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Author(s)	Sugie, Koji; Kuma, Kenshi; Fujita, Satoshi; Ikeda, Tsutomu
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Increase in Si:N drawdown ratio due to resting spore formation by spring bloom-forming diatoms under Fe- and N-limited conditions in the Oyashio region

Koji Sugie^{1†*}, Kenshi Kuma^{1,2}, Satoshi Fujita¹ and Tsutomu Ikeda²

1: Graduate School of Environmental Science, Hokkaido University, Kita 10-Nishi 5, Kita-ku, Sapporo, Hokkaido 060-0810, Japan

[†]Present address: Environmental Science Research Laboratory, Central Research Institute of Electric Power Industry, 1646 Abiko, Abiko-shi, Chiba 270-1194, Japan

2: Faculty of Fisheries Science, Hokkaido University, 3-1-1 Minato-cho, Hakodate, Hokkaido 041-8611, Japan

*: AUTHOR for CORRESPONDENCE

Koji Sugie

Graduate School of Environmental Science, Hokkaido University, Kita 10-Nishi 5, Kita-ku, Sapporo, Hokkaido 060-0810, Japan

E-mail: kojisugie@gmail.com

Phone & fax: +81-11-706-2324

Key Words

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Running Head

Fe- and N-limited resting spores

ABSTRACT: Resting spore formation and Si:N drawdown ratios were investigated under iron (Fe)- and nitrogen (N)-limited conditions using a unialgal culture of *Thalassiosira nordenskiöldii* and natural phytoplankton assemblages during the spring bloom in the Oyashio region. In the unialgal culture of *T. nordenskiöldii*, 20% and 100% of the cells formed resting spores under Fe- and N-limited conditions, respectively. The Si:N drawdown ratios were 2- and 14-fold higher in Fe- and N-limited conditions, respectively, compared to Fe- and N-sufficient conditions. At the start of the natural phytoplankton incubation, 18 among 47 identified diatom species were known resting spore-forming species. Approximately 15 common diatom species formed resting spores under Fe- and N-limited conditions. During the natural phytoplankton incubation, the percentage of the resting spores increased with time under both Fe- and N-limited conditions, reaching 25% and 40% of total diatom abundance, respectively. The Si:N drawdown ratios significantly increased with an increase in the contribution of resting spores in both the unialgal culture and natural phytoplankton incubations. These results suggest that if the bloom dominating by neritic, resting spore-forming diatom species declines by either Fe- or N-depletion, Si may be utilized preferentially to N in the upper mixed layer due to the formation of heavily silicified resting spores.

1. Introduction

The annual spring phytoplankton bloom in temperate to polar regions is a common phenomenon in which diatoms usually play a predominant role (Sarthou et al., 2005). Diatoms are a major component of biological pumps and biogenic silica flux in the ocean through sedimentation of unutilized phytodetritus, resting spores, and/or fecal pellets utilized by zooplanktons (Smetacek, 1999; Thompson et al., 2008). In the Western Subarctic Pacific (WSP) region, centric chain-forming diatoms dominate the phytoplankton community during the spring bloom period, and controlling macronutrient dynamics (Mochizuki et al., 2002; Liu et al., 2004). Some of these bloom-forming diatoms are known to form resting spores in response to adverse environmental conditions that are unfavorable for their growth (McQuoid and Hobson, 1996). Once the heavily silicified and fast-sinking resting spores are formed, they sink and can sequester nutrients without significant dissolution and grazing in the water column (Smetacek, 1985; McQuoid et al., 2002; Kuwata and Tsuda, 2005). Resting spore formation is reported to be induced primarily by nitrogen limitation

(Hargraves and French, 1983). We recently demonstrated the formation of resting spores in two strains of a unialgal culture of *Thalassiosira nordenskioeldii* under Fe- and N-limited conditions (Sugie and Kuma, 2008), suggesting that N was directly limited by substrate depletion under the N-limited condition and indirectly by intracellular Fe and N co-limitation under the Fe-limited condition. However, resting spore formation of natural diatom communities under Fe-limited conditions has not yet been reported.

Iron is one of the most important trace elements for phytoplankton growth, and it is essential for several biochemical processes such as photosynthetic and respiratory electron transport, and nitrogenous nutrient assimilation (Geider and La Roche, 1994). In general, one of the most bioavailable iron species for phytoplankton is dissolved inorganic Fe(III) [Fe(III)'] (Anderson and Morel, 1982; Morel et al., 2008). However, the thermodynamically stable oxidation state of iron in oxic surface seawater is Fe(III), which has an extremely low solubility (Stumm and Morgan, 1996; Waite, 2001). Furthermore, the presence of natural organic ligands such as siderophore in the surface mixed layer can reduce the concentration of bioavailable Fe(III)' by complexing strongly with Fe(III) in seawater (Rue and Bruland, 1995). Therefore, marine phytoplankton, especially diatoms in oceanic regions situated away from iron sources, often have limited Fe (Martin, 1990; Tyrrell et al., 2005). The WSP is one of the Fe-limited high-nutrient low-chlorophyll (HNLC) regions in the world's oceans (Banse and English, 1999), whereas the Oyashio region and some areas at the edge of the subarctic Pacific region are possible exceptions to the HNLC regime (Harrison et al., 2004; Whitney et al., 2005). However, Suzuki et al. (2002) and Nishioka et al. (2003) reported that the late spring-to-summer phytoplankton community in the Oyashio region was Fe-limited, with relatively low ambient dissolved Fe (D-Fe) concentrations ($<0.22 \mu\text{M}$, $\sim 0.1 \text{ nmol L}^{-1}$). In addition, the surface seawater in the Oyashio region during summer was a heterogeneous mixture of N-depleted and HNLC-like conditions (Saito et al., 2002). Therefore, it can be assumed that the spring phytoplankton bloom community in the Oyashio region would be affected and eventually regressed by either Fe- or N-deficient conditions, even in regions where relatively high levels of iron are supplied from Fe-rich intermediate waters and atmospheric Fe-rich dust deposition to the surface mixed layer (Nishioka et al., 2007).

Recent studies have demonstrated that Fe influences the macronutrient consumption ratio and elemental composition of diatoms, with the cellular Si:N ratio increasing under Fe-limited

conditions (Takeda, 1998). Other studies examining a variety of Fe-limited diatom cultures and natural phytoplankton communities have suggested a reduction in cellular N content; an increase in frustule silicification; and change in cell morphology, such as increased surface area-to-cell volume ratio, as possible mechanisms responsible for the elevated cellular Si:N ratio (Timmermans et al., 2004; Leblanc et al., 2005; Marchetti and Harrison, 2007). Similarly, the Si:N ratio is 8-fold higher in N-limited resting spores of *Chaetoceros pseudocurvisetus* than in vegetative cells (Kuwata et al., 1993). A few studies reported the sedimentation of resting spores in HNLC-like regions where seasonal blooms and resulting Fe-depletion were observed in the southeastern edge of the Western Subarctic Gyre (Onodera et al., 2002; Nishioka et al., 2003) and the Kerguelen Plateau in the Southern Ocean (Armand et al., 2008a, b). Although sedimentation of resting spores has been reported in HNLC-like regions that would experience Fe-depletion, the relationship between resting spore formation under N- or Fe-limited conditions and macronutrient dynamics during the spring bloom periods has not been closely investigated.

In this study, we investigated the formation of resting spores and Si:N drawdown ratios under Fe- and N-limited conditions in a unialgal culture of *T. nordenskiöldii* and natural phytoplankton community incubation during the spring bloom period in the Oyashio region. We hypothesized that many bloom-forming neritic diatom species form resting spores under Fe- and N-limited conditions. In addition, regardless of whether Fe- or N-depletion has a greater effect on the spring bloom, we predict that Si is preferentially utilized to N on the basis of the formation of heavily silicified resting spores.

2. Materials and methods

2.1. Unialgal culture experiment

A unialgal strain of *T. nordenskiöldii* was isolated from the surface seawater of the Oyashio region (42°00'N, 145°15'E) in the northwestern Pacific Ocean side of southern Hokkaido, Japan, by a capillary pipette. The unialgal strain was maintained in silicic acid-enriched [105 $\mu\text{mol L}^{-1}$ Si(OH)₄] f/2 medium (Guillard and Ryther, 1962) under 150 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ fluorescent light (QSL-100, Biospherical Instrument Inc.) in a 12-h L:12-h D cycle at 5°C. The maintenance

cultures were not axenic, but additional bacterial contamination was minimized by using sterile techniques and serial transfers during the exponential growth.

All equipment used in the culture experiment were acid-cleaned, followed by rinsing with Milli-Q water (Millipore), and all preparations and sampling for experiments were performed in a Class 100 laminar flow cabinet to avoid inadvertent trace metal contamination. Seawater for the culture experiment was collected from a coastal region near Hokkaido, in the northern Japan Sea (43°23' N, 141°02' E), and was filtered through an acid-cleaned 0.22- μ m GS cellulose membrane filter (Millipore). The filtered seawater was autoclaved for 20 min at 121°C (108 kPa) using an acid-cleaned glass Erlenmeyer flask, aged for ca. 1 week at room temperature in the flask, and re-filtered through the acid-cleaned 0.22- μ m membrane filter to eliminate particulate Fe(III) species. The concentrations of Fe, $\text{NO}_3 + \text{NO}_2 + \text{NH}_4$ (DIN), PO_4 (P) and $\text{Si}(\text{OH})_4$ (Si) in the double-filtered autoclaved seawater (base seawater) were 0.4 nmol L⁻¹, 6.2 μ mol L⁻¹, 0.3 μ mol L⁻¹ and approximately 250 μ mol L⁻¹, respectively. The Fe concentration in the base seawater was determined by an automated Fe analyzer (Kimoto Electric) using a combination of an 8-hydroxyquinoline chelating resin concentration and luminol-hydrogen peroxide chemiluminescence detection in a closed flow-through system (Obata et al., 1993). Macronutrient concentrations in the base seawater were measured by a QuAatro continuous flow analyzer (Bran+Luebbe). The Si:N drawdown ratios were calculated from delta Si divided by delta N during a certain growth interval.

Diatom stock cultures were maintained in silicic acid-enriched f/2 medium with three transfers (~30 doublings) during the exponential growth phase. Diatoms in the late exponential growth phase were inoculated into modified f/2 medium, which was prepared without adding f/2 metals, EDTA and vitamins to the medium. All f/2 nutrient stock solutions were passed through Chelex 100 ion-exchange resin (Bio-Rad) to remove trace metals (Morel et al., 1979). Diatoms were grown in modified f/2 media to which only ferric iron and manganese stock solutions were added to make final Fe and Mn concentrations of 100 and 25 nmol L⁻¹, respectively, and to obtain slightly Fe-stressed cells. Previous studies have found that addition of both Mn and Fe to the modified f/2 medium kept the cells in physiologically good state for a suitable length of time (Peers and Price, 2004; Ushizaka et al., 2008); hence, we added only Fe and Mn but eliminated other trace elements in the culture media in this study. Furthermore, late exponential growing cells in the modified f/2 media

were harvested by gravity filtration onto an acid-cleaned 0.2- μ m membrane filter and immediately resuspended in the base seawater to remove unused Fe in the pre-cultured media; these cells were used for the following experiments.

In the culture experiment, macronutrient stock solutions were added to obtain the final concentrations of 180 μ mol L⁻¹ DIN, 15 μ mol L⁻¹ P and 355 μ mol L⁻¹ Si (base medium), which were then determined by the approximate elemental ratio of macronutrients in the Oyashio region during winter (Saito et al., 2002; Saito and Tsuda, 2003). Resuspended *T. nordenskioeldii* were inoculated into base media (800 mL) in 1-L polycarbonate Erlenmeyer flasks, resulting in an initial cell density of approximately 1000 cells mL⁻¹. The effect of direct Fe and Mn inputs (Fe-replete treatment) was examined by adding Fe(III) and Mn(II) stock solutions to the control medium to obtain the final concentrations of 100 and 25 nmol L⁻¹, respectively. Fe-limited media (Fe-limited treatment) were prepared by adding only the acidic Mn(II) stock solution (final concentration of 25 nmol L⁻¹) to the control media. The N-limited medium (N-limited treatment) was prepared by adding the f/2 metal stock solution to the modified control media without nitrate. Culture experiments were conducted in triplicate. The light and temperature conditions were the same as those for the stock culture described earlier. During the experiments, the number of vegetative cells, resting spores and resting cells were monitored daily using unfixed cells by 6-replicate cell counts in a hemacytometer with a light microscope magnified $\times$ 100–200. Resting cells were identified by the chlorotic, shrunken, less abundant, and asymmetrically distributed chloroplasts without stored products within the cell. Resting spores have stored products such as carbohydrates within the cell (Kuwata et al., 1993), thus showing specific refraction under the light microscope. Some spores had both resting spore and daughter cell frustules (i.e., endogenous and semi-endogenous resting spores). In contrast, vegetative cells had symmetrically distributed swelling chloroplasts. The samples for nutrient analysis during cultivation (~20 mL) were obtained daily by filtration using a DISMIC 0.2- μ m filter, and were measured with a QuAAtro continuous flow analyzer.

2.2. Natural phytoplankton incubation experiment

Experiments were conducted in the Oyashio region (42°00'N, 145°15'E) on April 20, 2007 as part of the Ocean Ecodynamics Comparison in the subarctic Pacific research program during the

KH-07-01 cruise aboard R/V Hakuho-Maru. Seawater samples for the analysis of macronutrients and D-Fe were collected at depth ranging from 5 to 300 m, using acid-cleaned Teflon-coated 10-L Niskin X sampling bottles (General Oceanics) attached to a CTD-carousel multi-sampling system. Hydrographic data (salinity, temperature, and depth) were obtained using a CTD (Sea-Bird, Model 9-puls). Seawater for experiments was collected from a depth of 10 m and sieved by 100- μ m acid-cleaned Teflon-mesh to eliminate large herbivorous zooplankton. The prescreened seawater sample was mixed in an acid-washed 20-L polyethylene tank and then dispensed into acid-cleaned 320-mL polycarbonate bottles. The three treatments were carried out as follows: the unamended control; Fe-limited media with addition of 15 $\mu\text{mol L}^{-1}$ NO_3 , 1.9 $\mu\text{mol L}^{-1}$ P, 44 $\mu\text{mol L}^{-1}$ Si and 1 $\mu\text{mol L}^{-1}$ desferrioxamine B (DFB; Sigma Chem. Co. Ltd.); and N-limited media with addition of 1.9 $\mu\text{mol L}^{-1}$ P, 44 $\mu\text{mol L}^{-1}$ Si, and 5 nmol L^{-1} Fe. DFB is a small trihydroxamate molecule that complexes with inorganic Fe(III) with an extremely high conditional stability constant ($K'_{\text{FeL}, \text{Fe(III)}} = [\text{Fe(III)L}]/[\text{Fe(III)}][\text{L}] = 10^{16.5} \text{ M}^{-1}$) in seawater (Hudson et al., 1992). Thus, addition of an excess concentration of the siderophore DFB relative to iron (Fe:DFB = 1:10) prevents Fe uptake in phytoplankton by diminishing the concentration of bioavailable $[\text{Fe(III)}']$ (Wells, 1999; Iwade et al., 2006; Yoshida et al., 2006).

Triplicate and/or duplicate incubation bottles for 1-, 3-, 5-, 7- and/or 10-day cultivations for each treatment were incubated at 5°C under 150 $\mu\text{mol photon m}^{-2} \text{ s}^{-1}$ fluorescent light (12-h L:12-h D cycle). Bottles were sacrificed at each intervals. All experimental preparations were conducted in a clean room or on a clean bench (Class 100) on board. The chlorophyll *a* (chl-*a*) concentrations in the samples were measured at each intervals by using the Turner Design 10-AU fluorometer (Welschmeyer, 1994) after extracting the chl-*a* with *N, N*-dimethylformamide (Suzuki and Ishimaru, 1990). The methods for sample collection and analysis of macronutrients were the same as used in the unialgal culture experiment described earlier. Growth rates were calculated from the linear regression between the time and the natural log of chl-*a* concentrations. The samples (~100 mL) for diatom cell densities and species compositions were collected at 0, 5 and 10 days. They were then mixed with an equal volume of replicates of each treatment and fixed with formalin (1% final volume) for analysis in a laboratory on land. An adequate volume of fixed seawater was poured into a settling chamber (Hydro-bois) and was allowed to settle for at least 24 h before identification using a phase-contrast inverted microscope (Hasle, 1978). Diatom species were identified according to

Hasle and Syvertsen (1997). Cell volume of the dominant diatom species was measured as described by Hillebrand et al. (1999) and the cell volume was converted to carbon biomass as reported by Montagnes and Franklin (2001). We could not discriminate between resting and vegetative cells because the chloroplasts within the diatom cells had shrunk due to formalin fixation. Therefore, we counted both cell types as vegetative cells.

3. Results

3.1. Unialgal culture experiment

3.1.1. Vegetative cell and resting spore abundance

In the Fe-replete treatment, vegetative cells increased exponentially for 5–6 days before reaching the stationary growth phase. Vegetative cell density was almost constant for 4–10 days of cultivation in the Fe-limited treatment, whereas a sudden decrease was observed after 4 days of cultivation in the N-limited treatment (Fig. 1a). Resting cells in the Fe-limited treatment increased after 4 days during the stationary growth phase of vegetative cells, corresponding to a gradual increase in resting spores (Fig. 1b, c). In the N-limited treatment, there was a rapid decrease in vegetative cells after day 4, and resting spores increased rapidly between 4 and 6 days, whereas the resting cell density was much lower than that of the resting spores (Fig. 1b, c). The proportion of resting spores in the Fe-limited treatment gradually increased to ~20% between days 4 and 11, while the number of resting spores in the N-limited treatment reached >80% between 3 and 5 days (Fig. 1d). There was no resting spore and cell formation in the Fe-replete treatment during the 9-day experiment.

3.1.2. Nutrient dynamics

Nitrogen depletion ($<0.5 \mu\text{mol L}^{-1}$) was observed at days 3 and 9 in the N-limited and Fe-replete treatments, respectively (Fig. 2a). Si utilization rates in all three treatments were almost the same during 0–7 days of growth (Fig. 2b). Phosphate was not exhausted throughout the experiments in all treatments (Fig. 2c). The Si:N drawdown ratio of exponentially growing *T. nordenskiöldii* in the Fe-replete treatment was 0.59 (Table 1). Even after N depletion in the

N-limited treatment, Si uptake was maintained and the Si:N drawdown ratio reached ~64 during the spore-forming phase (transition phase during days 3–7). The Si:N drawdown ratio in the N-limited treatment reached ~8.5 by day 11, when the resting spore contribution was 100%. The Si:N drawdown ratio in the Fe-limited treatment was approximately two times higher than that in the Fe-replete treatment for all growth phases (Table 1).

3.2. Natural phytoplankton assemblage incubation experiment

3.2.1. Initial conditions

The upper mixed layer depth was 75 m, which was estimated from the first downward increase in $\sigma_t \geq 0.02 \text{ m}^{-1}$ (Fig. 3a). D-Fe, DIN, P, and Si concentrations in the surface water (10 m depth, 3.62°C) collected for the natural phytoplankton incubation experiment were 0.17 nmol L^{-1} , $14.8 \text{ } \mu\text{mol L}^{-1}$, $0.96 \text{ } \mu\text{mol L}^{-1}$, and $10.3 \text{ } \mu\text{mol L}^{-1}$, respectively. These micro- and macronutrient concentrations were vertically homogeneous in the upper 50 m and increased gradually with depth below 50 m (Fig. 3). The *in situ* Si:N ratio was ~1.1 in the upper mixed layer and increased up to ~2 below the 100 m stratum (Fig. 3b). Chl-*a* concentrations were also uniform in the upper 50 m (~4 $\mu\text{g L}^{-1}$) and decreased with depth (Fig. 3c). Therefore, water collected for the natural assemblage experiment was considered to be representative of the spring phytoplankton bloom in the Oyashio region of the WSP (Saito et al., 2002).

3.2.2. Phytoplankton dynamics

The chl-*a*-specific growth rate from day 0 to 3 in the N-limited treatment ($0.40 \pm 0.01 \text{ d}^{-1}$; avg. $\pm 1 \text{ SD}$) was significantly higher than that in the control ($0.14 \pm 0.01 \text{ d}^{-1}$; $p < 0.001$; ANOVA) and Fe-limited ($0.08 \pm 0.01 \text{ d}^{-1}$; $p < 0.001$) treatments (Fig. 4a), without exhaustion of macronutrients during the period (Fig. 5). Total diatom abundance was highest at day 10 with 19,850 cells mL^{-1} in N-limited treatment, followed by 7670 cells mL^{-1} in the Fe-limited treatment and 6230 cells mL^{-1} in the control (Fig. 4b). The total diatom abundances during days 5–10 were relatively constant in all treatments, probably due to Si depletion in the control, N depletion in N-limited culture media, and Si and/or Fe depletion in the Fe-limited culture media (Figs. 4b and 5).

Thirty-six centric and 11 pennate diatom species were identified in this study. Eighteen of

the forty-seven identified species have been reported previously to form resting spores (McQuoid and Hobson, 1996; Table 2), and resting spore-forming diatom species dominated in abundance, comprising up to ~85% of the diatom community at the start of the experiments (Table 3). In the initial phytoplankton community, *Chaetoceros* subgenus *Hyalochaete* spp. were dominant in abundance (~78%), whereas *Thalassiosira* spp. were dominant in biomass (~60%), followed by *Chaetoceros* subgenus *Hyalochaete* spp. (~30%) (data not shown). In all treatments, the resting spores increased with time (Fig. 4c). The relative order for the percentage of resting spores during the 10-day experiment was N-limited (40%) > Fe-limited (25%) >> control (5.2%) (Fig. 4d). Temporal changes in the percentage of resting spores of each species are shown in Fig. 6. *Chaetoceros compressus*, *Chaetoceros laciniosus*, *Chaetoceros similis* and *Chaetoceros socialis* were counted as *Chaetoceros* spp. 1, and *Chaetoceros cinctus*, *Chaetoceros furcellatus*, *Chaetoceros radicans* and *Chaetoceros tortissimus* were counted as *Chaetoceros* spp. 2, because they had indistinguishable morphology in their resting spores and vegetative cells, respectively (Table 3). The initiation of resting spore formation and the percentage of resting spores were remarkably different among species and treatments (Fig. 6). In the N-limited treatment, *Chaetoceros debilis* and *Chaetoceros diadema* rapidly formed resting spores during the first 5 days of the experiment (Fig. 6a, d), whereas the resting spore percentages of other species increased rapidly between days 5 and 10 (Fig. 6b, c, e, f). Lower resting spore percentages with lower sporulation rates were observed in the Fe-limited treatment when compared to the N-limited treatment for all diatom species, except for *Chaetoceros* spp. 1 and *T. nordenskioeldii* (Fig. 6b, f). The percentages of resting spores for four out of six species in the Fe-limited treatment increased continuously during the 10-day period. However, resting spore formation in the control (except for *Stephanopyxis nipponica*) increased only slightly throughout the experiment, probably due to Si exhaustion after day 5 (Figs. 5 and 6). Resting spores were observed for *C. cinctus* and *C. furcellatus*, but not for *C. radicans* (Table 3). *Leptocylindrus danicus* were sporadically observed at a very low abundance, and thus its ability to form resting spores under N- and/or Fe-limited conditions was not clear. *Probosira alata* were observed in all samples without resting spore formation (Table 3). It was difficult to distinguish the vegetative cells of some resting spore-forming species (e.g., *Thalassiosira* spp. and *Fragilariopsis* spp.) from those of non-spore-forming species to calculate the specific percentage of resting spores. It is notable that the diatom species that formed resting spores under Fe-limited conditions were the same as those that

formed resting spores under N-limited conditions (Table 3).

3.2.3. Nutrient dynamics

DIN was exhausted after 5 days in the N-limited treatment and after 7 days in the control. However, little DIN was utilized in the Fe-limited treatment with DFB (Fig. 5a). Si was depleted after 5 days in the control, linearly decreased over the 10-day period in the Fe-limited treatment, and suddenly decreased after 3 days in the N-limited treatment (Fig. 5b). Phosphate was not depleted in any treatment (Fig. 5c). The Si:N drawdown ratio in the control decreased from 1.17 during the first 3 days to ~0.7 over the 10-day cultivation period (Table 4). In the N-limited treatment, the Si:N ratio was 0.77 before N depletion and increased to ~2.7 during the 10-day period. The Si:N ratio in the Fe-limited treatment was relatively constant with a high value of ~2.4 throughout the experiment (Table 4).

4. Discussion

4.1. Resting spore formation under Fe-limited condition

Several studies have found that N deficiency is an important factor in the formation of resting spores in marine diatoms (Hargraves and French, 1983). This study is the first report on the formation of resting spores in *Chaetoceros teres*, *Fragilariopsis oceanica*, *Porosira* sp. cf. *pentaportula*, and *S. nipponica* under N-limited conditions, and in ~14 diatoms species under Fe-limited conditions. This implies that Fe-limitation is an important trigger for the formation of resting spores for many diatom species. In this study, D-Fe concentration in the surface mixed layer (0.14–0.19 nmol L⁻¹) was similar to previous reports for the Oyashio region in late spring to summer (~0.1–0.2 nmol L⁻¹; Nishioka et al., 2003; Takata et al., 2004). Furthermore, Fe addition (N-limited treatment) increased the phytoplankton growth rate during the first three days of the experiment and DFB addition (Fe-limited treatment) suppressed the growth rate and nitrate drawdown rate and increased the percentage of resting spores as compared to the control. These results suggest that the phytoplankton community during the spring bloom in the Oyashio region was Fe-limited without any intracellularly stored Fe. In addition, initial ambient macronutrient concentrations were much

higher than those required for vegetative growth by most diatom species (Sarthou et al., 2005). Therefore, the presence of resting spores at the start of the culture experiment in sunlit surface seawater was considered to be induced by Fe-limitation. We suggest that resting spore formation of diatoms under Fe-limited conditions occurs often during and after the spring diatom bloom in the Oyashio region, where the surface seawater during late spring to summer is sometimes in an iron-limited HNLC condition (Saito et al., 2002; Suzuki et al. 2002).

In addition, we found a discrepancy in the timing of spore formation, which probably depends on the various physiological responses among diatom species under Fe- and N-limited conditions (Fig. 6). Our results suggest that *C. diadema* and *Chaetoceros* spp. 2 will predominate in Fe-limited conditions, and *Chaetoceros* spp. 2 and *T. nordenskiöldii* will predominate in N-limited (Fe-replete) conditions. The susceptibility of resting spore formation to Fe- and/or N-depletions for each diatom species should be examined in the future. In such physically, chemically and biologically complex WSP regions (Saito et al., 2002; Oguma et al., 2008), the different characteristics of sporulation would affect the diatom community structure, especially after the later phase of the bloom, which may be subject to Fe- and/or N-limited conditions in the Oyashio region (Saito et al., 2002).

Resting spore formation in *Chaetoceros* subgenus *Hyalochaete* spp. and sedimentation without depletion in nitrate, but possibly a HNLC condition in the surface mixed layer, were observed around the Antarctic Peninsula (Bodungen et al., 1986; Leventer, 1991), the Kerguelen Plateau of the Southern Ocean (Armand et al., 2008a, b), the western and central subarctic Pacific Ocean, and the southern-central Bering Sea (Takahashi et al., 2002; Onodera et al., 2003; Onodera and Takahashi, 2009). The study by Armand et al. (2008a, b) and this study are the only two to report resting spore formation in neritic diatom-dominated blooms with a high-nitrate, low-iron, and mid- to high-chlorophyll environment (this study: D-Fe 0.17 nmol L^{-1} , chl-*a* $\sim 4 \text{ } \mu\text{g L}^{-1}$; Armand et al., 2008a, b: D-Fe $< 0.10 \text{ nmol L}^{-1}$, chl-*a* $> 1 \text{ } \mu\text{g L}^{-1}$). However, Armand et al. (2008a) considered Si-limitation to be the trigger for sporulation, and not Fe-limitation as observed in this study. In the natural phytoplankton incubation experiment, the Si concentration in the control after 5 days was significantly lower than in the Si-added, Fe-limited treatment, in which could continue further silicification to form the resting spores during days 5–10. Therefore, the amount of available Si during and after the bloom could critically regulate the number of diatom resting spores in the

spore-forming species, as demonstrated by Kuwata et al. (1993) under N-depleted conditions. We hypothesized that the formation of resting spores could be induced by Fe depletion in the HNLC coastal boundary regions if resting spore-forming diatoms were introduced to the regions such as the Oyashio region (Mochizuki et al., 2002; Liu et al., 2004).

4.2. Si:N drawdown ratio and phytoplankton dynamics

The Si:N drawdown ratios under Fe- and macronutrient-replete conditions in the unialgal culture of *T. nordenskioeldii* and the natural phytoplankton incubation experiment (N-limited treatment during days 0–3) were slightly lower than the Si:N ratio (~1) of vegetatively growing diatoms (Brzezinski, 1985). If diatoms continue to take up Si and N at the same lower Si:N ratio throughout the spring diatom bloom, Si will remain in the upper mixed layer with an increase in the water Si:N ratio after the bloom, because the Si:N supply ratio is >1 during winter in the subarctic Pacific regions (Harrison et al., 2004). It has been reported that the water Si:N ratio increased as the spring bloom progresses in the Fe-sufficient coastal region (Kudo et al., 2000). However, the Si:N drawdown ratios under N-limited conditions for both unialgal and natural phytoplankton experiments increased rapidly after N depletions, whereas the ratios under Fe-limited conditions were continuously higher during the cultivation periods as compared to those under Fe-replete conditions (Tables 1 and 4). These results suggest that the water Si:N ratio gradually decreases as the bloom progresses under Fe-limited conditions, whereas it increases under N-limited conditions. Therefore, change in the water Si:N ratio in the upper mixed layer during the spring phytoplankton bloom with an Si:N supply ratio of >1 during winter would be a significant indicator of whether the spring bloom-forming diatom community is influenced by Fe-limitation.

When we combined the results of both unialgal culture and natural phytoplankton incubation, the Si:N drawdown ratio increased significantly with an increase in the percentage of the resting spores (Fig. 7). The exponential relationship between the Si:N drawdown ratio and resting spore percentage indicates that the resting spores of *Chaetoceros* subgenus *Hyalochaete* spp. in the natural phytoplankton incubations (Table 3) and the resting spores and cells under Fe-limited conditions in the unialgal culture experiment (Table 4) would be lightly silicified as compared to the spores of *T. nordenskioeldii* under N-limited conditions in the unialgal culture experiment. Therefore,

Si was preferentially utilized due to the formation of resting spores either due to Fe or N depletion.

The Si:N drawdown ratios from winter to summer in the subarctic Pacific regions generally are always higher than the Brzezinski ratio of ~ 1 (Wong and Matear, 1999; Koike et al., 2001; Saito et al., 2002; Whitney et al., 2005) and also higher than that found in the Southern Ocean (Pondaven et al., 2000). The possible mechanisms for the increasing the Si:N drawdown ratio could involve an increase in the cellular Si:N ratio of diatoms by Fe-limitation (Takeda, 1998), a decrease in growth rate (Claquin et al., 2002; Saito and Tsuda, 2003), a high Si:N ratio of ambient water (Kudo, 2003), and preferential remineralization of N over Si, i.e., the Si pump (Dugdale and Wilkerson, 1998). This study demonstrates that the Si:N drawdown ratio increased with an increase in the percentage of heavily silicified resting spores under Fe- and N-depleted conditions. This could be one of the important mechanisms for increase in the Si:N drawdown ratio between winter and summer in which the resting spore-forming diatom species dominate in the spring bloom, such as in the WSP regions (Mochizuki et al., 2002; Liu et al., 2004, this study) and the Southern Ocean (Bodungen et al., 1986; Leventer, 1991; Abelmann et al., 2006; Armand et al., 2008a, b).

The formation of heavily silicified resting spores by diatoms under Fe- and N-limited conditions may be an important phenomenon in present biological and biogeochemical oceanography and could serve as a proxy for paleoproductivity (Abelmann et al., 2006). This study is the first to assess the role of resting spores in oceanic Si biogeochemistry under Fe- and N-limited conditions; however, further studies would be required to clarify the entire mechanisms of its role in coastal and oceanic regions. In addition to known heavily silicified but non-resting spore-forming diatoms such as *Neodenticula seminae* in the subarctic Pacific Ocean (Takahashi et al., 2002; Onodera and Takahashi, 2009), *Fragilariopsis kerguelensis* in the Southern Ocean (Abelmann et al., 2006; Armand et al., 2008b) and diatom aggregates (Smetacek, 1999; Michel et al., 2002), this study indicated that diatom resting spores could be an important component in the transport of Si to the depths in coastal and oceanic regions under the temporally and specially N-limited and HNLC-like conditions, such as in the Oyashio region (Saito et al., 2002; Harrison et al., 2004). Thus, species composition and physiological aspects of the diatom community may be among the most important factors influencing the biogeochemical Si cycle in the ocean.

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Table 1. Si:N drawdown ratio of unialgal culture experiment. Data represents means of triplicate experiments $\pm$ 1SD. Number in parenthesis represents the cultivation days. Transition phase means the duration when the sporulation was succeeded.

Treatment	Exponential	Transition	Total
Fe-replete	0.59 ± 0.00 (0–5 d)		
N-limited	2.05 ± 0.08 (0–2 d)	63.7 ± 6.24 (3–7 d)	8.46 ± 0.30 (0–11 d)
Fe-limited	0.81 ± 0.05 (0–3 d)	1.28 ± 0.04 (4–9 d)	1.17 ± 0.07 (0–11 d)

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632 Table 2. List of diatom species identified. Designated resting spore forming species are those with an
 633 asterisk. Genus species names are arranged alphabetically not systematically.

634	Centric diatoms	Pennate diatoms
635	<i>Asteromphalus flabellatus</i>	<i>Corethron criophilum</i>
636	<i>Astero. hookeri</i>	<i>Coscinodiscus asteromphalus</i>
637	<i>Attheya longicornis</i>	<i>Dactyliosolen fragilissimus</i>
638	<i>At. septentrionalis</i>	<i>Detonula confervacea</i> *
639	<i>Chaetoceros atlanticus</i>	<i>Eucampia groenlandica</i>
640	<i>C. cinctus</i> *	<i>Guinardia</i> sp.
641	<i>C. compressus</i> *	<i>Leptocylindrus danicus</i> *
642	<i>C. concavicornis</i>	<i>Odontella aurita</i>
643	<i>C. convoltus</i>	<i>Probosira arata</i>
644	<i>C. debilis</i> *	<i>Prosira</i> sp. cf. <i>pentaportula</i> *
645	<i>C. decipiens</i>	<i>Rhizosolenia</i> spp.
646	<i>C. diadema</i> *	<i>Stephanopyxis nipponica</i> *
647	<i>C. furcellatus</i> *	<i>Thalassiosira anguste-lineata</i>
648	<i>C. laciniosus</i> *	<i>T. antarctica</i> var. <i>borealis</i> *
649	<i>C. radicans</i> *	<i>T. nordenskiöldii</i> *
650	<i>C. similis</i> *	<i>Thalassiosira</i> spp.
651	<i>C. socialis</i> *	
652	<i>C. teres</i> *	
653	<i>C. tortissimus</i>	
654	<i>Chaetoceros</i> spp.	

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 662 Table 3. Contribution (%) and abundance ($\times 10^2$ cells L⁻¹) of resting spores at initial and 10 d of
 663 cultivation. *Chaetoceros* spp. 1: *C. compressus*, *C. laciniosus*, *C. similis* and *C. socialis*. *Chaetoceros*
 664 spp. 2: *C. cinctus*, *C. furcellatus*, *C. radicans* and *C. tortissimus* (see detail in text). +: Resting spore
 665 observed; -: No resting spore detected.

		0 day		10 day	
		Control N-limited Fe-limited			
668	Centric diatoms				
669	<i>C. debilis</i>	–	9.8	85.3	30.0
670		(0)	(410)	(23500)	(1350)
671	<i>C. diadema</i>	1.3	5.2	41.6	2.4
672		(9.6)	(187)	(9520)	(199)
673	<i>C. teres</i>	+	+	+	+
674		(0.8)	(3)	(43)	(3)
675	<i>Chaetoceros</i> spp. 1	9.7	1.1	25.5	7.7
676		(169)	(2160)	(21600)	(14500)
677	<i>Chaetoceros</i> . spp. 2 ^a	8.9	18.4	59.6	73.6
678		(391)	(429)	(24300)	(2660)
679	<i>D. confervacea</i>	–	–	+*	+*
680		(0)	(0)	(7)*	(3)*
681	<i>P. sp. cf. pentaportula</i>	–	+	+*	+*
682		(0)	(4)	(18)*	(5)*
683	<i>S. nipponica</i>	2.3	53.3	100	78.2
684		(0.4)	(40)	(181)	(97)
685	<i>T. antarctica</i> var. <i>borealis</i>	+	–	+	+
686		(0.1)	(0)	(2)	(2)
687	<i>T. nordenskioeldii</i>	–	3.9	40.0	43.2
688		(0)	(5)	(423)	(140)
689	<i>L. danicus</i>	–	–	–	–
690	<i>P. arata</i>	–	–	–	–

691	Pennete diatoms				
692	<i>F. oceanica</i>	–	+	+	+
693		(0)	(22)	(220)	(155)
694	<hr/>				

695 ^a: Resting spores of *C. radicans* was not detected.

696 *: Data at 5 d of cultivation because of no data at 10 d of cultivation.

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Table 4. Si:N drawdown ratio of natural phytoplankton community incubation experiment. Data represents means of triplicate experiments $\pm$ 1SD during 0–3 d and 0–5 d cultivations, and represent the ranges of duplicates for data during 0–10d cultivation period. --: No significant DIN uptake was observed.

Treatment	0–3 d	0–5 d	0–10 d
Control	1.17 ± 0.06	0.81 ± 0.01	0.69–0.70
N-limited	0.77 ± 0.10	1.70 ± 0.08	2.55–2.80
Fe-limited	--	2.48 ± 0.39	2.26–2.50

FIGURES CAPTIONS

Fig. 1. Temporal changes in (a) vegetative cells, (b) resting cells, (c) resting spores densities and (d) resting spores percentages for unialgal culture experiment. Data represents means $\pm$ 1 SD of triplicates.

Fig. 2. Temporal changes in (a) DIN, (b) Si(OH)₄ and (c) PO₄ for unialgal culture experiment. Data represents means $\pm$ 1 SD of triplicates.

Fig. 3. Vertical profiles of (a) sigma-*t* and temperature, (b) DIN, Si(OH)₄ and Si:N ratio, and (c) D-Fe and chlorophyll *a*.

Fig. 4. Temporal changes in (a) chlorophyll-*a* concentrations, (b) diatom abundances, (c) resting spore densities and (d) resting spore percentages for natural phytoplankton community incubation experiment. Data for (a) represents means $\pm$ 1 SD for triplicates and the range for duplicates.

Fig. 5. Temporal changes in (a) DIN, (b) Si(OH)₄ and (c) PO₄ for natural phytoplankton community incubation experiment. Data represents means $\pm$ 1 SD for triplicates and the range for duplicates.

Fig. 6. Temporal changes in the contribution of resting spores in (a) *C. debilis*, (b) *Chaetoceros* spp. 1, (c) *S. nipponica*, (d) *C. diadema*, (e) *Chaetoceros* spp. 2 and (f) *T. nordenskiöldii* for natural phytoplankton community incubation experiment. Note that scales of y axis were up to 100% for (a), (b) and (c), and up to 50% for (d), (e) and (f).

Fig. 7. Relationship between the Si:N drawdown ratio and the percentage of resting spores (RSP). Solid diamond represent unialgal culture experiment during 11 d cultivation period and open circle represent natural phytoplankton community incubation experiment under Fe- and N-limited treatment during 10 d cultivation period. The plotted line was obtained by least-square regression: [Si:N drawdown ratio] = $e^{0.022 \times (RSP)}$ ($r^2 = 0.91$, $n = 11$, $p < 0.001$). Y intercept was set at 1 according to the value of vegetative growing diatoms (Brzezinski, 1985).

Fig. 1

Fig. 2

Fig. 3

Fig. 4

Fig. 5

Fig. 6

Fig. 7