Resting spore formation in the marine diatom *Thalassiosira nordenskioeldii* under iron- and nitrogen-limited conditions

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Running Head

Iron limited resting spores

ABSTRACT

Resting spore formation was investigated in the neritic and oceanic strains of *Thalassiosira nordenskioeldii* under iron- and nitrate-depleted conditions at 5°C and 10°C. Both strains immediately formed resting spores under nitrate-depleted conditions with almost 100% composition after 4–8 day (d) and 3–6 d cultivation periods at 5°C and at 10°C, respectively. However, resting spore formation in both strains under iron-depleted conditions increased with incubation time more gradually, and 15 d of cultivation, spore composition ranged from 60% in the neritic strain at 5°C to 1% in the oceanic strain at 10°C. In addition, chlorotic cells with smaller cell volume compared with vegetative cells were observed under iron-depleted conditions. Sinking rates of vegetative cells, iron-limited cells and spores, and nitrate-limited resting spores cultivated at 5°C were 1.24 ± 0.14 m d^{-1}, 3.41 ± 0.43 m d^{-1} and 9.22 ± 1.04 m d^{-1}, respectively, slightly faster than those at 10°C. The faster sinking rates in iron-limited resting cells and resting spores than in vegetative cells may prevent their habitat from expanding to high-nitrate low-chlorophyll oceanic regions with low iron concentrations.

INTRODUCTION

Diatoms, a major group of siliceous organisms, play a predominant role in spring phytoplankton bloom formation, annual primary and export production and controlling marine biogeochemical cycling of biological elements (Smetacek, 1999; Ragueneau *et al*., 2000). In oceanic and coastal wasters of temperate to polar regions, the annual spring bloom in the phytoplankton community is a common phenomenon in which large chain-forming centric diatoms plays a predominant role (Smetacek, 1999; Sarthou *et al*., 2005). Spring bloom diatoms often form resting spores and resting cells (hereafter referred as “resting stages” for both types) in response to macronutrients limitation, decline in light intensity and fluctuations in salinity and/or temperature. In particular, nitrogen limitation is an important factor controlling diatom resting spore formation (McQuoid and Hobson, 1996). The centric diatom *Thalassiosira nordenskioeldii* Cleve is a major

component of temperate and boreal spring blooms and produces fast sinking resting spores following a bloom (Inoue and Taniguchi, 1999).

Resting stages have been considered as part of a long-term survival strategy since they may constitute the next growth season's seed population in seasonally blooming species and are more resistant to grazing than vegetative cells (Hargraves and French, 1983; McQuoid and Hobson, 1996; Lewis *et al*., 1999; McQuoid and Godhe, 2004; Kuwata and Tsuda, 2005). Diatom resting cells are morphologically similar to vegetative cells, but resting spores with heavily silicified valves are morphologically, biologically, ecologically and physiologically different from vegetative cells (Kuwata *et al*., 1993; Kuwata and Takahashi, 1999). Three main types of resting spores, designated endogenous, semi-endogenous and exogenous are based on whether the spores are completely, partly or not at all enclosed within the parent cell frustule, respectively (Syvertsen, 1979). The intracellular structures of resting cells differ from vegetative cells but are not as heavily silicified as resting spores (Sicko-Goad *et al*., 1989; Kuwata *et al*., 1993). Heavily silicified diatom resting spores have a faster sinking rate than vegetative cells, despite their smaller cell volume (CV) (Kuwata *et al*., 1993; McQuoid and Hobson, 1996), and sedimentation of resting spores has been used as a proxy for paleoproductivity (Abelmann *et al*., 2006).

Iron is an essential micronutrient for phytoplankton growth and an important component of several biochemical processes such as photosynthetic and respiratory electron transport, and nitrate assimilation (Weinberg, 1989; Geider and La Roche, 1994), all of which are inhibited by iron limitation (Milligan and Harrison, 2000). Bloom-forming diatoms are often dominated by neritic species that have higher iron requirements than oceanic species (Sunda and Huntsman, 1995). The thermodynamically stable oxidation state of iron in oxic surface seawater Fe(III) has extremely low solubility (Stumm and Morgan, 1996). In general, the phytoplankton iron uptake rate is related to the computed equilibrium concentration of Fe^{3+} in seawater and is dependent on the concentration of bioavailable dissolved inorganic Fe(III) species ([Fe(III)′]) (Anderson and Morel, 1982). In addition,

the presence of natural organic ligands complexes associated with Fe(III) in seawater reduces the bioavailable [Fe(III)′] (Rue and Bruland, 1995). Therefore, marine phytoplankton in offshore regions situated away from iron sources are in iron limited (Martin, 1990). Until date, there are no reports on resting spore formation by marine diatoms under iron-limited conditions.

In the present study, we hypothesized that iron-limited marine diatoms would form resting spores as a result of decreased nitrate assimilation caused by iron limitation. We investigated the formation ability of resting stages. Resting status was defined by the chlorotic, shrunken, less abundant and asymmetrically distributed chloroplasts without stored products, within a cell. This status was investigated in neritic and oceanic isolates of *T. nordenskioeldii* under iron- and nitrate-depleted conditions at 5°C and 10°C. In addition, we assessed the abundance and size of exogenous, semi-endogenous and endogenous resting spores and resting cells to investigate morphological divergences affecting the sinking rate, and commented on their biological significance.

METHODS

Algal strains

Two strains of *T. nordenskioeldii* were examined. A neritic strain (*T. n* A) was isolated by Pasteur-pipette from the residue of a sieved (20-μm nylon mesh) sediment sample, which was collected from the bottom (20 m) in Onagawa Bay (38°46′N, 141°46′E) northern Honshu, Japan. The Oyashio Current (OC, subarctic water) flows along the northwestern Pacific Ocean side of northern Honshu, Japan, and constitutes the southern range limit of *T. nordenskioeldii* (Hasle, 1976; Inoue and Taniguchi, 1999). An oceanic strain (*T. n* B) was isolated by capillary pipette from the surface seawater of the OC region (42°00′N, 145°15′E; depth 3800 m) on the northwestern Pacific Ocean side of southern Hokkaido, Japan. Frustules in the two strains of *T. nordenskioeldii* were cleaned using the method of Nagumo (Nagumo, 1995) and the cleaned samples were identified by scanning electron microscopy followed by isolation under light microscopy, according to Hasle and Syvertsen (Hasle

and Syvertsen, 1997). The unialgal strains were maintained by silicic acid-enriched f/2 medium (Guillard and Ryther, 1962) under 150 μmol photons m^{-2} s^{-1} fluorescent light (QSL-100; Biospherical Instrument Inc. San Diego, CA, USA) (12h light:12 h dark) at 10°C. The maintenance cultures were not completely axenic, but bacterial contamination was minimized by sterile techniques and serials transfer during exponential growth.

Culture experiment

Culture medium seawater 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-μm GS membrane filter (Millipore). The filtered seawater was autoclaved for 20 min at 121°C (108 kPa). The concentrations of Fe, NO_3+NO_2, PO_4 and $Si(OH)_4$ in the filtered autoclaved seawater were <2 nmol L^{-1}, <5 μmol L^{-1}, <0.5 μmol L^{-1} and approximately 250 μmol L^{-1}, respectively. Fe concentrations in the filtered autoclaved seawater, which was used in the laboratory culture experiments, were determined by an automated Fe analyzer (Kimoto Electric) with using a combination of chelating resin concentration and luminol-hydrogen peroxide chemiluminescence detection in a closed flow-through system (Obata *et al*., 1993). Nutrient concentrations in the collected seawater were measured by a QuAAtro continuous flow analyzer (Bran+Luebbe).

All equipment used in culture experiments was acid cleaned and followed by rinsing with Milli-Q water (Millipore: >18.0 MΩ cm^{-1}). All preparations and samplings for experiments were performed in a Class 100 laminar flow cabinet to avoid inadvertent trace metal contamination. Prior to culture experiments, diatoms were grown in silicic acid-enriched [105 μmol L^{-1}, $Si(OH)_4$] f/2 medium (Si-enriched f/2 nutrients plus f/2 metals) (Guillard and Ryther, 1962) with at least 2 transfers and >18 doublings during the exponential growth phase. The silicic acid-enriched f/2 medium contained 880 μmol L^{-1} NO_3, 38 μmol L^{-1} PO_4 and 355 μmol L^{-1} $Si(OH)_4$ as macronutrients and 11.7 μmol L^{-1} Fe(III), 0.44 μmol L^{-1} Co(II), 0.91 μmol L^{-1} Mn(II), 73 nmol L^{-1} Zn(II), 28 nmol L^{-1} Cu(II) and 29 nmol L^{-1} Mo(VI) with 15 μmol L^{-1} EDTA for trace metals. Diatoms at the late exponential growth

126 phase were inoculated into modified f/2 medium, which was prepared without adding f/2 metals,
127 EDTA and vitamins to the f/2 nutrients-added filtered autoclaved seawater (control medium). All f/2
128 nutrient stock solutions were passed through Chelex 100 ion-exchange resin (Bio-Rad) to remove
129 trace metals (Morel *et al.*, 1979). Diatoms were grown in modified f/2 media, to which ferric iron
130 stock solution (25 μmol L^{-1} Fe(III): $FeNH_4(SO_4)_2 \cdot 12H_2O$ in 5 mmol L^{-1} HCl, pH 2.3) and manganese
131 stock solution (25 μmol L^{-1} Mn(II): $MnCl_2$ in 5 mmol L^{-1} HCl, pH 2.3) were added to final Fe and
132 Mn concentrations of 100 and 25 nmol L^{-1}, respectively. Diatoms were grown at 5°C or 10°C under
133 150 μmol photon m^{-2} s^{-1} fluorescent light (12 h light:12 h dark) to obtain adequate cell densities for
134 the experiments. Addition of both Mn and Fe to filtered autoclaved seawater has been reported to
135 induce the highest growth rates with full physiological recovery for a long time in Mn-sufficient media
136 (Ushizaka *et al.*, 2008); therefore, Mn was added to the culture media in the present study.
137 A small amount of pre-cultured diatoms (~1 mL) in the late exponential growth phase was
138 inoculated into each control medium in polycarbonate Erlenmeyer flask. The effects of direct Fe and
139 Mn inputs (direct Fe treatment) and f/2 metal (f/2 treatment) inputs were examined by adding a small
140 amount of either Fe(III) and Mn(II) stock solutions and f/2 metal stock solution, respectively, directly
141 and together with the inoculation of diatom culture into control media. The growth rates and maximal
142 cell yields were not different between direct Fe and f/2 treatments at the same temperature. Fe-limited
143 media (Fe-limited treatment) were examined by adding only acidic Mn(II) stock solution directly, and
144 with the inoculation of culture into the control media. Nitrate-limited media (N-limited treatment)
145 were prepared by adding f/2 metal stock solution into the modified control media without the addition
146 of nitrate stock.
147 It has been reported that the addition of an excess concentration of the siderophore
148 desferrioxamine B (DFB) eliminated iron uptake in phytoplankton by diminishing the concentration of
149 bioavailable Fe(III)′ (Wells *et al.*, 1994). DFB is a small trihydroxamate molecule that complexes
150 inorganic Fe(III) with an extremely high conditional stability constant ($K'_{FeL,\ Fe(III)'}$ =

151 [Fe(III)L]/[Fe(III)′][L] = $10^{16.5}$ M^{-1}) in seawater (Hudson *et al*., 1992). In other studies (Iwade *et al*.,
152 2006; Yoshida *et al*., 2006), iron uptake by *Chaetoceros socialis* from external Fe was prevented by
153 adding DFB during cultivation. DFB–Fe-limited media (DFB–Fe-limited treatment) experiments were
154 performed by adding acidic Mn(II) stock solution into the control media and then adding DFB after
155 12–24 h of cultivation. DFB was added to a final concentration of 1 μmol L^{-1} in the Fe-limited media
156 to prevent further iron uptake by *T. nordenskioeldii* from ambient external Fe.

157 Cell densities at the start of the culture experiments were approximately 1,000 cells mL^{-1}.
158 Light and temperature conditions were the same as those for the stock culture described above. During
159 the experiments, numbers of vegetative cells and resting stages were monitored daily by triplicate cell
160 counts in a haemocytometer with a light microscope. Culture experiments were conducted in triplicate.
161 In *T. n* A culture experiments, the cultivation flasks were covered with aluminium foil after 15 d of
162 cultivation to monitor the percentage and composition of resting spores during 15–115 d dark
163 cultivation. In culture experiments at 5°C for *T. n* B and 10°C for *T. n* A, cell size (diameter and
164 height) measurements were taken of vegetative and/or resting stage cells in initial, exponential (6 d
165 cultivation) and stationary (15 d cultivation) growth phases by a calibrated ocular
166 micrometer-equipped light microscope to calculate CV and surface area (SA).

167 **Sinking rate**

168 The settling tube (Fisher borosilicate glass) was filled with control medium, tightly capped
169 with foil and acclimated at least 30 min at room temperature before measurement. A cell suspension
170 (200-μL) was diluted with a few drops of Milli-Q water, and a layer of a 100-μL aliquot was spread
171 with a micropipette on top of the temperature acclimated settling tube. Sinking rates of the vegetative
172 cells (6 d cultivation), Fe-limited resting stages (15 d cultivation) and N-limited resting spores (15 d
173 cultivation) for *T. n* B strain cultivated at 5°C and 10°C were measured at room temperature in
174 triplicate with a fluorometer (Turner Design AU-10) according to the method by Eppley *et al*. (Eppley
175 *et al*., 1967).

RESULTS

There was no formation of resting spores and resting cells in either *T. nordenskioeldii* strains in the direct Fe and f/2 treatments. A gradual increase in resting spores during the 15 d cultivation period was observed in Fe- and DFB–Fe-limited treatments. In contrast, there was a rapid and almost complete transformation of vegetative cells into resting spores after 4–8 d (5°C) and 3–6 d (10°C) cultivation observed in N-limited treatment (Fig. 1). In five culture treatments at 5°C and 10°C, direct Fe or f/2 treatments induced the highest maximal vegetative cell yields for both strains, while the Fe-, DFB–Fe- and N-limited treatments showed lower maximal vegetative cell yields than direct Fe or f/2 treatments (Fig. 1a-1, b-1, c-1 and d-1, Table I). However, the initial growth rate of each strain was almost the same among various treatments at each temperature with the lower growth rate occurring at lower temperatures (μ: 0.70 d^{-1} and 0.46 d^{-1} for the *T. n* A strain and 0.76 d^{-1} and 0.53 d^{-1} for the *T. n* B strain at 10°C and 5°C, respectively) (Table I). Vegetative cell densities during the stationary growth phase in Fe-limited treatment were relatively constant for several days after the transition phase, while those in N-limited treatment decreased suddenly after the late exponential growth phase (after 3 and 4–6 d cultivations at 10°C and 5°C, respectively).

In both strains, resting spore densities in N-limited treatment increased rapidly after 3 and 4–6 d cultivations at 10°C and 5 °C, respectively (Fig. 1a-2, b-2, c-2 and d-2), coincident with the sudden decrease in vegetative cell densities (Fig. 1a-1, b-1, c-1 and d-1). In contrast, Fe- and DFB–Fe-limited treatments resulted in a gradual increase in resting spore densities with time (at 10°C Fig. 1a-3, at 5°C Fig. b-3 and d-3) or a small increase of *T. n* B in DFB–Fe-limited treatment at 10°C (Fig. 1c-3) during 15 d cultivation. The resting spore compositions of *T. n* A and *T. n* B strains in N-limited treatment reached almost 90–100% after 6–8 and 4–6 d cultivations at both temperatures, respectively, while those in Fe- and/or DFB–Fe-limited treatments gradually increased to 19% at 10°C and 60% at 5°C for *T. n* A, and 1% at 10°C and 22% at 5°C for *T. n* B (Fig. 1a-3, b-3, c-3 and d-3).

During the long cultivation period (30–115 d) under dark conditions following 15 d of cultivation, resting spore composition of *T. n* A increased to 35% at 60 d (10°C) in Fe-limited treatment (Fig. 2a) with nearly double the number of resting spores, while the number of resting cells were constant, and increased to ~90% at 115 d of cultivation (5°C) in Fe- and DFB–Fe-limited treatments (Fig. 2b). The decrements in the number of resting cells (88% and 94% for Fe- and DFB–Fe-limited treatments, respectively) were much greater than those of resting spores (16% and 50%) at 5°C experiment. However, N-limited treatment maintained ~100% resting spore composition for a long period (30–115 d), even after 15 d of cultivation at both temperatures without a decrement in spore number (Fig. 2).

During cultivation of *T. n* A in N-, Fe- and DFB–Fe-limited treatments, the relative composition of exogenous, semi-endogenous and endogenous resting spores gradually shifted from semi-endogenous to endogenous with a nearly constant contribution of the exogenous type (Fig. 3a and b). In *T. n* B, however, exogenous resting spores were dominant and few endogenous spores were observed in Fe- and DFB–Fe-limited treatments, while in N-limited treatment at 5°C, exogenous and semi-endogenous resting spores were approximately equal while the endogenous type was low in relative abundance at the end of the experiment (49%, 51% and 0.3%, respectively) (Fig. 3c and d). During the long cultivation period (30–115 d) under dark conditions after 15 d of cultivation for *T. n* A (Fig. 4), endogenous resting spores decreased from 60% (15 d cultivation) to 50% (60 d cultivation) at 10°C (Fig. 4a), and from 35% (15 d cultivation) to 15% (115 d cultivation) at 5°C in Fe-limited treatment (Fig. 4b). However, N-limited treatment maintained the relative compositions of the three spore types for the duration of the experiment (15–115 d).

Sinking rate and cell size

Sinking rates of vegetative cells, Fe-limited resting cells and spores (78% and 22% at 5°C and 99% and 1% at 10°C for resting cells and spores, respectively), and N-limited resting spores for *T. n* B strain were 1.24 ± 0.14, 3.41 ± 0.43 and 9.22 ± 1.04 m d^{-1} at 5°C and 1.02 ± 0.30, 1.75 ± 0.08 and 7.04 ± 1.22 m d^{-1} at 10°C, respectively (Fig. 5).

226 In *T. n* B at 5°C (Fig. 1d-1, -2 and -3), no change in cell sizes was observed between initial
227 and vegetative cells at the late exponential growth phase (6 d cultivation) in direct Fe treatment (Table
228 II). However, sizes of both Fe-limited resting cells and N-limited resting spores decreased by about
229 8% and 40% in diameter and height, respectively (Table II). Consequently, CV of Fe-limited resting
230 cells and N-limited resting spores were approximately half those of the vegetative cells in the direct Fe
231 treatment, while resting cells and resting spores had about one-third higher SA:CV ratios than
232 vegetative cells (Table II). In *T. n* A, almost the same trends were observed in *T. n* B for vegetative
233 cells, Fe- and N-limited resting stages, respectively (data not shown).
234
235 **DISCUSSION**
236 **Formation of resting spores in Fe-limited treatment**
237 Several experiments with marine diatom culture have shown that changes in environmental
238 triggers, such as nutrients, pH, light and temperature may induce resting stages (McQuoid and Hobson,
239 1996). Some researchers have found that nitrogen deficiency is an important factor to induce
240 sporulation in marine diatoms (Durbin, 1978; Hargraves and French, 1983). In the present study,
241 N-limited treatment induced rapid resting spore formation of *T. nordenskioeldii* and achieved almost
242 100% spore contribution within 3–4 d of the start of sporulation (Fig. 1), similar to the results
243 previously reported (Durbin, 1978; Syvertsen, 1979). However, the current study is the first known
244 report on resting spore formation in a marine diatom under iron-depleted conditions (Figs. 1 and 2).
245 Both Fe- and DFB–Fe-limited treatments induced gradual spore formation in *T. nordenskioeldii* of
246 22%–60% at 5°C and 1%–19% at 10°C during 15 d cultivation periods (Figs. 1 and 2). It has been
247 reported that iron limitation significantly decreases nitrate assimilation by limiting photosynthetic
248 electron transport energy (Milligan and Harrison, 2000). In addition, Maldonado and Price
249 (Maldonado and Price, 1996) reported that severe iron limitation in marine diatoms induced iron and
250 nitrate co-limitation. Therefore, the slower resting spore formation in the iron-deficient treatments as

compared to N-limited treatment may have resulted from a gradual decrease in intracellular nitrate assimilation under iron-deficient conditions, which has been reported gradually reduced ambient bioavailable iron and/or intracellularly stored iron in iron-deficient media (Iwade *et al*., 2006). However, whether the sporulation trigger under iron-deficient conditions is affected directly by iron deficiency or indirectly by iron and nitrogen co-limitation is uncertain. This trigger needs to be investigated in future physiological and molecular assays.

The increase in the proportion of resting spores during the 15 d cultivation period in Fe-limited treatment was also higher at lower temperature (60% at 5°C and 19% at 10°C for *T. n* A strain; Fig. 1a-3 and b-3), similar to the higher resting spore contribution at lower temperatures under N-depleted conditions reported in a previous study [76%–96% at 5°C and 40%–52% at 10°C, (Durbin, 1978)] (Table III). Temperature, therefore, seems to be one of an important factor in the formation of diatom resting spores. It has been reported that resting spores tend to survive longer at colder temperatures and that spores of boreal species do not appear to tolerate temperatures higher than the tolerant limits of their vegetative cells (Hargraves and French, 1983; McQuoid and Hobson, 1996). Furthermore, it has been further reported that resting cell and resting spore formation usually occurs within the lower portion of the range of temperatures at which a given species grows (Durbin, 1978).

Diatom resting spores in the sediments have been used as a proxy for paleoproductivity in the Southern Ocean (e.g. Abelmann *et al*., 2006). Resting spore formation was induced by iron-deficient low-productivity conditions in the present study, suggesting that resting spore formation might be induced in Fe-depleted oceanic environments, such as Southern Ocean, if the neritic diatoms were introduce to the region.

Implications for biological oceanography of diatoms

Remarkably different percentage contributions of resting spores and different compositions of endogenous, semi-endogenous and exogenous resting spores were observed between *T. n* A and *T. n* B strains in Fe- and N-limited treatments in the present study, and between them and other strains [*T. n*

276 Narragansett Bay (Durbin, 1978) and *T. n* Oslofjord (Syvertsen, 1979)] in N- or P-limited treatments
277 (Table III). In *T. n* A (a neritic strain), approximately 60%–85% of the resting spores after a 15 d
278 cultivation period were composed of semi-endogenous and endogenous resting spores with
279 significantly lower SA:CV ratios than those of vegetative cells in N-, Fe- and DFB–Fe-limited
280 treatments ($p < 0.005$, one-way ANOVA), while almost 100% of the cells formed in *T. n* B (an oceanic
281 strain) were exogenous resting spores or resting cells with significantly higher SA:CV ratios than their
282 vegetative cells under iron-deficient conditions ($p < 0.001$, one-way ANOVA) (Figs. 3, 4 and 6). The
283 low SA:CV ratio of resting spores, formed by the neritic strain, suggest rapid sinking of spores to
284 relatively shallow, coastal seafloors. However, the present study suggests that the rapidly sinking
285 spores in a neritic strain would be at a competitive disadvantage to an oceanic strain in a pelagic
286 environment, because the resting spores of an oceanic strain need only to sink to the pycnocline before
287 resuspension and germination can take place (Hargraves and French, 1983). The temperature- and
288 strain-specific morphological diversity in the *T. nordenskioeldii* strains in the present study (Table III),
289 differed from the phylogenetic, species-specific morphology among the three spores types in *T.*
290 *nordenskioeldii*, suggested by Syvertsen (Syvertsen, 1979) and Hasle and Syvertsen (Hasle and
291 Syvertsen, 1997). These observations indicate that morphological adaptation in macro- and
292 micronutrient-depleted environments, may suit each habitat even within the same species. Moreover,
293 the relative higher temperature tolerance observed in autochthonous *T. nordenskioeldii* in Onagawa
294 Bay may indicate genetic diversity among local populations, as suggested by Inoue and Taniguchi
295 (Inoue and Taniguchi, 1999). This cryptic diversity in the biology of *T. nordenskioeldii* needs to be
296 elucidated.

297 Iron-limited treatments of both strains induced a large number of chlorotic resting cells in
298 addition to resting spores (Table I). Iron-limited cells decreased their cell height to a greater extent
299 than their diameter after 15 d of cultivation (Table II). An increment in cellular silicon content has
300 been reported (Takeda, 1998), which could interpret as increase the thickness of silicified cell walls in

response to iron limitation. This phenomenon may have contributed to the relatively quicker decrease in diameter with only 5.6 cell divisions during the 15 d cultivation period (Tables I and II). The decreased in cell height and diameter under iron-depleted conditions results in decreased CV and the increase in SA:CV ratios can both contribute to fast sinking rates of heavily silicified resting cells (Fig. 5). These morphological changes could result in an increase in the uptake of iron and macronutrients by allowing the diatom to sink to nutrient-rich deep water. In addition, the decrease in cell size may lower the cellular nutrient requirements and streamline the efficiency of intracellular material cycling as suggested previously (Pahlow *et al*., 1997; Raven, 1998; Raven and Waite, 2004). Therefore, silicification of resting stages, and of diatoms in general, could be a significant survival strategy acquired through evolution of Cretaceous ecosystems to the current iron and nutrient aquatic environments (Raven and Waite, 2004; Falkowski *et al*., 2004). The two main features of diatom resting spores are the heavily silicified spore frustules and compaction of cellular contents in the spores. A heavy spore frustule with an increased sinking rate not only would transfer the resting spores more quickly to new nutrient sources or remove them from dangerously high light intensity in the absence of such nutrients but also removes the cells at depth, sequester from potential pathogens (viruses) and/or predators (protozoa and crustaceans) (Raven and Waite, 2004). However, in the present study, resting cells did not survive as long as 115 days, even under lower temperature conditions (Table III), similar to the observations by Kuwata and Takahashi (Kuwata and Takahashi, 1999). Therefore, it appears to be difficult for the resting cells to survive in an oceanic environment and migrate from basin to basin directly by crossing the subarctic Pacific Front current system, a high-nutrient low-chlorophyll (HNLC) region. On the other hand, the almost cosmopolitan distribution of the *T. nordenskioeldii* metapopulation in the world could be achieved by expanding of the local populations in coastal regions. In addition, neritic diatoms that forms resting spores and cells under iron-deficient conditions with having faster sinking rates than vegetative cells may prevent their seeding populations from expanding to HNLC oceanic regions with low iron concentrations.

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501 **Table and Figure legends**

502 Table I: Maximal cell yield of vegetative cells, resting spores and resting cells of *Thalassiosira*
503 *nordenskioeldii* (*T. n* A and *T. n* B strains) during the 15 d cultivation period at 5°C and 10°C. Specific
504 growth rates (μ) during the exponential phase and cultivation days with maximal growth yields are
505 given in parentheses.

506

507 Table II: Cell diameter (r), height (h), r:h ratio, cell volume (CV), surface area (SA) and SA:CV ratio
508 of *T. n* B strain cultivated at 5°C. Cell sizes were measured at the exponential growth phase (6 d
509 cultivation) in direct Fe treatment, and at the stationary growth phase (15 d cultivation) in the Fe- and
510 N-limited treatments. Standard deviations (±1 SD) are given in parentheses.

511

512 Table III: Comparison of predominance and composition of the three types of resting spores in *T. n* A
513 and *T. n* B strains under Fe- and N-limited treatments, cultivated at 5°C and 10°C, and two strains [*T. n*
514 Narragansett Bay (Durbin, 1978) and *T. n* Oslofjord (Syvertsen, 1979)] in the N- or P-limited
515 treatments.

516

517 Fig. 1. Temporal changes in vegetative cell density (1), resting spore density (2) and resting spore
518 percentage (3) of the *T. n* A strain at 10°C (a), and at 5°C (b) and the *T. n* B strain at 10°C (c) and at
519 5°C (d) in the f/2, direct Fe, Fe-limited, DFB–Fe-limited and N-limited treatments. Data represent
520 means of triplicate experiments and the error bars indicate ±1 SD.

521

522 Fig. 2. Long-term changes in resting spore percentages in the *T. n* A strain at 10°C (a) and 5°C (b)
523 during 15-115 d cultivation periods in N-, Fe- and DFB–Fe-limited treatments. Data represent means
524 of triplicate experiments and the error bars indicate ±1 SD.

525

526 Fig. 3. Temporal changes in the composition of three resting spore types (exogenous,
527 semi-endogenous and endogenous) of the *T. n* A strain at 10°C (a) and 5°C (b), and the *T. n* B strain at
528 10°C (c) and 5°C (d) in N- (1), Fe- (2) and DFB–Fe-limited (3) treatments. Data represent means of
529 triplicate experiments and the error bars indicate ±1 SD.
530
531 Fig. 4. Long-term changes in the composition of the three resting spore types in the *T. n* A strain at
532 10°C (a) and 5°C (b) during 15–115 d cultivation periods in the N- and Fe-limited treatments. Data
533 represent means of triplicate experiments and the error bars indicate ±1 SD.
534
535 Fig. 5. Sinking rates of vegetative cells (6 d cultivation), Fe-limited resting stages (resting cells and
536 resting spores, 15 d cultivation) and N-limited resting spores (15 d cultivation) of the *T. n* B strain
537 cultivated at 5°C and 10°C. Data represent means of triplicate measurements and the error bars
538 indicate ±1 SD.
539
540 Fig. 6. Ratios of cell surface area to cell volume (SA:CV) in vegetative cells, the three resting spore
541 types, and Fe-limited resting cells of *T. n* A and *T. n* B strains. (RS: resting spores). Data represent
542 mean ±1 SD.
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551 Table I

Strain and Treatment (Specific growth rate: μ)	Maximal cell yield (cells mL^{-1}) Vegitative cells		Resting spores		Resting cells	
T. n A at 10°C	(μ: 0.70 d^{-1})					
Direct Fe	58900	(8 d)	-		-	
Fe-limited	4400	(6 d)	2700	(15 d)	12300	(15 d)
N-limited	5900	(3 d)	10900	(12 d)	1900	(4 d)
T. n A at 5°C	(μ: 0.46 d^{-1})					
f/2	62100	(11 d)	-		-	
Fe-limited	6900	(5 d)	19900	(14 d)	12800	(14 d)
DFB−Fe-limited	3100	(3 d)	5700	(15 d)	3900	(15 d)
N-limited	11600	(6 d)	31000	(11 d)	2700	(9 d)
T. n B at 10°C	(μ: 0.76 d^{-1})					
f/2	51200	(7 d)	-		-	
DFB−Fe-limited	10500	(3 d)	800	(14 d)	62800	(14 d)
N-limited	10700	(3 d)	26400	(14 d)	600	(4 d)
T. n B at 5°C	(μ: 0.53 d^{-1})					
Direct Fe	25600	(10 d)	-		-	
Fe-limited	11200	(5 d)	11000	(15 d)	38300	(15 d)
N-limited	6900	(4 d)	15800	(13 d)	1100	(5 d)

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557 Table II

	Initial	Direct Fe 6 d cultivation	Fe-limited 15 d cultivation	N-limited 15 d cultivation
Diameter (r) (μm)	27.15	27.39	25.07	25.93
	(1.08)	(0.77)	(0.79)	(1.31)
Height (h) (μm)	16.63	16.64	10.05	10.60
	(2.23)	(1.79)	(1.49)	(1.20)
r : h	1.66	1.67	2.54	2.48
	(0.24)	(0.20)	0.31	(0.32)
Cell Volume (CV) (μm^3)	9636	9801	4996	5613
	(1447)	(1081)	(1004)	(826)
Surface Area (SA) (μm^2)	2578	2611	1783	1922
	(230)	(168)	(188)	(178)
SA:CV (μm^{-1})	0.27	0.27	0.36	0.35
	(0.02)	(0.01)	(0.03)	(0.03)

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568 Table III.

Species and Treatment	Day	Resting spore composition (%)	Composition of three resting spore types (%)		
			Endogenous	Semi-endogenous	Exogenous
This study (5°C)					
Fe- or DFB−Fe-limited	~115	22.2−91.2	0-36.9	4.9−46.7	20.3−95.1
N-limited	~115	100	0.3−18.7	51.2−65.6	17.0−48.7
This study (10°C)					
Fe- or DFB−Fe-limited	~60	1.3−32.5	0−62.0	15.7−31.4	19.0−94.4
N-limited	~30	99.6−100	0−57.2	1.7−32.5	15.2−98.3
T. n Narragansett Bay at 5°C (Durbin, 1978)					
N-limited	-	76−96	-	-	-
T. n Narragansett Bay at 10°C (Durbin, 1978)					
N-limited	-	40−52	-	-	-
T. n Oslofjord (Syvertsen, 1979)					
N- or P-limited	-	-	<1	ca. 93	ca. 6

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Fig. 1

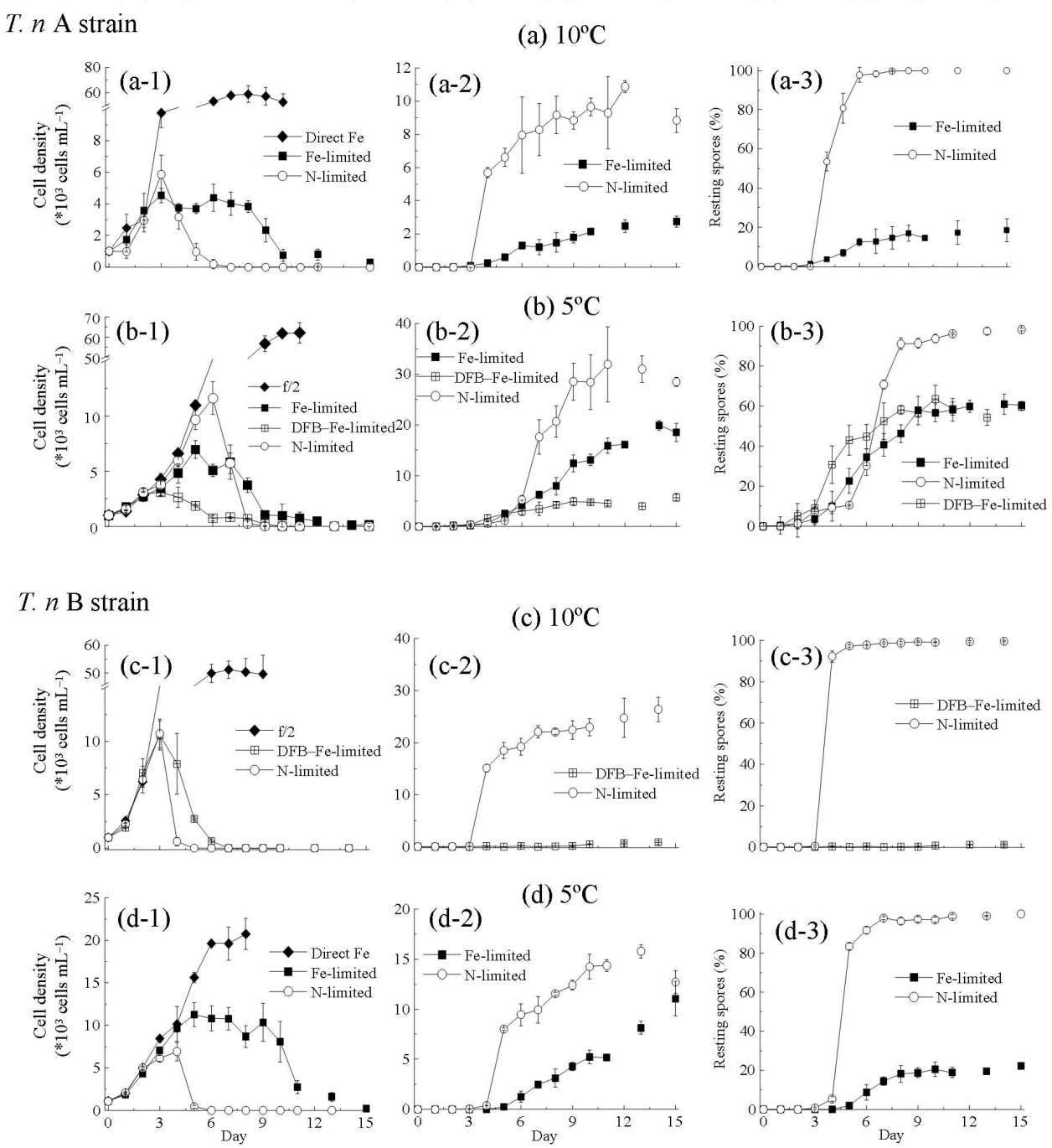

(-1) Vegetative cell density
(-2) Resting spore density
(-3) Resting spore percentage
T. n A strain
(a) 10ºC
(a-1)
(a-2)
(a-3)
Cell density (*10³ cells mL⁻¹)
Resting spores (%)
Direct Fe
Fe-limited
N-limited
(b) 5ºC
(b-1)
(b-2)
(b-3)
f/2
DFB–Fe-limited
T. n B strain
(c) 10ºC
(c-1)
(c-2)
(c-3)
(d) 5ºC
(d-1)
(d-2)
(d-3)
Day

Fig. 2

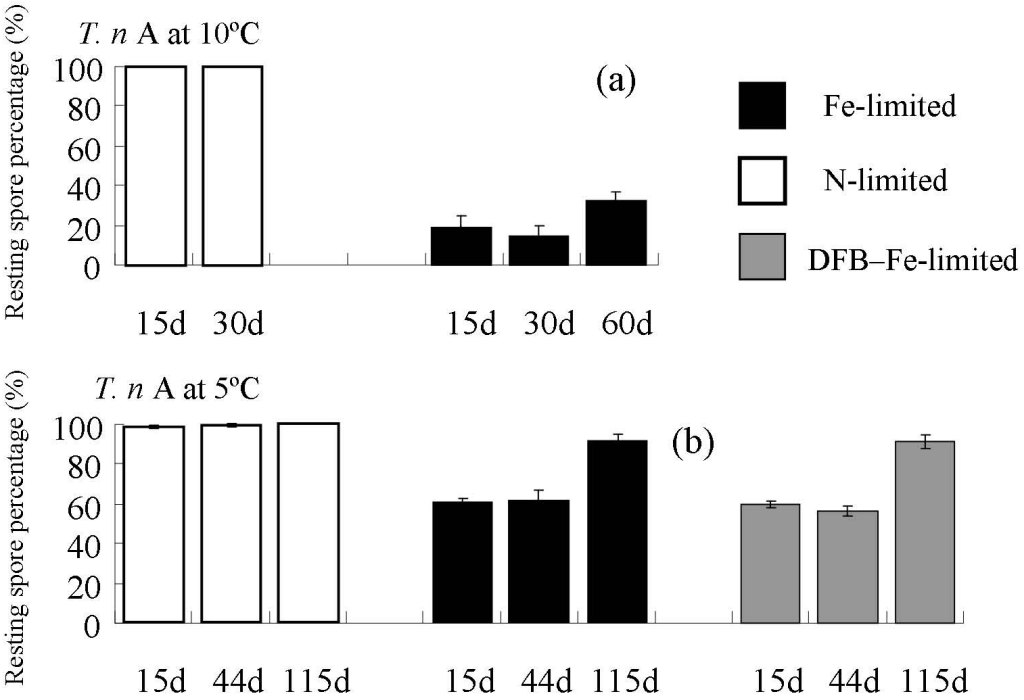

T. n A at 10ºC
Resting spore percentage (%)
100
80
60
40
20
0
(a)
Fe-limited
N-limited
DFB–Fe-limited
15d
30d
15d
30d
60d
T. n A at 5ºC
Resting spore percentage (%)
100
80
60
40
20
0
(b)
15d
44d
115d
15d
44d
115d
15d
44d
115d

Fig. 3

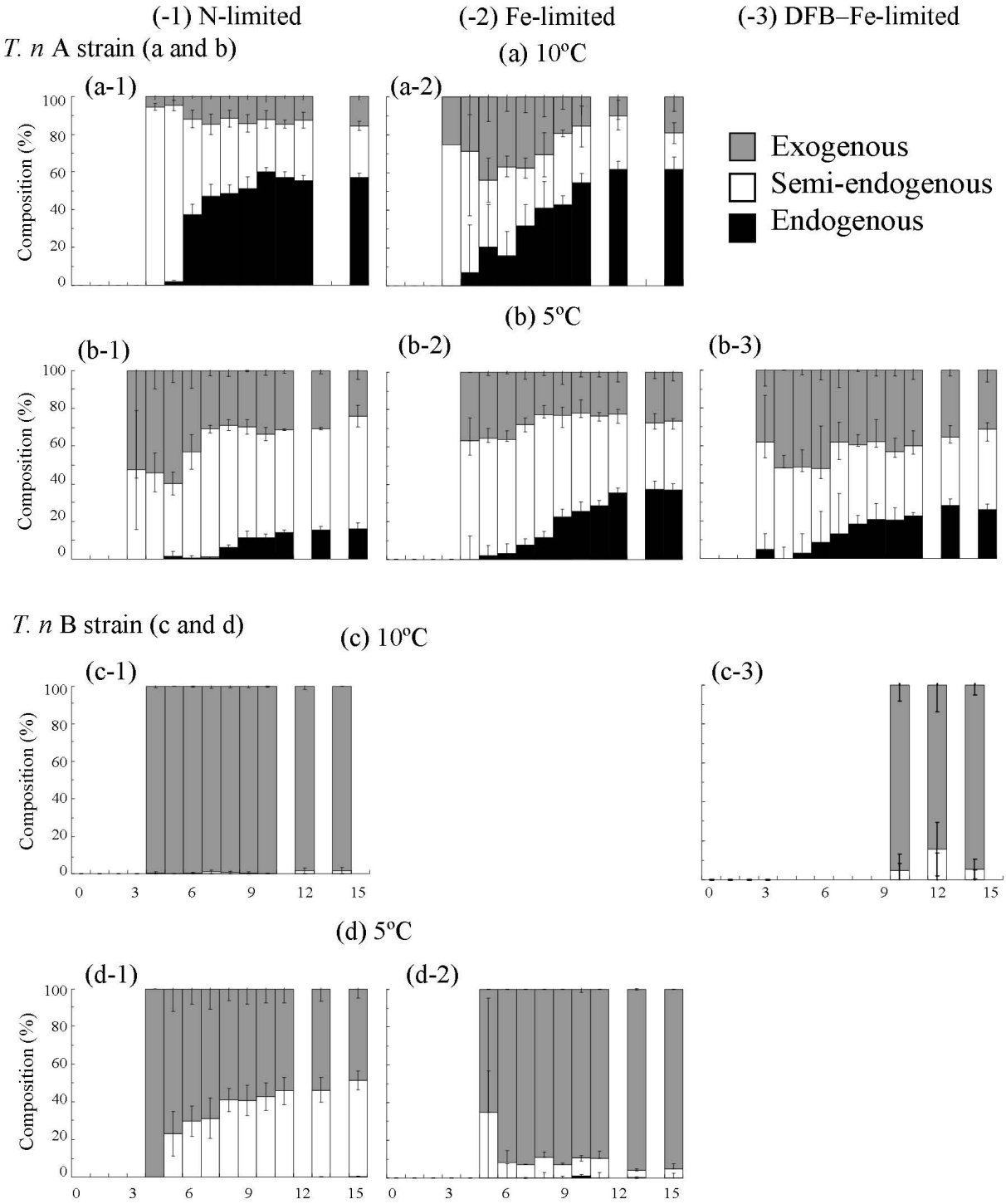

(-1) N-limited
(-2) Fe-limited
(-3) DFB–Fe-limited
T. n A strain (a and b)
(a) 10°C
(a-1)
(a-2)
Exogenous
Semi-endogenous
Endogenous
Composition (%)
(b) 5°C
(b-1)
(b-2)
(b-3)
T. n B strain (c and d)
(c) 10°C
(c-1)
(c-3)
(d) 5°C
(d-1)
(d-2)

Fig. 4

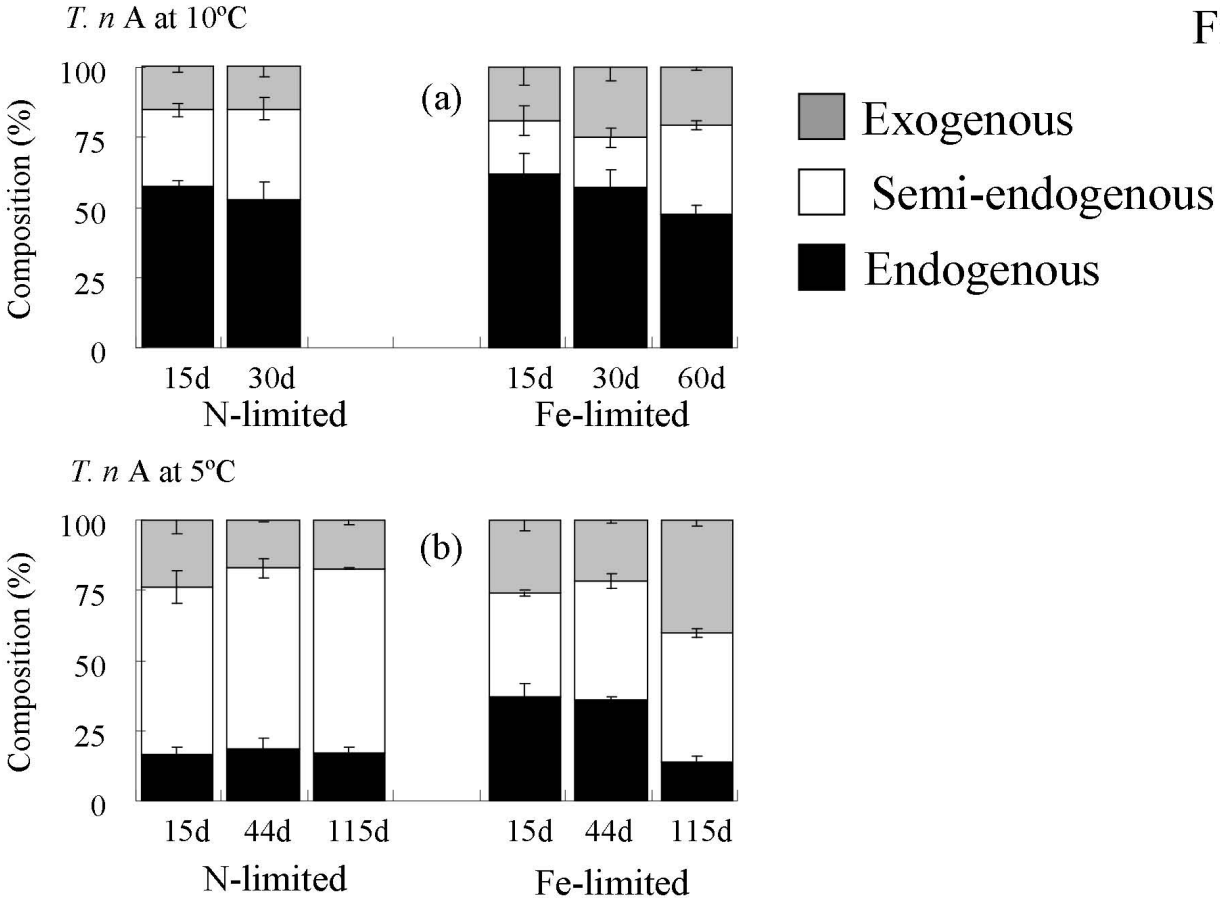

T. n A at 10ºC
Composition (%)
100
75
50
25
0
(a)
15d
30d
N-limited
15d
30d
60d
Fe-limited
Exogenous
Semi-endogenous
Endogenous
T. n A at 5ºC
Composition (%)
100
75
50
25
0
(b)
15d
44d
115d
N-limited
15d
44d
115d
Fe-limited

Fig. 5

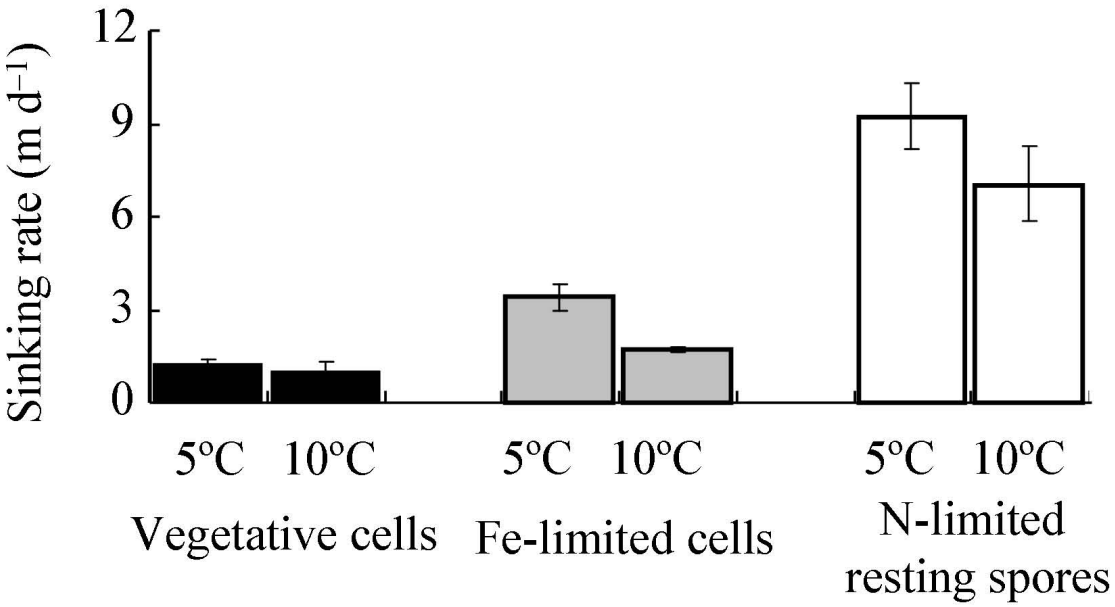
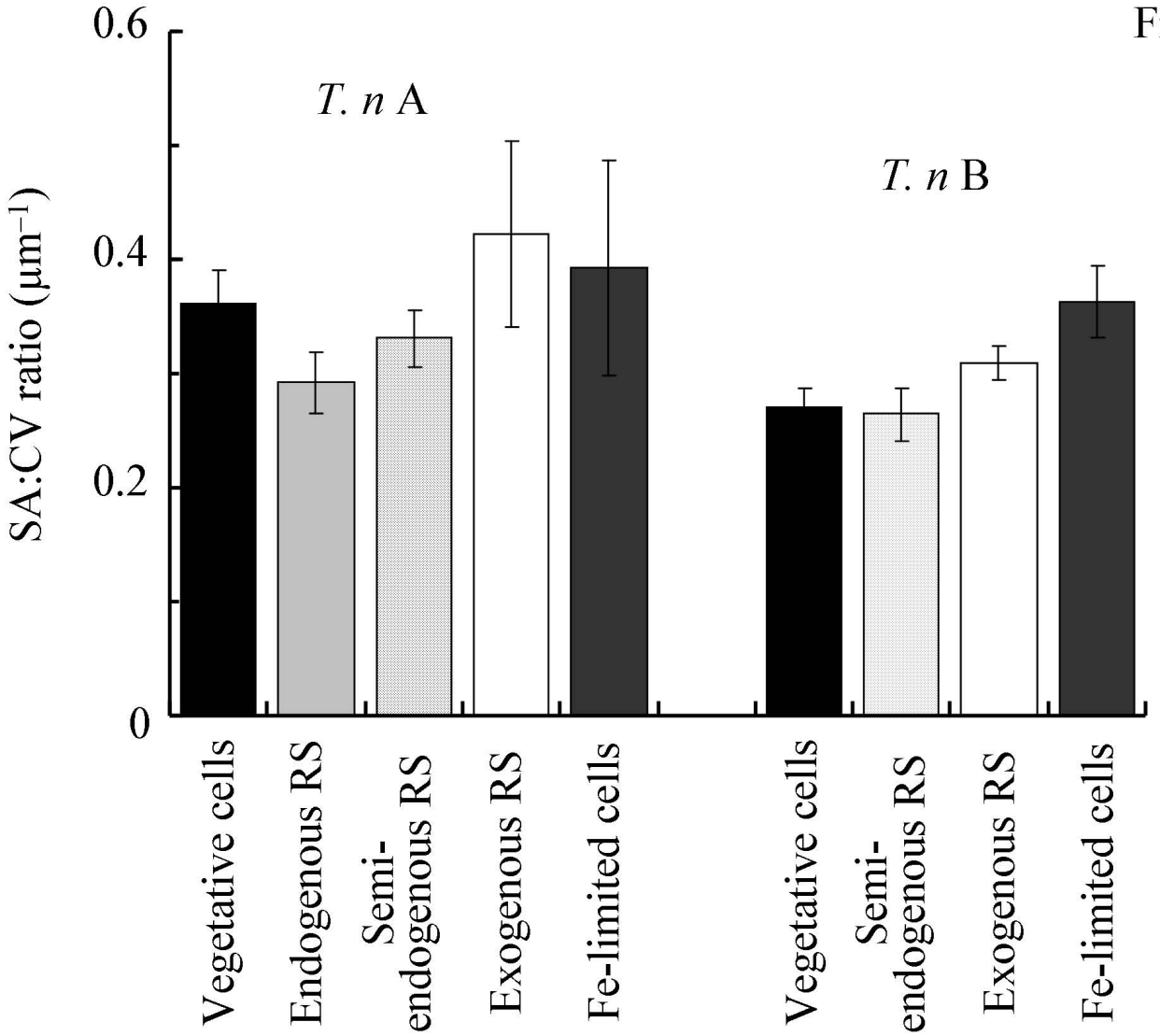

Sinking rate (m d^{-1})
12
9
6
3
0
5°C
10°C
5°C
10°C
5°C
10°C
Vegetative cells
Fe-limited cells
N-limited resting spores

Fig. 6


0.6
0.4
0.2
0
SA:CV ratio (μm⁻¹)
T. n A
T. n B
Vegetative cells
Endogenous RS
Semi-endogenous RS
Exogenous RS
Fe-limited cells
Vegetative cells
Semi-endogenous RS
Exogenous RS
Fe-limited cells