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TIME-COURSE MEASUREMENTS OF PHOSPHORUS DEPLETION AND CYST FORMATION IN THE DINOFLAGELLATE *GONYAULAX TAMARENSIS* Lebour[1]

DONALD M. ANDERSON

Biology Department, Woods Hole Oceanographic Institution, Woods Hole, MA 02543, U.S.A.

and

NIELS L. LINDQUIST

Scripps Institution of Oceanography, University of California–San Diego, La Jolla, CA 92093, U.S.A.

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Abstract: Time-course measurements were made during the transition from asexual to sexual reproduction and cyst formation in phosphorus-limited batch cultures of the dinoflagellate *Gonyaulax tamarensis* Lebour. The initial phase of growth was predominantly asexual, during which time the intracellular and extracellular phosphorus pools decreased steadily in the light and the dark. Cellular P dropped to a subsistence cell quota of 27 pg · cell^{-1} as division ceased. This gradual decrease in cellular P in the presence of micro-molar PO_4^{3-} concentrations suggests that this species may encyst in natural waters in response to nutrient limitation at concentrations above analytical detection limits. As the cells approached plateau phase, asexual growth ceased and sexual reproduction occurred, marked by: (1) an initial decrease in average cell diameter; (2) a subsequent increase in diameter through time as gametes fused, forming swimming zygotes (planozygotes); and (3) a possible surge in division rate. Although the motile population appeared to reach a typical plateau or stationary growth phase, the population was changing in composition as planozygotes formed and cysts fell from suspension. Planozygote numbers increased gradually over a 4-day interval, followed 6 days later by a parallel increase in cyst numbers. These gradual increases indicate that gamete formation and/or fusion did not occur in sudden, synchronized fashion, or alternatively that the duration of planozygote development varied as the batch cultures aged. Approximately 20% of all motile cells successfully completed encystment.

Key words: dinoflagellate phosphate depletion; dinoflagellate cyst formation; dinoflagellate reproduction; dinoflagellate growth

INTRODUCTION

The toxic dinoflagellate *Gonyaulax tamarensis* Lebour blooms regularly in many temperate coastal waters throughout the world. In recent years, a dormant cyst stage has been described in this organism's life history (Dale, 1977; Anderson & Wall, 1978) that can be important both as a "seeding" mechanism for bloom initiation (Steidinger, 1975; Wall, 1975; Anderson & Morel, 1979) and as a means of population dispersal (Wall, 1971; Anderson *et al.*, 1982).

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Details of *G. tamarensis* encystment remain obscure, due in part to the difficulty in culturing this sensitive organism. On a descriptive level, it is known that asexual reproduction by division can be replaced by the sexual fusion of gametes, yielding a swimming zygote (planozygote) which then becomes the non-motile resting cyst or hypnozygote (Anderson & Wall, 1978; Turpin *et al.*, 1978; Anderson, 1980). The physiological transformations that induce sexuality and cyst formation in this species are poorly understood and remain the subject of active research.

In many dinoflagellates, sexuality has been reported in response to stress – often, but not always, following nitrogen depletion (reviewed by Pfiester & Anderson, in press). There are also reports of sexuality under seemingly optimal conditions (Wall *et al.*, 1970; Zingmark, 1970; Beam & Himes, 1974; Morey–Gaines & Ruse, 1980; Anderson *et al.*, 1983), but comparisons between these conflicting observations is not possible due to the lack of data on nutrients, other environmental parameters, and the physiological status of the cells.

A recent study examined the total cyst yield of *G. tamarensis* in response to variations in temperature, light, and initial nutrient availability in batch cultures (Anderson *et al.*, 1984). Limitation by nitrogen or phosphorus resulted in cyst formation, whereas virtually no cysts were produced in nutrient-replete medium following substantial reductions in growth rate due to non-optimal light or temperature or to high batch culture densities. The results were consistent with a link between nutrient limitation and sexual induction, with no evidence for density-dependent or growth rate-related induction mechanisms. Since these data reflect "end-point" determinations of encystment success and did not include physiological measurements during the process, details of the dynamics of cyst formation remain obscure.

The objective of the study reported here was to monitor changes in intracellular and extracellular nutrient pools and the abundance of the four main *G. tamarensis* life cycle stages (vegetative cells, gametes, planozygotes, and resting cysts) in phosphorus-limited batch cultures. Although batch culture studies have the disadvantage that cell concentrations and nutrient pools change rapidly as exponentially growing populations utilize a limited resource, they nevertheless do simulate the process of nutrient depletion in natural waters. With frequent sampling throughout this process, such parameters as encystment efficiency (cysts produced per cell), planozygote longevity, and phosphorus uptake and utilization can be monitored as the dinoflagellate metabolism shifts from asexual to sexual reproduction.

METHODS

The experiment was conducted with *G. tamarensis* strain GTMP, established in 1978 using a single cyst from the sediments of Mill Pond, Orleans, Mass. The culture was unialgal, but not axenic. Repeated attempts to obtain cysts from crosses and single cultures of axenic (and non-axenic) isolates from this parent culture were all unsuccess-

ful. Therefore it was necessary to minimize the effects of bacteria by making frequent early exponential phase transfers of strain GTMP prior to the experiment and by using large pore-size (5 μm) filters for particulate phosphorus measurements. GTMP was maintained in f/2-Si medium (Guillard & Ryther, 1962) prior to inoculation into encystment medium in which PO_4^{3-} was reduced to 3.6 μM (10% of normal f/2 levels), with NO_3^- and all other components maintained at f/2 levels. Details of this medium and the precautions taken to minimize precipitation or chemical contamination are given in Anderson *et al.* (1984). Cultures were grown at 20 °C under 350 $\mu E \cdot m^{-2} \cdot s^{-1}$ illumination on a 14 : 10 L : D cycle.

A multi-tube approach was used to obtain more detailed information on the time course of encystment. One hundred thirty 25 × 150 mm culture tubes containing 25 ml of PO_4^{3-}-limited encystment medium were inoculated with exponentially growing cells from f/2-Si medium. At 12-h intervals, external PO_4^{3-}, cellular P, and total cell concentrations were determined using the combined contents of several tubes. At 24-h intervals, cyst concentration and a population size distribution were obtained from a single tube. Our decision to use the contents of numerous tubes each day rather than daily samples from one large batch culture was based on the relatively poor cyst yield obtained after frequent stirring and sampling from large flask cultures. Cyst counts were taken on a separate tube from those used for the other measurements since cysts tend to clump together, preventing accurate subsampling.

Samples for cell counts were preserved with formalin twice daily, settled, and counted with an inverted microscope. Approximately 400 cells were enumerated each time. Cysts were harvested by emptying and rinsing another tube into a plastic beaker after bottom residue was loosened with a rubber scraper. The harvested culture was then sonicated for 1 min at 1.4 A (Branson S-75 sonifier) to destroy most vegetative cells. Counts with and without sonication indicated that no cysts were destroyed by this process. The sonicated liquid was allowed to settle and the cysts counted with an inverted microscope.

Two methods were used to monitor life cycle stages, both based on daily photographs of the *G. tamarensis* population. 5 to 10 ml of culture were removed from a tube, sieved through 10-μm Nitex and the retained cells were washed into a vial with filtered sea water. This effectively immobilized the population without altering the cell size through fixation (Anderson *et al.*, 1983). The concentrated cells were placed on a slide and photographed at 100 × total magnification (5 to 10 photographs per sample, 40 to 100 cells per photograph). Negatives were analyzed using a Luzex Particle Analyzer which digitized the image and calculated the diameter of each cell that was not touching others. A minimum of 200 (and often as many as 600) cells were measured daily in this way. *G. tamarensis* vegetative cells and gametes are essentially spherical, but planozygotes are slightly longer than wide (46 × 40 μm; Anderson, 1980). For this reason, the photographs were also examined visually and planozygotes counted as a fraction of the total cells.

As the cultures aged, individual pairs of fusing gametes were photographed (at the

early stages of fusion) using a flash attachment and 250 × magnification. The diameters of 62 cells from these fusing pairs were measured using projected images of the negatives. Fusing *G. tamarensis* cells are relatively easy to distinguish from those in the process of division: dividing cells have their cingula orientated in a parallel configuration, while the cingula of fusing cells are typically at oblique angles to each other. Fusing cells also swim in tight circles while dividing cells swim in straight lines. These distinctions are especially clear in the early stages of fusion when individual cell dimensions were determined.

Total cellular phosphorus was assayed using a modification of the method of Menzel & Corwin (1965). Cells from one tube were filtered onto a distilled water-rinsed 5-μm membrane filter (Gelman Metricel GA), rinsed with 0.2-μm filtered Sargasso sea water, and dried with appropriate blank filters at 60 °C. Bacterial counts (acridine orange direct counts) before and after this process indicate that a negligible number of bacteria were retained on the filter or adhered to the dinoflagellate cells. Dried filters were autoclaved for 20 min (121 °C) in 15 ml of 0.17 M potassium persulfate. After cooling, 10-ml aliquots were analyzed for soluble phosphorus colorimetrically (Strickland & Parsons, 1972). In other experiments with *G. tamarensis*, we have obtained a 98% mean recovery of added phosphorus with this method.

Soluble reactive phosphate was determined using 0.45-μm filtrate from the particulate tube. Daily samples were frozen and later analyzed using the methods of Strickland & Parsons (1972). Disappearance of phosphate from the medium was used with daily cell counts to provide a second estimate of cellular P and an estimate of the specific uptake rate of phosphorus.

Results

The trends in Fig. 1 demonstrate that individual tubes in the multi-tube experiment were sufficiently similar to permit catenation of data for each parameter through time. *G. tamarensis* cell concentrations increased through Day 8 at an overall growth rate of 0.7 div · day^{-1} ($\mu = 0.5\ day^{-1}$), peaking at approximately 6500 cells · ml^{-1} and only decreasing after Day 15 (Fig. 1A). Planozygotes were present at very low concentrations between Days 4 and 7, increased rapidly thereafter to over 1100 · ml^{-1} on Day 12, and then decreased for the remainder of the experiment (Fig. 1B). This decrease was accompanied by an increase in cyst number between Days 13 and 16, reaching a maximum abundance of 823 · ml^{-1} (and apparently still increasing) when the last available tube was harvested (Fig. 1B).

Cellular P increased dramatically following inoculation and then decreased steadily after Day 2 to a subsistence quota of 27 pg P · $cell^{-1}$ on Day 8, a level $<10\%$ of the maximum (Figs. 1C, 2B). The subsistence quota is defined here for batch cultures as the cell phosphorus content which permits survival but no growth. This pattern was mirrored by decreasing extracellular PO_4^{3-} concentrations during both the light and the

dark periods, eventually dropping to undetectable levels on Day 7 (Fig. 1D). Phosphorus uptake rates calculated using the disappearance of PO_4^{3-} from the medium over each 12-h interval show a rapid initial uptake following inoculation and a sharp decrease to levels between 1 and 3 pg $P \cdot cell^{-1} \cdot h^{-1}$ between Days 2 and 6 (Fig. 1E). Specific uptake rates ranged from an initial high of $1.2 \cdot day^{-1}$ to $0.24 \cdot day^{-1}$ as PO_4^{3-} supplies diminished.

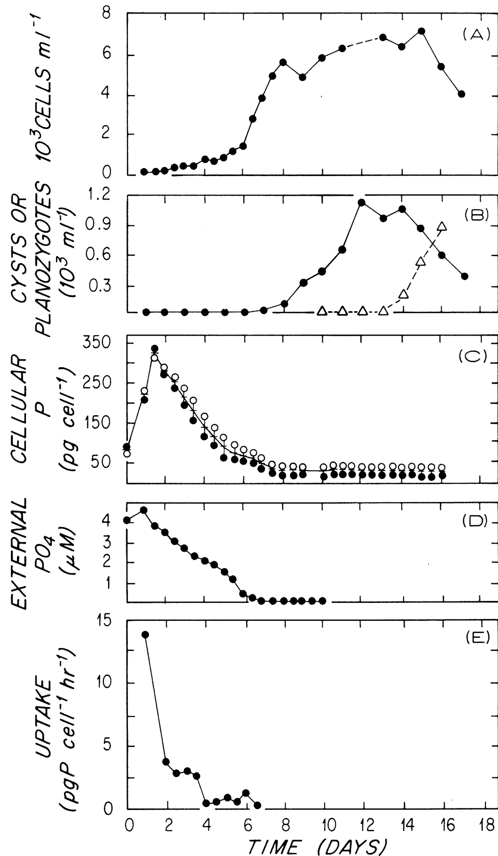

Fig. 1. Time-course measurements during asexual and sexual reproduction in *Gonyaulax tamarensis* batch cultures: A, motile cell concentration; ----, missing data point; B, planozygote (●——●) and cyst (△---△) concentrations; C, cellular phosphorus calculated from particulate P measurements (○) and solution data (●); D, external PO_4^{3-} concentration; E, phosphorus uptake rate calculated from the disappearance of PO_4^{3-} from the medium.

The validity of these cell quota and uptake rate estimates rests in part on bacterial abundance in the dinoflagellate cultures since bacteria have been shown to compete with phytoplankton for PO_4^{3-} (Currie & Kalff, 1984). We relied on frequent transfers of actively growing *G. tamarensis* cells prior to the experiment to keep bacterial biomass low and used well-rinsed 5-μm pore-size filters for particulate phosphorus measure-

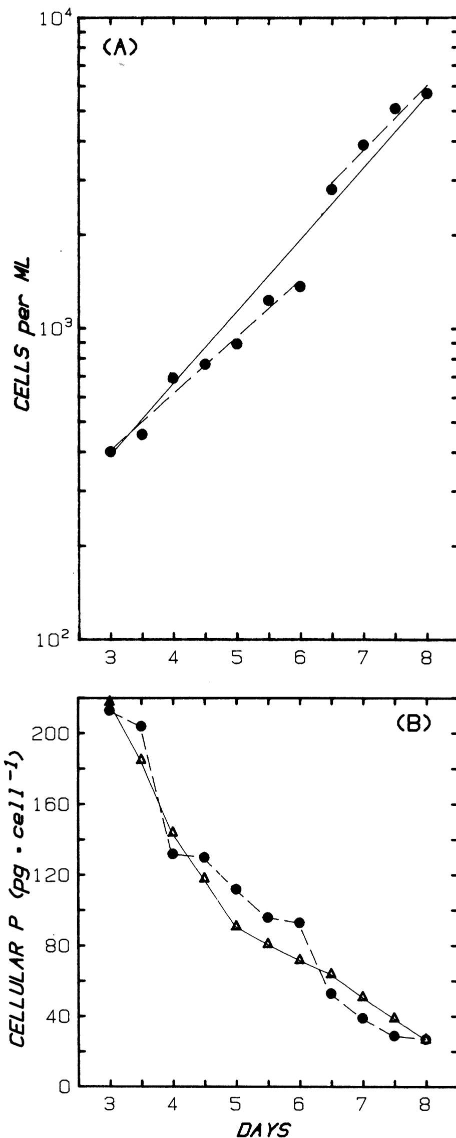

Fig. 2. Cell counts and phosphorus quota during the transition from asexual to sexual reproduction: A, twice-daily cell counts (●) between Days 3 and 8; dashed line is based on separate regressions of Days 3–6 a.m. and 6–8 p.m.; solid line represents ln-linear regression for entire exponential growth interval (Days 1–8); B, phosphorus cell quota calculated using mean value of particulate phosphorus measured directly and that calculated from solution data.

ments. Since direct counts showed that a negligible number of bacteria were retained with the *G. tamarensis* cells on those filters and there was excellent agreement between measured phosphorus cell quotas and other values calculated from the disappearance of PO_4^{3-} (Fig. 1C), our assumption of low bacterial uptake during the critical exponential and early plateau phases of growth seems justified.

Since it is clear that major changes in reproductive strategy occurred between Days 3 and 8, Fig. 2 provides more resolution of changes in *G. tamarensis* abundance and cellular phosphorus over this interval. At this scale, it is evident that division was generally exponential and that there was a possible surge in growth on Day 6 at a rate exceeding 1.5 div $\cdot$ day^{-1}. Fig. 2B shows the phosphorus cell quota dropping rapidly between Days 6 and 7, decreasing more gradually thereafter to the subsistence cell quota of 27 pg P $\cdot$ cell^{-1}.

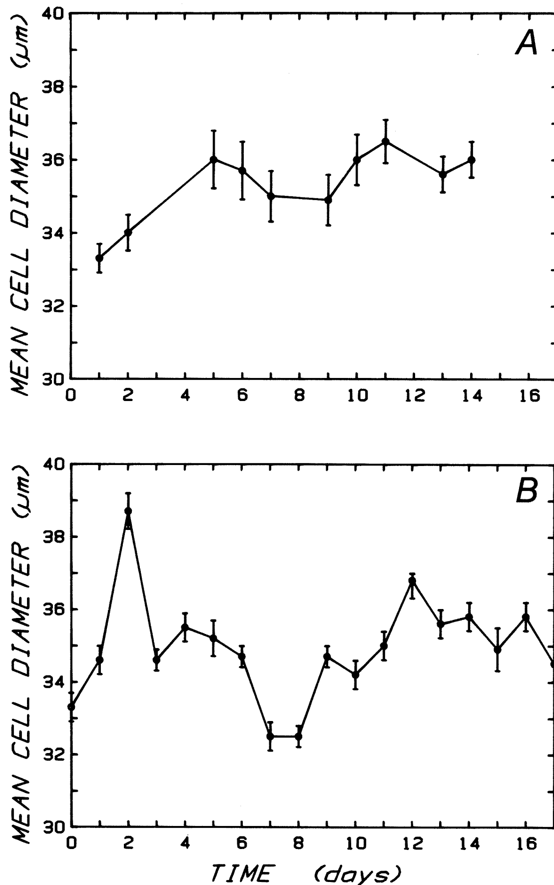

Fig. 3. *G. tamarensis* cell diameter through time: values plotted are the mean cell diameter (± SE) determined from 5–10 separate photographs of concentrated cell assemblages (40–100 cells/photograph); A, nutrient-replete f/2-Si medium with no sexuality; B, encystment medium.

An estimate of the reproducibility of individual cell counts from a multi-tube experiment was obtained by counting five replicate tube cultures at two times during exponential growth. These counts had means of 291 and 690 cells $\cdot$ ml^{-1}, with a coefficient of variation of $\approx 10\%$ in both cases.

Throughout the time-course, the size distribution of the motile population changed dramatically (Fig. 3B). Cells inoculated into encystment medium from f/2-Si were relatively small, but rapidly increased in size by Day 2. Only a slight increase in cell number occurred over this interval, so it is likely that the observed increase in cell size was due to a short lag phase in division. The average cell diameter of the population then decreased to a distinct minimum on Days 7 and 8, after which it increased steadily for the next four days. Comparable measurements in a nutrient-replete f/2-Si culture that did not produce cysts are presented in Fig. 3A. Following an initial increase in mean cell size during the first few days, the average diameter remained relatively constant as the cells completed exponential growth and entered the plateau phase (Day 11). Our previous experience with this species in f/2-Si cultures indicates that the population stopped growing due to density-related factors such as carbon limitation, pH, or excretion products and not nutrient limitation.

Photographs for size distribution measurements were taken at the same time every morning following the synchronized division pulse during the dark period. Fusing gametes were often observed, but never in large numbers. Those that were seen in the early stages of fusion were photographed alive using a strobe attachment and measurements made at a later date. The size of individual gametes (Fig. 4) ranged from 27 to 36 μm (mean = 31, n = 62), the largest difference between two fusing cells being 9 μm (mean = 1.9 μm).

DISCUSSION

A multi-tube culture of the dinoflagellate *G. tamarensis* was monitored throughout the transition from asexual to sexual reproduction, providing the first documentation of intracellular and extracellular nutrient pools and the succession of life cycle stages during dinoflagellate encystment. In our efforts to understand the factors regulating sexual induction in this species, we faced a significant obstacle common to many studies of cyst-forming dinoflagellates, namely that gametes were difficult to distinguish from vegetative cells unless they were in the process of fusing (Von Stosch, 1973; Pfiester, 1975, 1976, 1977; Walker & Steidinger, 1979). Although the first appearance of large numbers of the morphologically distinct *G. tamarensis* planozygotes provides some insight into induction mechanisms, uncertainties about the duration and synchrony of fusion and the time required for planozygote development preclude an accurate estimate of the onset of gametogenesis. For the purposes of this study, we attempted to quantify our general impression that fusing gametes are slightly smaller than normal *G. tamarensis* vegetative cells and to use this information and successive population size distributions as one indicator of the timing of gamete formation and fusion. This approach

provides a general indication of the timing of sexuality, but lacks the resolution needed to pinpoint the onset of sexual induction. Nevertheless, time-course measurements of intracellular and extracellular nutrient pools and frequent planozygote, cyst, and cell counts provide a valuable record of the dynamics of the transition from asexual to sexual reproduction.

The growth curve in Fig. 1A appears to have the normal lag, exponential, plateau, and death phases common to asexually-dividing batch cultures (Fogg, 1965). On closer examination, however, it is evident that a portion of the population switched from asexual to sexual reproduction during the experiment and that our cell counts actually are a measure of the motile population size following losses due to fusion and cyst deposition.

The first stage of growth following inoculation was, for the most part, asexual. Cell number increased at a rate of 0.7 $div \cdot day^{-1}$ ($0.5 \cdot day^{-1}$) and the bulk of the sexually-produced planozygote population only appeared as the motile population reached plateau phase. Some planozygotes ($10–20 \cdot ml^{-1}$) were observed before the population peaked, and fusing pairs were seen as early as Day 3, but the increase in planozygote numbers to $1130 \cdot ml^{-1}$ following Day 8 suggests a surge in sexual activity. The increase in cyst abundance lagged that of the planozygotes by 6–7 days, indicating that under the conditions of this culture, the swimming zygotes required approximately one week to mature before encysting. This interval is similar to that inferred from *G. tamarensis* blooms in natural waters (Anderson *et al.*, 1983).

There were insufficient tubes to continue this experiment beyond Day 17, so we cannot balance the planozygote and cyst abundances. However, cysts appeared in significant numbers as planozygotes decreased and the final cyst concentration ($823 \cdot ml^{-1}$) was still increasing towards the plateau abundance of planozygotes ($1130 \cdot ml^{-1}$) when the last tube was harvested (Fig. 1B). Unlike other studies where the fraction of the population that encysted could only be estimated from peak cell counts and final cyst yield (Watanabe *et al.*, 1982; Anderson *et al.*, 1984), our time-course data permit cyst yield to be expressed relative to the cumulative number of motile cells produced. To accomplish this, the planozygote concentration and two times the cyst concentration were added to the daily motile cell counts in Fig. 1A, (cysts and planozygotes were the product of the fusion of two cells and the planozygotes were already counted once). This "motile cell equivalent" count increased through time, eventually levelling-off on Day 15 at 7700 $cells \cdot ml^{-1}$. Approximately 21% of the motile population thus successfully encysted by the end of the experiment, corresponding to a cyst : cell ratio of 0.11.

Before discussing the timing of gamete formation and pairing, we wish to re-emphasize that *G. tamarensis* gametes are morphologically similar to vegetative cells. Furthermore, we have not observed a division sequence characteristic of gamete formation as was observed in *Gonyaulax monilata* by Walker & Steidinger (1979). Thus we do not know whether gametes are formed by the division of vegetative cells or whether vegetative cells with insufficient internal nutrient reserves or externally available supplies

are capable of sexuality directly. Given this uncertainty, the term "gametogenesis" must be loosely defined to include both of these possibilities for *G. tamarensis*.

Our best estimate of the onset of sexuality is between Days 6 and 8. This conclusion is based on: (a) the large number of relatively small cells on Days 7 and 8; (b) the steady increase in average cell size thereafter; (c) the internal phosphorus pool size at that time; and (d) an apparent surge in division rate immediately prior to Day 7. Taken separately, none of these factors can stand alone and be considered conclusive, yet together they provide a consistent indication that the reproductive mode of the population changed abruptly.

If we ascribe the initial surge in mean cell diameter in the encystment culture on Day 2 to a lag phase effect where little or no division occurred (Figs. 1, 3), we find that the exponentially dividing population subsequently produced relatively equal-sized daughter cells through Day 6 before the mean diameter decreased to a minimum of 32.5 μm on Days 7 and 8. Since fusing gametes averaged 31 μm in diameter (Fig. 4) and the average *G. tamarensis* cell size increased steadily from Day 8 to 12, the formation and subsequent fusion of gametes is suggested.

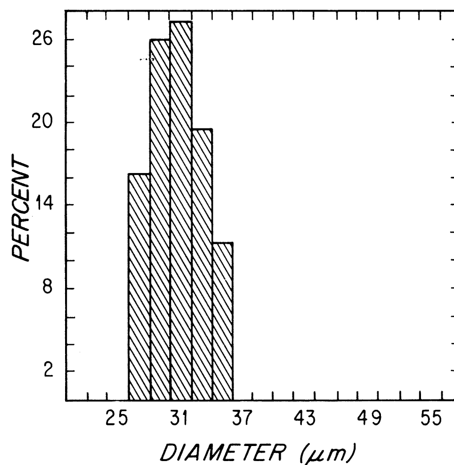

Fig. 4. Size distributions of individual gametes photographed during the early stages of fusion (n = 62).

In contrast, the mean cell diameter in a nutrient-replete f/2-Si culture remained relatively constant near 36 μm following an initial lag phase (Fig. 3A). This is consistent with other studies on changes in phytoplankton cell size with growth stage which indicate that during exponential growth with an excess of nutrients, metabolic processes needed for biomass synthesis are sufficiently synchronized to produce daughter cells of generally uniform size and composition (Fogg, 1965; Prakash *et al.*, 1973). As exponential growth ceases, suppression of cell division can produce larger cells, but not following the sudden and large decrease in mean cell size seen on Days 7 and 8 in encystment medium.

This minimum in mean cell diameter near Day 7 is consistent with an apparent surge in division rate at that time. As seen from our twice-daily cell counts in Fig. 2A, population growth increased at a rate in excess of 1.5 div $\cdot$ day^{-1} between Days 6 and 7. Given a 10% coefficient of variation for cell counts from replicate tubes in this experiment, the description by von Stosch (1973) of rapid "depauperating divisions" in *Gymnodinium pseudopalustre* prior to sexuality, and the documentation of two rapid divisions in 24 h immediately before fusion in a *Gyrodinium uncatenum* bloom population from the Potomac River (Coats *et al.*, 1984), the apparent burst of growth in Fig. 2A may be a result of gamete formation.

Despite these independent indications that gametes were present in large numbers near Days 7 and 8, planozygote numbers did not surge the next day but instead increased steadily over four days (Fig. 1B). Similarly, the population's mean cell diameter did not increase suddenly but increased gradually through Day 12 (Fig. 3). Our relatively infrequent observation of fusing pairs in daily samples and results from other dinoflagellates imply that plasmogamy should have been complete in < 24 h and in fact, probably lasted a few hours or less (Von Stosch, 1973; Pfiester, 1975; 1976; Coats *et al.*, 1984). The gradual increase in planozygotes (and cysts) thus indicates that gamete formation and/or fusion occurred over a 4-day interval as well, or alternatively that the duration of planozygote development following fusion varied as the culture aged. These data emphasize the need for cytological techniques that will permit *Gonyaulax tamarensis* planozygotes to be distinguished from the rest of the motile population at an early stage of development – well before they become morphologically distinct at the light microscope level.

It is possible that gametes were formed by division between Days 9 and 12. However, since extracellular and intracellular phosphorus pools reached minimum levels on Day 7, the subsequent appearance of over 1100 planozygotes $\cdot$ ml^{-1} by Day 12 would require that 20% of the plateau phase cells divided despite a constant and low population cell quota for phosphorus. An alternative possibility is that nondividing vegetative cells were able to function directly as gametes and that this metabolic transformation could have occurred over several days within a somewhat heterogeneous population. Given the similarity between fusing gametes and vegetative cells as well as the lack of a division sequence characteristic of gamete formation (e.g., Walker & Steidinger, 1979), it may not be possible to determine which of the two processes described above actually yields *G. tamarensis* gametes.

In this experiment, external PO_4^{3-} and cellular P dropped to minimum levels within 1 day of each other so there was no clear indication of which parameter was more closely linked to sexual induction. Evidently the bulk of the *G. tamarensis* population reproduced asexually until nutrient starvation was either imminent or had already occurred, at which time sexual reproduction ensued. These results are consistent with other laboratory studies on *G. tamarensis* that have documented cyst formation under N- or P-limited conditions, with no sexuality at high nutrient concentrations (Turpin *et al.*, 1978; Anderson *et al.*, 1984). This species thus falls into the category of those dino-

flagellates requiring some level of nutrient depletion as a prerequisite for sexuality in cultures (Anderson & Pfiester, in press). A similar relationship between nutrients and sexuality is common in other algal classes as well (Sager & Granick, 1954; Trainor, 1958; Biebel, 1964; Cain & Trainor, 1976; O'Kelley, 1983).

This conclusion seems inconsistent with field studies documenting *G. tamarensis* cyst formation (at 12–15 °C) when external nutrients were at or above levels that previously supported growth (Anderson & Morel, 1979; Anderson *et al.*, 1983). Those data were initially interpreted as evidence for sexual induction without nutrient stress. In our study, the steady decrease in cellular P after Day 2 (Fig. 1C) demonstrates that after an initial surge, the specific phosphate uptake rate was always lower than the specific growth rate. We recognize that our cultures were growing near their optimal growth rate at 20 °C and were not at steady state, but it is nevertheless noteworthy that such an imbalance between uptake and growth occurred with micro-molar PO_4^{3-} concentrations, forcing the cells to divide at the expense of stored reserves. We must now consider the possibility that *G. tamarensis* may be relatively inefficient at obtaining nutrients at low concentrations and that encystment may occur in natural waters due to nutrient limitation at concentrations above analytical detection limits.

Acknowledgements

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