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Survival and recovery of resting spores and resting cells of the marine planktonic diatom *Chaetoceros pseudocurvisetus* under fluctuating nitrate conditions

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Abstract Of the two resting life-forms of the planktonic diatom *Chaetoceros pseudocurvisetus* Mangin formed during periods of nitrate depletion, resting spores survived at least 1 month after spore formation at 24 °C, while resting cells survived only for about 10 d at the same temperature. Under nitrogen limitation, resting cells exhibited higher specific death rates than resting spores at temperatures ranging from 5 to 30 °C. After nitrogen replenishment, resting spores required a certain lag period of about 1 d to initiate vegetative growth at levels of nitrate supply from 0.5 to 20 μM, while resting cells initiated vegetative growth almost immediately. Resting spores exhibited an intracellular accumulation of the supplied nitrate during germination and initial vegetative growth. The resting cells, however, exhibited more active vegetative growth, closely coupled with the uptake of the supplied nitrate. The resting spores and resting cells appear to play different roles in the maintenance of populations under nutrient fluctuations depending on the interval length between nutrient fluxes in natural waters.

Introduction

Fluctuations in nutrient concentrations in the euphotic zone in association with water turbulence have been recognized as the most influential factor affecting localized phytoplankton populations (Margalef 1978). Phytoplankton living in such waters can therefore be expected to experience temporally variable nutrient conditions ranging from eutrophy to oligotrophy. Nutrient supply into the euphotic zone in the sea is associated with complex water dynamics, and fluctuations in nutrient supply often occur randomly with varying temporal and spatial scales. To maintain viable populations under such fluctuating nutrient environments, phytoplankton must be able to survive under nutrient-deficient conditions and to optimize nutrient replenishment for active growth.

Under nutrient depletion, many species of marine planktonic diatoms have been shown to form resting spores and/or resting cells (McQuoid and Hobson 1996). Resting spores have morphological structures characterized by heavily silicified frustules (Hargraves 1976). Resting cells have a similar appearance to vegetative cells but are characterized by physiological and cytoplasmic changes (Anderson 1975; Kuwata et al. 1993). Under nutrient depletion, these resting stages have been suggested to have two survival strategies (Gran 1912; Smetateck 1985; McQuoid and Hobson 1996). One is sinking out from the surface layer to avoid nutrient stress, settling on the sediment and serving as benthic seed banks for subsequent diatom bloom formation. The other is tolerating nutrient stress, remaining suspended and serving as a seed bank within the water column. The former strategy has been examined by several groups (Hargraves and French 1975; Garrison 1981; Hollibaugh et al. 1981; Pitcher 1986, 1990; Itakura et al. 1997), however, only a few studies have been done to assess the latter strategy (Dodson and Thomas 1977).

In *Chaetoceros pseudocurvisetus* Mangin, resting spore formation has been shown to be induced under nitrate depletion and to require a significant amount of silicon (Kuwata and Takahashi 1990). Under an insufficient supply of silicate, during a period of nitrogen deficiency, a part of this algal population forms resting spores and the remainder of the population forms resting cells. Both resting spores and resting cells of this

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species have been shown to be adaptive dormant stages under nutrient depletion, with different degrees of depressed metabolic activity and accumulation of glucan and/or neutral lipid as an energy store (Kuwata et al. 1993).

Chaetoceros pseudocurvisetus was originally described as a tropical or subtropical species (Cupp 1943). This species has recently been observed frequently in the warm waters of Japanese coastal regions, and blooms of this species occur from autumn to spring (Takano 1990). *C. pseudocurvisetus* is also found in areas of locally upwelled water around the Izu Islands, Japan, where the nutrient environment has been shown to change from eutrophic to oligotrophic conditions within periods of several days, and during oligotrophic periods nitrogen has been shown to be limiting for algal growth (Takahashi et al. 1986; Kuwata and Takahashi 1990).

As both resting spores and resting cells simultaneously occur in natural *Chaetoceros pseudocurvisetus* populations (Kuwata and Takahashi 1990) and each shows different characteristics (Kuwata et al. 1993), we suggest that each life-form plays a separate role in the maintenance of the algal population under the fluctuating nutrient environments in natural waters. To verify this hypothesis, survival under nutrient depletion and the subsequent recovery responses to the replenishment of nitrogen were examined for each life-form in an unialgal culture of *C. pseudocurvisetus*.

Materials and methods

Sample preparation

Isolation of the unialgal strain of *Chaetoceros pseudocurvisetus* Mangin and culture maintenance has been described previously (Kuwata and Takahashi 1990). Most experiments were carried out in batch culture using 4l polycarbonate bottles at 24 ± 1.5 °C under a 14 h light (ca. 600 μE m^{-2} s^{-1} provided by daylight-type fluorescent tubes): 10 h dark cycle. Light saturation occurred at ca. 200 μE m^{-2} s^{-1} (Suzuki and Takahashi 1995). Culture media in polycarbonate bottles were autoclaved at 120 °C under 2 atm, and cooled for at least 24 h at room temperature before inoculating cultured cells in logarithmic growth phase to an initial concentration of approximately 30 cells ml^{-1}. Resting spores and resting cells of *C. pseudocurvisetus* were induced under nitrate depletion as described in Kuwata et al. (1993).

Survival of the resting spores and resting cells

Samples with concomitantly occurring resting spores and resting cells were prepared by culturing in modified *f*/2 media (Guillard and Ryther 1962) with 10 μ*M* nitrate and 70 μ*M* silicic acid. An aliquot of the sample was removed from the culture container every day after the fourth day of the experiment, when formation of resting spores and resting cells ceased. The percentages of resting spores and resting cells were 38.8% and 61.2%, respectively. Samples for cell counting were fixed with glutaraldehyde (2.5%, v/v), allowed to settle in counting chambers, and counted with an inverted microscope (Utermöhl 1958). The rest of the sample was used for the determination of viability of cells (see following section) and for the examination of autofluorescence of chloroplast in individual cells as an index of the photosynthetic ability. Red autofluorescence emitted from the chloroplast of the individual cells was observed under blue (460 nm) excitation with a fluorescence microscope (Nikon Optiphoto).

To determine the effects of temperature on the death rate of resting spores and resting cells, aliquots of the culture containing the two life-forms were transferred into culture flasks of 100 ml on the sixth day after the formation of the life-forms and incubated at different temperatures ranging from 5 to 30 °C at ca. 200 μE m^{-2} s^{-1} for 1 week. The specific death rate of each life-form (d, d^{-1}) was obtained by using the following equation under the assumption of an exponential decrease with time:

$$d = \frac{1}{t_2 - t_1} \ln(N_1 - N_2) \quad , \tag{1}$$

where N_1 and N_2 are the viable cell numbers at time t_1 and t_2 in days, respectively.

Determination of cell viability

The viability of cells was evaluated using the neutral red staining technique modified from Crippen and Perrier (1974). Neutral red is absorbed only by metabolically active cells. Neutral red stock solution of 2.5 μl (1%, w/v) was added to 5 ml of samples to yield final concentrations of 5 μg dye ml^{-1} sea water. Samples were stained for 30 min in each settling chamber at room temperature, and the viability of 200 to >1000 cells in each sample was determined. The reliability of this staining method was confirmed by examining recovery of resting spores and resting cells after nutrient replenishment (Table 1). Around 80% of both resting spores and resting cells determined as viable by the neutral red staining methods were able to recover and initiated vegetative growth within 3 d of inoculation with *f*/2 medium. This result indicates at least an 80% reliability of this method in distinguishing viable cells.

Recovery responses of resting spores and cells

Resting spores and resting cells were obtained separately by culturing in modified *f*/2 media with 10 μ*M* nitrate and 160 μ*M* silicate and 10 μ*M* nitrate and 25 μ*M* silicate, respectively. Resting spores were harvested 2 to 4 d after their formation. The percentage of resting spores was 99.0%. Resting cells were obtained 3 d after entering the stationary phase. The percentage of resting cells was 99.8%. Resting spores and resting cells were then inoculated into the modified *f*/2 media containing 150 μ*M* silicate and various concentrations of nitrate from 0.5 to 20 μ*M* (100 ml culture flasks) to an initial concentration of approximately 200 cells ml^{-1}. Cultures were incubated at 22 ± 2 °C under 14 h light (ca. 500 μE m^{-2} s^{-1} provided by cool-white fluorescent tubes):10 h dark cycle for 3 or 4 d. An aliquot of sample was removed from each culture flask one to three times each day during the experiment. Samples for nutrient analysis were filtered through a cellulose acetate filter (Advantec, DISMIC-25cs), and the nitrate concentration was determined using an autoanalyzer according to Strickland and Parsons (1972). The initial specific growth rates

Table 1 *Chaetoceros pseudocurvisetus*. Comparison of the percentage of viable cells determined by neutral red staining and from the recovery to vegetative cells 3 d after inoculation into *f*/2 medium for resting spores and resting cells

	Neutral red staining cells (%)	Recovery to vegetative cells (%)
Resting spore	71	58
	61	49
Resting cell	59	50
	17	13

(μ, d^{-1}) of each life-form at each concentration of nitrate were obtained by using the following equation under the assumption of exponential increase:

$$\mu = \frac{1}{t_2 - t_1}\ln(N_2 - N_1) \ , \quad (2)$$

where N_1 and N_2 are the cell numbers at time t_1 and t_2 in days, respectively. The end of lag periods was identified as the point where the regression lines of the exponential growth phase intercept the initial cell number. The average nutrient uptake rates per cell between t_1 and t_2 ($\bar{\rho}_{t_1,t_2}$) were calculated by using the following equation:

$$\bar{\rho}_{t_1,t_2} = (S_1 - S_2)/\int_{t_1}^{t_2} N(t)\mathrm{d}t \ , \quad (3)$$

where S_1 and S_2 are the nutrient concentrations in the medium at times t_1 and t_2, respectively, and $N(t)$ is the population density at time t. Assuming that all nutrients that disappeared from the medium were taken up by cells and that the initial population density of inoculated cells was negligible compared to the density of resting spores or resting cells at harvesting time, the cellular nutrient content of resting spores or resting cells (Q_0) can be calculated using the following equation:

$$Q_0 = \frac{S_i - S_e}{N_e - N_i} \ , \quad (4)$$

where S_i and S_e are the nutrient concentrations in the culture at the initial time i and harvesting time e, respectively. N_i and N_e are the population densities at times i and e, respectively. After supplying nutrients to the resting spore or resting cell population, the average nutrient content per cell at a given time (Q_t) can be calculated using the following equation:

$$Q_t = \frac{N_0 Q_0 + S_0 - S_t}{N_t} \ , \quad (5)$$

where S_0 and S_t are the nutrient concentrations in the medium at times 0 and t, respectively. N_0 and N_t are the population density at times 0 and t.

Results

Survival of resting spores and resting cells of *Chaetoceros pseudocurvisetus* under nitrogen deficiency

The number of viable cells of the resting spores of *C. pseudocurvisetus* formed under nitrogen deficiency at 24 °C exhibited a gradual and almost exponential decrease during the experimental period (Fig. 1). Approximately 50% of the resting spore population had lost viability by the 22nd day of the experiment. The resting spores decreased by about 2% of the population every day and exhibited a death rate of 0.02 d^{-1} by regression. The subpopulation of viable resting cells formed under nitrogen deficiency at 24 °C decreased almost exponentially with time, and reached about 60% of the initial cell numbers on Day 12. The estimated death rate during this period was 0.04 d^{-1}. After Day 12, resting cells exhibited a much faster exponential decrease at a rate of 0.25 d^{-1}, and reached 5% of the initial cell numbers on Day 22. This indicates that the resting spores of *C. pseudocurvisetus* showed higher viability than the resting cells throughout the experimental period.

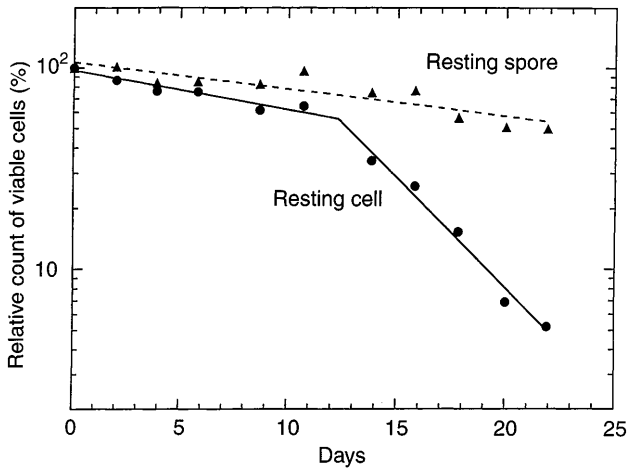

Fig. 1 *Chaetoceros pseudocurvisetus*. Changes in viable cell numbers of resting spores (▲) and resting cells (●) under nitrogen deficiency in unialgal batch culture experiments at 24 °C

Autofluorescence of chloroplasts was determined for both the resting spore and resting cell subpopulations (Fig. 2). All resting spores that were determined as viable by the neutral red staining method retained auto-

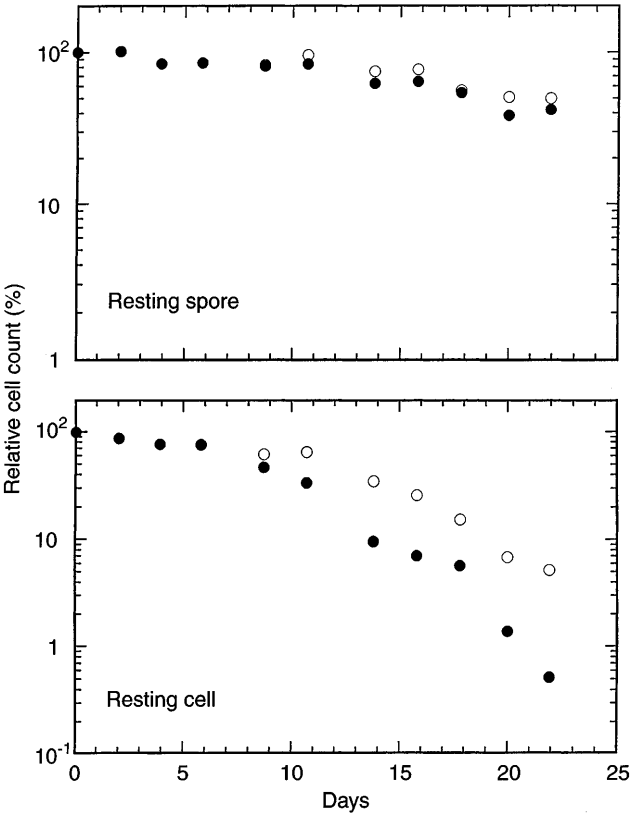

Fig. 2 *Chaetoceros pseudocurvisetus*. Changes in densities of viable resting spores (*upper panel*; ○) and those with autofluorescence in chloroplast (●) and resting cells (*lower panel*; ○) and those with autofluorescence (●) under nitrogen deficiency in unialgal batch culture experiments at 24 °C

fluorescence until Day 10. Even after this period, about 90% of the viable resting spores exhibited autofluorescence during the experiment. All viable resting cells exhibited autofluorescence until Day 7. The percentages of the viable resting cells with autofluorescence decreased rapidly and reached only 10% of the subpopulation on Day 22. This suggests that the resting spores and resting cells lose their viability after a decrease of photosynthetic ability.

The effect of temperature on the specific death rates of viable resting spores and resting cells was determined (Fig. 3). The specific death rates of resting cells were strongly affected by temperature, while those of resting spores were almost constant (0.01 d^{-1}) at temperatures between 10 and 30 °C. At 5 °C, the death rate of both life-forms was higher than that at 10 °C. Comparison of death rates of resting spores and resting cells at each temperature shows that values for resting cells are from two to ten times higher than for resting spores. In particular, the differences in the death rate between the two life-forms were large at 5, 25 and 30 °C.

Recovery responses of resting spores and resting cells to nitrogen replenishment

Unialgal culture experiments were conducted to evaluate the recovery responses of *Chaetoceros pseudocurvisetus* to nitrate supply that have been observed in natural upwelled water. Recovery response of this algal population, consisting solely of viable resting spores, after the addition of 20 μM nitrate into culture media was examined at 22 °C. The population of resting spores took about 1 d to initiate vegetative growth (Fig. 4). After this lag phase, the cell number of the population increased exponentially, and reached eight times the initial population size on the third day. Resting spores began to germinate, casting off their heavily silicified valves half a day after nutrient replenishment, and 80% of the resting spores germinated within 1 d. The remainder of the resting spores germinated gradually. The specific growth rate of vegetative cells from the germinated spores was 1.0 d^{-1} during the logarithmic growth period.

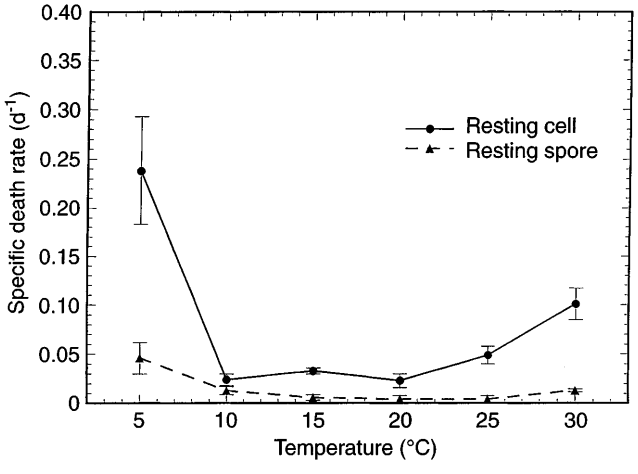

Fig. 3 *Chaetoceros pseudocurvisetus*. Changes in the specific death rates of resting spores and resting cells at different temperatures under nitrogen deficiency in unialgal batch culture experiments. Error bars represent standard deviations

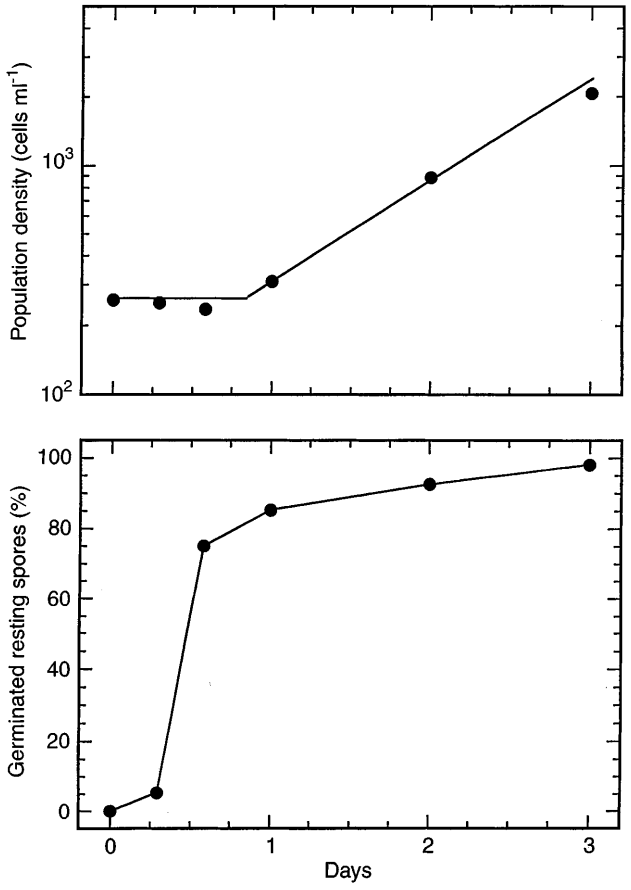

Fig. 4 *Chaetoceros pseudocurvisetus*. Changes in the densities of the total population of viable resting spores and germinated spores after the addition of 20 μM nitrate in unialgal batch culture experiments at 22 °C

Resting cells fully expanded their chloroplasts and initiated vegetative growth after the nutrient replenishment of 20 μM nitrate (Fig. 5). The resting cells exhibited exponential growth immediately after nitrate replenishment, and within 3 d the cell number of the population reached more than 50-times that at the beginning of the experiment. The specific growth rate obtained was 1.4 d^{-1} during the logarithmic growth period.

The lag periods for vegetative growth and initial specific growth rates of each life-form were determined at various concentrations of nitrate supply (Fig. 6). Resting spores exhibited lag periods of around 1 d over all levels of nitrate supply, while the resting cells had almost no lag period. Initial specific growth rates of the resting cells were almost constant at around 1.6 d^{-1}, and were higher than those of the resting spores at all levels of nitrate addition. Specific growth rates of the vegetative cells from resting spores elevated from 0.2 to 1.0 d^{-1}

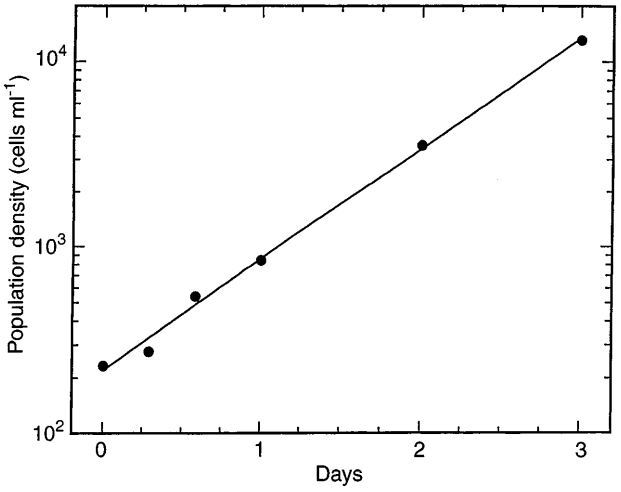

Fig. 5 *Chaetoceros pseudocurvisetus*. Changes in the densities of the total population of viable resting cells after the addition of 20 μ*M* nitrate in unialgal batch culture experiments at 22 °C

as the concentrations of supplied nitrate increased from 0.5 to 20 μ*M*. When the initial growth rates of resting spores were fitted to the equation of Monod (1950), they gave: $y = 1.23x/(2.89 + x)$, $r = 0.96$. This result indicates that resting cells recovered and increased the vegetative population size more quickly than the resting spores at all levels of nutrient supply.

The estimated nitrate uptake rates of resting spores were higher than those of resting cells at almost all levels of nitrate supply (Fig. 7). Assuming that the uptake kinetics followed the Michaelis–Menten equation (Dugdale 1967), the data were fitted to that equation. The maximum uptake rates (r_{max}) and the half-saturation constants of the uptake (K_s) of the resting spores and the resting cells were 30 and 14 pmol cell^{-1} d^{-1} and 6.7 and 4.7 μ*M*, respectively. When the estimated cellular nitrate contents (cell quota) of the resting spores and the resting cells at various levels of nitrate supply were compared, those of the resting spores were again larger than those of the resting cells at all levels of nitrate supply; on the second day after nutrient replenishment the difference was less pronounce (Fig. 8). This indicates that the resting spores exhibited a more rapid uptake than the resting cells. The resting spores had a lower affinity for nitrate but accumulated more nitrate than the resting cells. Furthermore the cells from resting spores retained a larger cellular nitrogen content.

As germination is a critical process for the recovery of resting spores, the germination process of the resting spores after supplying various concentrations of nitrate was examined (Fig. 9). The resting spores took several hours to initiate germination in all cases. After this time lag, germination occurred rapidly during the first day of the experiment and then gradually leveled off. About 70% of the resting spores had germinated even at the lowest concentration of 0.5 μ*M* nitrate on the third day. This indicates that most of the resting spores germinated rapidly. If more than 0.5 μ*M* nitrate was supplied, their germination was almost completed within a day.

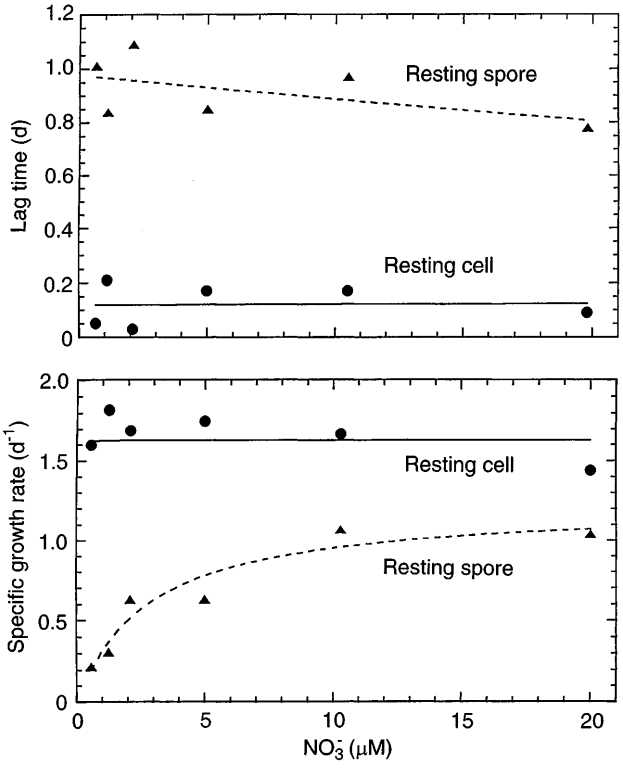

Fig. 6 *Chaetoceros pseudocurvisetus*. Changes in lag periods of resting spores (*upper panel*; ▲) and resting cells (●) and the initial growth rates of resting spores (*lower panel*; ▲) and resting cells (●) at various concentrations of nitrate supplied in unialgal batch culture experiments at 22 °C. Lines are fitted by linear regression. The lag period of resting spores: $y = 0.98 - 0.0087x$, $r = 0.553$; that of resting cells: $y = 0.12 + 0.00016x$, $r = 0.119$; and the initial growth rate of resting cells: $y = 1.75 - 0.013x$, $r = 0.75$. The initial growth curve of resting spores is fitted to Monod's equation

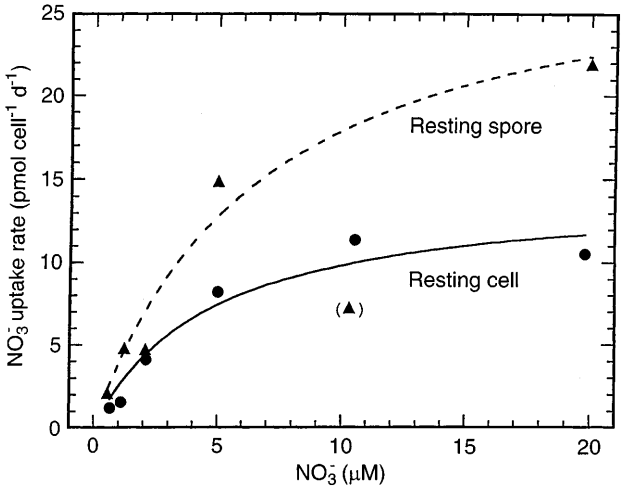

Fig. 7 *Chaetoceros pseudocurvisetus*. Changes in the average nitrate uptake rates of resting spores (▲) and resting cells (●) at various concentrations of nitrate supplied in unialgal culture experiments at 22 °C. The curves in this figure are fitted to the Michaelis–Menten equation. The data point in parentheses was omitted during fitting, as the value may have been caused by contamination during the analysis of nitrate concentration

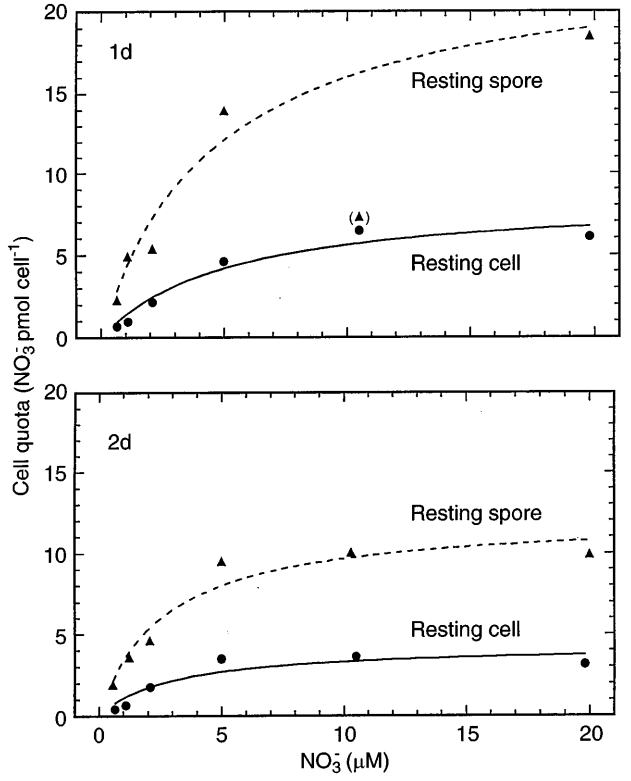

Fig. 8 *Chaetoceros pseudocurvisetus*. Changes in the average contents of nitrate per cell (cell quota) of resting spores (▲) and resting cells (●) on the first and second day after nitrate supply at various concentrations in unialgal culture experiments at 22 °C

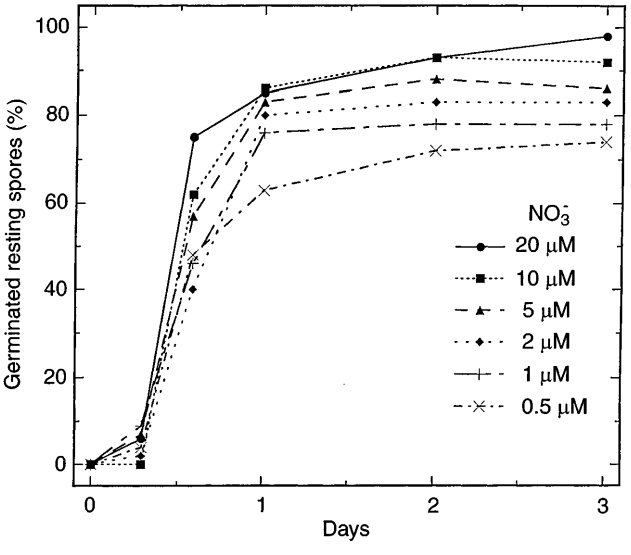

Fig. 9 *Chaetoceros pseudocurvisetus*. Changes in percentages of germinated spores at various concentrations of nitrate supplied in unialgal batch culture experiments at 22 °C

Discussion

In this study, resting spores of *Chaetoceros pseudocurvisetus* had a greater capacity to survive a longer period of time than the resting cells under nitrate depletion. Demographic analysis using unialgal culture experiments revealed that the resting cells of *C. pseudocurvisetus* are able to maintain low death rates until about 10 d after they were formed, but the death rates increased greatly after this time. Resting spores however survived at least 1 month with an almost constant, low death rate (Fig. 1). This species has been reported to be common in local upwelled water masses around the Izu Islands, Japan (Furuya et al. 1986; Kuwata and Takahashi 1990). These local upwelling events supply nutrients into the euphotic zone at a time interval of several days (Atkinson et al. 1987). Among the nutrients, nitrogen has been shown to be limiting for algal growth (Takahashi et al. 1986; Kuwata and Takahashi 1990), and nitrate has been suggested to be the main nitrogen source (Kanda et al. 1985). Therefore, both the resting spores and resting cells of this algal population could survive under nitrate depletion, as long as local upwelling events subsequently occur.

The formation process of resting spores involves suppression of respiratory activity and accumulation of storage products, leading to a profound dormant stage. The formation process of resting cells is accompanied by small changes of metabolic activity and cellular components which then lead to a resting state that requires the consumption of small amounts of cellular carbon to survive (Kuwata et al. 1993). On the basis of the daily negative carbon budget and cellular carbon content found, resting cells were estimated to maintain dark respiration by consuming cellular organic carbon for about 10 d (Kuwata et al. 1993). These estimations agree well with the present study (Fig. 1). Chlorophyll autofluorescence of resting spores and resting cells under nitrogen deficiency indicates that mortality is closely coupled with the loss of in vivo chlorophyll fluorescence under unfavorable nutrient conditions (Fig. 2). The negative carbon budget observed in resting cells was considered to be due to a slight excess in dark respiratory rates compared to gross photosynthetic rates (Kuwata et al. 1993). Reduction of the photosynthetic activity is considered to be a cause of the increased death rate of cells under nitrogen depletion.

The resting cells exhibited higher death rates than the resting spores at all temperatures ranging from 5 to 30 °C under nitrogen depletion (Fig. 3). The death rates of both resting cells and resting spores increased with temperature from 10 to 30 °C as expected. However, both resting cells and resting spores of *Chaetoceros pseudocurvisetus* showed a high death rate at 5 °C . This might reflect observations that this species is commonly found in warm waters (Cupp 1943).

In cultured populations of *Chaetoceros pseudocurvisetus* of solely resting spores or resting cells, vegetative cells from resting cells grew more rapidly than those from resting spores after replenishment of the limiting nutrient (Figs. 4 to 6). The different responses shown by resting spores and resting cells to the supply of nitrate can be considered to be the result of the two following processes. The first is the recovery process of

resting stages to vegetative cells. Resting spores of *C. pseudocurvisetus* took about 1 d to germinate following a single addition of nitrate at any concentration level after a period of deficiency (Figs. 4, 6). Some *Chaetoceros* and *Leptocylindrus* species seem to have a short mandatory dormancy period of several days to a few weeks during which the resting spores do not germinate (Hargraves and French 1983; Itakura et al. 1993). However, resting spores of *C. pseudocurvisetus* did not exhibit a dormancy period of more than 2 d in the present study. The mandatory dormancy of spores may be species specific among diatoms. Resting cells rejuvenated into vegetative cells within several hours, and initiated vegetative growth immediately after nutrient replenishment (Figs. 5, 6).

The second is the initial vegetative growth after the recovery from the resting stages. Resting cells of *Chaetoceros pseudocurvisetus* exhibited more rapid growth than resting spores at all nitrate replenishment concentrations (Figs. 4 to 6). This difference in growth rate reflects nutrient utilization. Germinating resting spores exhibited a more rapid uptake but had a lower affinity for nitrate; they also showed a greater accumulation of nitrate than rejuvenating resting cells. The vegetative cells from the resting spores retained larger quantities of cellular nitrogen and showed slower growth rates than those from resting cells for at least a few days after germination (Figs. 6 to 8). Luxury consumption of nitrate appeared to occur in the germinating resting spores, and a considerable cellular rearrangement using stored nitrogen may be necessary to germinate and initiate normal vegetative growth for a few days. Phytoplankton species have been described as showing two essential responses to increase in nutrients, i.e., the "growth" response and the "storage" response (Sommer 1985; Collos 1986). According to Collos (1986), in the first case rapid growth is stimulated by a close coupling between the cellular uptake of nutrients and cellular growth. The storage response, however, shows a time lag in cell division, while nutrients are being accumulated into internal nutrient pools; this lag possibly reflects a decoupling of nutrient uptake and growth. The recovery behavior of resting spores and resting cells after a period of limited nutrient supply can be regarded, respectively, as a storage response and a growth response. Collos (1986) suggested that the storage response type has an ecological advantage when the nutrient-pulsing frequency is lower than the cell division rate, and the growth response type would provide a competitive advantage at high-frequency pulses.

In this study, we only assessed the effect of a limiting nutrient (nitrate) on the recovery processes of the resting spores and resting cells. The effects of light intensity, photoperiod and temperature on the germination of resting spores have been reported by Hollibaugh et al. (1981), Eilertsen et al. (1995) and McQuoid and Hobson (1995). Further studies on the effects of temperature and light conditions on the recovery processes of resting spores and resting cells following a period of limited nutrient supply are necessary.

The present study shows that resting spores have a greater capacity to survive than resting cells under nitrate depletion, while resting cells can respond more rapidly and achieve more active vegetative growth than resting spores after nitrate replenishment. The resting spores and resting cells can be expected to exhibit significantly different behaviors corresponding to nutrient fluctuations over various time intervals and to play different roles in the maintenance of this algal population under the fluctuations observed in natural waters.

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