authors. Von Stosch (1973) suggested that they should result from "depauperating" divisions, i.e. division giving rise to cells lower in mass and poorer in plastids than the vegetative ones. Other workers considered that "small" cells must be produced by budding of normal cells (Apstein 1911, Silva 1971). Cytologically, the budding would be the result of an amitosis, i.e. a fragmentation of the nucleus by simple constriction (Borgert 1910). According to Silva (1971), this particular division provides two daughter nuclei of different sizes, originating from an unequal distribution of the chromosomes. The largest nucleus stays in the mother cell, whereas the smallest one is isolated in a bud appearing or already formed on the cell surface. The bud is released after partial or complete recovery of the specific characters (flagellae, theca, etc.), giving a "small" cell (Silva 1971). A third hypothesis, evoked for the formation of "small" cells in *Goniodoma pseudogonyaulax, Gonyaulax tamarensis* and *Gyrodinium instriatum*, suggests that the "small" cell is formed inside the mother cell and released by rupture of the cell wall (Silva 1971).

In this paper, we describe cell size differentiation in cultures of the naked European dinoflagellate *Gymnodinium* cf. *nagasakiense* (currently known as *Gy-*

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rodinium aureolum Hulburt, Partensky et al. 1988) and examine the effects of nutrient conditions on its occurrence. In contrast, we report the absence of such differentiation within cultures of the Japanese species *Gymnodinium nagasakiense* Takayama et Adachi, which is morphologically similar to *G.* cf. *nagasakiense* (Partensky et al. 1988).

MATERIALS AND METHODS

Algal strains. Three unialgal strains of *Gymnodinium* cf. *nagasakiense*, PLY-497A (courtesy of Dr. J. C. Green, Plymouth Marine Laboratory, U.K.), Iroise and Tinduff (isolated, respectively, off and along the coast of Brittany, France) were used in this study. Three clones of the Japanese species *Gymnodinium nagasakiense* (Buzen-'82, Buzen-'85-2 and Katsuura) were kindly provided by Prof. M. Kodama (Kitasato University, Japan). A fourth clone of the same species (NIES-249) was purchased from the National Institute for Environmental Studies (Tsukuba, Japan).

Samples of wild *G.* cf. *nagasakiense* were obtained in August 1986 during a cruise off Ushant and in July 1987 in Vilaine Bay (Brittany). Strain Tinduff was isolated by cell sorting (see below) from a third wild sample obtained in July 1987 from aquaculture tanks (Tinduff shellfish farm, Brest Bay, Brittany) naturally contaminated by this species.

Culture conditions. Batch cultures were grown in 250 or 500 mL Erlenmeyer flasks at 20 ± 1° C in K medium (Keller et al. 1987) under a 12:12 h L:D cycle. Illumination was provided by Grolux and cool-white fluorescent tubes giving a mean photon flux density of 100 $\mu E \cdot m^{-2} \cdot s^{-1}$. These culture conditions are defined as "standard conditions" throughout the text. Attempts to maintain the strains under continuous light generally led to a significant reduction in growth rate, as previously noted by Dixon and Holligan (1989).

The effect of nutrients on population growth, cell size differentiation and induction of sexuality was examined in *G.* cf. *nagasakiense* (strain Tinduff) by inoculating exponentially growing samples in K medium lacking either PO_4^{3-}, NO_3^-, NH_4^+ or both NO_3^- and NH_4^+. All cultures were sampled daily at the beginning of the light period for cell number and volume measurements until the late stationary phase. Samples were transferred into a 2 mL sedimentation chamber and examined using an Olympus CK2 inverted microscope (Tokyo, Japan) to look for abnormal forms and sexual stages (i.e. zygotes and cysts).

Several stages of normal and unequal cell divisions were photographed using either interferential or phase contrast. Whenever possible, dividing forms of both types were pipetted from the sedimentation chamber and transferred onto a slide. After DNA staining with 0.1% of a 1 $mg \cdot mL^{-1}$ solution of the vital dye Hoechst 33342 (Sigma, Saint Louis, Missouri), cells were photographed under UV light, using an epifluorescence Olympus BH2 microscope.

Cell concentration, volume and size. Cell concentration and volume were measured at least three times for each sample with a Coulter counter model ZM (Coulter Electronics, Hialeah, Florida) equipped with a 100 μm aperture tube. Cell volume distributions were analyzed with a Coulter Channelyzer C1000 interfaced to an IBM-PC compatible microcomputer.

Optical measurements of cell size also were made on several cultures of both species using a microscope equipped with an ocular micrometer.

Flow cytometric analyses. All the analyses were performed with an EPICS 541 (Coulter Electronics) flow cytometer. Excitation was provided by the 488 nm laser line of an argon laser (Innova 90-6, Coherent, Palo Alto, California) delivering 100 mW, unless specified otherwise. Forward angle light scatter (FALS), 90° light scatter (90LS) and red fluorescence above 690 nm (proportional to chlorophyll content, Partensky 1989) were determined on live *G.* cf. *nagasakiense* populations.

For analyses of DNA versus protein, about 10^6 cells were centrifuged (2000 × *g*, 10 min) at 0° C, and cell pellets were fixed in cold methanol according to Olson et al. (1983) for at least 2 days. Fixed cells were centrifuged (2000 × *g*, 10 min), rinsed twice with phosphate buffered saline (PBS) and stained for DNA and proteins using a mixture of 20 $\mu g \cdot mL^{-1}$ propidium iodide (PI), 40 $\mu g \cdot mL^{-1}$ RNase (DNA staining) and 1 $\mu g \cdot mL^{-1}$ fluorescein isothiocyanate (FITC, protein staining) in PBS (final concentrations; all chemicals from Sigma), as described in Vaulot et al. (1986). Protein-FITC fluorescence was measured between 488–560 nm and DNA-PI fluorescence above 630 nm.

Cloning. A drop of dense culture containing both "small" and "large" specimens of *Gymnodinium* cf. *nagasakiense* (strain Tinduff) was put on a slide and examined with an inverted microscope under a laminar flow hood. Cell size was estimated visually and individual "small" or "large" cells were transferred by pipetting into a drop of their initial culture medium, which had been previously sterilized by filtration through a Flowpore D filter unit (Sartorius, West Germany). Those cells which were still motile were then transferred individually into a 96-well plate (Nunclon, Denmark) filled with the same medium. Cell number and size were determined during the week following isolation and after 18 days. Thereafter, cells were inoculated into 5 mL tubes filled with fresh K medium.

Cell sorting. Pure cultures of "small" and "large" cells of *Gymnodinium* cf. *nagasakiense* were sorted from bimodal, exponentially growing populations using the flow cytometer. Three different laser powers were tested: 250, 100 and 20 mW. "Large" cells survived after cell sorting at 250 mW, whereas "small" cells did not. In contrast, "small" cells survived after sorting either at 100 and 20 mW laser power, but the lag phase was shorter in the latter case. Cell populations were sorted directly into 16 mL sterile polystyrene tubes (Falcon, Oxnard, California) containing K medium. Two mL subsamples were transferred immediately after cell sorting into 24-well Nunclon plates; cell size and morphology were recorded during one week following isolation, using an inverted microscope and a time-lapse video cassette recorder Panasonic NV-8051. In parallel, flow cytometric measurements (cell number, FALS, 90LS and chlorophyll content) were made daily on 0.5 mL samples from the remaining 10 mL. Attempts to block the normal and budding-like divisions were made by adding in some tubes 0.1% of a 1 $mg \cdot mL^{-1}$ nocodazole (Janseen, Beerse, Belgium) stock solution in dimethylsulfoxide (Serva, New York).

Transmission electron microscopy. Pure cultures of "large" or "small" cells of *G.* cf. *nagasakiense* were concentrated by leaving them under an intense light source (200 $\mu E \cdot m^{-2} \cdot s^{-1}$) for 1 h. Under these conditions, cells accumulate naturally near the surface (phototaxis), where they may be easily pipetted. This concentrate (0.5 mL) was fixed for 20 min in 1.4% glutaraldehyde, 1% OsO_4, 0.2 M cacodylate and 0.7 M sucrose (Merck, Germany), buffered at pH 7.2. Cells were rinsed twice with 0.2 M cacodylate and 0.7 M saccharose (2 × 5 min) and post-fixed for 1 h with 1% OsO_4 and 0.25 M cacodylate. After being rinsed for 5 min with cacodylate, cells were dehydrated through graded ethanol and propylene oxide series, then embedded progressively in Spurr's medium. Sections were cut with an ultramicrotome Nova (LKB, Bromma, Sweden) and stained with a mixture of 2% aqueous uranyl acetate and lead acetate prior to TEM examination on a JEOL JEM-100C microscope.

RESULTS

"Large" vs. "small" cells. The range of cell size in the European species *Gymnodinium* cf. *nagasakiense* (16–37 μm long, 13–36 μm wide) is greater than in

the Japanese species *G. nagasakiense* (21–34 μm long, 16–29 μm wide, Partensky et al. 1988). Cell volume distributions also differed between these two species. *G. nagasakiense* populations were always distributed unimodally in volume whatever the growth phase and strain (Fig. 1A), even during division, which is restricted to the dark period (Partensky 1989). In contrast, both in natural samples and in cultures of *G.* cf. *nagasakiense*, we observed volume distributions with either a single peak formed by "large" (Fig. 1B, C) or "small" cells (Fig. 1E, F) or double peaks (Fig. 1D). In culture, the relative proportion of each cell type generally changed with the growth phase; however, populations of "large" or "small" cells, which were initially pure sometimes remained so during growth. "Large" cells of *G.* cf. *nagasakiense* were similar in volume and morphology to typical *G. nagasakiense* cells (Partensky et al. 1988). In the latter species, smaller specimens appeared to be newly divided cells. In contrast, "small" cells of *G.* cf. *nagasakiense* constituted an individualized subpopulation able to divide, as established below. The range of cell size of "small" and "large" cells of *G.* cf. *nagasakiense* (body length = 16–26.5 and 25.5–37 μm, respectively) was such that the largest "small" cells were not different from the smallest "large" cells. This was confirmed by the partial overlap of the two peaks in bimodal volume distributions (Fig. 1D). Mean cell volume varied during exponential growth between 4500–7000 μm^3 for the "large" cells and 1500–2500 μm^3 for the "small" cells (Partensky 1989). The mean volume of both forms generally increased during the stationary phase.

When a live population of *G.* cf. *nagasakiense*, which was bimodally distributed in volume (Fig. 2A), was analyzed by flow cytometry, both FALS and 90LS histograms appeared unimodal (Fig. 2C, D), whereas red fluorescence histograms were bimodal (Fig. 2B). FALS and 90LS are a function of cell size and refractive index (Ackleson and Spinrad 1988), but also of orientation in the case of *G.* cf. *nagasakiense*, owing to its flattened shape. "Large" cells illuminated by the laser from the side and "small" cells illuminated from the front can produce scattering signals similar in intensity. In contrast, red fluorescence appeared to be well correlated to volume, and using cell sorting we checked that cells having low and high fluorescences corresponded to "small" and "large" cells, respectively, as measured by the Coulter counter (Partensky 1989). In methanol fixed samples, the two cell types could be identified on the basis of their different protein content (Fig. 3).

Flow cytometric DNA analyses of exponentially growing mixed populations revealed that both "small" and "large" subpopulations may have cells within all the stages of the cell cycle (G_1, S and G_2 + M; Fig. 3). The relative percentage of cells within the different stages varied for each cell type during the L:D cycle and also between the two forms at a given time (Fig. 3), demonstrating that the two forms cycled independently. Both forms were presumably haploid, since all the dinoflagellates studied to date but one (*Noctiluca*, a questionable case) are known to be so (Pfiester 1984). Therefore, the hypothetical sexual nature of "small" cells could not be checked on the basis of their DNA content, which is similar for vegetative cells and gametes in haplontic species, in contrast to diplontic species. However, the occurrence of "small" cells in the S phase, i.e. undergoing DNA synthesis, shows that they were in a vegetative state, since sexual stages may only be in G_1 (single gametes) or in an apparent G_2 stage (fusing gametes or zygotes). Furthermore, the absence of DNA replicating cells beside G_2 as well as of tetraploid cells suggests that meiosis did not occur as would be expected if "small" cells were gametes. The DNA content of "small" and "large" forms in G_1 appeared to be identical (around 44 $pg \cdot cell^{-1}$, Partensky et al. 1988); however, "small" cells, which seemed to contain a little more DNA than "large" ones were sometimes observed (Fig. 3A). This increase could be due to a variation in the staining ability of the nuclei related to their degree of condensation, rather than reflect an actual difference in DNA amounts. This hypothesis is supported by

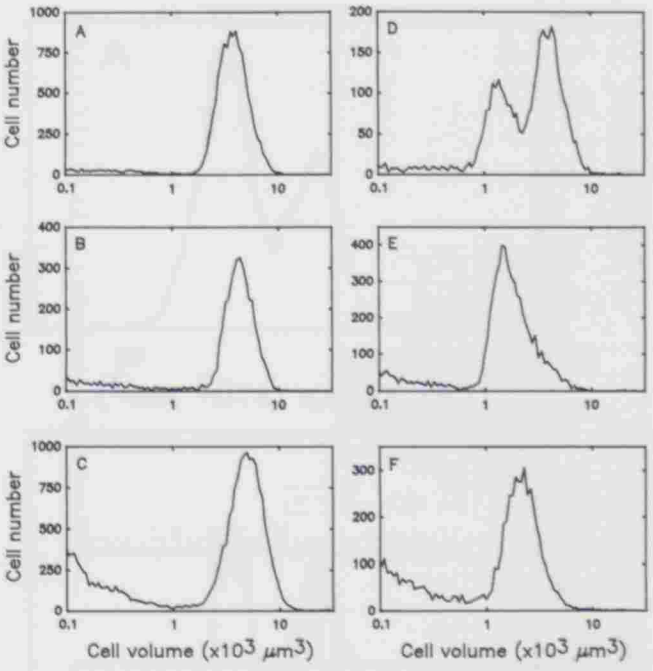

FIG. 1A–F. Cell volume distributions of the Japanese species *Gymnodinium nagasakiense* (A) and the European species *G.* cf. *nagasakiense* (B–E). A) Typical cultured population of *G. nagasakiense* (strain Katsuura). B) Cultured population of "large" cells of *G.* cf. *nagasakiense* (strain PLY-497A). C) Natural population of "large" cells of *G.* cf. *nagasakiense* (from Tinduff, Brittany). D) Cultured population of *G.* cf. *nagasakiense* comprising both cell types (strain PLY-497A). E) Cultured population of "small" cells of *G.* cf. *nagasakiense* (strain Iroise). F) Natural population of "small" cells (from Vilaine Bay).

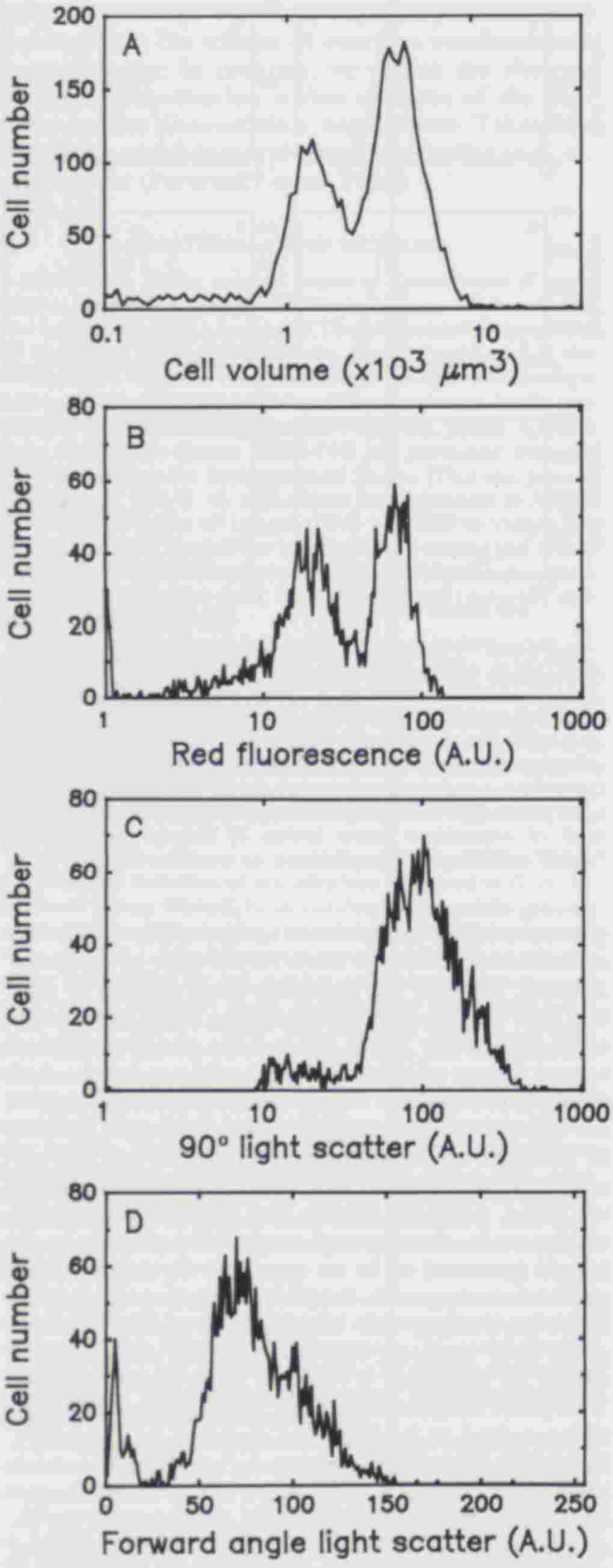

FIG. 2A–D. Cellular parameter distributions in a cultured population of *Gymnodinium* cf. *nagasakiense* comprising "small" and "large" cells (strain PLY-497A), measured by Coulter counting (A) and flow cytometry (B–D). A) Cell volume. B) Red fluorescence (proportional to chlorophyll). C) 90° light scatter. D) Forward angle light scatter. A.U. = arbitrary units.

the observation that, during interphase, the nucleus of "large" cells was generally elongated with the chromosomes in loose parallel bundles, whereas "small" forms had a small, condensed, round or slightly oval nucleus (Partensky et al. 1988). This resulted in a marked increase of the density of the nuclear material of "small" cells with respect to typical cells, as previously noted by other workers (von Stosch 1964, Silva 1971). This discrepancy was confirmed at the ultrastructural level. The chromosomes of "large" forms were dispersed in a loosely condensed nucleoplasm (Fig. 4A, C); in contrast, "small" cell chromosomes were packed together in a dense nucleoplasm (Fig. 4B, D). The internal structure of chromosomes also appeared to differ between "large" and "small' forms (Fig. 3C, D), but this could also result from differences in cell cycle positions (Puiseux-Dao 1981) or be an artifact of fixation. The other ultrastructural features, which have been recently documented by Kite and Dodge (1988), do not differ significantly between the two forms of *G.* cf. *nagasakiense* (Partensky 1989).

Cell sorting and cloning. Flow cytometric cell sorting was used to get pure cultures of "small" and "large" cells and elucidate the interrelationships between these two subpopulations. They were identified on flow cytometric histograms on the basis of their differences in red fluorescence (Fig. 2B). "Small" cells sorted at 100 mW of laser power displayed a 12-day long lag phase and divided quickly afterwards, remaining "small" during the whole exponential phase. At the same laser power, "large" sorted cells rapidly regenerated a bimodal population, and "small" cells became dominant at the end of the exponential phase. In further cell sorting experiments using lower laser power (20 mW), we found contradictory results. "Small" cells enlarged within 2–3 days, dividing normally after they became "large" whereas, with a few exceptions, sorted populations of "large" cells remained "large" during the whole growth phase. Despite this variability, each population ("small" or "large"), which remained pure following cell sorting, finally gave back mixed populations after being subcultured from one to three times in Erlenmeyer flasks. It is noteworthy that populations of "large" cells sorted at 250 mW, a laser power which selectively killed the "small" forms, did not lose their ability to differentiate.

"Small" cells may regenerate "large" forms by simple enlargement; this phenomenon occurred currently in culture as soon as a population of "small" cells reached the stationary phase. In contrast, the regeneration of "small" cells from "large" forms took a variable number of generations, ranging from one (immediately after cell sorting) to more than 50

(after 3 subcultures). This indicates that the formation of "small" cells was probably not continuous, except perhaps at an extremely low background level, but appeared sporadically during the life cycle of *G.* cf. *nagasakiense.*

A large variability was also found with manual cloning experiments. Each form could either give back the other one after 5–7 generations, divide keeping its initial size, or even give anomalously large forms, probably resulting from a karyokinesis not followed by cell division. Only one clone (started initially from a "large" cell) survived after being transferred and, again, gave back a mixed population.

Equal vs. unequal division. Both "small" and "large" cells of *G.* cf. *nagasakiense* were able to divide asexually, according to a classical pattern similar to that described in *Gyrodinium uncatenum* by Coats et al. (1984). During interphase, "large" cells generally possessed a single antero-posteriorly elongated or crescent-shaped nucleus, located in the left side of the cell (Fig. 5A, B). During division, the nucleus rounded up and seemed to migrate toward the center of the cell, then two identical sets of chromosomes which became the nuclei of the daughter cells moved progressively aside (Fig. 5D, F); at the same time, the mid part of the hypocone grew hollow until the two daughter cells were completely separated (Fig. 5C, E).

Besides typical forms of division, which gave rise to two cells of similar sizes, a few forms of unequal division were observed (Fig. 6). They were very rare in batch cultures but were found several times in sorted populations of "large" cells. Conclusions about the eventual triggering role of cell sorting on the formation of "small" cells could not be made, since this phenomenon was not systematically observed. When present, unequal forms of division were visible 12–48 h after cell sorting and represented 10–50% of the dividing cells but were scarce or absent thereafter. "Small" cells appeared to result from a budding of the left epicone (Fig. 6A). Very early in its development, the bud was encircled with a transverse flagellum (Fig. 6A, B), beating in phase with that of the mother cell and probably continuous with it in the earliest stages. The longitudinal flagellum was rarely visible as long as the bud was still linked to the mother cell, but it was clearly observed when the bud was released. The newly formed "small" cell was subspherical (Fig. 6C, D), always smaller than the mother cell, and could swim immediately after separation. Bud release was recorded using a video-recorder and therefore was clearly not a misinterpretation of a fusing process between a "small" and a "large" cell. Further stages of the evolution of "small" cells were not observed in detail, but these cells are thought to grow rapidly and divide normally to give back the typical "small" cell described earlier (Partensky et al. 1988).

At the nuclear level, budding appeared to begin with a constriction of the nucleus, separating two subequal sets of chromosomes; this phenomenon was visible in the cell shown in Figure 6A but was not photographed. Thereafter, one daughter nucleus moved into the newly formed bud (Fig. 6D, E). The

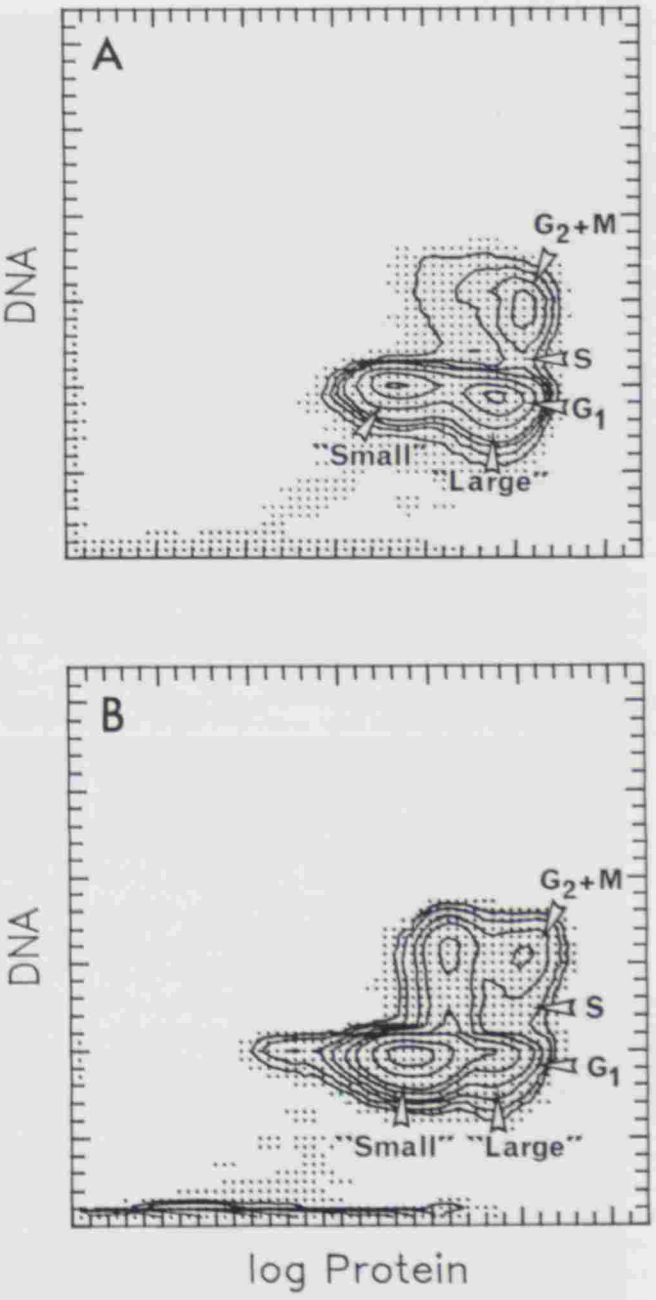

FIG. 3A, B. Distribution of DNA and protein contents (expressed in arbitrary units) in two mixed populations of *Gymnodinium* cf. *nagasakiense,* as measured by flow cytometry. "Large" and "small" cells are distinguished on the basis of their different protein contents. A) "Large" cells are dividing, as shown by the numerous "large" cells within the S and G_2 + M stages of the cell cycle, whereas "small" cells are in stationary phase (few cells in S and G_2 + M). B) Both cell types are dividing; a large number of "small" cells are undergoing DNA synthesis. Note the presence of a population with a very low protein content, which is only present when "small" cells are dividing actively (B).

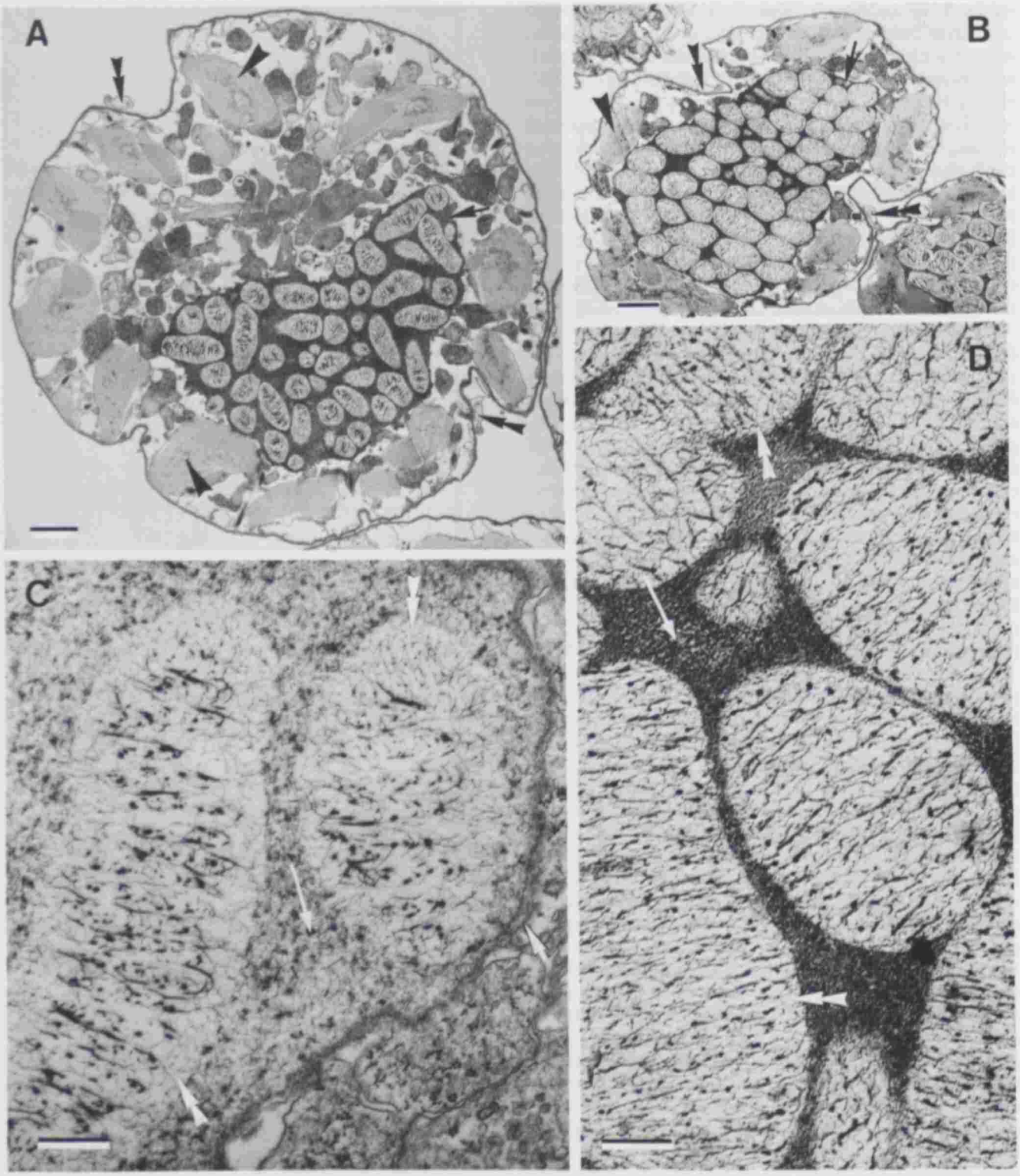

FIG. 4.A–D. Ultrastructure of "large" (A, C) and "small" (B, D) cells of *Gymnodinium* cf. *nagasakiense* during interphase. A, B) General views. Scale bars = 2 μm. C, D) Details of nuclei. Scale bars = 0.5 μm. Note the highly vacuolized cytoplasm in both cell types, the chloroplasts set up at the cell periphery (arrowheads), the ribbon-like transverse flagellum (double black arrowheads) and the large nucleus (black arrow). The latter is surrounded with a nuclear membrane (short white arrow) and contains permanently condensed chromosomes (double white arrowheads) dispersed in the nucleoplasm (long white arrows), which is extremely dense in "small" cells.

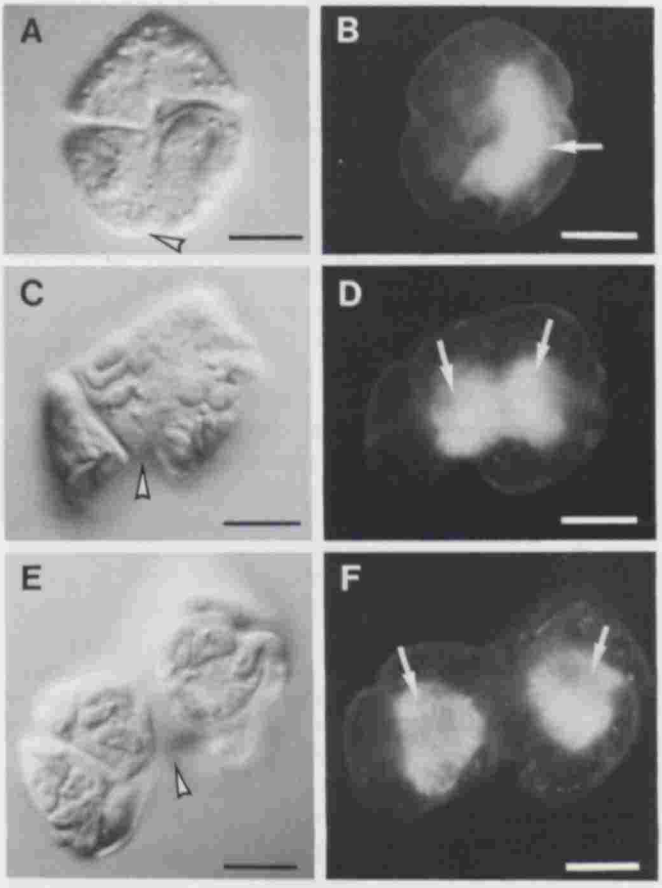

FIG. 5A–F. Normal division in "large" forms of *Gymnodinium* cf. *nagasakiense*. (A, B), (C, D), and (E, F) represent pairs of photographs of identical cells. A, C, E) Interferential contrast. B, D, F) Visualization of nuclei under UV fluorescence, after DNA staining with Hoechst 33342. A, B) Interphasic cells have an elongated nucleus with clearly visible chromosomes (white arrow). C–F) During division, the longitudinal furrow (arrowheads) grows hollow, while the chromosomes of each daughter nuclei (white arrows) move aside. All scale bars = 10 μm.

position of the daughter nuclei after karyokinesis suggests that the division plane was oriented at a right angle from the antero-posterior cell axis and not along this axis as in the normal mode. It is noteworthy that "small" cells could also divide unequally, but the bud was then situated in the axis of the mother cell and not laterally (not shown).

Effect of nutrients on cell size differentiation. Removing essential nutrients (N, P) from the standard medium (K medium) did not seem to have any drastic effect on the formation of "small" cells (Fig. 7). The division of both forms was almost completely inhibited in the absence of phosphorus (Fig. 7A, Table 1). This was accompanied by a sharp increase in volume (Fig. 7B), especially in the "small" cells, which became rapidly indistinguishable from the "large" ones on cell volume histograms. In contrast, the N-depleted medium supported limited growth (Fig. 7A, C, E). Final cell densities were not significantly different between the control medium, the medium

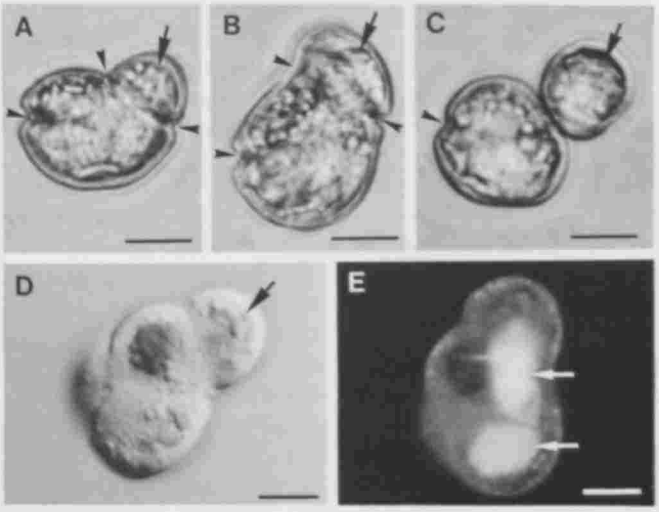

FIG. 6A–E. Budding-like division in *Gymnodinium* cf. *nagasakiense*. A–C), taken in phase contrast, represent three stages of the budding process. A) Formation of a superficial bud (black arrow) on the left hypocone of the "mother" cell. Note the presence of cingular furrows (black arrowheads) encircling both the "mother" cell and the bud. B) Growth of the bud. C) Isolation of the bud. D, E) represent the same cell, which has been blocked in an early stage of atypical division with nocodazole and stained for DNA with Hoechst 33342, as viewed with interferential contrast (D) and UV fluorescence (E). Note that the nucleus has recently divided by constriction (white arrows) and the upper daughter nucleus is migrating inside a newly formed bud on the cell surface of the "mother" cell. Scale bars = 10 μm.

with NH_4^+ omitted, and the medium with an initial concentration of PO_4^{2-} three times higher than in K (Fig. 7A). The lower generation time observed in the latter medium (Table 1) may be due to an initial lag phase of both cell types. Population growth of *G.* cf. *nagasakiense* was slowed when NH_4^+ was the sole source of N (Fig. 7A and Table 1), as a result of a significantly decreased growth rate of the "small" cells (Fig. 7C and Table 1). This apparently means that "small" cells preferentially use NO_3^- as a nitrogen source, whereas "large" cells can use either NO_3^- or NH_4^+. However, the latter cell type displayed a longer lag phase in all media supplemented with NH_4^+ than in the one where NH_4^+ had been omitted. In this experiment, average volumes of both

TABLE 1. *Generation times of the total population and of the "small" and "large" subpopulations of* Gymnodinium *cf.* nagasakiense *cultures in standard K medium or with one or several enrichment solutions omitted or more concentrated (same cultures as in Fig. 7). Values represent average generation times calculated on a 4-day period at the beginning of the exponential phase (days 1–5). Generation times of "small" and "large" subpopulations have been omitted in K−PO_4^{2-} medium, in which the two cell types were not distinguishable.*

	Average generation time (days)					
	K Control	K−N	K−NO_3^-	K−NH_4^+	K−PO_4^{3-}	K + (2 × PO_4^{3-})
Total cells	1.75	1.73	2.18	1.61	8.46	1.98
"Small" cells	1.58	1.51	2.73	1.56	—	2.25
"Large" cells	2.17	2.04	1.70	1.76	—	1.72

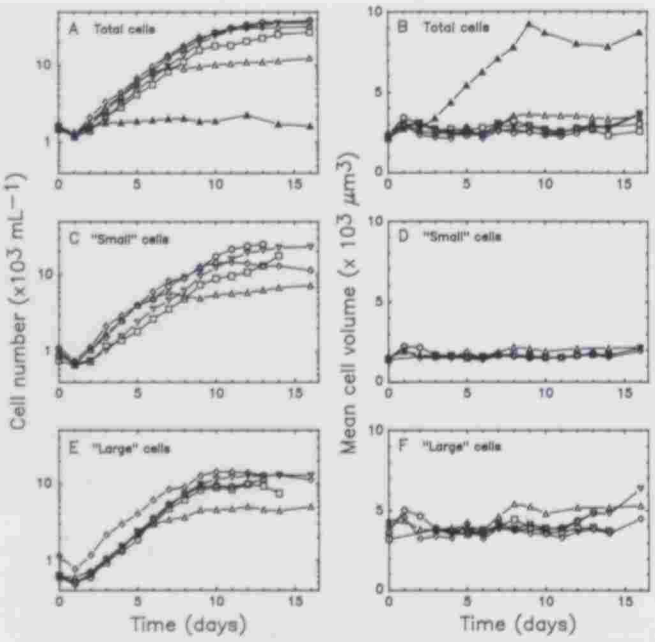

FIG. 7A–F. Change in cell number (A, C, E) and volume (B, D, F) after inoculation at t = 0 of a bimodal population of *Gymnodinium* cf. *nagasakiense* (strain Tinduff) in complete and nutrient-depleted K medium. A, B) Total population; C, D) "Small" cells; E, F) "Large" cells. (○) K medium (control); (▲) K − PO_4^{2-}; (□) K − NO_3^-; (◇) K − NH_4^+; (△) K − (NO_3^-, NH_4^+); (▽) K + (2 × PO_4^{2-}). Note that the curves representing "small" and "large" cells in K − PO_4^{2-} have been omitted, because these two cell types were not distinguishable in this medium.

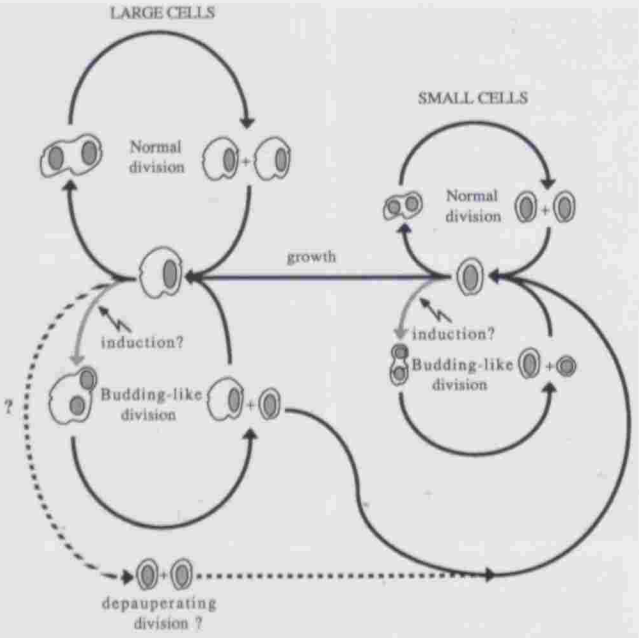

FIG. 8. Typical and atypical division modes in "large" and "small" cells of *Gymnodinium* cf. *nagasakiense*. The dashed arrow represents a possible but not observed division process, whereas gray arrows correspond to observed atypical division modes, which probably require induction.

"small" and "large" subpopulations displayed limited changes during growth (Fig. 7D, F). In general, cell volume increased during lag phase, decreased during exponential growth, and increased again during stationary phase (Partensky 1989).

Under standard conditions, "small" cells generally divided quicker than "large" ones (Table 1). In some cultures, "small" cells could reach generation times as short as 1 day (not shown). In contrast, generation times shorter than 1.7 were never observed for "large" cells (Partensky 1989).

N and/or P starvation did not seem to induce any kind of sexuality, since neither quadriflagellated zygotes nor cysts were observed during the course of this experiment. In contrast, we did observe some rare budding forms in nitrogen-depleted cultures.

DISCUSSION

Both cloning and cell sorting experiments clearly demonstrated that the two cell types ("small" and "large"), which may be observed in cultures of *Gymnodinium* cf. *nagasakiense*, are not two subspecies or different populations of the same species coexisting in the same culture but belong to a single genetically homogeneous population. The relationships among the different cellular forms of *G.* cf. *nagasakiense* are summarized in Figure 8. Subpopulations of "small" cells appear to result from a budding-like division of "large" cells, similar to that described by Silva (1971). However, the hypothesis of depauperating divisions suggested by von Stosch (1973) may not be eliminated as an alternative process for the production of "small" cells (Fig. 8), since it would be extremely difficult in practice to distinguish cells dividing by this particular process from normal stages of division of "large" or "small" cells. In contrast, the hypothesis of the "small" and "large" cells belonging to two cohorts of distinct age from a single homogeneous population can be eliminated. Indeed, double peaks in volume distributions are expected for a synchronized culture with a generation time longer than one day (Vaulot and Chisholm 1987, Homma and Hastings 1989), which was the case in our cultures (Partensky 1989). "Large" cells would then represent individuals born during a first division period (24 h < ages < 48 h) and "small" cells, individuals born during the second division period (ages < 24 h). However, if this hypothesis held, then when "small" cells are isolated, they should always enlarge and give back "large" cells before division (Homma and Hastings 1989), whereas we demonstrated that pure populations of "small" cells may divide and remain "small" during the whole exponential phase (Fig. 8). Also, the ratio of cell volume between "large" and "small" cells is larger than 2, whereas in the case of division synchrony it should be less than 2. Finally, the Japanese dinoflagellate *Gymnodinium nagasakiense* does not form bimodal distributions, even though this species is also syn-

chronized by L:D cycles and has a generation time of about 2 days (Partensky 1989).

In contrast to the hypotheses of Silva (1971), we showed that "small" and "large" cells have similar DNA contents. If the budding-like division is assumed to be the main, if not the sole, mode of formation of "small" cells, nuclear division occurring during this atypical process must provide two equal sets of chromosomes. Alternatively, if the distribution of DNA is slightly unequal between the two daughter cells, both the regeneration of new nuclear material in the "small" cell and the deletion of surnumeral DNA in the "large" cell must occur rapidly. Another crucial finding was the ability of "small" cells to duplicate DNA and divide, since there is a possible confusion between dividing and fusing forms (Silva 1971). Therefore, the nature of "small" cells appears to be vegetative, in contrast to gametes of most dinoflagellate species. In general, gametes rapidly lose their ability to divide once they have been induced sexually (Pfiester 1975, 1976, 1977). Nevertheless, in a few species of dinoflagellates, such as *Woloszynskia apiculata*, in which sexuality may be induced under unfavorable culture conditions, gametes (i.e. cells identified by their ability to fuse) were found to revert to a vegetative stage and divide, when put back under conditions favorable to normal growth (von Stosch 1973). This shows the potential duality of dinoflagellate gametes, a trait which could be linked to their low degree of differentiation as compared to vegetative cells. Thus, despite their ability to divide, "small" cells produced by *G.* cf. *nagasakiense* and by many other species (Braarud 1957, Silva 1965, 1967, 1969, 1971) could well be gametic or rather "pre-gametic" stages. In a variety of dinoflagellates, the induction of gamete formation appears to be controlled by environmental factors such as unfavorable nutrient conditions, which also trigger the other stages of sexuality, i.e. fusion of gametes and production of planozygotes and resting cysts. In contrast, we did not observe any drastic effect of nutrient depletion on the formation of "pre-gametes" in *G.* cf. *nagasakiense*, which occurs under seemingly favorable conditions; the latter could induce these cells to divide rather than fuse. The potential role of this uncoupling between gamete formation and fusion in this species could be to multiply the number of gametes before they are induced to couple. Conditions necessary to trigger the fusion have not been met in our cultures but may exist *in situ*. Alternatively, since no resting cyst has been identified yet in *G.* cf. *nagasakiense*, this species may reproduce only by vegetative division, as suggested by the discovery of *Gymnodinium* cells in low concentration during the winter months off the coasts of Brittany (Birrien 1987). Production of "small" vegetative cells could then be an archaic feature, representing an ancestral ability to reproduce sexually, although this mode of reproduction is not currently used by this species. This assumption cannot explain, however, why the Japanese species *G. nagasakiense* apparently cannot produce "small" cells, except if we assume that despite the remarkable morphological similarity of these two taxa, their genomes are not completely similar, as suggested by their significantly different DNA content (Partensky et al. 1988).

A limited number of observations on natural samples suggest that cell size differentiation also occurs *in situ* (Fig. 1). Previously, workers had noted that average cell dimensions decrease during the course of *G.* cf. *nagasakiense* blooms, due to the significant reduction of the number of large specimens toward the end of blooms (Ballantine and Smith 1973, Pybus 1980). Although these authors did not clearly mention the presence of two subpopulations, their observations suggest that during these red tides, "large" cells appear first and then generate "small" forms, which become dominant toward the end of blooms. "Small" cells of *G.* cf. *nagasakiense* are potentially proliferative forms, with a maximum growth rate as high as 1 day^{-1} (Partensky 1989). This finding apparently contradicts the general tendency in unicellular algae of a given genotype to be smaller at lower growth rates (Raven 1986), but it is consistent with the inverse relation existing between cell size and maximum growth rate noted in a range of microalgae belonging to different species (Raven 1986). The reduced size of the "small" subpopulation allows an optimization of photon capture and nutrient uptake (Raven 1986), such that they may take optimal advantage of the conditions generally prevailing during blooms (e.g. availability of nutrients, stability of the water column, etc.). In contrast, the "large" form appears to be more adapted for survival under non-bloom conditions, since it is less fragile and its abundant cytoplasm probably contains more food reserves. Whatever the true origin of cell size differentiation, either sexual or vegetative, this biological mechanism could account for a large part of the success of *G.* cf. *nagasakiense* in the field.

We cannot conclude definitively about the identity of "small" vegetative cells of *G.* cf. *nagasakiense* and gametes, since we did not observe sexuality in our cultures. However, a similar study made on a dinoflagellate species which forms "small" cells and reproduces sexually at the same time would allow this riddle to be solved. Bimodal distributions of cell volume, similar to our own observations on *G.* cf. *nagasakiense*, were documented during the growth of a clone of *Protogonyaulax tamarensis* var. *excavata* Braarud (= *Gonyaulax excavata* (Braarud) Balech) (Yentsch et al. 1985). Although this phenomenon was attributed to simple cell division, it is likely that it was in fact due to cell size differentiation. Previously, the same species had been shown to exhibit sexual reproduction (Anderson and Wall 1978). This

dinoflagellate could therefore be used to demonstrate the probable relationships between "small" cells and gametes.

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Ackleson, S. G. & Spinrad, R. W. 1988. Size and refractive index of individual marine particulates: a flow cytometric approach. *Appl. Opt.* 27:1270–7.

Anderson, D. M., Kulis, D. M. & Binder, B. J. 1984. Sexuality and cyst formation in the dinoflagellate *Gonyaulax tamarensis*: I. Cyst yield in batch cultures. *J. Phycol.* 20:418–25.

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

Apstein, C. 1911. Biologische Studie über *Ceratium tripos* var. *subsalsa* Ostf. *Wiss. Meeresunterers., Abt. Kiel* 12:135–62.

Ballantine, D. & Smith, F. M. 1973. Observations on blooms of the dinoflagellate *Gyrodinium aureolum* Hulburt in the river Conwy and its occurrence along the North Wales coast. *Br. Phycol. J.* 8:233–8.

Birrien, J. L. 1987. Cycles des variations des éléments nutritifs et du phytoplancton en baie de Douarnenez et dans les secteurs adjacents: importance du front côtier de l'Iroise. Thèse Doctorat Univ. Bretagne Occid., 160 pp.

Borgert, C. 1910. Kern und Zellteilung bei marinen *Ceratium* Arten. *Arch. Protistenk* 20:1–46.

Braarud, T. 1957. Observations on *Peridinium trochoideum* (Stein) Lemm. in culture. *Nytt Mag. Bot.* 6:39–41.

Coats, D. W., Tyler, M. A. & Anderson, D. M. 1984. Sexual processes in the life cycle of *Gyrodinium uncatenum* (Dinophyceae): a morphogenetic overview. *J. Phycol.* 20:351–61.

Dixon, G. K. & Holligan, P. M. 1989. Studies on the growth and nitrogen assimilation of the bloom-forming dinoflagellate *Gyrodinium aureolum* Hulburt. *J. Plankton Res.* 11:105–18.

Homma, K. & Hastings, J. W. 1989. Cell growth kinetics, division asymmetry and volume control at division in the marine dinoflagellate *Gonyaulax polyedra*: a model of circadian clock control of the cell cycle. *J. Cell Sci.* 92:303–18.

Keller, M. D., Selvin, R. C., Claus, W. & Guillard, R. R. L. 1987. Media for the culture of oceanic ultraphytoplankton. *J. Phycol.* 23: 633–8.

Kite, G. C. & Dodge, J. D. 1988. Cell and chloroplast ultrastructure in *Gyrodinium aureolum* and *Gymnodinium galatheanum*. Two marine dinoflagellates containing an unusual carotenoid. *Sarsia* 73:131–8.

Kofoid, C. A. 1909. Mutations in *Ceratium*. *Bull. Mus. Comp. Zool., Harvard College* 52:13.

Lohmann, H. 1908. Untersuchungen zur Feststellung des vollständigen Gehaltes des Meeres an Plankton. *Wiss. Meeresunters., Abt. Kiel* 10:129–370.

Olson, R. J., Frankel, S. L., Chisholm, S. W. & Shapiro, H. M. 1983. An inexpensive flow cytometer for the analysis of fluorescence signals in phytoplankton: chlorophyll and DNA distributions. *J. Exp. Mar. Biol. Ecol.* 68:129–44.

Partensky, F. 1989. Stratégies de croissance et toxicité de deux Dinoflagellés responsables d'"eaux colorées" nuisibles: *Gyrodinium* cf. *aureolum* et *Gymnodinium nagasakiense*. Thèse Doctorat Univ. Paris 6, 165 pp.

Partensky, F., Vaulot, D., Couté, A. & Sournia, A. 1988. Morphological and nuclear analysis of the bloom-forming dinoflagellates *Gyrodinium* cf. *aureolum* and *Gymnodinium nagasakiense*. *J. Phycol.* 24:408–15.

Pfiester, L. A. 1975. Sexual reproduction of *Peridinium cinctum* f. *ovoplanum* (Dinophyceae). *J. Phycol.* 11:259–65.

——— 1976. Sexual reproduction in *Peridinium willei* (Dinophyceae). *J. Phycol.* 12:234–8.

——— 1977. Sexual reproduction in *Peridinium gatunense* (Dinophyceae). *J. Phycol.* 13:92–5.

——— 1984. Sexual reproduction. *In* Spector, D. L. [Ed.] *Dinoflagellates*. Academic Press, New York, pp. 181–99.

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

Puiseux-Dao, S. 1981. Cell-cycle events in unicellular algae. *In* Platt, T. [Ed.] *Physiological Bases of Phytoplankton Ecology. Can. Bull. Aquat. Sci.* 210:130–49.

Pybus, C. 1980. Observations on a *Gyrodinium aureolum* (Dinophyta) bloom off the south coast of Ireland. *J. Mar. Biol. Assoc. U.K.* 60:661–74.

Raven, J. A. 1986. Plasticity in algae. *In* Jennings, D. H. & Trewavas, A. J. [Eds.] *Plasticity in Plants*. The Company of Biologists Ltd, Cambridge, pp. 347–72.

Silva, E. S. 1965. Note on some cytophysiological aspects in *Prorocentrum micans* Ehr. and *Goniodoma pseudogonyaulax* Biech. from cultures. *Notas Est. Inst. Biol. Madrid* 30:1–30 (26 pl.).

——— 1967. *Cochlodinium heterolobatum* n. sp.: structure and some cytophysiological aspects. *J. Protozool.* 14:745–54.

——— 1969. Cytological aspects on multiplication of *Goniodoma* sp. *Bot. Mar.* 12:233–43.

——— 1971. The "small" form in the life cycle of dinoflagellates and its cytological interpretation. *In* Farinacci, A. [Ed.] *Proceedings of the 2nd Plankton Conference, Roma 1970*. Tecnisienza, Roma, pp. 1157–67.

Turpin, D. H., Dobell, P. E. R. & Taylor, F. J. R. 1978. Sexuality and cyst formation in Pacific strains of the toxic dinoflagellate *Gonyaulax tamarensis*. *J. Phycol.* 14:235–8.

Vaulot, D. & Chisholm, S. W. 1987. A simple model of the growth of phytoplankton populations in light/dark cycles. *J. Plankton Res.* 9:345–66.

Vaulot, D., Olson, R. J. & Chisholm, S. W. 1986. Light and dark control of the cell cycle in two phytoplankton species. *Exp. Cell Res.* 167:38–52.

von Stosch, H. A. 1964. Zum Problem der sexuellen Fortpflanzung in der Peridineengattung *Ceratium*. *Helgol. Wiss. Meeresunters.* 10:140–51.

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

Yentsch, C. M., Cucci, T. L., Phinney, D. A., Selvin, R. & Glover, H. E. 1985. Adaptation to low photon flux densities in *Protogonyaulax tamarensis* var. *excavata*, with reference to chloroplast photomorphogenesis. *Mar. Biol. (Berl.)* 89:9–20.