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HIGH ENCYSTMENT SUCCESS OF THE DINOFLAGELLATE *SCRIPPSIELLA* CF. *LACHRYMOSA* IN CULTURE EXPERIMENTS[1]

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Close to 100% encystment efficiency and a yield above 10^5 cysts·mL^{-1} were routinely achieved in full strength f/2 medium-based batch cultures (883 μM NO_3^- and 36 μM PO_4^{-3}) of the marine dinoflagellate *Scrippsiella* cf. *lachrymosa* Lewis. Increases in cell density led to nutrient depletion in this enriched medium, which was the most likely cause for initiation of cyst formation. Lowering the concentration of either nutrient to 1/10 the initial levels decreased the encystment efficiency, whereas use of ammonium as the N source resulted in both low cell yield and low encystment efficiency. The mandatory dormancy period was ca. 60 days and was not affected by cold dark storage of the cysts. Cysts produced in the initial phase of sexual reproduction were relatively large (length 47 μm, width 31 μm) with a heavy calcareous cover. Cysts produced thereafter lacked apparent calcareous cover and were smaller (length 29 μm, width 19 μm). The decrease of cyst volume (by a factor of 0.24–0.4) suggested strong resource limitation during the course of encystment. However, after the mandatory dormancy period, germination success of the smaller cysts was higher (80%), compared with the larger cysts that had been produced initially (50%). Germling survival (74%) was independent of cyst type but was enhanced by higher nutrient concentration during incubation. The ratio of initial nutrient concentration in the medium to the cyst yield was used as a proxy to estimate the cellular nutrient quota. The conservative estimates of 9 pmol N·cyst^{-1} and 0.4 pmol P·cyst^{-1} obtained in this manner are at the low end of the range of previous published estimates for other dinoflagellate cysts. Given the high encystment observed in laboratory experiments, we have no reason to assume an inherently lower encystment success in dinoflagellate field populations. Our results do not challenge the low nutrient paradigm for dinoflagellate sexuality. We believe that the high encystment success and cyst yield of this particular species is at least partly due to its ability to achieve very high cell densities in cultures, which evidently leads to nutrient depletion even in f/2 medium.

Key index words: **Dinophyceae; encystment; germination; life cycle; resting cysts; *Scrippsiella* cf. *lachrymosa***

A wide variety of phytoplankton species has been reported to form cysts, spores, or other types of resting stages as part of their life cycle (Fryxell 1983). Many dinoflagellate species spend a major portion of their life cycle as resting cysts that are resistant and nonmotile, capable of undergoing true dormancy (Pfiester and Anderson 1987, Dale 1993). Dormancy enables the population to survive fluctuations of one or several environmental variables that exceed the tolerance range for vegetative growth of the species. When a mandatory dormancy period is completed, germination can take place if environmental conditions are permissive (e.g. Anderson et al. 1987). Otherwise, the cysts remain in an environmentally controlled resting state called quiescence (Pfiester and Anderson 1987).

Numerous issues concerning the encystment of dinoflagellates remain to be resolved. One of the most important unknowns is the factor or factors that trigger sexuality. In many species, sexuality and subsequent cyst formation have been linked to stress (e.g. Anderson and Lindquist 1985, Pfiester and Anderson 1987). In most studies, this stress has been nutrient limitation (von Stosch 1973, Anderson et al. 1984, Coats et al. 1984, Binder and Anderson 1987, Blackburn et al. 1989, Doucette et al. 1989, Park and Hayashi 1993, Bravo and Anderson 1994, Blanco 1995). In culture experiments, encystment has been induced by resuspending actively growing cells into "encystment medium" containing reduced concentrations of one essential nutrient, usually nitrogen (Pfiester 1975, Turpin et al. 1978, Walker and Steidinger 1979) or phosphorus (Anderson et al. 1985, Anderson and Lindquist 1985). A common routine has been to use 1/10 of full strength culture medium nutrient concentration (either N or P), substituting ammonium for nitrate as the N source (Anderson et al. 1985, Binder and Anderson 1987, 1990). Some studies have indicated that dinoflagellate sexuality does not occur under nutrient-replete conditions, even when growth rate is reduced by nonoptimal temperatures or by high batch culture density (e.g. carbon limitation, see Anderson et al. 1984, 1985).

There are occasional reports of spontaneous cyst formation in high nutrient dinoflagellate cultures (Zingmark 1970, Beam and Himes 1974, Morey-Gaines and Ruse 1980), and many field studies do not support the view that nutrient limitation is the main stimulus for dinoflagellate sexual reproduction (Wall et al. 1970, Anderson et al. 1983, Kremp and Heiskanen 1999). Anderson et al. (1983) observed the timing of cyst formation in three Cape Cod (Massachusetts, USA) salt ponds and speculated that sexual reproduc-

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tion was induced after a minimal number of cell divisions after germination. They observed a remarkable consistency in net cell divisions that occurred between the time the first *Alexandrium tamarense* (Lebour) Balech cells were observed and the time that planozygotes appeared despite a 1-month difference in population development between the ponds and nutrient-replete conditions. The possibility of gradual depletion of some storage product after cyst germination was considered a possible trigger for sexuality, with replenishment of that stored reserve during the nondividing planozygote stage (Anderson et al. 1983, Anderson 1998).

Perhaps the most challenging aspect of dinoflagellate life cycle studies has been to estimate encystment success. Here we use the term encystment success to specify the proportion (range, 0–1) of newly formed cysts in the total population at any given time. Encystment percentage (range, 0%–100%) is used as a parallel term. Numerous laboratory experiments where special "encystment media" have been used to produce resting cysts of dinoflagellates have yielded a modest 10%–20% final encystment percentage (e.g. Anderson et al. 1984, Binder and Anderson 1987, Montresor and Marino 1996), indicating that most cells failed to form cysts. However, the often large residual planozygote population in cultures (Anderson et al. 1984, 1985, Coats et al. 1984, Anderson and Lindquist 1985) argues that the total cyst yield may seriously underestimate the extent of sexual induction and gamete pairing. If gametes are formed when nutrients are depleted in batch cultures, how then does the planozygote obtain sufficient resources to complete the transition to cyst, to support prolonged dormancy, quiescence, germination and growth? Anderson (1998) suggested that possibly only the first planozygotes to form are able to complete the transition to cysts, perhaps because they were able to take up additional nutrients before concentrations became too low in the batch cultures to permit significant uptake. Only those with adequate nutrients were then able to complete the transition to cysts.

The present study demonstrates that the encystment success of *Scrippsiella* cf. *lachrymosa*, grown in batch culture with full strength f/2 growth medium, can be close to 100%. *Scrippsiella lachrymosa* is a coastal dinoflagellate species that produces resting cysts covered with flattened calcareous plates (Lewis 1991, D'Onofrio et al. 1999). Given its high encystment efficiency, we investigated whether sexual reproduction was initiated by a specific number of cell divisions after germination, independent of nutrient concentration, and how shortage of either N or P affected encystment success. In the course of these investigations, we observed an interesting phenomenon of decreasing cyst size and the disappearance of the calcareous outer layer of cysts as the vegetative cultures aged. The germination success of these different types of cysts was therefore determined to see if the two morphotypes were equally viable.

MATERIALS AND METHODS

Culture. A culture of *S.* cf. *lachrymosa* was obtained by germinating a single resting cyst originating from the surface sediments of Casco Bay (Gulf of Maine, northeast coast of the United States). The culture was unialgal but not clonal or axenic. Species identification was accomplished using light microscopy based on the descriptions of Lewis (1991) and D'Onofrio et al. (1999). *Scrippsiella lachrymosa* can be easily distinguished from other *Scrippsiella* species by cyst morphology (M. Montresor, personal communication). Cysts were larger than vegetative cells, with an elongated oval to rhomboidal shape (Fig. 1, A–C). The size of the cysts and the thickness of the calcareous cover varied. The first cysts produced in batch cultures had a thick calcareous cover (Fig. 1; hereafter termed thick-walled cysts). In some specimens the calcite crystals were large, up to 4 μm in diagonal dimension, compared with ca. 2 μm in most cysts (Fig. 1D). Cysts produced in a later phase of batch culture growth lacked an evident calcareous layer, as did cysts produced in a still later phase, which were also smaller in size (Fig. 1, B and C; hereafter termed thin-walled cysts). All cysts were true hypnozygotes with a large red accumulation body in the middle of the cell, sometimes near one wall or toward the apex. After germination, the accumulation body was expelled from the germling cell and remained at the bottom of the culture vessel. This could occur either before or after the first cell division; in the latter case the accumulation body was passed to just one daughter cell. Gametes were equal sized and notably smaller than vegetative cells (Fig. 1, E and F). Planozygotes were larger than vegetative cells and more elongate.

Cultures were maintained in 50-mL borosilicate glass tubes filled with 25 mL f/2 medium (Guillard and Ryther 1962) without silica addition, with Vineyard Sound seawater as a base (hereafter referred to as f/2 medium, see also Table 1 and Anderson et al. 1994). Cultures were grown at 15° C, 200 μmol photons·m^{-2}·s^{-1}, and a 12:12-h light:dark photoperiod (hereafter referred to as standard growth conditions). Maximum growth rate in f/2 medium was about one doubling per day.

Cultures maintained at standard growth conditions encysted completely after approximately 50 days of incubation. This was evidenced by a characteristic granular reddish layer at the bottom of the tube, clearly different from the brownish hue of dense motile populations. Microscopic examination revealed that the reddish deposit contained almost exclusively resting cysts; the reddish color was caused by the accumulation bodies in the cysts. Cells other than cysts were extremely rare at this advanced stage of culture growth.

Estimating mandatory dormancy period. Mandatory dormancy period is defined as the minimum length of time elapsing from the formation of cysts to the first germination event. Cysts used for dormancy period estimation were produced in f/2 medium and harvested 3 days after the onset of encystment (day 0). This ensured that all the cysts used were approximately the same age. Aliquots were dispensed into 1.5-mL centrifuge tubes and stored in darkness at 4° C. At 20-day intervals (starting from day 0), one tube was removed and 24 cysts were isolated into individual wells of a 96-well tissue culture plate filled with 0.2 mL f/2 medium. Plates were incubated at standard growth conditions. Plates were inspected for cyst germination every 4 to 9 days using an inverted microscope (100× magnification). The frequency of observations determined the uncertainty in the estimation of the dormancy period.

Germination success and germling survival. We hypothesized that the small size of the thin-walled cysts was a result of severe resource limitation, which could lead to poor germination success and viability compared with the thick-walled cysts. Cysts, which had been at 4° C for 3 months to complete dormancy, were isolated into individual tissue culture plates. Plate wells were filled with 0.2 mL (96-well plate) and 1 mL (48-well plate) of medium at different concentrations (f/2, f/4, and f/8; for definitions of media see Table 1). First, 144 cysts were isolated by scanning a transect in a Sedgwick-Rafter counting slide and isolating all the cysts on a transect, without discriminating be-

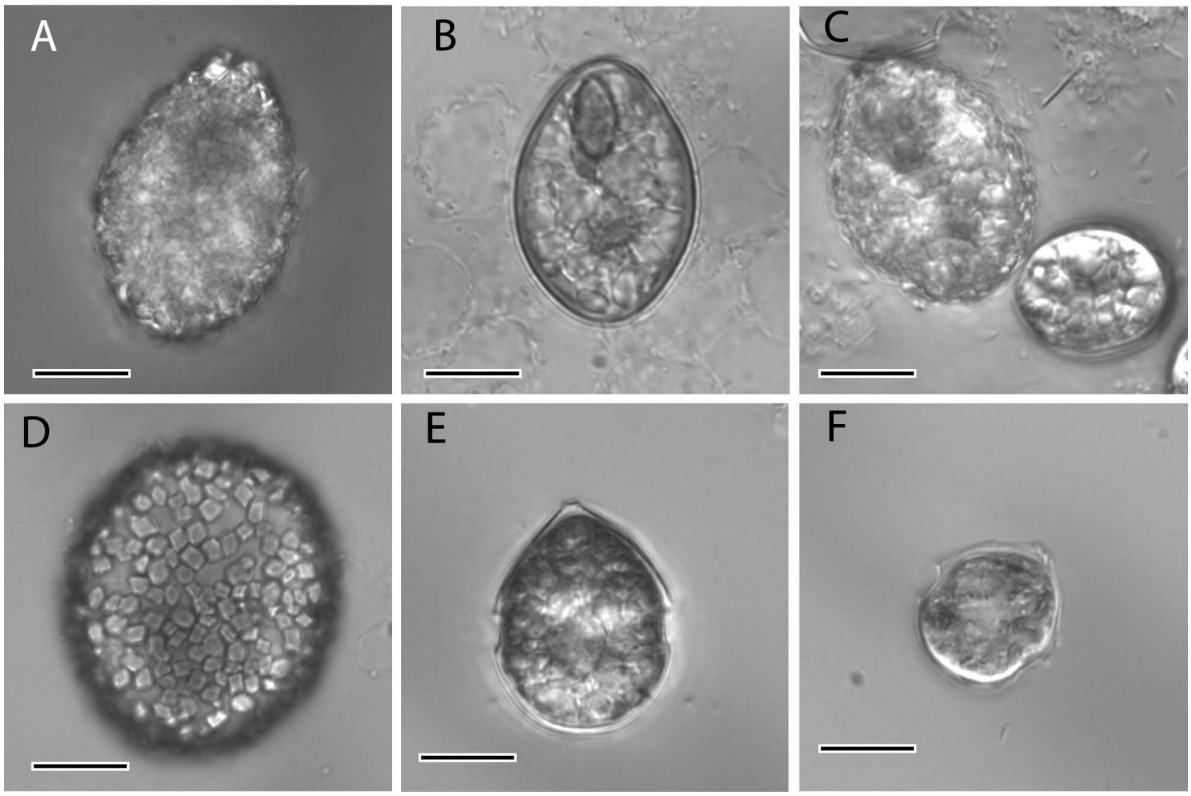

FIG. 1. Light micrographs of cysts and motile cells. Scale bars, 10 μm. (A) Cyst with calcareous plates. (B) Cyst without calcareous plates but initial cell size. (C) Two cysts showing the extent of size decrease. The large one with strong calcareous cover corresponds to a cyst produced in early stage of culture; the smaller, with no calcareous cover, corresponds to a cyst produced in later stage of the culture and belongs to the smallest size range. (D) Large rounded cyst showing the structure of the calcareous plates. (E) Vegetative cell. (F) A small motile cell, probably a gamete.

tween size and appearance unless cysts were obviously damaged or dead. It was assumed that this procedure gives a representative sample of the cyst population from a given culture. Wells with thick-walled cysts were marked separately. Because thick-walled cysts were less frequent, an additional 36 thick-walled and 60 thin-walled cysts were isolated into a separate 96-well plate filled with f/2 medium. During a 35-day period, the plates were monitored daily to document the germination event, follow the survival of germlings, and record the appearance of new cysts.

The associations between germination, cyst type (thick- and thin-walled), and medium type were analyzed with a logit model, which is a variant of log-linear models where the response variable is clearly defined (Agresti 1984). The germination success, as a response variable, was expressed as the odds of germination (the ratio of the relative probability of germination to the relative probability of germination failure) and the germling survival success as odds of survival (the ratio of survival probability to the probability of survival failure). Survival was considered positive when the germling gave rise to a new vegetative population, which in all cases led to new cyst formation. Survival was negative when the cyst had germinated, as defined by an empty cyst with a longitudinally split wall, but with no vegetative population growth. The model fit was evaluated through a log-likelihood ratio, and the resulting G statistic was used for chi-square testing of the model with the appropriate number of degrees of freedom (df). The additivity feature of the G statistic was used to test the adequacy of simpler models (i.e. with fewer parameters) relative to more complex ones. The difference in G statistics between the best fit of a simpler and a more complex model has an asymptotic chi-square distribution with df equal to the difference in the df of the two models. This property was used in conditional tests of the interaction between cyst and medium types on germination success and the ordinal effect of medium type on germling survival.

TABLE 1. Mineral N and P concentrations (in μM) and the molar N:P ratio in the nutrient media used in the experiments. All the other constituents are as in f/2 medium except that no silicate was added.

Medium type	NO_3^-	NH_4^+	PO_4^{-3}	N:P
f/2	883	0	36.3	24.3
f/4	442	0	18.2	24.3
f/8	221	0	9.1	24.3
f/20 NO_3^-	88.3	0	36.3	2.4
f/20 PO_4^{-3}	883	0	3.63	243.3
f/2+NH_4^+	0	176.6	36.3	4.9

Nutrient concentration and induction of sexual reproduction. We hypothesized that encystment starts a certain number of cell divisions after germination and is independent of nutrient concentration. To test this hypothesis we used culture plates from the germination success experiment, where the surviving populations in the wells were started from single cysts. As soon as the appearance of new cysts was recorded during the daily inspections, the well in which cysts were seen was emptied with a pipette followed by several rinses with filtered seawater. All the cells and cysts were transferred to separate tubes and preserved with Utermöhl solution (Utermöhl 1958) for later quantification. The culture plate was returned to the incubator. All counting was done with a Nikon light microscope (100× total magnification, bright field) in Sedgwick-Rafter counting slide.

To achieve convenient counting concentration, the samples were usually diluted with filtered seawater. At least 400 cells or cysts were counted, when feasible. Cells were grouped into three classes: haploid cells (i.e. vegetative cells and gametes), planozygotes (relatively rare), and mature cysts. The distinction between the first two classes during routine counting was based subjectively on the shape and size of the cells. Planozygotes were larger, more pigmented, and often more elongated, suggestive of a transition in shape between vegetative cells and cysts. To facilitate comparisons between treatments, all counts of different cell types were converted to "haploid cell equivalents" ($2 \times$ cysts + $2 \times$ planozygotes + vegetative cells + gametes) because cysts are products of the conjugation of two cells (Anderson and Lindquist 1985, Montresor 1995, Montresor and Marino 1996) and are hereafter referred to as haploid cells.

Mean growth rate of the population was calculated from the final population size and the time from germination to first cyst formation (when the wells were emptied). The population size at the time of new cyst appearance was used to evaluate hypotheses on the effects of nutrient concentration and number of cell divisions before encystment.

Encystment dynamics and changes in cyst morphology. This experiment was conducted to study the time course of cyst production in relation to the motile population size and the change in the morphology of cysts produced at different time intervals. We followed the population dynamics in test tubes filled with 10 mL of f/2 and f/4 medium for a period of 54 days. Two sets of 24 test tubes were filled with either of the two medium types and inoculated with cells in exponential growth phase to reach initial concentrations of 70 cells·mL^{-1} (f/2) and 120 cells·mL^{-1} (f/4). Initially, at 4-day intervals, and later, at longer intervals, three tubes were sacrificed at random from both sets and the cells and cysts harvested and preserved with Utermöhl solution for quantification. Before harvesting, the tubes were briefly sonicated (model 250, Branson Ultrasonics Corp., Danbury, CT) at low power (7 W) for a few seconds to agitate cysts that had settled to the bottom. Aliquots were reserved for future measurements of cyst size distribution on an image analysis system composed of a Zeiss Axioscope compound microscope (Zeiss, Jena, Germany) (400× magnification), Princeton Instruments digital CCD camera (Princeton Scientific Instruments, Monmouth Junction, NJ) model RTE/CCD-1317K/2), and IPLab software (www.IPLab.com). Mineral nutrient concentrations ($NO_2^- + NO_3^-$ and PO_4^{-3}) were measured at the end of the experiment (days 37 and 54) following the standard procedures of Eppley (1978) and Murphy and Riley (1962). Encystment percentage during the course of the experiment was expressed as [$2 \times$ cysts/(all motile cells + $2 \times$ cysts)] $\times$ 100.

Effect of single nutrients on encystment success. Aliquots of exponentially growing culture were resuspended into f/2, f/20 NO_3^-, f/20 PO_4, and f/2+NH_4^+ medium (Table 1) to a final concentration of 365 cells·mL^{-1}. The inoculated medium was then gently mixed and dispensed into 96-well culture plates in 0.2-mL volumes (average 73 cells·well^{-1}). The plates were monitored daily using an inverted microscope to register the appearance of cysts. The experiment was terminated when the cyst abundance in all the wells had reached a plateau (visual judgement). Upon termination, the populations were harvested and quantified as described above. Cellular nutrient quotas were estimated by dividing the total amount of nutrients in the initial medium (given by volume times the concentration) with the number of haploid cell equivalents in the well, assuming that all the nutrients were taken up by the cells (see Results).

RESULTS

Dormancy period and germination frequency. The dormancy period of the cysts was estimated to be ca. 60 days regardless of temperature and light preconditioning (Fig. 2). Newly produced cysts, when transferred into fresh f/2 medium, were able to germinate after 60 days of storage at 15° C and 200 μmol photons·m^{-2}·s^{-1}. Our results suggest that the dormancy period was not prolonged or shortened by dark cold storage.

Once the cysts had been stored in darkness at 4° C for over 60 days, germination usually occurred within a few days after transfer to fresh f/2 medium and standard growth conditions. Germination did not occur during the first 2 days but increased thereafter with a peak on day 6 (Fig. 3). The average germination time was not significantly different between the thin- and thick-walled cysts or among different nutrient concentration.

Germination success and germling survival, as a function of medium strength and cyst type, are given in Table 2. Out of the 240 isolated cysts, 167 germinated within a period of 35 days, equivalent to an overall germination percentage of 70%. Germination among the 71 thick-walled cysts was only 48%, compared with 79% among the 169 thin-walled cysts (Table 2).

A logit model with germination as the response variable and cyst and medium types as categorical independent variables gave a reasonably good agreement with the data ($G = 1.2$, $df = 2$, $P = 0.54$) without the need to add a cyst type–medium type interaction parameter. The modeled odds of germination were very close to observed values (Table 3). The model parameter estimates (Table 4) indicate that the odds of germination were $\exp(1.395) = 3.82$ times higher among thin-walled cysts compared with the thick-walled cysts. The negative parameter estimates indicate that in f/2 medium the odds of germination were $\exp(-0.889) = 0.411$ times and in f/4 medium $\exp(-1.4517) = 0.234$

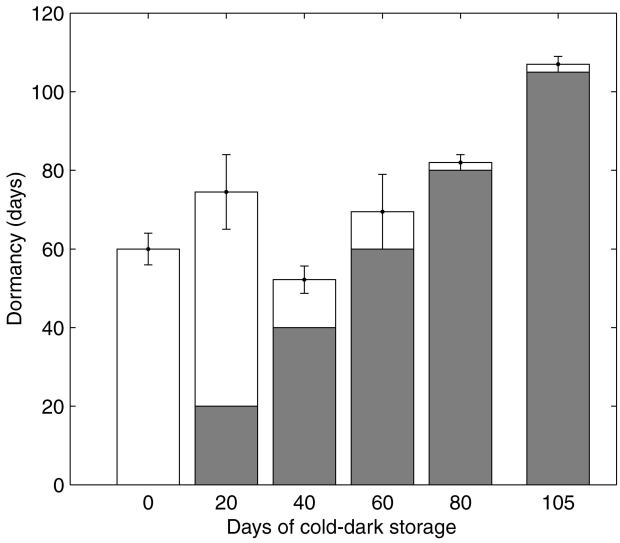

FIG. 2. Length of dormancy for *Scrippsiella* cf. *lachrymosa* cysts after different periods of dark storage at 4° C. Gray bars, time spent in dark cold conditions; white bars, time spent under light-exposed conditions at 15° C. Error bars indicate the range of days when germination occurred, that is, the uncertainty imposed by frequency of checking the culture plates.

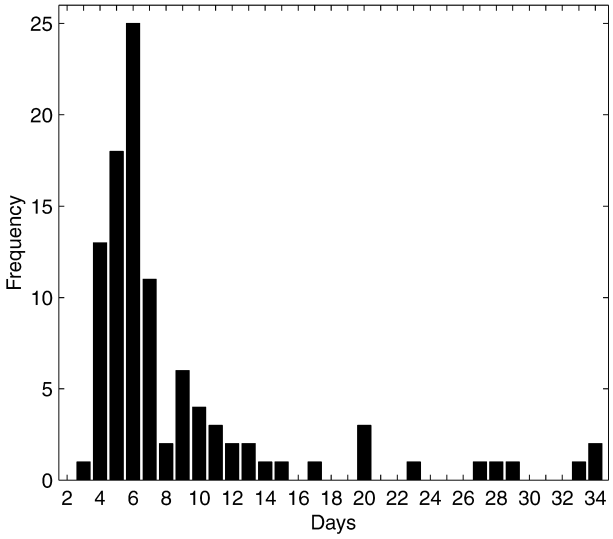

FIG. 3. Germination frequency of *Scrippsiella* cf. *lachrymosa* cysts over a period of 35 days. Cysts were first stored in darkness at 4° C for 3 months to complete dormancy and transferred to light-exposed conditions at 15° C.

times the odds of germination in f/8 medium. Thus, germination success was higher among thin-walled cysts and in f/8 medium, and the effect of cyst type was reasonably uniform among the tested medium types. Conditional tests of significance of cyst and medium types on germination success revealed that both were highly significant ($G = 20.7$, $df = 2$, $P < 0.001$ and $G = 8.5$, $df = 1$, $P < 0.005$, respectively). Adding the cyst type–medium type interaction term would saturate the model ($G = 0$, $df = 0$), and the difference between the present model and saturated model was used to show that the interaction term was not significant ($P > 0.5$).

Germling survival. Because of the mortality of germlings, not all germinated cysts gave rise to healthy populations and new cyst formation. Out of the 167 germinated cysts, 123 (74%) gave rise to successful population growth and production of new cysts (Table 2); thus, the overall odds of survival were 2.8. The odds of survival increased with increasing nutrient concentrations in the incubation medium (Table 3) but were not significantly different between populations originating from the two types of cysts ($G = 0.2$, $df = 1$, $P > 0.6$). A logit model that assumes independence of germling survival from medium type gave a poor fit ($G = 5.0768$, $df = 2$, $P = 0.079$), whereas a model accounting for the ordered nature of medium strength gave a very good fit ($G = 0.0007$, $df = 1$, $P = 0.98$) without still being saturated. The model had two independent parameters: the estimated effect of survival in f/2 medium (1.3628), which corresponded to the odds of germling survival of $\exp(1.3628) = 3.9$ (Table 3), and the effect of medium strength −0.4568. Within the tested range of medium types the odds of new cyst formation were estimated to be proportional to $\log_2$ of nutrient concentration, decreasing by a factor of $\exp(-0.4568) = 0.633$ when moving from f/2 medium to f/4 or from f/4 to f/8 (Table 3). Thus, germling survival increased with increasing nutrient concentration in the medium.

TABLE 2. Success (%) of germination of different cyst types (thin- and thick-walled) and germling survival in different strength media and different cyst types (thin- and thick-walled) and the respective row and column totals.

Germination	f/2	f/4	f/8	Total
Thin wall	77.1 (96)	71.4 (35)	89.5 (38)	78.7 (169)
Thick wall	50.0 (48)	23.1 (13)	70.0 (10)	47.9 (71)
Total	68.1 (144)	58.3 (48)	85.4 (48)	**69.6 (240)**
Survival	79.6 (98)	71.4 (28)	61.0 (41)	73.6 (167)

The grand total for germination is shown in bold. Numbers in parenthesis denote the number of cysts in each category.

TABLE 3. Observed/modeled odds of germination in combinations of cyst and medium types.

Germination	f/2	f/4	f/8
Thin wall	3.36/3.44	2.5/1.96	8.5/8.4
Thick wall	1/0.9	0.3/0.51	2.33/2.2
Survival	3.9/3.9	2.5/2.5	1.56/1.56

Number of cysts in each class is the same as in Table 2.

Nutrient availability and induction of encystment. The median of haploid cell yield at the time when the first cysts were produced ranged from 4 to 95 × 10^3 cells·well^{-1} and was proportional to the total amount of available nutrients (Fig. 4). The number of cell doublings from germination to reencystment can be calculated as $\log_2$ of the total haploid cell yield and ranged from 12 to 16.7 doublings. The time from germination to reencystment ranged from 18 to 23 days. The exponential growth rate (from germination event to new cyst formation) varied between 0.49–0.50 d^{-1} in f/2 and f/4 and 0.44–0.46 d^{-1} in f/8. The median of haploid cells per nmol N ranged from 80 to 124 (Fig. 4).

The haploid cell yield (Fig. 4), number of cell doublings, and time from germination to reencystment were all different between the treatments. However, much of the variability between the treatments was explained by the differences in the total amount of nutrients. Haploid cell yield per unit nutrient amount was not significantly different between the six treatments (Kruskal-Wallis analysis of variance by ranks; $H = 7.4$, $df = 5$, $P > 0.19$). However, pooling the data within medium types revealed a significant difference ($H = 6.5$, $df = 2$, $P < 0.04$) due to the lower cell yield in f/8 medium (see Fig. 4).

Encystment dynamics and cyst morphology. Population growth and encystment dynamics are shown in Figure 5. The vegetative population size and the number of resting cysts were quite variable among the replicate tubes. After an initial lag period, the population of vegetative cells started to increase rapidly. Some en-

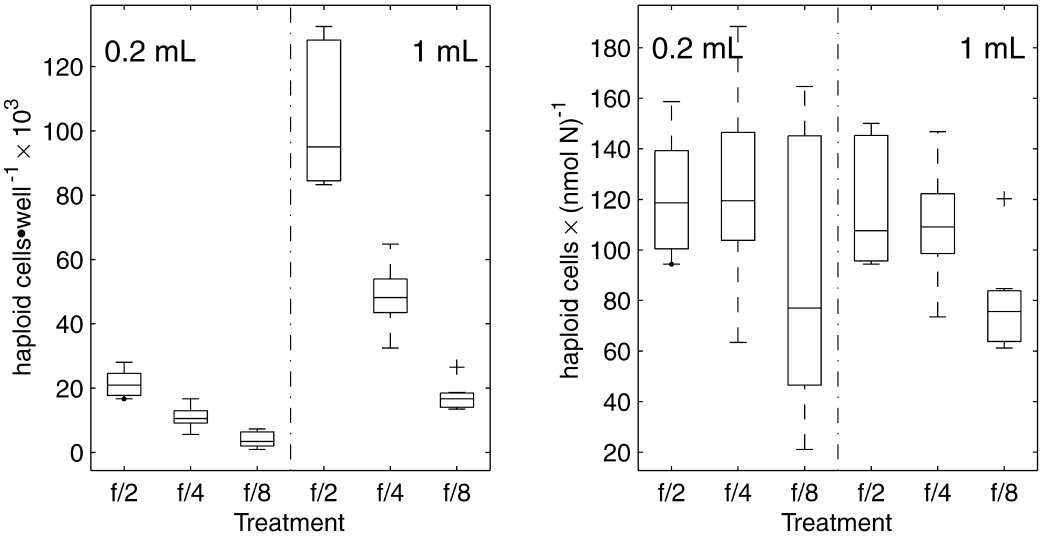

FIG. 4. Box and whisker plots showing the haploid cell yield in 96-well (0.2-mL volume; left part of each panel) and 48-well (1-mL volume; right part of each panel) tissue culture plates filled with different strength media. The left panel shows the absolute cell numbers per well, and the right panel shows cell numbers normalized by dividing with the total nutrient amount in the particular well. The box has lines at the lower quartile, median, and upper quartile values. The whiskers are lines extending from each end of the box to show the extent of the rest of the data. Outliers are marked with +.

cystment occurred before day 4. Cysts accounted for 10%–30% of the total haploid cell numbers between days 4 and 16, but a considerable increase in cyst numbers occurred after day 16 (f/4 medium) or day 24 (f/2 medium). These results indicate that encystment occurred before the vegetative cell population peaked. At the end of the experiment, inorganic nutrients were depleted (0.026–0.5 μm P; 0.03–0.55 μm N) and there were no consistent differences between the treatments.

Cyst volume decreased during the course of the experiment by a factor of 0.24–0.4 (Fig. 5). From the digital images we estimated that only a minor part of the mean cyst volume decrease was due to absence of calcareous cover, and most of it was due to a true decrease of the protoplast volume.

Effect of nutrients on encystment success. Starting with an initial concentration of 365 cells·mL^{-1}, new cysts appeared in the culture plate wells after 10 days of incubation under standard growth conditions. Encystment proceeded very quickly, and the experiment was terminated on day 14. As expected, the haploid cell yield was highest in f/2 medium (Table 5). However, both in f/20 NO_3^- and f/20 PO_4^{-3} medium, which had only 10% of the concentration of N and P, respectively, the haploid cell yield was 18% of that in f/2 medium, suggesting a nonlinear relationship between a single nutrient limitation and cell yield. In contrast, the f/2+NH_4^+ medium had 20% of the f/2 N concentration but yielded only 3% of the f/2 haploid cell yield. Cyst concentration (up to 10^5 cysts·mL^{-1}) and encystment percentage were very high (94%) in f/2 medium and considerably lower in the N or P deficient medium (ca. 3 × 10^3 cysts·mL^{-1}; 15% and 20%, respectively). Ammonium, as the sole N source, resulted in lowest encystment success (7%; Table 5). Assuming that the cell yield was limited by N in f/20 NO_3^- medium and by P in f/20 PO_4^{-3} medium, the N and P quota should approximate the minimum cellular requirements of these limiting nutrients. Thus, the lowest N quota was on average 2.5 pmol N per haploid cell, and the lowest P quota was 0.09 pmol P per haploid cell.

TABLE 4. Parameter estimates (±SE) of fitting a logit model to cyst germination data.

Parameter	Estimate
Germination (thick wall; f/8)	0.7833 ± 0.4533
Thin-wall effect	1.3953 ± 0.3111
f/2 medium effect	−0.8892 ± 0.462
f/4 medium effect	−1.4517 ± 0.5228

DISCUSSION

The present study documents an unprecedented high encystment success (close to 100%) of *S.* cf. *lachrymosa* in batch cultures. The high encystment success was achieved in full strength f/2 culture medium, and the cyst yield (ca. 10^5 cysts·mL^{-1}) indicated that virtually all the available mineral nutrients were transferred into the resting cyst biomass (see discussion on cyst nutrient quota below). These findings give rise to two possible interpretations. First, our *Scrippsiella* strain is a superb cyst producer, distinct in this respect from all other hitherto cultured dinoflagellates. Alternatively, high encystment success is commonplace in na-

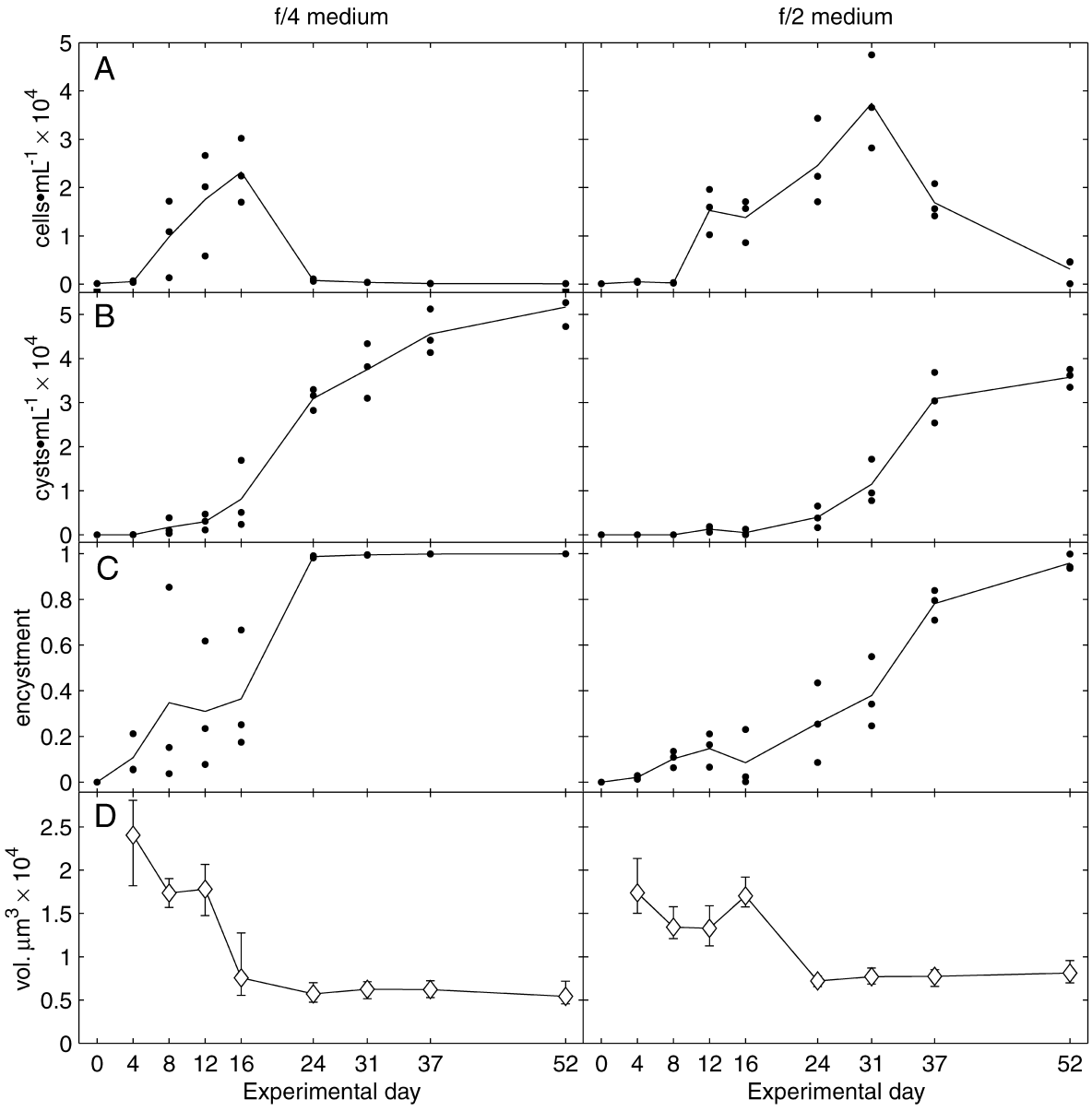

FIG. 5. Time course of *Scrippsiella* cf. *lachrymosa* encystment in f/2 (right) and f/4 (left) media. (A–C) Dots denote values from replicate tubes, line is the mean of the three replicates. (A) Motile cell concentration. (B) Cyst concentration. (C) Proportion of cysts in the total population [2 × cysts/(2 × cysts + motile cells)]. (D) Median of cyst volume (diamonds). Error bars denote the 25th and 75th percentile.

ture, and the low encystment success reported in laboratory cultures reflects a mismatch between the physiological requirements of the cells and culture conditions. Laboratory observations would generally not be indicative of the true encystment potential of populations in natural waters. Clearly, an unambiguous explanation is not possible at this time, though we favor the second simpler alternative.

Encystment success has been an intriguing but elusive aspect of dinoflagellate population dynamics. The ability of some dinoflagellate species to form cysts was observed in natural plankton samples over a hundred years ago. However, only during the last few decades have developments in culturing methods allowed biologists to observe the entire life cycle of dinoflagellates in laboratory cultures (e.g. von Stosch 1964, 1965, 1973, Pfiester 1975, 1976, 1977). Laboratory experiments suggest that only a relatively small fraction of the dinoflagellate populations form cysts in cultures. Estimates vary but usually are well below 40% (Dale 1983, Anderson et al. 1984, Anderson and Lindquist 1985, Binder and Anderson 1987, Lirdwitayaprasit et al. 1990, Park and Hayashi 1993, Montresor and Marino 1996).

The causes for the low to moderate encystment success in batch cultures remain unclear. Physiologically, cyst formation is preceded by accumulation of

TABLE 5. Mean haploid cell (vegetative cells + 2 × cysts) yield (10^3 cells·mL^{-1}), encystment percentage, and the average cellular N and P quota of the culture (pmol N and P per haploid cell) as a function of medium type.

Medium	Haploid cells 10^3	Encystment percentage	N quota	P quota
f/2	191.8 (±12.3)	93.9 (±1.1)	4.6	0.19
f/2+NH_4^+	5.8 (±0.4)	6.5 (±2.0)	N/A	N/A
f/20 NO_3^-	35.3 (±2.8)	14.7 (±3.5)	2.5	N/A
f/20 PO_4^{-3}	35.5 (±2.2)	19.5 (±5.4)	N/A	0.09

Standard error of the mean is shown in parenthesis. N/A, not applicable (nutrient depletion cannot be assumed).

storage products such as lipids and carbohydrates (Binder and Anderson 1990). Studies in which the planozygote stage was observed indicate that this accumulation occurs before encystment (von Stosch 1973, Chapman et al. 1982, Lirdwitayaprasit et al. 1990). Anderson (1998) proposed that the rapid decrease of mineral nutrients in batch cultures of *Alexandrium tamarense* might prevent all but the first formed planozygotes to complete encystment, leaving the bulk of fused gametes arrested in the planozygote stage. This ultimately results in low encystment percentages.

The cysts of *S. lachrymosa* are larger than the vegetative cells (Lewis 1991, D'Onofrio et al. 1999), perhaps as a result of accumulation of reserves before encystment. The volume decrease of cysts produced in later growth stages in the present study suggests that the population did not have sufficient resources to produce normal-sized cysts. We believe that the first thick-walled cysts were produced in relatively resource-replete medium, and the smaller size of the thin-walled cysts produced in later stages indicate that the medium was depleted. However, unlike *Alexandrium tamarense* (e.g. Anderson and Lindquist 1985), all *S.* cf. *lachrymosa* vegetative cells went through the gametic stage, formed planozygotes, and encysted successfully despite the severe resource limitation. The apparent mismatch between nutrient availability and population size resulted in decreased volume of the cysts by a factor of 0.25–0.4. This volume decrease was not simply due to the lack of a calcareous cover; the cyst protoplasm was smaller in the cysts formed late in the culture process. We are also confident that the smaller cysts without a calcareous cover were true sexual cysts rather than temporary pellicle cysts (e.g. Anderson and Wall 1978, Garcés et al. 1998) that were never observed in the cultures. This confidence is based on the morphological similarity (i.e. the overall shape, presence of accumulation body, and other cellular constituents). The long mandatory dormancy period is also indicative of true sexual cysts.

Germination success. We hypothesized that the small cysts produced under severe resource deficiency would exhibit poor viability and low germination success. The germination percentage of all *S.* cf. *lachrymosa* cysts (70%) was comparable or slightly lower than maximal germination percentages (70%–90%) found in other species (Anderson 1980, Lirdwitayaprasit et al. 1990, Bravo and Anderson 1994, Montresor and Marino 1996). The high germination success of thin-walled cysts (79%) falsified our hypothesis that these cysts were aberrant and had poor viability. Surprisingly, germination success of the thick-walled cysts was only 48%. One possible explanation to this paradoxical finding could be that for maximum germination success, the thick-walled cysts required a longer maturation or mandatory dormancy period compared with the thin-walled cysts. When estimating the length of the mandatory dormancy period, we did not discriminate between the cyst types nor did we study any possible change in the germination success as a function of storage conditions during the dormancy. It remains to be seen if the thick-walled cysts demonstrate better viability after prolonged storage times.

Nutrient effects on encystment success. The high encystment success was achieved using full strength f/2 medium, which is in sharp contrast with previously published works (Pfiester 1975, Turpin et al. 1978, Walker and Steidinger 1979, Anderson et al. 1985, Binder and Anderson 1987, 1990). In the present study, encystment percentage dropped dramatically when either N or P were supplied at 1/10th of the f/2 concentration. This suggests a dependence of encystment success on the type of nutrient depletion. Success is low if exponential growth is interrupted by the depletion of only one major nutrient and high if nutrients are supplied close to preferred ratio (see below) and colimitation occurs. Encystment in small volumes, like the tissue culture plate wells, was remarkably synchronous, and transition from motile population to resting cysts took about 2 days. The experiments were terminated 4 days after the appearance of cysts, and we are therefore confident that shortage of a single nutrient had a clear negative effect on encystment success. Ammonium as the sole N source resulted in poor total cell yield and impaired encystment efficiency. Encystment percentage was highest in f/2 medium, which has an N:P (molar) ratio of 24. Even more, equivalent reduction of either N or P resulted in identical reductions in cell yield. Interestingly, the ratio 24 is quite close to 28, which was the ratio of the cellular N quota in N-limited culture (2.5 pmol N·haploid cell^{-1}) to the cellular P quota in P-limited culture (0.09 pmol P·haploid cell^{-1}; Table 5). We lack direct evidence, but it is tempting to speculate that 28 is the preferred N:P uptake ratio for *S.* cf. *lachrymosa*. Although there could be a difference in preferred N:P for vegetative cells and cysts, our results suggest that much more N relative to P was taken up compared with the Redfield ratio of 16.

At this point, a note of caution is warranted. Although close to 100% encystment occurred routinely in f/2 medium, there are many poorly understood factors that can decrease encystment success. Most remarkably, in the months of August and September, encystment success declined to negligible levels (data not shown). This behavior was consistent in 2 consecutive

years, 1999–2000. An internal clock type of regulation (Anderson and Keafer 1987) could play a role here, but more likely the decrease depends on some poorly understood and seasonally changing property of the natural seawater used in the culture medium. This inference is based on simultaneous encystment experiments conducted in September with a fresh batch of Vineyard Sound seawater and a batch originating from the same location the prior spring, both enriched to f/2 level and inoculated with the same cell culture. Although high encystment occurred in the spring batch, very few cysts were formed in the September medium.

Nutrient quota of cysts. Another noteworthy result was the high final cyst yield, which often reached 10^5 cysts·mL^{-1} and above. Assuming that the inorganic nutrient concentration in the Vineyard Sound seawater, used as the culture medium base, was low compared with f/2 medium and given that the measured final residual concentrations of N and P were below 0.5 μmol, we can use the initial nutrient concentrations of the medium to roughly approximate the total N and P quota of the cells and cysts. Using a conservative cyst yield of 10^5 cysts·mL^{-1} in f/2 medium, these calculations give ca. 9 pmol N·cyst^{-1} (126 pg N·cyst^{-1}) and 0.4 pmol P·cyst^{-1} (12.8 pg P·cyst^{-1}). When either nutrient was supplied at lower concentrations, the quota for the limiting nutrient was even lower: 5 pmol N diploid·cell^{-1} (70 pg N·diploid cell^{-1}) and 0.18 pmol P diploid·cell^{-1} (5.8 pg P·diploid cell^{-1}; see also Table 5). Note that the latter are mean values for all cell types. We cannot rule out that the subpopulation which successfully encysted (15% and 20% with N and P shortage, respectively) had cellular quota different from the mean. Nevertheless, these are considerably lower values than reported in the few studies of dinoflagellate cell composition during encystment. New cysts of a congeneric species, *Scrippsiella trochoidea* (Stein) Loeblich III, contained 100 pmol N·cyst^{-1}, which decreased to 60 pmol N·cyst^{-1} after cold storage; total P content was 19 pmol P·cyst^{-1} (Lirdwitayaprasit et al. 1990). Volume normalized N and P content of *S. trochoidea* cysts, based on 28 μm length and 23 μm width (Binder 1986), was ca. 6 and 50 times higher, respectively, in *S. trochoidea* (Lirdwitayaprasit et al. 1990) compared with *S.* cf. *lachrymosa* in our study.

In a study of *Gyrodinium uncatenum* Hulburt, Anderson et al. (1985) found a subsistence quota of 230 pg N·cell^{-1} and 29 pg P·cell^{-1}. Using a cell volume of 45,000 μm^3 for *G. uncatenum* (Coats et al. 1984), we calculate that the volume normalized N and P quota in Anderson et al. (1985) exceed our estimates for *S.* cf. *lachrymosa* by a factor of 4 and 12, respectively. Rengefors et al. (1996, 1999) measured the P content of cysts of cultured *S. trochoidea* (28–72 pg P·cyst $^{-1}$) and natural populations of *Ceratium hirundinella* (O. F. Müller) Dujardin cysts (16–37 pg P·cyst $^{-1}$). The above comparisons are dependent on both the accuracy of the nutrient content and cyst volume measurements and thus are only approximations. Nevertheless, our calculated estimates (5 pmol N and 0.18 pmol P diploid·cell^{-1}) are clearly at the lower range reported in the literature. We did not measure directly the N and P content of the cells, but it is obvious that the cellular nutrient quota cannot exceed the total amount provided by the medium divided by the total cell yield.

Encystment induced by nutrient limitation. Numerous laboratory studies suggest that the induction of sexuality occurs as a result of nutrient limitation (von Stosch 1973, Pfiester 1975, Turpin et al. 1978, Anderson and Lindquist 1985, Blackburn et al. 1989, Doucette et al. 1989, Park and Hayashi 1993), yet this is not well supported by field measurements (Anderson et al. 1983, Kremp and Heiskanen 1999). Given the fact that cyst formation in our study was highly successful in f/2 medium, in sharp contrast most previously published culture experiments, we hypothesized that encystment occurred in nutrient-replete conditions and was induced by some other factor, like culture density or number of cell divisions since germination. To test the former hypothesis, we compared the total haploid cell yields produced from one cyst while varying the total nutrient amount 20-fold (5-fold difference in medium volume combined with 4-fold difference in medium concentration). If a certain nutrient concentration was to initiate sexuality, we would expect the total haploid cell yield at the onset of cyst formation to be in the ratio of 4:2:1 in f/2, f/4, and f/8 media, respectively. In addition, the total haploid cell yield in 1-mL and 0.2-mL volumes should have a ratio of 5:1. Alternatively, if sexuality is triggered by other factors like the number of divisions or population density, we would expect the haploid cell yields to be approximately equal in all treatments or equal within a given volume treatment, respectively.

The results revealed that the haploid cell yield, by the time the first cysts were seen, was indeed proportional to the total amount of nutrients. Thus, sexuality was not induced until the nutrients were depleted to a certain level. Once this critical concentration was reached, encystment occurred in all cases. One caveat to this scenario was the earlier encystment in f/8 medium, that is, the first cysts appeared at a lower nutrient-normalized cell concentration compared with other media. We believe this discrepancy was due to the slightly lower growth rate in the most dilute medium. In other words, during the time interval from induction of encystment to the appearance of the first cysts, the faster growing populations in f/2 and f/4 media reached higher cell densities than the populations in f/8 medium. Our results do not support the idea that encystment starts in nutrient-replete medium after a certain number of divisions since germination.

Low nutrient-induced encystment seems to contradict the results from our test tube experiment where cysts were formed in a relatively early phase before the peak of the vegetative population. One possible explanation could be that the dense aggregation of cells in the upper part of the test tubes, which was always observed in the exponential and early stationary phases

of growth during the light interval, created local nutrient minima that were sufficient to induce sexuality. Ultimately, the gradual diffusive erosion of nutrient gradients and/or possible vertical migration of the cells within the tube during the dark interval made all the nutrients available over a longer time period. In contrast, the small volumes in tissue culture plates do not support significant cell aggregation and concentration differences. This could explain the relatively gradual cyst accumulation in test tubes, compared with the remarkably rapid and synchronous encystment in culture plates. Although the culture plate experiment indicated that a certain low nutrient concentration had to be reached to induce sexuality, we cannot rigorously rule out the possibility that in the test tubes, encystment was initiated by other factors in nutrient replete conditions.

Dormancy and activation time. The dormancy period of newly formed cysts was approximately 2 months. This is well within the very broad range from a few hours (Pfiester 1977) to several months (Anderson 1980) to ca. 1 year (Perez et al. 1998) reported for various dinoflagellates. Our data indicate that the dormancy period was not significantly affected by dark cold storage. Cold conditioning can affect the length of cyst dormancy period in different directions. Montresor and Marino (1996) showed that the dormancy period of *Alexandrium pseudogonyaulax* (Biecheler) Horiguchi ex Yuki et Fukuyo shortened from 190 days to 75 days when cysts were exposed for 40–60 days to cold dark conditions. In contrast, Anderson (1980) found that 22° C temperature led to rapid maturation of the hypnozygotes of a congeneric species *Alexandrium tamarense* (formerly *Gonyaulax tamarensis* Lebour). In that study, germination was possible 1 month after formation, whereas with 5° C storage, the first revivals occurred after nearly 4 months.

In the present study, germination of mature cysts, once transferred to favorable conditions, was remarkably synchronous. Germination frequency peaked 5 days after the transfer, which also matched the lag period of the original cyst isolated from the Casco Bay. This is in accord with Binder and Anderson (1990), who showed that cysts of *Scrippsiella trochoidea* stored at 3° C and transferred to 18° C germinated within a few days. Large interspecific variation is demonstrated by much longer lag periods (19–25 days) reported by Montresor and Marino (1996) for cysts of *A. pseudogonyaulax*.

Proportion of successfully encysting population. The proportion of vegetative population, which successfully forms cysts, has a distinct biological meaning. If this proportion is small, much of the resources acquired by the vegetative population are lost and cannot be passed to the next bloom generation. In several studies, the ratio of total cyst yield to peak cell concentration has been used as the fraction of the population that encysts successfully (Anderson et al. 1984, 1985). However, as correctly noted by Anderson et al. (1985), this index is only an approximation, because some cysts can be formed before the motile cell maximum occurs in batch culture. More importantly, the above ratio can be used to approximate the fraction of the successfully encysting population only if the population ceases vegetative division after the motile cell maximum. This was clearly not the case in our study and probably is not true generally, which invalidates the interpretation of the above ratio as the fraction of the population that successfully encysts. It can easily be imagined that with parallel vegetative and sexual production, the cumulative amount of cysts can exceed the peak motile population size. In the present study the motile population peaked on day 16 in f/4 medium (2.3×10^4 cells·mL^{-1}) and on day 31 in f/2 medium (3.7×10^4 cells·mL^{-1}). The final cyst yields were 5.2×10^4 cysts·mL^{-1} and 3.6×10^4 cysts·mL^{-1} in f/4 and f/2, respectively. Acknowledging that each diploid cyst was produced by the fusion of two haploid cells, we calculate that the cumulative amount of cysts accounted for 195% and 452% of the maximum vegetative cell populations in f/2 and f/4, respectively.

There is a clear need for a biologically meaningful index to quantitatively express cyst formation in relation to the vegetative population size. In culture experiments we suggest the use of the terms "encystment success" and "encystment percentage" interchangeably, as defined here, and discourage the comparison of final cyst yield with maximum motile population size, because it has no well-defined upper limit. The final encystment percentage, estimated when population growth has ceased and cyst numbers have stabilized, can be interpreted as the fraction of successfully encysting population under a given culture condition.

CONCLUSIONS

We found unusually high encystment efficiency and cyst yield in a cultured strain of *S.* cf. *lachrymosa.* We believe that our results reflect a good match between the culture conditions and the physiological preferences of the strain. High encystment efficiency could be commonplace in natural waters, being mainly constrained by other losses such as grazing, turbulent mixing to deeper layer, or low encounter rates of compatible gametes in low density populations. Estimating encystment efficiency reliably in field conditions is notoriously difficult, leaving laboratory experiments as a more rigorous alternative.

Sexual reproduction was induced at a certain low nutrient concentration, even in batch cultures that started with f/2 levels. This indicates that the growth of the particular strain in not density limited, at least up to cell numbers as high as the f/2 medium could yield. The generally reported lack of encystment in high nutrient batch cultures of other dinoflagellates might well be interpreted as the inability of a particular species to reduce the initial nutrient concentrations to limiting levels, possibly because of density-limited growth. *Alexandrium tamarense*, for example, appears to be carbon limited in dense cultures grown in f/2 medium (Anderson et al. 1983). The ability of any partic-

ular dinoflagellate species to produce cysts in f/2 medium might thus depend on its potential to grow to the very high densities, which inevitably lead to nutrient depletion, not on its ability to reproduce sexually under high nutrient conditions.

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