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Significance of Mn and Fe for growth of coastal marine diatom *Thalassiosira weissflogii*

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ABSTRACT: The significance of Mn and Fe for the growth of a coastal marine diatom *Thalassiosira weissflogii* was investigated by performing culture experiments containing macronutrients with either Mn or Fe, or both. Only the addition of both Mn and Fe induced the highest growth rates and maximal cell yields. Maximal growth was maintained in continuous culture media, which were repeatedly prepared by an inoculation of pre-culture and the addition of both Mn and Fe to the control culture medium containing macronutrients. In particular, it was found that the full growth recovery in Mn-sufficient medium (without added Fe) is accomplished by the addition of Fe even after several days' incubation. On the contrary, there was no sufficient growth recovery by the addition of Mn after a long incubation time in Fe-sufficient medium but without additional Mn. These results suggest that *T. weissflogii* in Mn-sufficient waters retains the ability for full physiological recovery for a long time, probably resulting from the decrease in the oxidative stress of phytoplankton by the production of antioxidant enzyme Mn superoxide dismutase during a long incubation period.

KEY WORDS: **coastal marine diatom, iron, manganese, maximal cell yields, specific growth rate, *Thalassiosira weissflogii*.**

INTRODUCTION

Iron is an essential micronutrient for phytoplankton growth, as it is an important component of such biochemical processes as photosynthetic and respiratory electron transport, nitrate and nitrite reduction, chlorophyll synthesis and other biosynthetic or degradative reactions.[1,2] However, iron in oxic sea water is in the thermodynamically stable 3+ oxidation state and presents predominantly in insoluble particulate form.[3,4] Therefore, iron supply is thought to regulate primary production in coastal and oceanic regions where the surface waters are deficient in iron. Manganese is also essential to all organisms and has particular importance in plant nutrition. In general, manganese is required for oxidation of water in photosynthesis and for detoxification of superoxide radicals.[5] Manganese in sea water occurs dissolved in the 2+ oxidation state. Observed concentrations for dissolved Mn in open ocean waters are normally 0.1–3 nM.[6–9] These higher concentrations than the equilibrium concentration of dissolved Mn with solid $Mn^{IV}O_2$ or $Mn^{III}OOH$ are partially maintained by the relatively slow oxidation kinetics of Mn^{2+} and by photochemical reduction processes and photoinhibition of microbial oxidation of Mn^{2+} in oceanic surface waters.[10,11] Typical dissolved Mn vertical profiles in the open ocean show surface dissolved Mn maxima of approximately 1 nM, evidence of atmospheric input to the surface layer.[8,9,12] Therefore, manganese could potentially limit oceanic productivity in remote oceanic regimes with high nutrients and low atmospheric input without an adequate external supply from the atmosphere or from horizontal mixing and phytoplankton growth in culture experiments.[13,14] Conversely, some trace metals have been shown to be toxic to organisms at very low concentrations. Therefore, trace metals can have either a positive or negative effect on phytoplankton growth in culture media and in the oceans.

In the present study, it has been found that sufficient phytoplankton growth is not accomplished in continuous cultivations by the addition of only Fe and macronutrients (NO_3^-, PO_4^{3-} and SiO_2).

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Therefore, the effects of especially Mn in addition to other trace metals such as Co, Ni, Cu and Zn on the growth of a coastal marine diatom *Thalassiosira weissflogii*, which has been investigated in many studies for the rates of growth and trace metal uptake, were examined by the addition of these trace metals in culture media in the presence of Fe and macronutrients.

MATERIALS AND METHODS

Chemical analysis for natural sea water

Sea water was collected from a coastal region near Hokkaido, Japan, northern Sea of Japan (salinity = 33.8) and was filtered through an acid-cleaned 0.22-μm cellulose membrane filter (Millipore, Billerica, MA, USA). The 100-mL filtrate for Fe concentration analysis was buffered at pH 3.2 with a 10 M formic acid–2.4 M ammonium formate buffer solution (0.5 mL per 100 mL filtrate). The Fe concentration in buffered filtrate was determined by an automated Fe analyzer (Kimoto Electric, Osaka, Japan) by use of a combination of concentration onto an 8-hydroxyquinoline chelating column and luminal–hydrogen peroxide chemiluminescence detection in a closed flow-through system.[15,16] The Fe concentration was approximately 2 nM. The concentrations of $NO_3^- + NO_2^-$, PO_4^{3-}, and SiO_2 in the filtered sea water measured by an autoanalyzer (Technicon, Buffalo Grove, IL, USA) were less than 0.5, 0.1 and 5 μM, respectively, which are negligible values compared with the concentrations added in the culture experiments. All bottles, flasks and tubes used in our experiments were acid-washed (soaked for at least 24 h in 1 or 3 M HCl solution or 1 M HCl for polycarbonate apparatuses) followed by repetitive washes with Milli-Q water (>18.0 MΩ/cm, Millipore). All preparation and sampling for experiments was performed in a Class 100 laminar flow cabinet to avoid inadvertent trace metal contamination.

Culture experiments

The filtered sea water was autoclaved for 20 min at 121°C, and the culture medium was prepared by adding modified f/2 nutrient and f/2 metals[17] to the autoclaved filtered sea water. The modified f/2 medium contained 880 μM NO_3^-, 38 μM PO_4^{3-} and 105 μM SiO_2 for nutrients and 11.7 μM Fe^{III}, 0.44 μM Co^{II}, 0.91 μM Mn^{II}, 73 nM Zn^{II}, 28 nM Cu^{II} and 29 nM Mo^{VI} with 15 μM EDTA for metals. The chain-forming coastal marine diatom *T. weissflogii* was grown in 50 mL of the f/2 medium in a 100 mL polycarbonate Erlenmeyer flask. Cells were grown at 20°C under 150 μmol photons/m^2 per s fluorescent light (12 h:12 h light–dark). An f/2 nutrient stock solution and then a small amount of culture (~250–500 μL) in an initial stationary growth phase (≥100 000 cells/mL after 5 days incubation) were added to 50 mL of the first culture medium [Direct-Fe^{III} (first)], to which acidic ferric iron stock solution [25 μM Fe^{III}; $FeNH_4(SO_4)_2 \cdot 12H_2O$ in 5 mM HCl, pH 2.3] was added to make an iron concentration of 100 nM. The pH of the sea water after the acidic iron addition was 8.0. All major nutrient stocks were passed through Chelex 100 ion-exchange resin to remove trace metals.[18] Cells in the Direct-Fe^{III} (first) medium, to which only macronutrients and Fe^{III} was added, were grown to obtain the cell concentration (1000 cells/mL) expected at the start of the following culture experiments. In our previous studies,[19–22] the effect of direct Fe input was examined by adding a small amount of acidic ferric iron stock solution directly together with an inoculation of culture into the control culture medium, resulting in the highest phytoplankton growth rate and the highest maximal cell yields. In the following culture experiments, all culture media contained 880 μM NO_3^-, 38 μM PO_4^{3-}, and 105 μM SiO_2 for nutrient. In addition, metals (except for Mn and/or Fe) and EDTA in the f/2 metal medium are diluted to extremely low concentrations (<0.1 nM for each metal and <1.5 nM for EDTA with low conditional stability constant, $K'_{M\text{-}EDTA,M'} = [M\text{-}EDTA]/[M'][EDTA']$; M, metal) in sea water, negligible values in the following culture experiments[19,20]) in the second culture medium with twice inoculations of culture. It has been reported that the concentrations of natural Fe-binding organic ligands in surface sea waters are normally in the low range of 0.3–3.6 nM,[20] which are negligible values compared with the Fe concentration (100 nM) in the culture experiments.

Continuous cultivations by only addition of Fe and macronutrients

A small amount of culture (~250–500 μL) at an initial stationary growth phase in the Direct-Fe^{III} (first) medium was added to the Direct-Fe^{III} (second) medium, to which only both Fe^{III} and nutrient were added, to make the cell concentration of 1000 cells/mL at the start of the culture experiment. Continuously, cells at an initial stationary growth phase in the Direct-Fe^{III} (second) medium was also inoculated to the Direct-Fe^{III} (third) medium, to which only both Fe^{III} and nutrient were added [Table 1 (1)], to make a cell concentration of 1000 cells/mL at the start.

Table 1 Growth rate (μ) and maximal cell yields (cells/mL) of *Thalassiosira weissflogii* in f/2 metal medium, direct trace metals (Mn^{II}, Fe^{III}, Co^{II}, Ni^{II}, Cu^{II}, Zn^{II}) media with/without the addition of Fe^{III} and direct Fe^{III} media with Mn^{II} ($n = 3$)

Medium		Addition of trace metals (concentration, nM)	Specific growth rate (μ, /day)	Maximal cell yields ($\times 10^3$ cells/mL)
(1) Growth rate experiments in f/2 metal medium and continuous direct Fe^{III} media				
f/2 metal	0	f/2 metals	0.95–0.98 (0–5 d)	171–208
Fe^{III}	first	Fe^{III} (100 nM)	0.90–0.94 (0–5 d)	157–169
Fe^{III}	second	Fe^{III} (100 nM)	0.58–0.70 (0–5 d)	25–28
Control	second	no Fe	0.28–0.41 (0–5 d)	5–6
Fe^{III}	third	Fe^{III} (100 nM)	0.20–0.70 (0–5 d)	36–40
(2) Growth rate experiments in trace metals media				
(a) Fe^{III}	second	Fe^{III} (100 nM)	0.66–0.73 (0–5 d)	22–29
Mn	second	Mn^{II} (25 nM)	0.65–0.70 (0–5 d)	33–38
Co	second	Co^{II} (25 nM)	0.64–0.66 (0–5 d)	22–23
Ni	second	Ni^{II} (25 nM)	0.64–0.70 (0–5 d)	21–24
Cu	second	Cu^{II} (25 nM)	0.62–0.66 (0–5 d)	21–22
Zn	second	Zn^{II} (25 nM)	0.63–0.66 (0–5 d)	22–23
(b) Fe+Mn	second	Fe^{III}+Mn^{II} (100 + 25 nM)	0.92–0.94 (0–5 d)	140–163
Fe+Co	second	Fe^{III}+Co^{II} (100 + 25 nM)	0.60–0.61 (0–5 d)	23–24
Fe+Ni	second	Fe^{III}+Ni^{II} (100 + 25 nM)	0.58–0.59 (0–5 d)	21–23
Fe+Cu	second	Fe^{III}+Cu^{II} (100 + 25 nM)	0.47–0.52 (0–5 d)	13–16
Fe+Zn	second	Fe^{III}+Zn^{II} (100 + 25 nM)	0.60–0.62 (0–5 d)	23–25
(3) Growth rate experiments in continuous direct Fe^{III} media with the addition of Mn (25 nM) and in direct-Fe^{III} (second) media by addition of different Mn concentrations (0, 1, 5, 10, 25 and 50 nM)				
(a) Fe+Mn	first	Fe^{III}+Mn^{II} (100 + 25 nM)	0.92–0.95 (0–5 d)	146–154
Fe+Mn	second	Fe^{III}+Mn^{II} (100 + 25 nM)	0.94–1.00 (0–5 d)	133–159
Fe+Mn	third	Fe^{III}+Mn^{II} (100 + 25 nM)	0.90–0.93 (0–5 d)	132–163
Fe+Mn	fifth	Fe^{III}+Mn^{II} (100 + 25 nM)	0.93–0.98 (0–5 d)	146–163
(b) Fe^{III}	second	Fe^{III} (100 nM)	0.59–0.63 (0–4 d)	10–11
Fe+Mn	second	Fe^{III}+Mn^{II} (100 + 1 nM)	0.69–0.71 (0–5 d)	40–43
Fe+Mn	second	Fe^{III}+Mn^{II} (100 + 5 nM)	0.92–0.94 (0–5 d)	161–171
Fe+Mn	second	Fe^{III}+Mn^{II} (100 + 10 nM)	0.89–0.90 (0–5 d)	167–171
Fe+Mn	second	Fe^{III}+Mn^{II} (100 + 25 nM)	0.90–0.93 (0–5 d)	164–166
Fe+Mn	second	Fe^{III}+Mn^{II} (100 + 50 nM)	0.88–0.89 (0–5 d)	167–169
(4) Growth rate experiments in Direct Fe^{III} (second) media by different timing of Mn addition (25 nM) or Fe+Mn addition (100 nM + 25 nM) and Direct Mn (25 nM) media by different timing of Fe addition (100 nM)				
(a) Fe+Mn	second (0 d)	Fe^{III}+Mn^{II} (100 + 25 nM)	0.95–0.97 (0–5 d)	140–171
Fe+Mn	second (4 d)	Fe^{III}+Mn^{II} (100 + 25 nM)	0.67–0.76 (4–6 d)	156–169
Fe+Mn	second (6 d)	Fe^{III}+Mn^{II} (100 + 25 nM)	0.40–0.57 (6–8 d)	70–83
Fe × 2+Mn	second (6 d)	Fe^{III} × 2+Mn^{II} (100 × 2 + 25 nM)	0.23–0.57 (6–8 d)	66–79
Fe+Mn	second (8 d)	Fe^{III}+Mn^{II} (100 + 25 nM)	ND (8–9 d)	20–22
Fe × 2+Mn	second (8 d)	Fe^{III} × 2+Mn^{II} (100 × 2 + 25 nM)	ND (8–9 d)	17–22
(b) Mn+Fe	second (0 d)	Mn^{II}+Fe^{III} (25 + 100 nM)	1.00–1.05 (0–5 d)	139–170
Mn+Fe	second (2 d)	Mn^{II}+Fe^{III} (25 + 100 nM)	0.79–0.84 (2–6 d)	148–173
Mn+Fe	second (4 d)	Mn^{II}+Fe^{III} (25 + 100 nM)	0.66–0.68 (4–8 d)	154–161
Mn+Fe	second (6 d)	Mn^{II}+Fe^{III} (25 + 100 nM)	0.56–0.62 (6–10 d)	146–159
Mn+Fe	second (8 d)	Mn^{II}+Fe^{III} (25 + 100 nM)	0.68–0.69 (8–11 d)	146–149

ND, Not determined.

Effect of other trace metals in second culture media

The effect of trace metals such as Mn^{II}, Co^{II}, Ni^{II}, Cu^{II} and Zn^{II} (metal chloride or sulfate) on the growth of *T. weissflogii* was examined for culture media with and without the addition of Fe^{III} [Table 1 (2)]. A small amount of culture at an initial stationary growth phase in the Direct-Fe^{III} (first) medium was added to the Direct-Metal (second) medium without any added Fe^{III} and Direct-Fe + Metal (second) medium with Fe^{III} to which only both nutrient and one of the trace metals were added. Just after an inoculation of culture into the

control culture medium containing nutrient, iron and/or one of other trace metals were directly added to make concentrations of 100 nM for Fe^{III} and 25 nM for each other trace metal.

Continuous cultivations by addition of Fe, Mn and macronutrients

An inoculation of culture in the f/2 metal medium to the control culture medium containing nutrient was followed by the addition of both Fe^{III} and Mn^{II} as Direct-Fe + Mn (first) medium. Repeatedly, that in the Direct-Fe + Mn (first) medium was also added to the second culture medium with only the addition of both Fe^{III} and Mn^{II} as Direct-Fe + Mn (second) medium. Similarly, the third, fourth and fifth [Direct-Fe + Mn (third, fourth and fifth)] culture media were also prepared by an inoculation of pre-culture and the addition of both Fe^{III} and Mn^{II} to the control culture medium [Table 1 (3a)]. The final concentrations of Fe^{III} and Mn^{II} in each culture medium are 100 and 25 nM, respectively. In addition, the effect of Mn^{II} concentrations on the growth of *T. weissflogii* was examined in the Direct-Fe + Mn (second) medium with concentrations of 1, 5, 10, 25 and 50 nM Mn^{II} and of 100 nM Fe^{III} [Table 1 (3b)].

Effect of Mn and/or Fe addition with different timing

The effect of direct Mn input to the Direct-Fe^{III} (second) medium with different timing on the growth of *T. weissflogii* was examined by the addition of Mn^{II} after 0, 4, 6 and 8 days incubation and by the addition of both Fe^{III} and Mn^{II} after 6 and 8 days incubation in the Direct-Fe^{III} (second) medium. In addition, the effect of direct Fe input to the Direct-Mn (second) medium was carried out by the addition of Fe^{III} after 0, 2, 4, 6 and 8 days incubation in the Direct-Mn (second) medium [Table 1 (4)]. The final concentrations of Mn^{II} and Fe^{III} in each culture medium are 25 and 100 nM, respectively.

Control (without any added Fe and other trace metals), [Control (second)] culture medium ($\leq$ 3 nM Fe) was prepared to compare the growth rates and cell yields with those containing Fe and other trace metals. The light, temperature and nutrient conditions were the same as those of the stock culture described above. During the experiments, cell growth was monitored daily by triplicate cell counts (cells and not chains) using an optical microscope. Culture experiments were conducted in triplicate.

RESULTS

Growth rate and maximal cell yields

Continuous cultivations by only addition of both Fe and macronutrients

In the present study, the Direct-Fe^{III} (first) medium induced the highest growth rate (μ = 0.90–0.94/day) and the highest maximal cell yields (157 000–169 000 cells/mL), showing values similar to those in the f/2 metal medium [Table 1 (1), Fig. 1]. In the continuous culture experiments by an inoculation of pre-culture and then addition of Fe into the control culture medium containing nutrient, however, the growth rates and maximal cell yields in the Direct-Fe^{III} (second) and Direct-Fe^{III} (third) media were approximately one-half and one-sixth to one-fourth, respectively, of those in the f/2 metal and Direct-Fe^{III} (first) media. The relative order for the growth rates and maximal cell yields in the continuous culture experiments was f/2 metal ≈Direct-Fe(Fe^{III} (first) >> Direct-Fe^{III} (second) ≈Direct-Fe(Fe^{III} (third) > Control (second) [Table 1 (1), Fig. 1].

Effect of other trace metals in second culture media

In the Direct-Metal (second) media for trace metals without any added Fe, a little growth of *T. weissflogii* was observed with the growth rates of 0.6–0.7/day and the maximal cell yields of 33 000–38 000 cells/mL for Mn and 21 000–24 000 cells/mL for Co, Ni, Cu and Zn [Table 1 (2a), Fig. 2a].

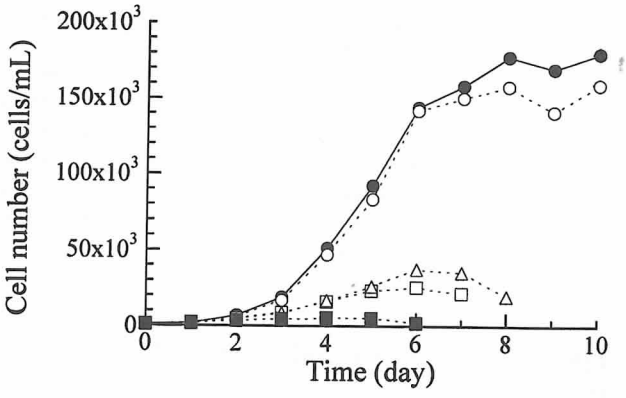

Fig. 1 Change in cell numbers of cultures supplied with f/2 metal (●) and with/without the addition of Fe by the continuous cultivations in the Direct-Fe^{III} [first (○), second (□) and third (△)] media and control culture medium without any added Fe (■).

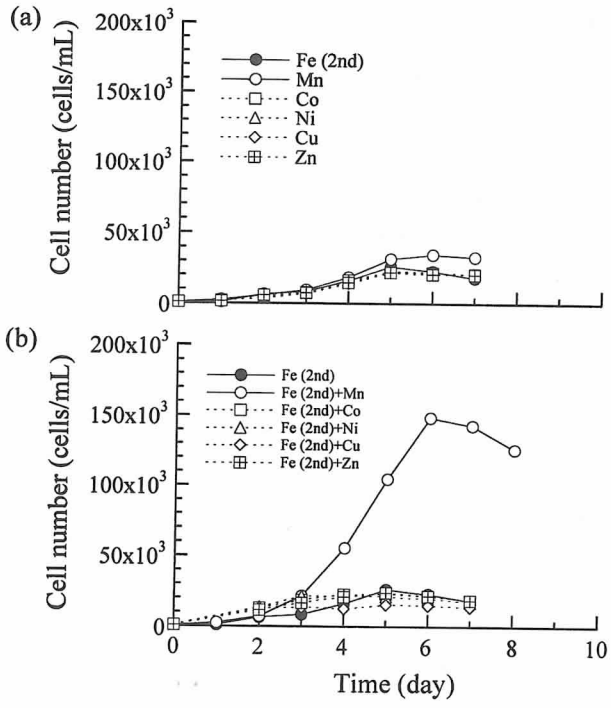

Fig. 2 Changes in cell numbers of cultures supplied with (a) each trace metal (Fe^{III}, Mn^{II}, Co^{II}, Ni^{II}, Cu^{II}, Zn^{II}) in the control culture media without any added Fe in the Direct-Metal (second) media and (b) each trace metal (Mn^{II}, Co^{II}, Ni^{II}, Cu^{II}, Zn^{II}) in the Direct-Fe^{III} (second) media with Fe in the Direct-Fe^{III} + Metal (second) media.

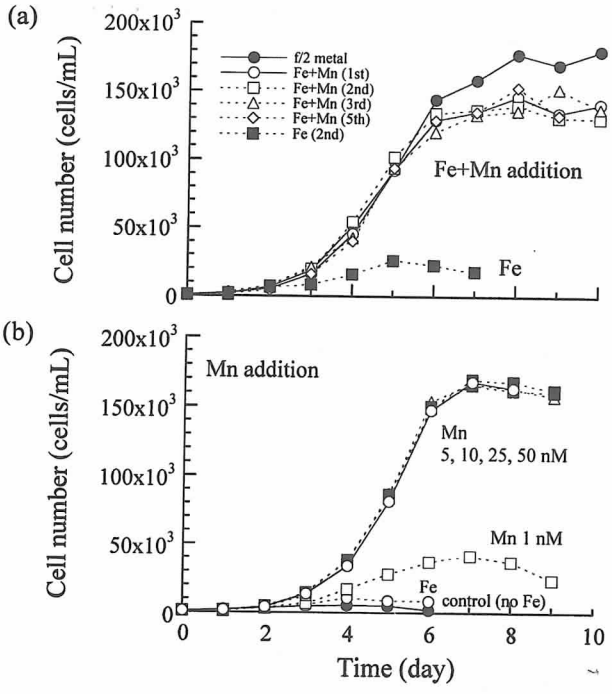

Fig. 3 Changes in cell numbers of cultures by the continuous culture experiments in (a) the Direct-Fe + Mn (first, second, third and fifth) media with 100 nM Fe and 25 nM Mn and (b) the Direct-Fe + Mn (second) media with different concentrations of 1, 5, 10, 25 and 50 nM Mn.

Among the Direct-Fe + Metal (second) media, only Direct-Fe + Metal (second) media with the addition of both Fe and Mn induced the highest growth rate ($\mu = 0.92$–0.94/day) and the highest maximal cell yields (140 000–163 000 cells/mL), almost the same as those obtained in the Direct-Fe^{III} (first) medium [Table 1 (2b), Fig. 2b]. The growth rates ($\mu \approx 0.6$/day) and the maximal cell yields (21 000–25 000 cells/mL) in the Direct-Fe + Metal (second)]media for Co, Ni and Zn were extremely low, similar to those in the Direct-Metal (second) media without any added Fe, while those in the Direct-Fe + Cu (second) medium for Cu were a little lower than those in the Direct-Fe + Metal (second) media for Co, Ni and Zn. The relative order for growth rates and maximal cell yields was Direct-Fe + Mn (second) >> Direct-Fe + Co (second) ≈ Direct-Fe + Ni (second) ≈ Direct-Fe + Zn (second) > Direct-Fe + Cu (second)] media [Table 1 (2b), Fig. 2b].

Continuous cultivations with addition of Fe, Mn and macronutrients

All continuous culture experiments in the Direct-Fe + Mn (first, second, third and fifth) media with 100 nM Fe and 25 nM Mn, which were prepared by an inoculation of pre-culture and the addition of both Fe^{III} and Mn^{II} to the control culture medium, induced almost the same maximum growth rate and maximal cell yield at the ranges of 0.90–1.0/day and 132 000–163 000 cells/mL, respectively [Table 1 (3a), Fig. 3a].

Among the Direct-Fe + Mn (second) media with different Mn concentrations, the Mn concentrations of 5, 10, 25 and 50 nM promoted nearly the same maximum growth rates ($\mu = 0.88$–0.94/day) and maximal cell yields (164 000–171 000 cells/mL) as those in all continuous culture experiments in the Direct-Fe + Mn (first, second, third and fifth) media, while those in the Direct-Fe + Mn (second) medium with 1 nM Mn concentration and in the Direct-Fe^{III} (second) medium without any added Mn were remarkably lower than those with Mn concentrations ≥5 nM. The order of growth rates and maximal cell yields was Direct-Fe+Mn (second) media with 5, 10, 25 and 50 nM Mn >> Direct-Fe + Mn (second) medium with 1 nM Mn > Direct-Fe^{III} (second) medium without any added Mn ≥ Control medium without any added Fe and Mn [Table 1 (3b), Fig. 3b].

Effect of Mn and/or Fe addition with different timing

In direct Mn input and Fe + Mn input to the Direct-Fe^{III} (second) medium [Table 1 (4a), Fig. 4a], the growth rates and maximal cell yields decreased rapidly with incubation time (0–8 days) in the Direct-Fe^{III} (second) medium although the maximum cell density (140 000–171 000 cells/mL) by Mn input after four days incubation in the Direct-Fe^{III} (second) medium was the same as that after 0 days incubation. Only Mn and both Fe + Mn inputs after 8 days incubation induced only a little increase in the cell density (Fig. 4a).

In direct Fe input to the Direct-Mn (second) medium [Table 1 (4b), Fig. 4b], the maximal cell yields were high (~ 150 000 cells/mL, almost the same in all media) and independent of incubation time (0–8 d) in the present study. The growth rates by Fe input after 2–8 days incubations were almost the same in all media with one-half to one-third lower values than that after 0 days incubation.

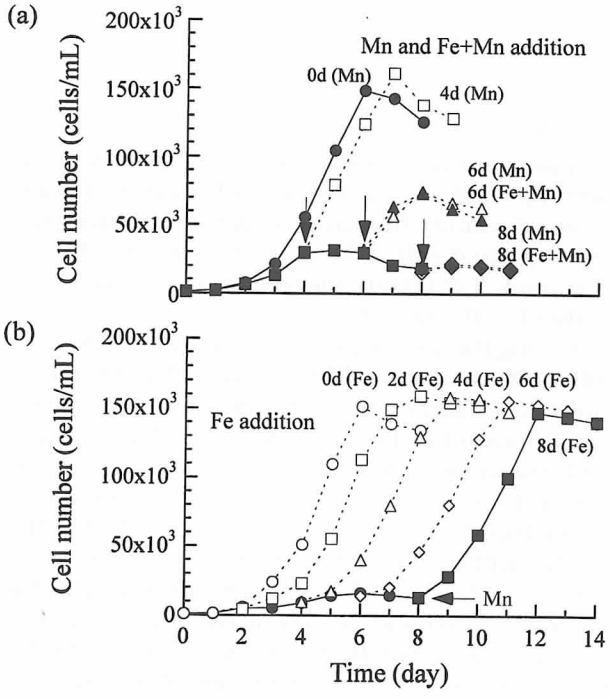

Fig. 4 Changes in cell numbers of cultures by (a) addition of Mn or Fe + Mn with different timing during incubation for several days in the Direct-Fe^{III} (second) media and (b) addition of Fe with different timing during incubation for several days in the Direct-Mn (second) media.

DISCUSSION

Significance of Mn and Fe for growth of coastal marine diatoms

Trace metals influence phytoplankton production and community structure. These biological influences are particularly important for several metals of the first transition series which are required by phytoplankton for various metabolic functions: Mn, Fe, Co, Ni, Cu and Zn. Fe is the most important of all the bioactive trace metals as an important component of such biochemical processes as photosynthetic and respiratory electron transport, nitrate and nitrite reduction, and other biochemical reactions.[1] However, sufficient growth of a coastal diatom *T. weissflogii* was not observed in the continuous second and third culture experiments by an inoculation of pre-culture to the control medium, to which only Fe and macronutrients were added (Fig. 1). However, the addition of both Mn and Fe in the culture experiments (Fig. 2b) induced the highest growth rates (μ = 0.92–0.94/day) and maximal cell yields of *T. weissflogii* [Table 1 (2)]. Moreover, the highest growth was maintained in the continuous first, second, third, fourth (data not shown) and fifth media to which both Mn and Fe were added (Fig. 3a). Conversely, the addition of both Cu and Fe to the culture medium had a negative effect on phytoplankton growth probably because of Cu toxicity at high concentration of 25 nM in the present study.

The observed concentrations for dissolved Mn in open ocean surface waters are normally between 0.1 and 3 nM,[8,9,12] resulting from atmospheric inputs to the oceans. However, the main sources of Mn to coastal waters are generally from river runoff and a dissolved Mn flux from shelf sediments. It has been reported that dissolved Mn in the coastal surface waters reached approximately 10–25 nM during the coastal upwelling conditions over the shelf.[23] These observed concentrations of dissolved Mn in surface waters are much higher than the equilibrium concentration (<<1 nM) of dissolved Mn with solid MnO_2 or MnOOH in oxygenated sea water and are partially maintained by the relatively slow oxidation kinetics of Mn^{2+}.[8,11,24] In the present culture experiments, the filtered coastal water was autoclaved for 20 min at 121°C to prepare the culture medium. The autoclaving treatment (121°C) probably promoted the formation of solid MnO_2 or MnOOH with low solubility by hastening the oxidation rate of dissolved Mn^{2+} and then changing to larger and more stable solid MnO_2 or MnOOH phases, resulting in the decrease in bioavailable dissolved Mn concentration in the

medium (data not shown). In a previous study,[25] the autoclaving treatment of the freshly precipitated amorphous Fe^{III} hydroxide (am-Fe^{III}) in sea water accelerated the conversion to a more stable solid am-Fe^{III} phase by loss of water and increased crystallization. The conversion to a stable am-Fe^{III} phase with low solubility and dissolution rate greatly decreased iron availability of phytoplankton.[25,26] In the present study, high growth of *T. weissflogii* was observed in the nutrient enrichment culture experiments (except for the first culture media with only Fe addition, Fig. 1) with only the addition of both Mn and Fe (Table 1). Manganese (≥5 nM) and Fe (≥100 nM)[21] in the direct metal input media were both required to support the sufficient growth (maximum cell yields) of *T. weissflogii*. Therefore, it may be suggested that dissolved Mn and Fe in coastal waters are reduced to extremely low levels (low bioavailable Mn and Fe) by autoclaving although other dissolved trace metals such as Co^{II}, Ni^{II}, Cu^{II} and Zn^{II} are probably not influenced.

Among the Direct-Fe + Mn (second) media with different Mn concentrations (1–50 nM), the media with Mn concentrations ≥5 nM promoted nearly the same maximum growth rate and maximal cell yield of *T. weissflogii* while the growth in the media with 1 nM Mn concentration and without any added Mn was remarkably low (Fig. 3b). It has been reported that the reproductive rate of oceanic diatom *Thalassiosira oceanica* was limited at Mn activities below 10^{-10} M (0.1 nM) using EDTA-trace metal ion buffer systems while that of neritic diatom *Thalassiosira pseudonana* estuarine isolate was limited below 10^{-9} M (1 nM).[13,27] The lowest free manganese ion concentration for the growth of *T. pseudonana* was nearly consistent with the least Mn concentration (5 nM Mn^{2+}), which promoted the maximal growth rate and cell yield of *T. weissflogii* in the present study. In addition, Brand *et al.*[13] reported that there is a clear distinction between neritic and oceanic phytoplankton for Fe. All of the neritic phytoplankton had substantially decreased reproductive rates below 10^{-7} M Fe while the oceanic phytoplankton were either not or only slightly limited at the lowest Fe concentrations (~10^{-9} M). Recently, it has been reported that the oceanic diatom had up to several-fold lower photosystem I (PS I) and lower cytochrome b_6f complex concentrations than a coastal diatom and these changes to the photosynthetic apparatus markedly decrease the cellular Fe requirements of the oceanic diatom but not its photosynthetic rates.[28,29] In the present study, Mn (≥5 nM) and Fe (≥100 nM) in the direct metal input media without any other trace metals were both required to support sufficient growth of *T. weissflogii* in continuous culture experiments with sufficient macronutrients. In addition, it may be suggested that other trace metals (Co, Ni, Cu and Zn) except for Mn and Fe are present at levels sufficient to support the high growth for coastal diatoms in the control media, which were produced from natural coastal waters in the present study.

Full growth recovery in Mn-sufficient waters

Manganese is an essential micronutrient required for oxidation of water in photosynthesis and for detoxification of superoxide radicals.[5] In photosynthetic reactions, manganese is used to produce molecular oxygen, protons and electrons from water by a Mn-based enzyme system in a photosystem II (PS II).[30] It is now known that each Mn in the four-Mn cluster each loses an electron to PS II, with replacement of these electrons from the oxidation of two water molecules to molecular oxygen. This photosynthetic pathway couples the generation of an excited state on chlorophyll molecules for electron transport. In addition to ferredoxins (iron–sulfur proteins), redox-active molecules such as quinones, blue copper proteins and cytochromes (iron hemoproteins) can also be involved in the electron transfer chain throughout the photosystems (PS I and PS II). In addition, Mn also appears to be used in the antioxidant enzyme superoxide dismutase (SOD) to detoxify the reactive oxygen species (ROS) such as superoxide, hydroxyl radical, hydrogen peroxide and organohydroperoxide, which are a by-products of normal cellular metabolism and are commonly generated under conditions of metal overload by metal redox reactions.[31] Of the four known SOD isoforms, distinguished by their metal cofactors (Fe, Mn, Cu/Zn and Ni), Mn-SOD is the dominant form in the diatom *Thalassiosira pseudonana* and chloroplastic Mn-SOD accounts for 10–20% of cellular Mn, depending on incident light intensity and cellular growth rate.[32]

In the present study, direct Mn and Mn + Fe inputs after 6–8 days incubation in Fe-sufficient medium without any added Mn in the Direct-Fe^{III} (second) medium showed only a small effect on the growth of *T. weissflogii* (Fig. 4a) while direct Fe input after 0–8 days incubation in Mn-sufficient medium without any added Fe in the Direct-Mn (second) medium promoted growth with full recovery (Fig. 4b). The sufficient growth by direct Fe input even after 6–8 days incubation probably results from maintaining the high physiological recovery activity of a cell for a long incubation period in Fe-deficient and Mn-sufficient media. It has been reported that coastal and oceanic

diatoms require more Mn to grow in Fe-deficient than in Fe-sufficient sea water.[33] Fe-limitation of diatom growth increases the rate of production of ROS, and the corresponding up-regulation of the superoxide dismuting enzyme Mn-SOD increases the requirement for Mn. According to Peers and Price,[33] SOD enzyme activity increased in Fe-deficient *T. weissflogii* compared with Fe-replete cells and the Mn content of the SODs of *T. weissflogii* increased in Fe-limited cells. Therefore, *T. weissflogii* in Fe-deficient and Mn-sufficient media such as the Direct-Mn (second) medium in the present study could retain the ability for full physiological recovery for several days, probably by decreasing the oxidative stress of phytoplankton with high rates of consumption of ROS. However, Mn-limited *T. weissflogii* in the Fe-sufficient medium could not recover full physiological activity probably by simultaneously increasing the rates of production of ROS. Chrichton[31] reported that excess Fe accumulated by the cells could participate in oxygen radical generation. According to our laboratory results, the addition of only Mn and Fe allows for the full recovery of photosynthesis and the detoxification of superoxide, allowing maximal growth. Fe enrichments stimulated phytoplankton growth without losing the ability for full physiological recovery in Fe-deficient and Mn-sufficient waters. Therefore, Mn may play an important role in maintaining the ability for full physiological recovery for a long time in low Fe regions of the ocean. Future work will aim to measure the activity level of ROS and SOD in Mn-deficient and Fe-sufficient media and Mn-sufficient and Fe-deficient media.

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