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FORMATION OF RESTING SPORES BY *LEPTOCYLINDRUS DANICUS* (BACILLARIOPHYCEAE) IN A CONTROLLED EXPERIMENTAL ECOSYSTEM[1]

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ABSTRACT

Leptocylindrus danicus *Cleve became the dominant phytoplankton species, comprising 70–80% of the total assemblage, in one of the CEPEX Controlled Experimental Ecosystems (CEE) at Saanich Inlet, British Columbia (Canada). In the first week of June, when nitrate levels were reduced below 0.5 μm, the majority of the* L. danicus *cells present in the CEE formed resting spores. The spores were heavily armored with spines and appeared to sink unmolested to the bottom of the CEE. Four continuous cultures were started with an inoculum from the CEE and, in a 24 h period when N became depleted, 86% of the* L. danicus *present (~80% of the total phytoplankton assemblage) formed resting spores. A daily dilution culture with nutrients kept at saturating levels was started at the same time from the same inoculum and continued for 2 wk beyond the spore formation event in the N-limited cultures. No spores were observed in the nutrient-saturated culture, indicating that N limitation was necessary to trigger spore formation. Spores were kept in the dark at 3 and 10 C. After 36 and 97 days, a large percentage of the spores germinated. After 214 days, >1% of the spores were still capable of germination. Laboratory studies with* L. danicus *isolated from the CEE confirmed that N limitation was the primary factor triggering spore formation. Microscope observations of these cultures indicated that the spores were formed by auxospores following sexual reproduction.*

Key index words: CEPEX; controlled experiment ecosystem; diatom; Leptocylindrus; *nitrogen limitation; resting spore,* Leptocylindrus

The formation of resting spores by *Leptocylindrus danicus* Cleve (Gran 1915a) and by other marine centric diatoms (Drebes 1966, Hargraves 1976), is a well-known and apparently common phenomenon. Spores are frequently reported in coastal phytoplankton samples from the water column (Hargraves and French 1975) and from the sediments (Hargraves and French 1975, Gucluer and Gross 1964). Some success has been achieved in inducing spore formation in laboratory cultures (Durbin 1978) and in testing the survival and germination of spores under a variety of laboratory conditions (Hargraves and French 1975). The above approaches have provided a great deal of useful but fragmented data. To fully assess the importance of spores in the life cycle of diatoms requires a wide ranging set of data including: i) environmental conditions which induce spore formation; ii) biological processes involved in spore formation; iii) sinking and survival of spores including resistance to grazing and environmental stresses, such as temperature changes, darkness, and anoxic conditions; and, iv) conditions for germination of spores and production of the new vegetative population.

In the marine environment, it is difficult to associate spores with the parent population or the environmental condition which induced spore formation since the spores sink 5–6 times faster than the parent population (P. Bienfang, Oceanic Institute, Hawaii, pers. comm.) and are therefore quickly separated vertically and subsequently horizontally (by advection) from the vegetative population. Conversely, laboratory experiments are useful in establishing a range of conditions for spore formation,

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survival, or germination, but it is very difficult to relate these data to the specific suite of variables encountered by that species in the natural environment.

Resting spore formation was frequently observed in the CEPEX Controlled Environmental Ecosystems (CEE; Menzel and Case 1977). The CEEs offer a unique opportunity in that they are large enough (1,300 m^3) to observe the formation and sinking out of the spore population, but they exclude advection which masks cause and effect relationships in the natural environment. This paper reports the observation of one such spore formation event in a CEE in May and June 1977, including a number of auxilliary experiments that examined conditions which induced spore formation, the biology of the spore formation process, and the survival and germination of the spores.

MATERIALS AND METHODS

CEE experiments. The CEPEX Controlled Experimental Ecosystems are large plastic cylinders used to enclose representative water columns in Saanich Inlet, British Columbia (Canada). The spore formation event described in this paper occurred in CEE 77-3 which was the control CEE for a trace metal experiment. The CEE did not receive any trace metal additions, but deep water was upwelled from the bottom of the CEE to the surface and nutrients (N, P, Si) were added as noted in Table 1 and Fig. 1. All analyses were performed on integrated samples collected biweekly from five depth intervals (0–4, 4–8, 8–12, 12–16, and 16–20 m) using a peristaltic pump.

Nutrients from the CEE samples were analyzed by hand methods following Strickland and Parsons (1972). Specifically, ammonia was analyzed by the phenol-hypochlorite method (Solórzano 1969), nitrate by the cadmium-copper reduction method (Wood et al. 1967), phosphate by the molybdate-ascorbic acid method (Murphy and Riley 1962) and silicate by the molybdate-metoloxalate method (Mullin and Riley 1955).

Phytoplankton samples were taken as part of the routine sampling procedure, preserved in Lugol's solution, settled overnight, and phytoplankton species enumerated using a phase contrast inverted microscope (Utermöhl 1958, Thomas and Seibert 1977). Cell volumes were calculated from appropriate measurements of cell dimensions and used to determine cell carbon content.

Outdoor continuous cultures. A water sample taken from 4–8 m in CEE 77-3 on 2 June and filtered through a 143 μm net to remove large zooplankton was used as an inoculum for outdoor continuous culture studies. Four N-limited continuous cultures were incubated outdoors in 2 l borosilicate flasks in a water bath incubator at 10.5 + 0.5 C with a blue Plexiglas light shield designed to approximate 5 m underwater light. The irradiance was about 50% of incident radiation (~300 $\mu Ein \cdot m^{-2} \cdot s^{-1}$). Millipore filtered (0.45 μm) water from the CEE was enriched and used for the feed water for the continuous cultures. Details of the culture methods and the results of these continuous culture experiments are published elsewhere (Harrison and Davis 1979), and only observations on spore formation by *L. danicus* in these cultures are included in this paper.

A fifth culture was started from the same inoculum and was kept in the same incubator. The culture was diluted daily with high nutrient water (Millipore filtered CEE water with nutrients added to approximate f/2; Guillard and Ryther 1962) so that the culture always had an adequate supply of nutrients. This culture was used to estimate the maximal growth rate of the phytoplankton assemblage from the CEE.

Spore survival and germination. Leptocylindrus danicus spores were collected from a sediment sample from the bottom of CEE 77-3 on 17 June using a peristaltic pump. A portion of the sediment sample was washed 2× with filtered seawater and 10 ml of the sedimented material added to 200 ml of filtered seawater and stored at either 3 or 10 C in the dark. Four months later, the samples were transported to the Scripps Institution of Oceanography (SIO) on ice in the dark, and stored at 2 C in the dark thereafter. They were washed periodically (ca. monthly) by decanting the supernatant and replacing it with filtered seawater.

At various times after collection, samples from these cultures were tested to see if the spores were viable. This was accomplished by placing 1 ml of the spore suspension in 50 ml of medium contained in 125 ml Pyrex Erlenmeyer flasks and then incubating these cultures in the light. When the tests were performed at CEPEX, the medium was Saanich Inlet seawater filtered through a 0.45 μm millipore filter and enriched with 60, 60, and 6 μM of NO_3^-, $Si(OH)_4$, and PO_4^{3-}, respectively. Illumination was provided by a bank of cool-white fluorescent lights operated on a 17/7 LD cycle providing 120 $\mu Ein \cdot m^{-2} \cdot s^{-1}$ irradiance, and the incubation temperature was 12 C. At SIO, the medium was f/2 (Guillard and Ryther 1962), illumination was provided by a bank of cool-white fluorescent light providing 120 $\mu Ein \cdot m^{-2} \cdot s^{-1}$ of continuous illumination, and the incubation temperature was 20 C. Viability was judged by inspecting 2.2 ml samples for vegetative cells at 200× on a Zeiss Inverted Microscope after the cultures had incubated for 5 days.

RESULTS

Spore formation in the CEE. The CEE experiment was initiated on 2 May. *Leptocylindrus danicus* was first observed in the CEE on 6 May and increased at a modest rate reaching peak abundance in early June (Table 1, Fig. 1a). Spores were first encountered in significant numbers on 6 June. Both vegetative cells and spores remained in high numbers through 9 June and then both declined rapidly. Vegetative growth of *L. danicus* and spore formation occurred in the upper 8 m (Figs. 1a, b). The vertical profiles show that the spores were formed over a short time period and sank rapidly to the bottom (Fig. 1b). The peak abundance of spores was in the 4–8 m depth interval on June 6. However, by June 9, the abundance peak was in the 16–20 m depth interval.

The spores observed in the water column on June 20 and 23 were spores resuspended by upwelling of the sediments from the bottom of the CEE to the surface on June 16 (after sampling was completed for that day). Both sets of observations (6–9 and 20–23 June) suggest that the spores were sinking at 2–3 $m \cdot d^{-1}$. By comparison, the change in the depth distribution of the vegetative cells of *L. danicus* (Fig. 1a) during late May suggests that the vegetative cells sink much more slowly (<1.0 $m \cdot d^{-1}$). Studies of vegetative cells and spores of several *Chaetoceros* spp. in CEE experiments in 1978 showed a similar relationship with the vegetative cells sinking at 0.5 $m \cdot d^{-1}$ and the spores 2.75 $m \cdot d^{-1}$ (P. Bienfang, pers. comm.).

The nutrient environment in the CEE is difficult to interpret due to the addition of nutrients and upwelling of sediments (including regenerated nutrients, especially NH_4^+ and PO_4^{3-}). Spore forma-

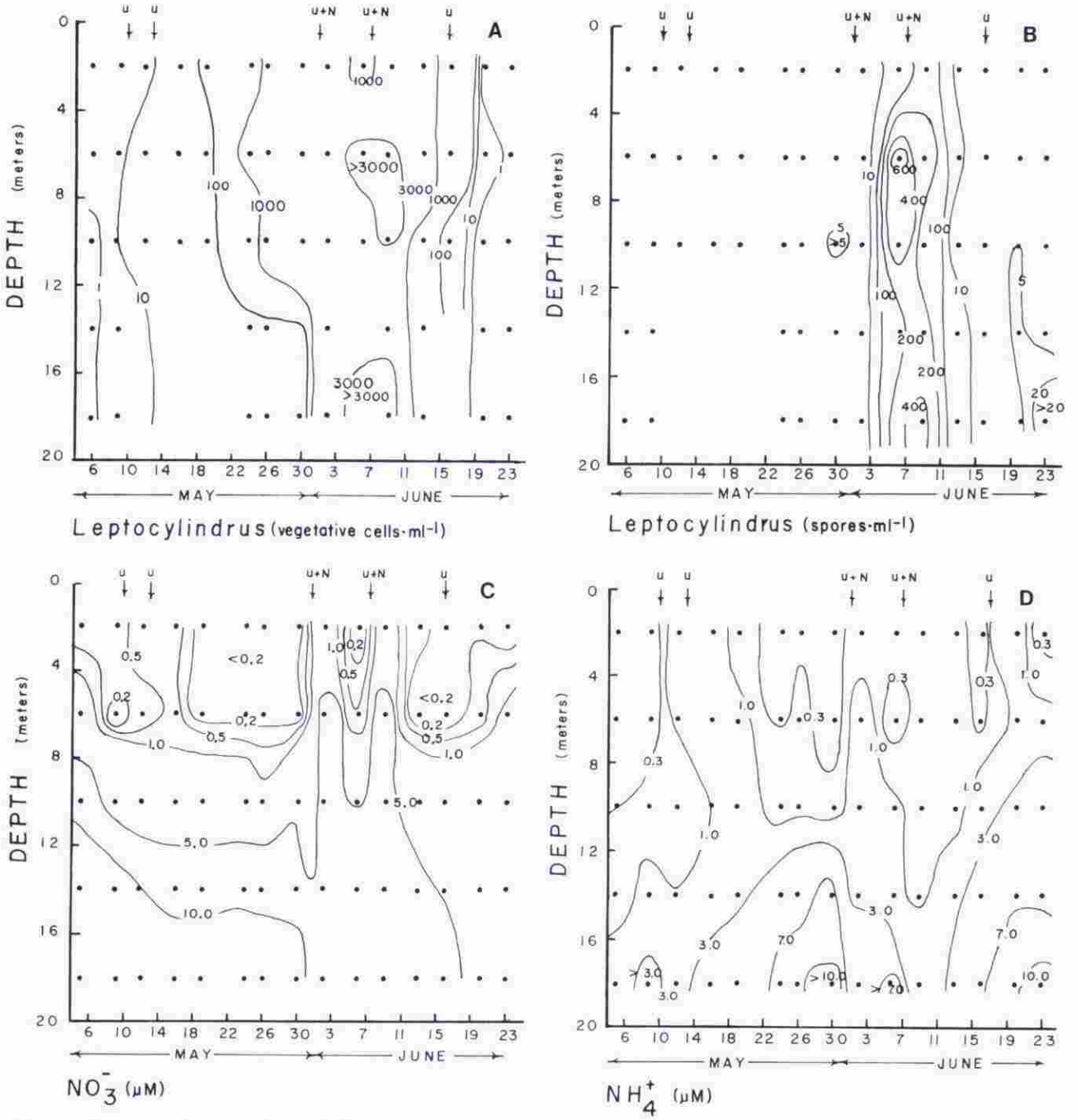

FIG. 1. Depth distribution of *Leptocylindrus danicus* and nutrient conditions in CEE 77-3 during the spore formation event in May and June 1977: A, *L. danicus* vegetative cells; B, *L. danicus* resting spores; C, nitrate; D, ammonia.

tion took place on 6 June in the upper 8 m of the CEE and can be correlated with the combination of two conditions. First, *L. danicus* was in bloom concentrations and was clearly the dominant phytoplankton species (>86% of the total phytoplankton C) and secondly, the total available inorganic N was near zero (Table 1, Figs. 1c, d). The importance of this combination of factors was verified by the continuous culture experiments described below.

A third factor, that the cells were first N-limited then N was resupplied and again depleted before spore formation began may have effected the spore formation process. It is doubtful that this pattern is necessary to induce spore formation, however, the rapid depletion of the added N by the large standing stock of phytoplankton present at that time may have increased the synchrony of spore formation. The same pattern was used in our previous study to

TABLE 1. *Population growth and spore formation by* Leptocylindrus danicus *in CEE 77-3. Treatment U indicates artificial upwelling of water and sedimented material from bottom of CEE to top 5 m: volume upwelled varied from 0.5 to 1% of volume of CEE, depending on amount of sediment material present. Treatment N indicates addition of sufficient nutrients to increase concentrations in entire CEE on 31 May by 1.9 μM of nitrate, 0.16 μM of phosphate, and 7.3 μM of silicate; and, on 7 June by 1.5 μM of nitrate: CEE was mixed thoroughly on these days, and therefore, increase in nutrient levels in top 8 m was greater than additions.*

		Leptocylindrus danicus (0–20 m)					Nutrients μM (0–8 m)		
		Carbon		Cells/ml					
Treatment	Date	μg C/ℓ	% of total	Vegetative	Spores	Vegetative apparent doublings/day	NH_4^+	NO_3	$Si(OH)_4$
	6 May			1	0		.2	1.02	3.96
	9			6	0	.86	.16	.21	1.54
U	10								
	12			11	0	.29	1.24	.52	3.07
U	13								
	16			25	0	.29	1.81	.63	3.42
	19			71	0	.50	1.07	0	4.59
	24			466	0	.54	.24	.10	2.78
	26	128	66	831	0	.42	.34	0	1.10
	30	211	94	1,380	1	.18	.04	.08	.54
U + N	31 May								
	2 June	329	84	2,376	0	.26	.99	4.72	15.0
	6	265	93	2,150	374	−.04	.30	.28	7.76
U + N	7								
	9	440	91	3,042	255	.17	.29	4.50	22.8
	13	139	92	999	12	−.40	.62	.19	14.3
	16			498	0	−.33	−.02	.09	11.6
U	17								
	20			.6	3	−2.42	1.52	.31	15.6
	23 June			.2	7	−.53	.60	.61	10.9

induce synchronized sexual reproduction in *Skeletonema costatum* under silicate limitation (Davis et al. 1973).

Continuous and semi-continuous culture experiments. In the continuous cultures, the inoculum which was obtained from the CEE (2 June) was allowed to grow for two days as a batch culture and deplete the ambient N supply in the reactor. Then the pumps were started and the cultures continued growing under N-limited conditions. The phytoplankton assemblages were clearly dominated by *L. danicus*, and the vast majority of the *L. danicus* cells formed spores one day after pumping was initiated on 4 June (Table 2). By contrast, no *L. danicus* spores were observed in the semi-continuous culture started from the same inoculum and incubated under identical conditions, but with excess nutrients. This latter culture was continued for 14 days beyond the spore formation event in the N-limited cultures and in the CEE.

In February 1978, a *L. danicus* culture which had been isolated from CEE 77-3 and maintained at the University of British Columbia, was tested for spore formation ability. When a batch culture was grown at 15 C and an irradiance of 75 $\mu Ein \cdot m^{-2} \cdot s^{-1}$, the majority of the vegetative cells present formed spores when the culture became N-limited. Detailed observations with a phase contrast light microscope indicated that the spores were formed by auxospores following fertilization of egg cells by male gametes (Figs. 2a–d).

Some evidence from the CEE and continuous culture data also supports the idea that the resting spores were formed by auxospores. The total number of *L. danicus* cells (vegetative cells plus spores) was greatly reduced following the spore formation event (Tables 1, 2). This suggests that spore formation is a sexual process requiring several spermatogonial cells and perhaps several oogonial cells for each successful auxospore.

In the 1978 CEE experiments, the formation of resting spores by several *Chaetoceros* spp. was observed and, in all instances, there was not marked decrease in cell numbers of *Chaetoceros* at the time of the spore formation event. In this genus spores are known to be formed asexually (von Stosch et al. 1973). Long chains with a spore inside each vegetative cell were observed in the *Chaetoceros* spore forming events, however, in the case of *L. danicus*,

TABLE 2. *Changes in the* Leptocylindrus danicus *populations in four N-limited continuous cultures at time of spore formation.*

Date	Culture	Dilution rate (d^{-1})	Vegetative cells $\cdot ml^{-1}$ (% of total assemblage)	Spores $\cdot ml^{-1}$ (% of total *Leptocylindrus*)
4 June	1	0.5	10,100 (78%)	0
	2	0.25	10,100 (78%)	0
	3	0.1	10,100 (78%)	0
	4	0.1	11,700 (84%)	0
5 June	1	0.5	200	1,950 (91%)
	2	0.25	0	2,620 (100%)
	3	0.1	1,960	6,250 (76%)
	4	0.1	600	1,990 (77%)

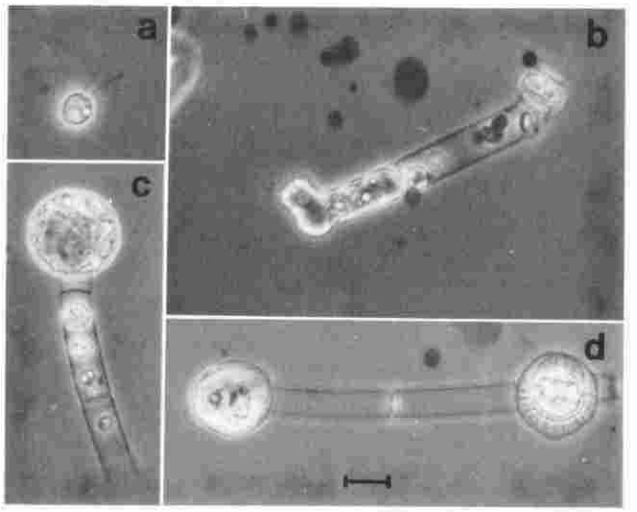

FIG. 2. Stages of resting spore formation by *Leptocylindrus danicus*, suggesting resting spores form from an auxospore: a, male gamete; b, early stage of auxospore formation; c, mature auxospore; d, pair of auxospores, one of which has developed silicon spines as first step to becoming resting spore. Scale = 10 μm.

only single spores or pairs of spores were observed. It should also be noted that *Chaetoceros* resting spores formed inside individual cells, while the *L. danicus* spores were twice as wide as the vegetative cells that produced them. Also the vegetative cells which developed when the spores germinated were wider than the original vegetative cells, a characteristic result of sexual reproduction and auxospore formation.

Spore survival and germination. The spores were formed in the CEE between 3 and 8 June, and by 16 June essentially all the spores were in the sediment (Fig. 1b). When the spores in the suspensions from the 17 June sediment sample were tested on 30 June, >50% of the spores germinated. Over the next 7 mo, viability decreased with >1% of the *L. danicus* spores germinating on 17 January. Subsequent attempts to induce germination failed to produce vegetative cells.

Additional sediment samples were removed from CEE 77-3 on 21 June and 7 July and were tested to see if the spores could survive longer periods (5 and 22 days) in the sediments in the bottom of the CEE. In those tests no viable spores were detected, suggesting that the conditions in the sediments in the CEEs (dark, ~10 C, anoxia, high concentrations of NH_4^+, H_2S and organic matter) greatly accelerated the loss of viability. The spore suspensions from the 17 June sediment sample kept in the laboratory were also in darkness at low temperatures, however, care was taken to avoid high concentrations of organics and anoxic conditions in these suspensions. The comparison of these two results suggests that the high level of decomposing organic matter and/or the anoxic conditions which resulted were responsible for the early demise of the spores in the sedimented material in the bottom of the CEE.

Immediately following the spore formation event in the nitrogen-limited continuous cultures, samples of the spores were collected and incubated in batch cultures with saturating nutrients in the outdoor incubator used for the continuous cultures. The spores in these cultures did not germinate. Successful germination was only achieved in spore suspensions which had experienced a period of darkness at low temperatures as described above for the 17 June sample.

DISCUSSION

Gran (1915b) first observed that *Leptocylindrus danicus* appeared to form resting spores subsequent to sexual reproduction and auxospore formation. We have confirmed that observation (Fig. 2) and our observations that the population of *L. danicus* was greatly reduced during spore formation, that spores were only formed singly or in pairs, and that the post resting spore cells were wider than the parent population all support that conclusion. However, further detailed laboratory studies such as those conducted by von Stosch and Drebes (1964) for *Stephanopyxis turris* (Grev.) Ralfs would be useful to make a definitive statement. Typically, resting spores are formed vegetatively (Drebes 1977), and the transformation of the auxospore directly into a resting spore, as is apparently the case for *L. danicus*, is rare among centric diatoms and could prove interesting for further study.

Manipulations of physical or chemical factors have typically been used to initiate auxospore formation, including changes in light intensity, photoperiod, temperature, and salinity (Drebes 1977). Davis et al. (1973) induced auxospore formation in *Skeletonema costatum* (Grev.) Cleve by rapidly depleting cultures of silicon in the medium. A few experiments and observations of resting spore formation implicate the same range of factors (reviewed by Hargraves 1976). In all, the common pattern is that a sharp change in the controlling factor (temperature, light, salinity, nutrients) is required to initiate spore formation.

We contend from the results of this study and other recent studies that N limitation is the most likely trigger for resting spore formation in the natural environment. Nitrogen limitation was clearly the triggering mechanism in this study, especially as verified by the outdoor N-limited continuous cultures and the nutrient-saturated culture (Table 2). The CEE results are the closest approximation to observations in nature available at this time. Spore formation was also observed for several species of *Chaetoceros* in the CEEs in 1977 (Hollibaugh et al. unpubl.) and in 1978 (Davis and Parsley unpubl.). In each, the spore formation event occurred at the end of a bloom terminated by nutrient limitation.

Durbin (1978) used N limitation in laboratory cul-

tures to trigger resting spore formation in *Thalassiosira nordenskioeldii* Cleve and *Detonula confervacea* (Cleve) Gran. More importantly, recent observations of resting spores in nature equate them with the end of phytoplankton blooms (Hargraves 1972, Hargraves and French 1975). As such blooms are often terminated by nitrogen limitation (Ryther and Dunstan 1971), there is a strong suggestion from the field observations that nitrogen limitation is the likely trigger mechanism.

All of the laboratory studies conducted where spore formation was induced have one common feature—it takes a sudden shock or change to initiate spore formation. In the ocean, light, temperature, and salinity typically vary over seasonal cycles but sudden changes are rare. Nutrient concentrations, on the other hand, can change very rapidly. Because of the exponential nature of phytoplankton growth, the limiting nutrient is reduced from a considerable level to near zero during the last doubling of the phytoplankton at the end of a bloom (in one day or less for many diatoms). Thus, a sudden nutrient shock is a typical feature at the end of a diatom bloom (assuming the bloom is terminated by nutrient depletion and grazing is not significant, as is often the case for winter and spring blooms in temperate zone coastal waters; Smetacek et al. 1978, Taguchi and Hargrave 1978). This again strongly implicates nutrient limitation as the trigger for resting spore formation.

Our observations suggest that spore formation is a transient event which would frequently be missed by typical field sampling procedures and, therefore, we suggest that the frequency of spore formation is greatly underestimated. The spores were first observed in significant numbers on 6 June. By 13 June they had essentially sunk out of the 20 m water column (Fig. 1). Thus, a weekly or monthly sampling program, as is typically used, would likely miss the spore formation event. Further, if the spores were not observed during the formation process (i.e., if one did not happen to sample during that 1–5 day window) then it is highly unlikely that one would be able to relate the spores to the vegetative cells or environmental conditions under which they were formed. The spores sink 5–6× as fast as the vegetative cells (Fig. 1; P. Bienfang, pers. comm.) and thus advection would quickly separate the two populations 2–3 days after the spores were formed. The CEE observations presented here, and from other years, suggest that spore formation is the typical end to a diatom bloom in the absence of significant grazing by zooplankton, and we believe it is not reported more widely because of the transient nature of the event.

The role of resting spores in the life cycles of those diatom species that have resting spores continues to be a subject of speculation. *Leptocylindrus danicus* spores are heavily silicified and covered with sharp stout siliceous spines (Hargraves 1976). The spores sank rapidly in the CEE and were found in great abundance in the sediment, indicating that they were not being grazed by the zooplankton in the CEE. There is also some evidence from experiments by Hargraves and French (1977) that spores can survive grazing. This is a valuable feature but the true value of the spores must be as a survival mechanism (presumably on the bottom since they sink rapidly and they appear to need a period of cold and darkness before they can germinate) until more favorable conditions induce germination to produce vegetative cells once again.

In two temperate latitude embayments where spore formation has been studied, Saanich Inlet, British Columbia, and Narragansett Bay, Rhode Island, spores have been observed to be abundant in the sediments (Saanich Inlet, Gucluer and Gross 1964; Narragansett Bay, Hargraves and French 1975). In the latter study, the spores were observed in the water column during a bloom of the spore forming species and then in the surficial sediment, a month or so later. Hargraves and French also tested the survival of the resting spores at various temperatures and found that the spores survived only a few weeks at 15 C but longer periods at 2 C. Thus spores may aid species over-wintering in Narragansett Bay, but probably are not of much survival value during the summer, when water temperatures are higher.

In Saanich Inlet, both vegetative cell frustules and resting spores were found in great abundance in the sediments (Gucluer and Gross 1964); it was estimated that phytoplankton remains accounted for ca. 34% of the sediments, 9% as organic material and 25% as biogenic opal from diatom frustules, including a large proportion of resting spores. As noted in that study, the deep water and the sediments in the deeper parts of Saanich Inlet were anoxic. Our results on spore survival suggest that resting spores may not survive well under anoxic conditions. However, many parts of the inlet, such as Patricia Bay where this study was conducted, do have oxygenated surficial sediments and bottom temperatures below 10 C. Spores could survive long periods under such conditions, and vegetative populations arising from these spore populations could easily be mixed throughout the inlet.

Resting spores may be important in a number of environments such as fjords similar to Saanich Inlet, arctic conditions as suggested by Durbin (1978), or possibly the continental shelf environment. In recent studies of storm events in the New York Bight, Walsh et al. (1978) noted that storms resuspended a significant amount of chlorophyll *a* containing material from the sediment surface in April 1975 and March 1976. This was from a depth of 60–70 m and at a temperature of 6 C (i.e., conditions under which spores of many species of diatoms could have survived the winter). They also noted that this subsequently became a region of high productivity,

suggesting that seeding of this area by spores from the sediment was possibly an important factor. We conclude that spore formation is an important, but often missed, event; that it may have significance in a wide range of environments and, therefore, that it is a subject which should receive increased emphasis in future phytoplankton research.

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