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

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20 *nordenskiöldii*

21

22 *Running Head*

23 Iron limited resting spores

24

25

26 **ABSTRACT**

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

39

40 **INTRODUCTION**

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

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

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

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

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

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

88

## 89 **METHODS**

### 90 **Algal strains**

91 Two strains of *T. nordenskiöldii* were examined. A neritic strain (*T. n* A) was isolated by  
92 Pasteur-pipette from the residue of a sieved (20-µm nylon mesh) sediment sample, which was  
93 collected from the bottom (20 m) in Onagawa Bay (38°46'N, 141°46'E) northern Honshu, Japan. The  
94 Oyashio Current (OC, subarctic water) flows along the northwestern Pacific Ocean side of northern  
95 Honshu, Japan, and constitutes the southern range limit of *T. nordenskiöldii* (Hasle, 1976; Inoue and  
96 Taniguchi, 1999). An oceanic strain (*T. n* B) was isolated by capillary pipette from the surface  
97 seawater of the OC region (42°00'N, 145°15'E; depth 3800 m) on the northwestern Pacific Ocean side  
98 of southern Hokkaido, Japan. Frustules in the two strains of *T. nordenskiöldii* were cleaned using the  
99 method of Nagumo (Nagumo, 1995) and the cleaned samples were identified by scanning electron  
100 microscopy followed by isolation under light microscopy, according to Hasle and Syvertsen (Hasle

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

## 106 **Culture experiment**

107 Culture medium seawater was collected from a coastal region near Hokkaido, in the northern  
108 Japan Sea (43°23'N, 141°02'E) and was filtered through an acid cleaned 0.22- $\mu\text{m}$  GS membrane filter  
109 (Millipore). The filtered seawater was autoclaved for 20 min at 121°C (108 kPa). The concentrations  
110 of Fe,  $\text{NO}_3+\text{NO}_2$ ,  $\text{PO}_4$  and  $\text{Si}(\text{OH})_4$  in the filtered autoclaved seawater were  $<2 \text{ nmol L}^{-1}$ ,  $<5 \text{ }\mu\text{mol}$   
111  $\text{L}^{-1}$ ,  $<0.5 \text{ }\mu\text{mol L}^{-1}$  and approximately  $250 \text{ }\mu\text{mol L}^{-1}$ , respectively. Fe concentrations in the filtered  
112 autoclaved seawater, which was used in the laboratory culture experiments, were determined by an  
113 automated Fe analyzer (Kimoto Electric) with using a combination of chelating resin concentration  
114 and luminol-hydrogen peroxide chemiluminescence detection in a closed flow-through system (Obata  
115 *et al.*, 1993). Nutrient concentrations in the collected seawater were measured by a QuAAtro  
116 continuous flow analyzer (Bran+Luebbe).

117 All equipment used in culture experiments was acid cleaned and followed by rinsing with  
118 Milli-Q water (Millipore:  $>18.0 \text{ M}\Omega \text{ cm}^{-1}$ ). All preparations and samplings for experiments were  
119 performed in a Class 100 laminar flow cabinet to avoid inadvertent trace metal contamination. Prior to  
120 culture experiments, diatoms were grown in silicic acid-enriched [ $105 \text{ }\mu\text{mol L}^{-1}$ ,  $\text{Si}(\text{OH})_4$ ] f/2 medium  
121 (Si-enriched f/2 nutrients plus f/2 metals) (Guillard and Ryther, 1962) with at least 2 transfers and  $>18$   
122 doublings during the exponential growth phase. The silicic acid-enriched f/2 medium contained  $880$   
123  $\mu\text{mol L}^{-1} \text{ NO}_3$ ,  $38 \text{ }\mu\text{mol L}^{-1} \text{ PO}_4$  and  $355 \text{ }\mu\text{mol L}^{-1} \text{ Si}(\text{OH})_4$  as macronutrients and  $11.7 \text{ }\mu\text{mol L}^{-1}$   
124  $\text{Fe}(\text{III})$ ,  $0.44 \text{ }\mu\text{mol L}^{-1} \text{ Co}(\text{II})$ ,  $0.91 \text{ }\mu\text{mol L}^{-1} \text{ Mn}(\text{II})$ ,  $73 \text{ nmol L}^{-1} \text{ Zn}(\text{II})$ ,  $28 \text{ nmol L}^{-1} \text{ Cu}(\text{II})$  and  $29$   
125  $\text{nmol L}^{-1} \text{ Mo}(\text{VI})$  with  $15 \text{ }\mu\text{mol L}^{-1} \text{ EDTA}$  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 ( $25 \mu\text{mol L}^{-1}$  Fe(III):  $\text{FeNH}_4(\text{SO}_4)_2 \cdot 12\text{H}_2\text{O}$  in  $5 \text{ mmol L}^{-1}$  HCl, pH 2.3) and manganese  
131 stock solution ( $25 \mu\text{mol L}^{-1}$  Mn(II):  $\text{MnCl}_2$  in  $5 \text{ mmol L}^{-1}$  HCl, pH 2.3) were added to final Fe and  
132 Mn concentrations of 100 and  $25 \text{ nmol L}^{-1}$ , respectively. Diatoms were grown at  $5^\circ\text{C}$  or  $10^\circ\text{C}$  under  
133  $150 \mu\text{mol photon m}^{-2} \text{ s}^{-1}$  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 ( $\sim 1 \text{ mL}$ ) 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 ( $K'_{\text{FeL}, \text{Fe(III)'}} =$

151  $[\text{Fe(III)L}]/[\text{Fe(III)}][\text{L}] = 10^{16.5} \text{ M}^{-1}$ ) 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  $1 \mu\text{mol L}^{-1}$  in the Fe-limited media  
156 to prevent further iron uptake by *T. nordenskiöldii* from ambient external Fe.

157 Cell densities at the start of the culture experiments were approximately  $1,000 \text{ cells mL}^{-1}$ .  
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  $5^\circ\text{C}$  for *T. n* B and  $10^\circ\text{C}$  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- $\mu\text{L}$ ) was diluted with a few drops of Milli-Q water, and a layer of a 100- $\mu\text{L}$  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  $5^\circ\text{C}$  and  $10^\circ\text{C}$  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).

176

## 177 **RESULTS**

178           There was no formation of resting spores and resting cells in either *T. nordenskiöldii* strains  
179 in the direct Fe and f/2 treatments. A gradual increase in resting spores during the 15 d cultivation  
180 period was observed in Fe- and DFB–Fe-limited treatments. In contrast, there was a rapid and almost  
181 complete transformation of vegetative cells into resting spores after 4–8 d (5°C) and 3–6 d (10°C)  
182 cultivation observed in N-limited treatment (Fig. 1). In five culture treatments at 5°C and 10°C, direct  
183 Fe or f/2 treatments induced the highest maximal vegetative cell yields for both strains, while the Fe-,  
184 DFB–Fe- and N-limited treatments showed lower maximal vegetative cell yields than direct Fe or f/2  
185 treatments (Fig. 1a-1, b-1, c-1 and d-1, Table I). However, the initial growth rate of each strain was  
186 almost the same among various treatments at each temperature with the lower growth rate occurring at  
187 lower temperatures ( $\mu$ : 0.70 d<sup>-1</sup> and 0.46 d<sup>-1</sup> for the *T. n* A strain and 0.76 d<sup>-1</sup> and 0.53 d<sup>-1</sup> for the *T. n*  
188 B strain at 10°C and 5°C, respectively) (Table I). Vegetative cell densities during the stationary growth  
189 phase in Fe-limited treatment were relatively constant for several days after the transition phase, while  
190 those in N-limited treatment decreased suddenly after the late exponential growth phase (after 3 and  
191 4–6 d cultivations at 10°C and 5°C, respectively).

192           In both strains, resting spore densities in N-limited treatment increased rapidly after 3 and  
193 4–6 d cultivations at 10°C and 5 °C, respectively (Fig. 1a-2, b-2, c-2 and d-2), coincident with the  
194 sudden decrease in vegetative cell densities (Fig. 1a-1, b-1, c-1 and d-1). In contrast, Fe- and  
195 DFB–Fe-limited treatments resulted in a gradual increase in resting spore densities with time (at 10°C  
196 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  
197 (Fig. 1c-3) during 15 d cultivation. The resting spore compositions of *T. n* A and *T. n* B strains in  
198 N-limited treatment reached almost 90–100% after 6–8 and 4–6 d cultivations at both temperatures,  
199 respectively, while those in Fe- and/or DFB–Fe-limited treatments gradually increased to 19% at 10°C  
200 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).

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

209 During cultivation of *T. n A* in N-, Fe- and DFB–Fe-limited treatments, the relative  
210 composition of exogenous, semi-endogenous and endogenous resting spores gradually shifted from  
211 semi-endogenous to endogenous with a nearly constant contribution of the exogenous type (Fig. 3a  
212 and b). In *T. n B*, however, exogenous resting spores were dominant and few endogenous spores were  
213 observed in Fe- and DFB–Fe-limited treatments, while in N-limited treatment at 5°C, exogenous and  
214 semi-endogenous resting spores were approximately equal while the endogenous type was low in  
215 relative abundance at the end of the experiment (49%, 51% and 0.3%, respectively) (Fig. 3c and d).  
216 During the long cultivation period (30–115 d) under dark conditions after 15 d of cultivation for *T. n A*  
217 (Fig. 4), endogenous resting spores decreased from 60% (15 d cultivation) to 50% (60 d cultivation) at  
218 10°C (Fig. 4a), and from 35% (15 d cultivation) to 15% (115 d cultivation) at 5°C in Fe-limited  
219 treatment (Fig. 4b). However, N-limited treatment maintained the relative compositions of the three  
220 spore types for the duration of the experiment (15–115 d).

### 221 **Sinking rate and cell size**

222 Sinking rates of vegetative cells, Fe-limited resting cells and spores (78% and 22% at 5°C  
223 and 99% and 1% at 10°C for resting cells and spores, respectively), and N-limited resting spores for *T.*  
224 *n B* strain were  $1.24 \pm 0.14$ ,  $3.41 \pm 0.43$  and  $9.22 \pm 1.04$  m d<sup>-1</sup> at 5°C and  $1.02 \pm 0.30$ ,  $1.75 \pm 0.08$  and  
225  $7.04 \pm 1.22$  m d<sup>-1</sup> 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. nordenskiöldii* 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. nordenskiöldii* 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

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

257         The increase in the proportion of resting spores during the 15 d cultivation period in  
258 Fe-limited treatment was also higher at lower temperature (60% at 5°C and 19% at 10°C for *T. n A*  
259 strain; Fig. 1a-3 and b-3), similar to the higher resting spore contribution at lower temperatures under  
260 N-depleted conditions reported in a previous study [76%–96% at 5°C and 40%–52% at 10°C, (Durbin,  
261 1978)] (Table III). Temperature, therefore, seems to be one of an important factor in the formation of  
262 diatom resting spores. It has been reported that resting spores tend to survive longer at colder  
263 temperatures and that spores of boreal species do not appear to tolerate temperatures higher than the  
264 tolerant limits of their vegetative cells (Hargraves and French, 1983; McQuoid and Hobson, 1996).  
265 Furthermore, it has been further reported that resting cell and resting spore formation usually occurs  
266 within the lower portion of the range of temperatures at which a given species grows (Durbin, 1978).

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

## 272 **Implications for biological oceanography of diatoms**

273         Remarkably different percentage contributions of resting spores and different compositions of  
274 endogenous, semi-endogenous and exogenous resting spores were observed between *T. n A* and *T. n B*  
275 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. nordenskiöldii* strains in the present study (Table III),  
289 differed from the phylogenetic, species-specific morphology among the three spores types in *T.*  
290 *nordenskiöldii*, 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. nordenskiöldii* 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. nordenskiöldii* 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

301 response to iron limitation. This phenomenon may have contributed to the relatively quicker decrease  
302 in diameter with only 5.6 cell divisions during the 15 d cultivation period (Tables I and II). The  
303 decreased in cell height and diameter under iron-depleted conditions results in decreased CV and the  
304 increase in SA:CV ratios can both contribute to fast sinking rates of heavily silicified resting cells (Fig.  
305 5). These morphological changes could result in an increase in the uptake of iron and macronutrients  
306 by allowing the diatom to sink to nutrient-rich deep water. In addition, the decrease in cell size may  
307 lower the cellular nutrient requirements and streamline the efficiency of intracellular material cycling  
308 as suggested previously (Pahlow *et al.*, 1997; Raven, 1998; Raven and Waite, 2004). Therefore,  
309 silicification of resting stages, and of diatoms in general, could be a significant survival strategy  
310 acquired through evolution of Cretaceous ecosystems to the current iron and nutrient aquatic  
311 environments (Raven and Waite, 2004; Falkowski *et al.*, 2004). The two main features of diatom  
312 resting spores are the heavily silicified spore frustules and compaction of cellular contents in the  
313 spores. A heavy spore frustule with an increased sinking rate not only would transfer the resting spores  
314 more quickly to new nutrient sources or remove them from dangerously high light intensity in the  
315 absence of such nutrients but also removes the cells at depth, sequester from potential pathogens  
316 (viruses) and/or predators (protozoa and crustaceans) (Raven and Waite, 2004). However, in the  
317 present study, resting cells did not survive as long as 115 days, even under lower temperature  
318 conditions (Table III), similar to the observations by Kuwata and Takahashi (Kuwata and Takahashi,  
319 1999). Therefore, it appears to be difficult for the resting cells to survive in an oceanic environment  
320 and migrate from basin to basin directly by crossing the subarctic Pacific Front current system, a  
321 high-nutrient low-chlorophyll (HNLC) region. On the other hand, the almost cosmopolitan distribution  
322 of the *T. nordenskiöldii* metapopulation in the world could be achieved by expanding of the local  
323 populations in coastal regions. In addition, neritic diatoms that forms resting spores and cells under  
324 iron-deficient conditions with having faster sinking rates than vegetative cells may prevent their  
325 seeding populations from expanding to HNLC oceanic regions with low iron concentrations.

326

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330 this study. We acknowledge two anonymous reviewers for valuable comments with significantly  
331 improving the paper. The SEM analysis of *T. nordenskioldii* frustules were carried out with Hitachi  
332 S-4200 for neritic isolated strain and with JSM-6360LA for pelagic isolated strain at Tohoku  
333 University and at the OPEN FACILITY, Hokkaido University Sousei Hall, respectively.

334

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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 *nordenskioldii* (*T. n* A and *T. n* B strains) during the 15 d cultivation period at 5°C and 10°C. Specific  
504 growth rates ( $\mu$ ) 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 ( $\pm 1$  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  $\pm 1$  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  $\pm 1$  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  $\pm 1$  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  $\pm 1$  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  $\pm 1$  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  $\pm 1$  SD.

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551 Table I

Strain and Treatment (Specific growth rate: $\mu$ )	Maximal cell yield (cells mL <sup>-1</sup> )					
	Vegetative cells		Resting spores		Resting cells	
<i>T. n A</i> at 10°C	(μ: 0.70 d <sup>-1</sup> )					
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)
<i>T. n A</i> at 5°C	(μ: 0.46 d <sup>-1</sup> )					
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)
<i>T. n B</i> at 10°C	(μ: 0.76 d <sup>-1</sup> )					
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)
<i>T. n B</i> at 5°C	(μ: 0.53 d <sup>-1</sup> )					
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) ( $\mu\text{m}$ )	27.15 (1.08)	27.39 (0.77)	25.07 (0.79)	25.93 (1.31)
Height (h) ( $\mu\text{m}$ )	16.63 (2.23)	16.64 (1.79)	10.05 (1.49)	10.60 (1.20)
r : h	1.66 (0.24)	1.67 (0.20)	2.54 0.31	2.48 (0.32)
Cell Volume (CV) ( $\mu\text{m}^3$ )	9636 (1447)	9801 (1081)	4996 (1004)	5613 (826)
Surface Area (SA) ( $\mu\text{m}^2$ )	2578 (230)	2611 (168)	1783 (188)	1922 (178)
SA:CV ( $\mu\text{m}^{-1}$ )	0.27 (0.02)	0.27 (0.01)	0.36 (0.03)	0.35 (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
<i>T. n</i> Narragansett Bay at 5°C (Durbin, 1978)					
N-limited	-	76–96	-	-	-
<i>T. n</i> Narragansett Bay at 10°C (Durbin, 1978)					
N-limited	-	40–52	-	-	-
<i>T. n</i> Oslofjord (Syvertsen, 1979)					
N- or P-limited	-	-	<1	ca. 93	ca. 6

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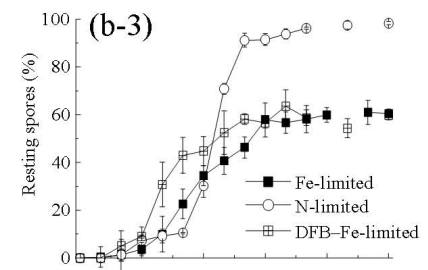
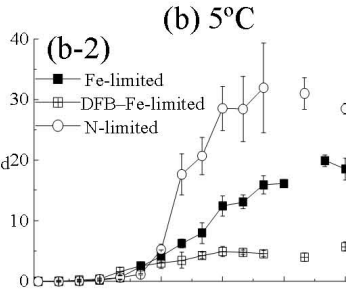
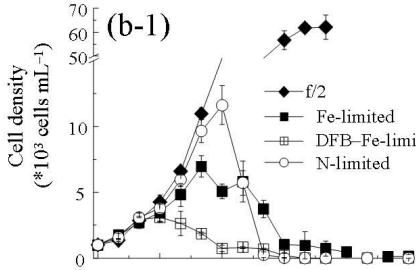
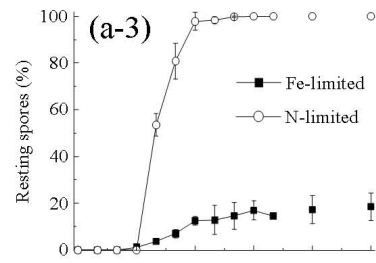
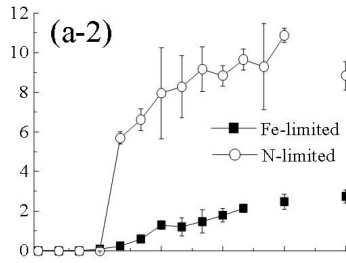
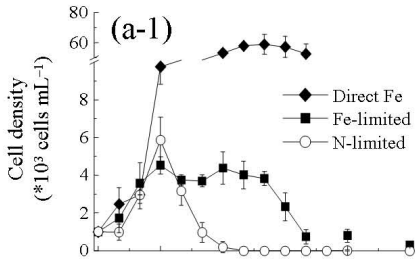
(-1) Vegetative cell density

(-2) Resting spore density

(-3) Resting spore percentage

*T. n* A strain

(a) 10°C



*T. n* B strain

(c) 10°C

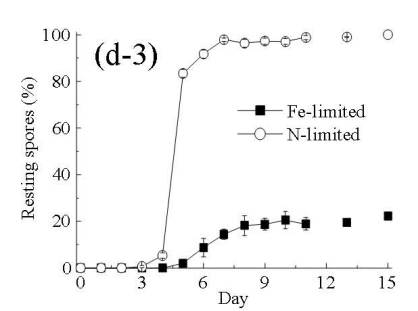
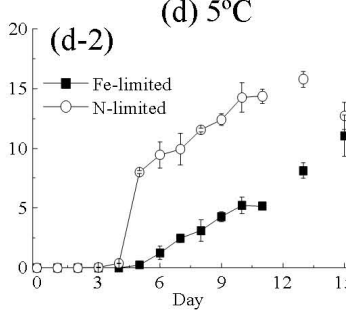
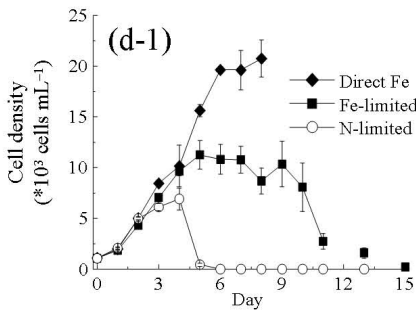
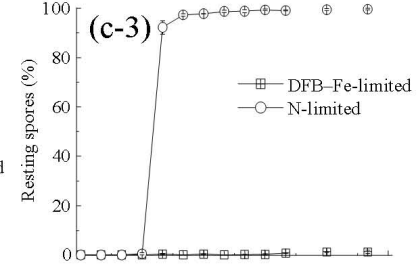
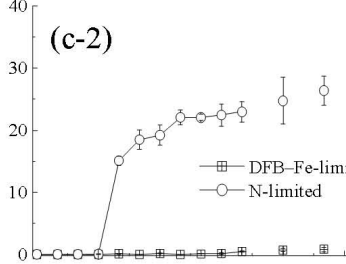
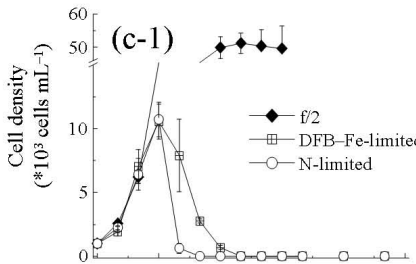
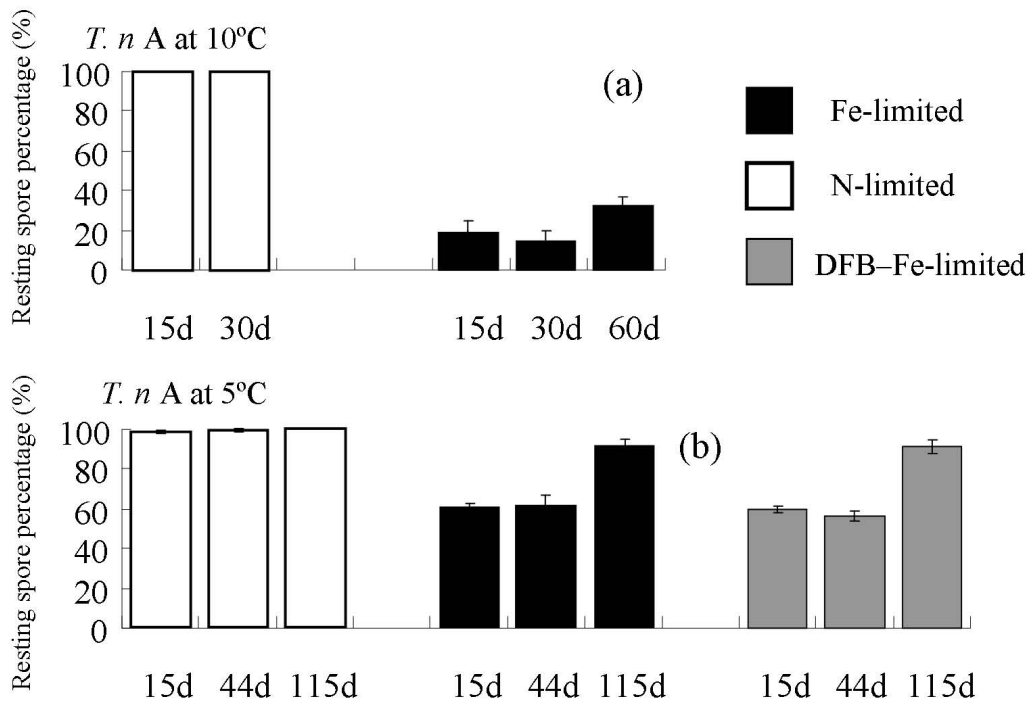


Fig. 2



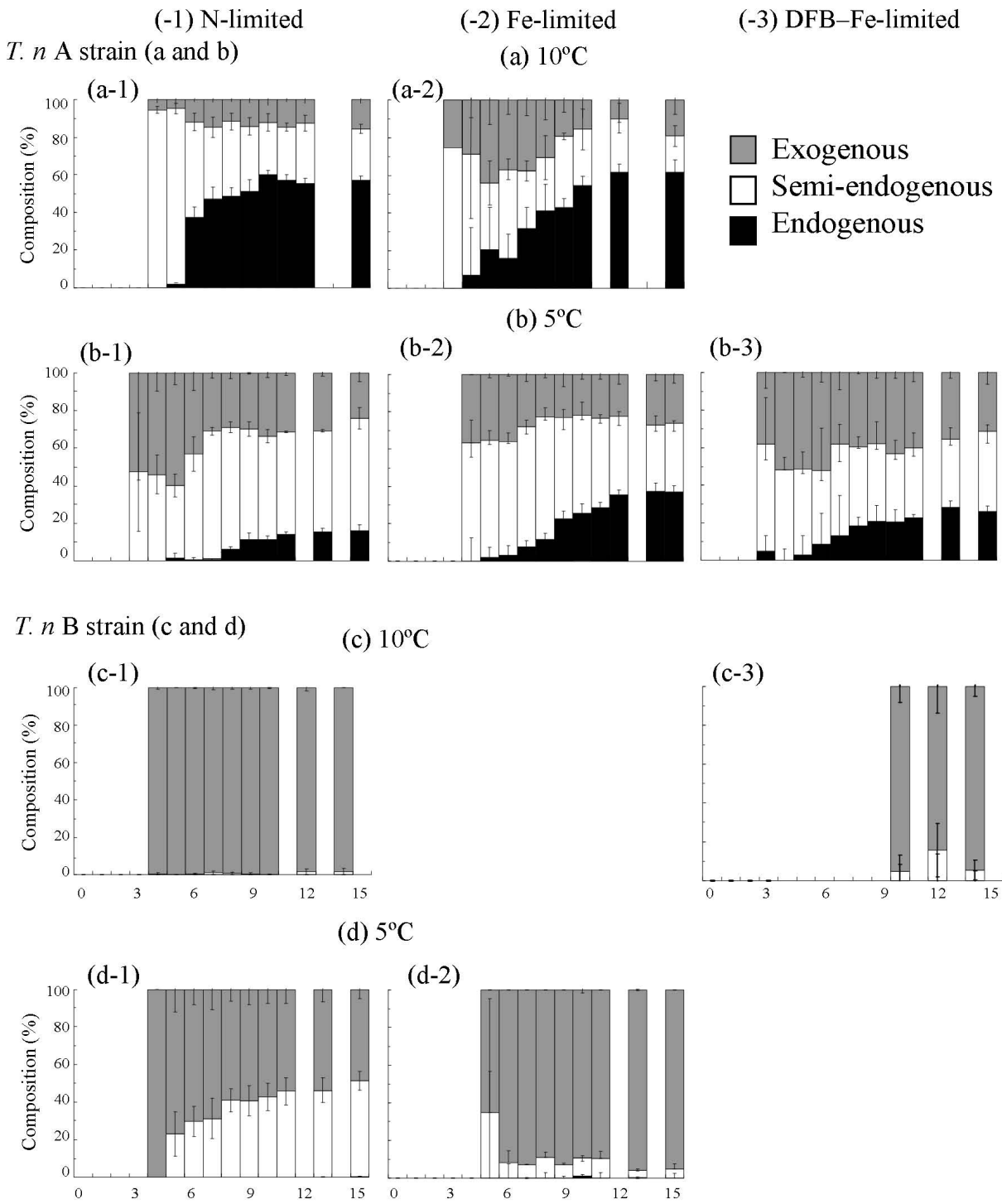


Fig. 4

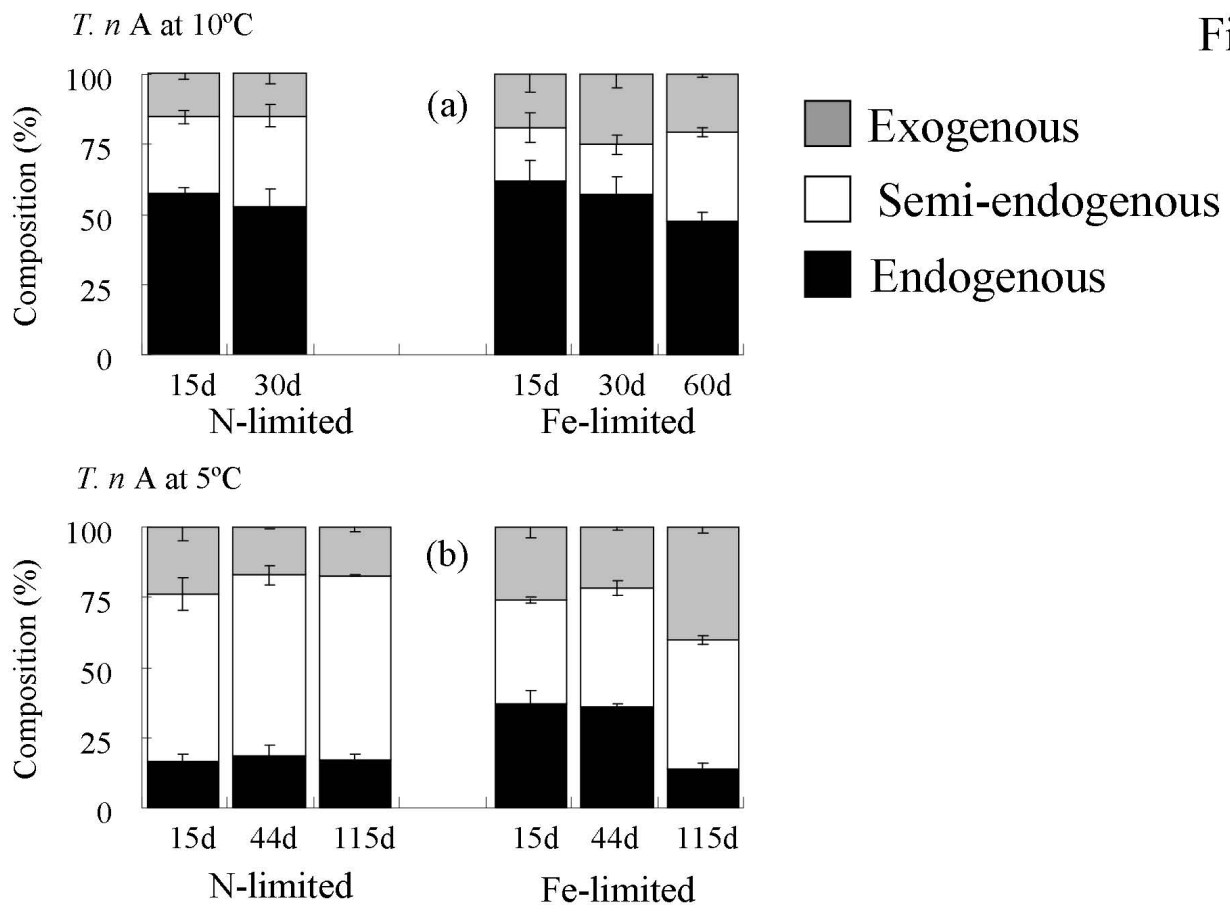


Fig. 5

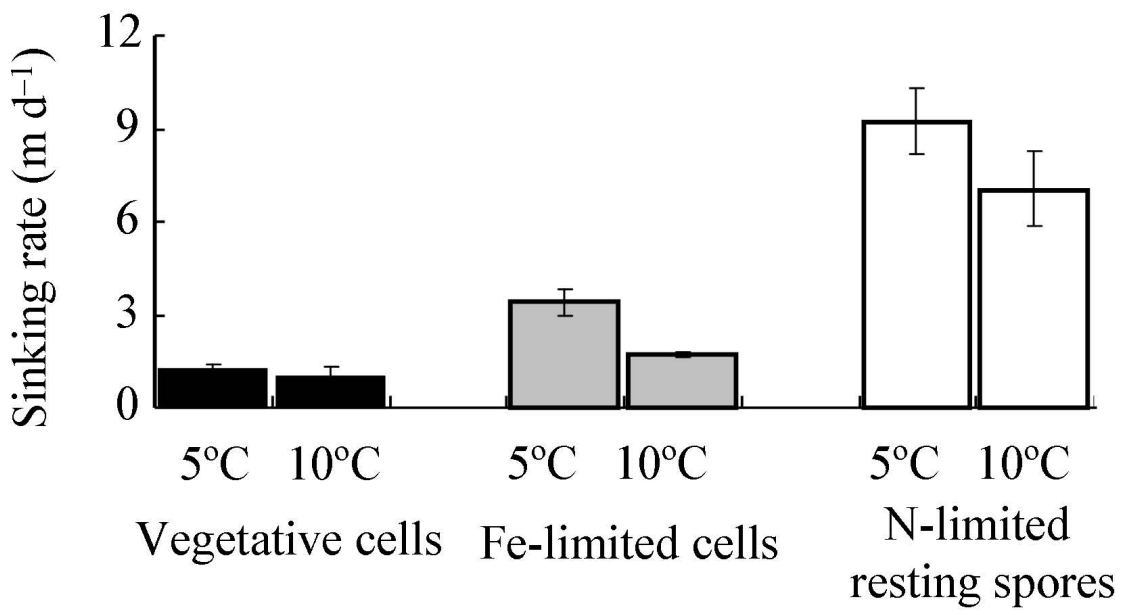


Fig. 6

