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Different responses of phytoplankton to Fe manipulation in Fe-limited waters with contrasting surface mixed layer depths in the western subarctic Pacific

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Abstract

In the western subarctic Pacific (WSP), iron (Fe) can enhance the biological carbon pump by stimulating phytoplankton photosynthesis. However, little is known about how Fe and light availability controls the phytoplankton photophysiology in the WSP near the Kuril Islands, where water mixing is sometimes enhanced, even in summer. Here, we conducted on-deck Fe-manipulated incubation experiments at two stations where surface mixed layer depths were distinct but showed similar initial macronutrient and dissolved Fe concentrations and phytoplankton community composition even experienced light availability for phytoplankton in the water column could be different. An Fe addition to water samples from the deeper surface mixed layer enhanced the phytoplankton biomass and the photosynthetic competence in photosystem II (F_v/F_m), suggesting an Fe-light co-limitation, which was supported by the results from high functional absorption cross-section of photosystem II (σ_{PSII}) and low light saturation index (E_k). At the shallower mixed layer station, Fe amendment did not stimulate phytoplankton biomass and photosynthesis, indicating that Fe was sufficient for the phytoplankton. Although the centric diatom Chaetoceros species were predominant at both stations throughout incubation, the pennate diatoms Pseudo-nitzschia spp. and Cylindrotheca closterium significantly increased at the deeper- and shallower-mixed layer stations, respectively, after Fe enrichment. The elongation of the pennate diatom Neodenticula seminae chains was observed under low Fe availability caused by a chelator. This could be related to the fact that N. seminae is abundant in sediment trap samples from the WSP under Fe-limited conditions and contributed to the high biological pump efficiency.

1. Introduction

Iron (Fe) plays a central role in algal photosynthetic processes such as pigment synthesis, electron transfer via iron-related proteins, nitrate reduction, and detoxification of reactive oxygen (Sunda and Huntsman, 1995; Twining and Baines, 2013; Behrenfeld and Milligan, 2013). *In situ* mesoscale Fe fertilization experiments have revealed that Fe availability controls phytoplankton biomass and primary productivity in High Nutrient, Low Chlorophyll (HNLC) waters (e.g., Boyd et al., 2007). The western subarctic Pacific (WSP) is known as an HNLC region with unique biogeochemical features from spring to summer (Harrison et al., 1999; Tsuda et al., 2003; Suzuki et al., 2009; Hattori-Saito et al., 2010; Fujiki et al., 2014; Nishioka et al., 2020). An *in situ* Fe fertilization in the Western Subarctic Gyre of the WSP during summer stimulated the growth of large diatoms (> 10 μm) and their export (Tsuda et al., 2003), which suggests that the WSP has significant potential to

sequestrate carbon after natural Fe fertilization compared with other major HNLC waters (de Baar et al., 2005). Indeed, during springtime, it has been reported that the WSP has high transport efficiencies of particulate organic carbon (Honda, 2003; Kawakami et al., 2004; 2015) and consequently has the most prominent biological drawdown of CO₂ among the world's oceans (Takahashi et al., 2002).

Nishioka et al. (2007, 2013, 2014, 2020) and Nishioka and Obata (2017) demonstrated that the Dense Shelf Water (DSW) in the Sea of Okhotsk transports sedimentary Fe from the continental shelf to the North Pacific Intermediate Water (NPIW) via the straits of the Kuril Islands where intense vertical mixing occurs. This upward Fe supply fertilizes coastal waters along the Kuril Islands in the WSP (Nishioka et al., 2020). Yoshida et al. (2020) also confirmed that, by conducting photophysiological measurements, coastal communities on the shelf around the Kuril Island Chain were in Fe-repleted conditions for their growth, whereas offshore communities were starved for Fe availability. Yoshida et al. (2020) also reported that, even though the macronutrient and Fe concentrations were similar among the HNLC waters, the offshore phytoplankton showed diverse primary productivity, ranging from 19.0-161.4 mg C m⁻³ d⁻¹. The various photosynthetic status implies that other environmental parameters regulate the photosynthesis activity of the Fe-limited phytoplankton communities. In general, primary productivity (photosynthetic rate) can be defined by the combination of biomass, light availability, and photosynthetic physiology (Bannister, 1974). Yoshida et al. (2020) suggested that light availability, in terms of the ratios of euphotic zone and mixed layer depths, was a crucial controlling factor for primary productivity in the WSP during summer.

Low Fe availability reduces the activity and amount of photosystems I and II (Greene et al., 1992; Geider and LaRoche, 1994). The resultant reduced abundance of Fe-related photosynthetic components consequently hinders electron transport and algal pigment synthesis in the photosystems, as mentioned above. Sunda and Huntsman (1997) reported, by conducting culture-based incubation experiments, that co-limitation of Fe and light further limited the photosynthetic processes of phytoplankton; algal cells at lower light conditions require more Fe to maintain their photosynthesis. This antagonistic Fe-light co-limitation has also been reported on natural phytoplankton communities in subarctic waters and Antarctic diatoms (Maldonado et al., 1999; Strzepek et al. 2012; 2019; Alderkamp et al., 2019). Also, in the WSP, the antagonistic Fe-light co-limitation has been suggested in well-mixed water columns near the straits around the Kuril Islands (Yoshimura et al., 2010; Sugie et al., 2013; Suzuki et al., 2014). For the water mixing processes, (1) strong vertical mixing in straits (Ono et al., 2010; Yagi and Yasuda, 2012), and (2) mesoscale eddies along the Kuril-Kamchatka Trench (Yasuda, 2000; Kaneko et al., 2015) have been documented. It is, thus, needed to understand how Fe and light availability

interact to disentangle the diverse photosynthetic physiology of phytoplankton in Fe-limited waters in the WSP.

We conducted on-deck Fe-manipulated incubation experiments combined with multiple photosynthetic measurements to discuss how Fe and light availabilities affect the photosynthetic physiology of phytoplankton in the WSP near the Kuril Islands. Then, we collected seawater from two stations with similar macronutrient concentrations but contrasting surface mixed layer depths (MLD; 28.5 m and 12.8 m). Effects of Fe amendment plus light acclimation states were assessed with carbon-based photosynthesis—irradiance relationships and variable chlorophyll *a* fluorescence, which can provide powerful insights into the physiological acclimation of phytoplankton (Sakshaug et al., 1997; MacIntyre et al., 2002; Suggett et al., 2009; Bouman et al., 2018). The community composition of phytoplankton was also monitored with scanning electron microscopy and flow cytometry during the Fe-manipulated incubation experiments to investigate which species/groups respond to enhanced Fe availability.

2. Materials and methods

2.1. Experimental setup

During the R/V Professor Multanovskiy cruise (Mu14 expedition) in the summer of 2014, using trace-metalclean techniques, surface seawater was collected for two Fe-manipulated on-deck incubation experiments in the western subarctic Pacific off the eastern Kamchatka Peninsula (Station C5, 52° 00' 07 N, 160° 10' 34 E) and the Kuril Islands (Station A5, 45° 26' 39 N, 152° 58' 38 E) on 18 and 28 June 2014, respectively (Fig. 1). Seawater was obtained from 5 m layer and 5% light depths around noon using a CTD (SBE 9 plus, SeaBird Ltd.) and carousel multi-sampler (SBE32, SeaBird Ltd.) system (CTD-CMS) with acid-cleaned Teflon-coated 10 L Niskin-X bottles (Obata et al., 1993; Takeda and Obata, 1995; Nishioka et al., 2001). Mixed layer depths were defined where the potential density anomaly ($\Delta \sigma_{\theta}$) of the water column was 0.125 kg m⁻³ larger than the sea surface at 10 m (Monterey and Levitus, 1997). The 5% light depths were determined based on the diffusive attenuation coefficients of photosynthesis available radiation (PAR) (K_d (PAR)) calculated from *in situ* vertical PAR profiles using a Compact-Optical Profiling System (Biospherical Instruments Inc.) (Hooker et al., 2013) and the National Aeronautics and Space Administration Processing of Radiometric Observations of Seawater using Information Technologies (PROSIT) software (Hooker et al., 2018) with reference to the incident PAR $(E_0(PAR))$ above the sea surface. Euphotic zone depths were defined as 1% light depth compared to $E_0(PAR)$ (Kirk, 2010). The surface seawater from 5 m was dispensed into eight trace-metal-cleaned 9 L polycarbonate (PC) bottles without pre-filtration. Two out of the eight bottles were Fe-enriched by adding FeCl₃ solution (final Fe concentration of 10 nM; hereafter, +Fe treatments). For another set of two PC bottles, the Fe chelator deferoxamine B was amended (final concentration of 1 μ M, hereafter +DFB treatments) to reduce Fe availability for phytoplankton (e.g., Wells, 1999; Hutchins et al., 1999; Kondo et al., 2013). The other two bottles were used as controls without any addition (hereafter Control), whereas another set of two bottles were time-zero (hereafter Initial). The six bottles, except for Initials, were double-wrapped with transparent plastic bags to prevent contamination and incubated in a temperature-controlled on-deck incubator at *in situ* temperature and natural light environment (100% surface light) for 3–4 days. Unfortunately, one of the +DFB bottles incubated at station A5 was contaminated due to water seeping into the bottle (discussed in section 3.4).

Vertical water-column stability (i.e., stratification) at MLD was estimated from Brunt-Väisälä frequency (N^2) , which was calculated on 3-m moving-averaged temperature-salinity profiles (Wahl and Teague, 1983; Schaffer et al., 2012):

$$N^{2} = (g / \rho) \cdot (\partial \rho / \partial z) \tag{1}$$

where g is gravitational acceleration, ρ is seawater density at each depth (kg m⁻²), and z is depth. Besides, upward fluxes, across the mixed layer of nitrate and dissolved iron (DFe) were quantified as F_{NO3} and F_{DFe} based on the vertical diffusivity according to Osborn (1980) and Kaneko et al. (2013):

$$F_{\rm NO3} = K_{\rm \rho} \cdot \partial {\rm NO}_3 / \partial z \tag{2}$$

$$F_{\rm DFe} = K_{\rm \rho} \cdot \partial \rm DFe / \partial z \tag{3}$$

$$K_{\rm p} = \Gamma \varepsilon \,/\, N^2 \tag{4}$$

where K_{ρ} is vertical diffusivity to estimate upward nutrient flux derived from the micro-temperature observations using MicroRider MR6000 (Rockland Scientific) attached to the CTD-frame. The MR6000 was equipped with FP07 (Fastip Probe #7) fast-response thermistor. Turbulent energy dissipation rate ε and turbulent thermal dissipation rate χ were estimated from temperature gradient spectra following the procedures with the time-constant corrections of double-pole 3 ms (Goto et al. 2016, 2018, 2021; Yasuda et al. 2021), in which the measurements were confirmed to be valid in the ranges of $10^{-11} < \varepsilon < 10^{-8}$ (W kg⁻¹) and $10^{-11} < \chi < 10^{-7}$ (°C² s⁻¹). Vertical diffusivity K_{ρ} is then computed as $K_{\rho}= 0.2 \varepsilon N^{-2}$ (m² s⁻¹) (Osborn, 1980) where 10-dbar mean squared buoyancy frequency N^2 using CTD density data. The shallow part (< 100 m depth) of the ε data at stations C5 and A5 was expected to be underestimated, considering the measurement of the upper limit ($\varepsilon =$ ~ 10^{-8} W kg⁻¹) near the surface. In this study, for the upper 100 m, temperature dissipation χ was alternatively used for the estimate of the vertical diffusivity (Osborn and Cox, 1972):

$$K_{\rm T} = 0.5 \ \chi \ \theta_{\rm z}^{-2} \tag{5}$$

where θ_z is the vertical gradient of potential temperature θ because χ is less underestimated than using ε (Goto et al., 2018). Differences in average nitrate and DFe concentration from 10–50 m depths were represented as $\partial NO_3/\partial z$ and $\partial DFe/\partial z$, respectively. Also, we used the averaged K_T value from 10 m to 50 m depths to estimate F_{NO3} and F_{DFe} . To discuss the potential Fe limitation for phytoplankton photosynthesis, the upward flux ratio, F_{DFe}/F_{NO3} , was calculated:

$$F_{\rm DFe} / F_{\rm NO3} = (\partial \rm DFe / \partial z) / (\partial \rm NO_3 / \partial z)$$
(6)

2.2. Total dissolvable Fe analysis

Before and after the incubation experiments, total dissolvable Fe (TFe) samples in each 9 L PC bottle were collected into an acid-cleaned 125 mL LDPE bottle. The samples were acidified with 20% ultrapure HCl (Tamapure AA-10, Tamapure Co., Ltd.) to solubilize all labile Fe in seawater (Obata et al., 1997). Samples were buffered with 10 M formic acid - 2.4 M ammonium formate buffer to adjust pH to 3.2 just before measurements on land. Concentrations of TFe were determined with an automatic Fe (III) flow injection analytical system (Kimoto Electric, Ltd.) with pre-concentration by a chelating resin following the chemiluminescence method (Obata et al., 1993; 1997). The concentration of TFe in the DFB treatments was not able to be quantified due to the strong ligand complexation. All ambient DFe (< 0.2 μ m) data used in the discussion section of this study were cited from Nishioka et al. (2020) and Nishioka et al. (2021).

2.3. Phytoplankton pigment analysis

Phytoplankton biomass in each treatment was estimated from the sum of chl *a* and its derivatives (i.e., chl *a*allomer, chl *a*-epimer, and chlorophyllide *a*) determined with High-Performance Liquid Chromatography (HPLC). Triplicated water samples taken from each 9 L PC bottle, before and after the incubation experiments, were filtered onto 25 mm GF/F filters (Whatman) under gentle vacuum (<0.013 Mpa). The filters were flashfrozen in liquid nitrogen and stored in a deep freezer (-80 °C) until analysis on land. The frozen filter was blotted with a qualitative filter paper, snipped, and sonicated in 3 mL of *N*,*N*-dimethylformamide (DMF) containing a known amount of canthaxanthin as an internal standard to extract algal pigments (Suzuki et al., 2002). The pigment extracts were passed through a 0.45 μ m PTFE filter to remove fine particles. The pigment extracts of 250 μ L were mixed with 28 mM tetrabutylammonium acetate aqueous solution of the same quantity, and the mixture was injected into an HPLC (CLASS-VP System, Shimadzu) with an Agilent Eclipse XDB C8 column (3.5 μ m particle size, 4.6×150 mm) following Endo et al. (2013).

2.4. Photosynthesis-irradiance (P-E) relationship measurement

The seawater from each 9 L PC bottle was dispensed into twelve 275-mL polystyrene bottles. A ¹³C-labelled sodium bicarbonate (NaH¹³CO₃, 99 atom% ¹³C, Cambridge Isotope Laboratories, Inc.) solution was added to ten bottles (ca. 10% of total dissolved inorganic carbon). The remaining two bottles were initial samples used as a ¹³C background for each bottle. A series of ¹³C-labeled seawater in 10 bottles was incubated for 2 h in a temperature-controlled incubator at 10 different light intensities from 1.44 to 3,000 µmol photons m⁻² s⁻¹ and at the *in situ* temperatures (Table 1). After 2 h incubation, the samples were filtered onto pre-combusted 25 mm GF/F filters (Whatman) with a gentle vacuum in the dark (<0.013 MPa) and stored in a deep freezer (-80 °C) until further analysis on land. The amount of ¹³C on the filters was measured with an online element analyzer (FlashEA1112, Thermo Finnigan)/Isotope ratio mass spectrometer (EA/IRMS) (Delta-V, Thermo Finnigan). The photosynthesis rates of phytoplankton were calculated following Hama et al. (1983). The resultant photosynthesis rate was normalized with chl *a* concentration to obtain chl *a*-specific photosynthesis-irradiance (*P*-*E*) relationship model of Platt et al. (1980) (Fig. 3).

2.5. Variable chlorophyll *a* fluorescence

Photosynthetic physiological responses, including light acclimation, to Fe manipulation were also assessed with the maximum photochemical quantum yield and functional absorption cross-section of photosystem II (PSII) for phytoplankton using variable chl *a* fluorometry. Variable chl *a* fluorescence *in situ* was measured with a Fluorescence Induction and Relaxation (FIRe) fluorometer (Satlantic Inc.). Natural seawater samples at 5 m and 5% light depth (C5: 26.0 m; A5: 23.6 m) were collected into a 30 mL shading polyethylene bottle, whereas seawater from the Fe-manipulated incubation bottles was dispensed into another shading bottle. The samples were incubated in darkness at *in situ* temperature for 30 minutes for dark acclimation. A single turnover excitation protocol was applied to the dark-acclimated cells with 80 µs flash at 450 nm from a light-emitting

diode (LED) equipped with the FIRe fluorometer. Chl *a* fluorescence induction curves were iterated 10 times after 1000 ms intervals between each flash. The obtained induction curves were averaged and fitted to the model proposed by Kolber et al. (1998) using the MATLAB-based program fireworx developed by Audrey Barnett (Dalhousie University). The maximum quantum yield of PSII photochemistry (F_v/F_m) and functional absorption cross-section of PSII (σ_{PSII} ; Å² quanta⁻¹) were obtained from the resultant fitted curves. The difference in σ_{PSII} values between samples from the surface and the depth of the 5% light level was defined as $\Delta \sigma_{PSII}$.

2.6. Microscopy

Armored phytoplankton cells were enumerated with scanning electron microscopy (SEM) to examine community shifts after Fe manipulation. Five hundred milliliters of seawater in each bottle were fixed with a neutral formaldehyde solution buffered with sodium acetate (final concentration 2%). A subsample of the fixed sample was filtered onto a 25 mm, 0.4 µm Nuclepore membrane filter set on a glass funnel under gentle vacuum (< 0.013 Mpa). After rinsing the filter with distilled water for desalination, the filter was coated with Au-Pb alloy using an MSP-1S magnetron spatter device (Vacuum Device Inc.). Armored protistan cells were counted with a scanning electron microscope (VE-8800, Keyence Co., Ltd.) with a magnification of >1000×, according to Nosaka et al. (2014, 2017). For each sample, >3000 cells were enumerated. Species identification was based on Tomas (1997), Round et al. (2007), Konno et al. (2007), and Medvedeva and Nikulina et al. (2014). The number of plastids in *Chaetoceros* cells was enumerated by bright-field microscopy (Keyence BZ-9000).

2.7 Flow cytometry

Ultraphytoplanktonic (< 10 µm in size) cryptophytes and *Synechococcus* were enumerated with flow cytometry (FCM) to complement SEM, which cannot identify small unarmoured phytoplankton. Two milliliters of seawater samples from each bottle were fixed with 40 µL of 10 % paraformaldehyde (PFA) in a cryovial (final concentration 0.2 %). The seawater samples in the vials were flash-frozen in liquid nitrogen and stored in a deep freezer (-80 °C) until analysis on land. Enumeration of ultraphytoplankton cells was performed with an EPICS flow cytometer (XL ADC system, Beckman Coulter) equipped with 15 mW air-cooled laser exciting at 488 nm. The FCM samples were melted gradually in distilled water and prefiltered through a 35 µm nylon-mesh-capped Falcon cell strainer (Becton-Dickinson) to remove larger cells before loading to the FCM. Each sample was analyzed with the EXPO32 software (Beckman Coulter) as described in Suzuki et al. (2005).

3. Results

3.1. Hydrography

Sea surface temperature and salinity at 5 m (hereafter, SST and SSS, respectively) were similar and generally low at both stations (C5: 6.05 °C, 32.77; A5: 6.80 °C, 32.81, Fig. 1 and Table 1). Macronutrient concentrations at 5 m of the two stations were also similar to each other and relatively high. Fe concentration was low at the two stations (C5: 0.05 nM DFe; A5: 0.06 nM DFe, Nishioka et al., 2020, 2021) (Table 1). Both stations showed rather similar euphotic layer depths (Z_{eu} ; C5: 40.3 m; A5: 36.2 m). In contrast, the surface mixed layer depths (MLDs) were contrastive; station C5 showed a deep MLD (28.5 m), whereas station A5 showed a shallow MLD (12.8 m) (Table 1). Note that MLDs at stations C5 and A5 were the deepest and the second shallowest throughout the Mu14 expedition (Yoshida et al., 2020). Although the vertical water-column stability (N^2) within the MLDs was comparable between the two stations (C5: $9.82 \times 10^{-2} \text{ s}^{-1}$; A5: $9.82 \times 10^{-2} \text{ s}^{-1}$; Table 1), the upward fluxes of NO₃ and DFe at station C5 were 10-times higher than those at station A5 (C5: $F_{NO3} = 2.82 \times 10^3 \text{ mmol m}^{-2} \text{ day}^{-1}$, $F_{DFe} = 3.11 \text{ µmol m}^{-2} \text{ day}^{-1}$, Table 1), where F_{NO3} and F_{DFe} were comparable with those reported in Nishioka et al. (2020), although their flux data were calculated based on different depths from the present study. The resultant upward flux ratios of DFe to NO₃ (F_{DFe}/F_{NO3}) were similar between the two stations (C5: 0.0112 mM µM⁻¹; A5: 0.0105 mM µM⁻¹, Table 1).

Concentrations of TFe after the incubation experiments showed similar patterns at both stations (Fig. 2). The highest TFe was derived from the Fe-added bottles, followed by the initial (i.e., time-zero) and control treatments (Fig. 2).

3.2. Responses of photosynthetic parameters to Fe and DFB addition

A large increase in chl *a* concentration after Fe addition was observed at station C5 compared to the control treatment (2.2-times higher, Fig. 4). Of all the bottles at station C5, the +DFB bottles had the lowest chl *a* value, although little difference was found as compared to the Control bottles (Fig. 4). At station A5, on the other hand, the concentrations of chl *a* in the Control and +Fe bottles were increased by 30% from the Initial, while the DFB bottle showed the second lowest following the Initial (Fig. 4). At station A5, one out of the +DFB bottles was contaminated with external Fe due to the immersion of waters into bottles, the results from the contaminated bottle were thus excluded from further discussion.

The F_y/F_m values between stations C5 and A5 behaved similarly with the response of chl *a* after the Fe and DFB amendment (Fig. 5A). After Fe addition at station C5, the F_v/F_m values increased by 43.7% compared with the Control treatment (Fig. 5A), while DFB amendment showed the lowest $F_{\rm v}/F_{\rm m}$ value (0.175), which was far lower than the Control and +Fe treatments. At station A5, a small difference (10%) in F_v/F_m was observed between the Control and +Fe treatments (Fig. 5A). The F_v/F_m values for +DFB treatments at both stations were almost identical or lower than the initial values. FIRe fluorometry showed that the values of σ_{PSII} decreased after the incubation experiments compared to the Initial at both stations. However, the σ_{PSII} further decreased in the +Fe bottles at station C5, whereas those in the Control and +Fe bottles were comparable at station A5 (Fig. 5B). Also, the σ_{PSII} in the +DFB bottles showed relatively high, which was comparable to the Initial at station C5, while the σ_{PSII} values were almost comparable among the treatments (Fig, 5B). FIRe fluorometry also provided σ_{PSII} values in the Initial at the surface and 5% light depths at both stations. Little variation in σ_{PSII} was found at station C5 between the samples collected from the surface and 5% light depth (5 m: 328 ± 10 Å² quanta⁻¹, 5%: 326 ± 28 Å² quanta⁻¹, i.e., $\Delta\sigma_{PSII}=2$ Å² quanta⁻¹), whereas the $\Delta\sigma_{PSII}$ at station A5 was large (5 m: 332 ± 18 Å² quanta⁻¹, 5%: 403±34 Å² quanta⁻¹, i.e., $\Delta \sigma_{PSII}=71$ Å² quanta⁻¹) (Fig. 7). The 5% light depth (26.0 m) at station C5 was comparable to the MLD, whereas the 5% light depth at station A5 was much deeper than the MLD. Photosynthetic-irradiance (P-E) parameters showed different responses to the Fe and DFB amendment between stations C5 and A5 (Fig. 6). At station C5, P-E parameters little changed between the Control and +Fe treatments, whereas the DFB amendment significantly lowered only P^{B}_{max} (2.38±0.73 mg C mg chl a^{-1} hr⁻¹) compared to the Control $(9.09\pm1.58 \text{ mg C mg chl } a^{-1} \text{ hr}^{-1})$ and +Fe treatments $(10.08\pm1.75 \text{ mg C mg chl } a^{-1} \text{ hr}^{-1})$ hr⁻¹). The Fe amendments to the A5 seawater largely increased the $\alpha^{\rm B}$ values (Control: 0.045±0.013 (mg C mg chl a^{-1} hr⁻¹) (µmol photons m⁻² s⁻¹)⁻¹, +Fe: 0.053±0.003 (mg C mg chl a^{-1} hr⁻¹) (µmol photons m⁻² s⁻¹)⁻¹, Fig. 6A); however, the other *P*-*E* parameters did not respond to the Fe addition (Fig. 6B, C, D). The increment of E_k

3.3. Community responses to Fe and DFB addition

The abundance of coccolithophores and Parmales decreased during incubation at station C5, while *Chaetoceros* species dominated in the Control and +Fe bottles at the station (Tables 2 and S1). *Pseudo-nitzschia* spp. was the only species showing a remarkable increase after +Fe addition. Interestingly, in the +DFB treatment, *Cheatoceros* species were replaced with large and robust diatoms (e.g., *Corethron pennatum*, *Neodenticula seminae*, and *Thalassionema nitzschioides*) with an increase in *Fragilariopsis* species and armored

between Initial and Control was doubled at station C5, while that at station A5 was small (Fig. 6D).

ciliates at station C5 as well as resting spores of *Chaetoceros* (Tables 2 and S1). The abundance of *Chaetoceros* species, which were initially dominant, was also the highest in the control bottles, followed by +Fe bottles at station C5. *Pseudo-nitzschia* spp. also increased both in the Control and +Fe bottles, while *Cylindrotheca closterium* greatly increased only in the +Fe treatment.

At station A5, *Chaetoceros* species were predominant both in the Control and +Fe bottles. In contrast, the large and robust species (i.e., *C, pennatum, N. seminae*, and *T. nitzschioides*) outcompeted the community in +DFB bottles, replacing the *Chaetoceros* species except for *Ch. concavcornis* and *Ch. convolutus*, which increased in abundance (Tables 2 and S1). Length of chains of *Neodenticula seminae* were often long (>5 up to 23 cells in a chain) in the +DFB bottles at both stations, although solitary cells or short chains of the species were generally observed at the other stations and in the other bottles (Fig. 8, Table 2).

The Fe addition enhanced the abundance of cryptophytes, whereas *Synechococcus* did not respond to Fe manipulations at the two stations (Fig. 9). Cryptophytes considerably grew up in the +Fe treatments compared with the Control and +DFB treatments at station C5 (~2.5-times and ~2-times higher compared with Control and +DFB treatments, respectively). Also, at station A5, the abundance of cryptophytes largely increased in the +Fe treatment compared with the Control treatment (1.90-times higher) (Fig. 9A). The abundance of *Synechococcus*, on the other hand, did not change throughout the incubation both at stations with slight decreases (Fig. 9B).

4. Discussion

The initial community structures at both stations were relatively similar (Tables 2 and S1), although their photosynthetic responses to Fe or DFB additions differed. At station C5, a remarkable increase in chl *a* was observed after the Fe addition as compared with that of the Control treatment (Fig. 4), suggesting that Fe limitation occurred. The large increase in F_v/F_m values after Fe addition supported this notion (Fig. 4A). The Fe addition to the algal assemblages at station A5 did not show little variation in chl *a* (Fig. 4) between the Control and +Fe bottles. The F_v/F_m values also responded less to the Fe amendment at station A5 (Fig. 5A), indicating Fe repletion for the phytoplankton assemblage, in spite of the low dissolved Fe concentration (DFe=0.05 nM; Table 1, Nishioka et al., 2020; 2021). However, the upward fluxes of NO₃ and DFe were much higher at station C5 than those at station A5 (Table 1). The phytoplankton assemblage at station C5 would thus be more accessible to Fe from below the MLD than at station C5 (Table 1). Note that the upward flux ratios (F_{DFe}/F_{NO3}) were relatively low between the two stations (Table 1). Browning et al. (2017) and Shaked et al. (2021) suggested that Fe could be the primary limiting nutrient even if DFe/NO₃ ratios were low (0.01-0.016), indicating that the phytoplankton assemblages at both stations in this study were potentially Fe-limited compared with nitrate availability even if the deepwater uplifted.

Even potential Fe availability was low at both stations, the ¹³C-based photosynthetic performance interestingly showed little changes after Fe addition. Interestingly, the ¹³C-based photosynthetic performance showed little changes, even though the biomass was enhanced after Fe addition at station C5 (Figs. 4 and 6). These physiological responses indicate the phytoplankton assemblages at this station prioritized pigment synthesis over carbon fixation when Fe was supplied. Indeed, the increased Fe availability reduced the lightharvesting antennae size of PSII, σ_{PSII} (Fig. 5B) with the F_v/F_m and chl *a* enhancement (Figs. 4 and 5A), which are similar to PSII reorganization to improve the energy funneling from light-harvesting antennae to the reaction center of PSII (Suggett et al. 20009). D'Haene et al. (2015) and Yoshida et al. (2018) demonstrated that PSII antennae are restructured with a decrease in σ_{PSII} and an increase in F_v/F_m with significant increases in the number of the reaction center of PSII (n_{PSII}). This relative increase in n_{PSII} consequently reduces σ_{PSII} . This is because the +DFB bottles showed relatively high σ_{PSII} values due to unsuccessful antennae reorganization under severe Fe-limitation (Fig. 5B). In another aspect from bio-optics, deeper MLD had smaller E_k and vice versa in the studied area (Yoshida et al. 2020), indicating that phytoplankton in the study area had been well-acclimated to light environments. The phytoplankton assemblages at station C5, where the MLD was 28.5 m, had a low E_k for the Initial (i.e., low light-acclimated), whereas that at station A5, where the MLD was 12.8 m, was higher (i.e., high light-acclimated). The inherent photophysiological status of the phytoplankton assemblages (e.g., pigmentation and cellular Fe requirement) might differ between the two stations. The difference in the degree of light acclimation between the phytoplankton at stations C5 and A5 was supported by the results of $\Delta \sigma_{PSII}$ (Fig. 8). The smaller $\Delta \sigma_{PSII}$ value at station C5 suggested the water column was well mixed, therefore the phytoplankton would experience lower average light intensity in the water column, which supports the low E_k value for the Initial. The large $\Delta \sigma_{PSII}$ value at station A5 suggested the phytoplankton acclimated to the light intensity at each depth, specifically due to the shallow MLD (Figs. 6 and 8). The phytoplankton assemblages at station C5 were thus acclimated to a dimmer environment, indicating that Fe-light co-limitation likely occurred at the station as suggested by Suzuki et al. (2014) in the well-mixed water columns in the WSP near the Kuril Islands., which could exacerbate Fe-light co-limitation for the phytoplankton assemblage. This notion was supported by the doubled increment of E_k between Initial and Control at station C5. The results suggest that the initial phytoplankton cells suffered from light limitation (Fig. 6D). However, the phytoplankton assemblage at

station A5 might employ other strategies, enhancing the carbon fixation activity (i.e., a significant increase in α^{B} ; Fig. 6A). Greene et al. (1992), Geider and LaRoche (1994), and McKay et al. (1997) discussed that phytoplankton could decrease the ratio of more Fe-requiring PSI/cytochrome *b₆/f* complex to less Fe-requiring PSII. In addition, Strzepek et al. (2012, 2019) demonstrated that phytoplankton increases photosynthetic antenna size (i.e., σ_{PSII}) rather than the number of antennae under Fe-deficient conditions, which does not associate with little change in chl *a* concentration. This notion was also supported by the comparable σ_{PSII} values among the treatments (Fig. 5B), indicating that phytoplankton did not need to reorganize the PSII antennae even with Fe manipulations. The Fe-replete (or slightly Fe-starved) phytoplankton at station A5 might thus prioritize modifying their photosynthetic architecture without chl *a* increase or changes in σ_{PSII} and n_{PSII} (Figs. 4 and 5B). At both stations, DFB inhibited the growth of phytoplankton compared to the Control due to its strong Fe complexation capability even though the Fe concentration was low (Table 1). However, chl *a* in the DFB treatment at station A5 increased than the Initial condition (Fig. 4) despite the Fe chelation. This increase might be related to the community shifts from the natural assemblage to large, heavily-silicified diatoms (Tables 2 and S1, discussed below).

The diatom community responded little to Fe addition at both stations, although the communities after on-deck incubation were surely shifted from the Initial. From the microscopic observations, changes in the community composition of phytoplankton at the genus level were few after Fe addition at both stations (Tables 2 and S1). The genus Chaetoceros, which was dominated in each bottle, might outcompete under Fe-replete conditions, as observed in other onboard Fe amendment incubations (Timmermans et al., 2001) and in situ Fe fertilization experiments (Tsuda et al., 2003). Our study demonstrated that Fe addition stimulated some specific diatom species (e.g., Pseudo-nitzschia spp. and Cylindrotheca closterium at stations C5 and A5, respectively), which were minor in the initial Chaetoceros-dominated communities (Tables 2 and S1). Smaller cell sizes could benefit the efficiency of their nutrient uptake because of their higher cell surface area to volume ratio (Taguchi, 1976; de Baar et al., 1995; Sunda and Huntsman, 1997). The Chaetoceros spp., except the larger Ch. concavicornis and resting spores, were not abundant in the +DFB bottles but replaced by much larger diatoms such as Thalassionema nitzschioides, Neodenticula seminae, Corethron pennatum, and Asteromphalus spp. (Table S1). The larger cells might be capable of storing surplus Fe in their larger vacuoles (Sicko-Goad et al., 1984; Raven, 1998; Behrenfeld et al., 2021), which can become an important intracellular Fe source (Maranón et al., 2012, 2015) and support their growth even under Fe-deficient conditions (Wells, 1999; Sugie et al., 2011). Also, Maldonado and Price (1999) demonstrated that Fe bound to DFB could be utilized via the reduction of Fe

by large phytoplankton (> $3 \mu m$) such as diatoms. Therefore, the larger diatoms were able to survive in the +DFB bottles.

The abundance of ultraplanktonic cryptophytes was enhanced by Fe addition, whereas Synechoccocus little changed. Increases in the abundance of cryptophytes after Fe addition (Fig. 9) were similar to the results from an in situ Fe-manipulated experiment in the WSG during summer (Suzuki et al., 2009). Camoying et al. (2022) demonstrated that cryptophytes are vulnerable to Fe limitation due to low affinity to Fe with reduced Cumediated Fe reductase (Behnke and LaRoche, 2020); in turn, Fe amendment stimulates the growth of cryptophytes (Camoying et al., 2022) as observed in our study. Sato et al. (2009) reported an increased abundance of Synechoccocus after the Fe enrichment during an in situ Fe fertilization experiment of SEEDS II in the WSG. In contrast, Kudo et al. (2006) showed a minor increase in Synechoccocus by conducting Fe amendment bottle incubation in the WSG. The different responses to Fe enrichment were partly due to physiological or phylotypic differences in Synechoccocus before the Fe amendment and higher microzooplankton grazing pressure in Kudo et al. (2006). Our experiments were also conducted in 9 L bottles, and the high abundance of ciliates and choanoflagellates could enhance the grazing pressure on picoplankton. The abundance of both *Synechoccocus* and cryptophytes in the incubation bottles might be somewhat underestimated, although significant increases in cryptophytes were observed in the +Fe bottles (Fig, 9). Some relatively abundant dinoflagellates (Prorocentrum spp. and Gymnodimium spp.) could also play as grazers (Table S1).

This study first reported the elongation of *Neodenticula seminae* chains with +DFB treatment (Fig. 8). In the WSP, *Neodenticula seminae* was often observed in sediment traps (Katsuki et al., 2003; Katsuki and Takahashi, 2005; Onodera et al., 2005). This pennate diatom species might have a strategy to sink rapidly under Fe-limited conditions by their chain elongation, which could play a significant role in the high biological pump efficiency in the western subarctic Pacific (Honda, 2003).

5. Conclusions

This study demonstrated that the photosynthesis of phytoplankton responded differently to increased Fe availability in respect to the mixed layer depths, which consequently controlled their biomass and community composition in HNLC waters of the WSP. Even though the macro-nutrient and Fe concentrations were similar, vertical mixing significantly affected the photo-acclimation status and, consequently, Fe requirement of phytoplankton communities: low light acclimation caused Fe limitation in the deep-mixed water. The upward fluxes of DFe and NO₃ were much higher in the deep-mixed water than in the shallow-mixed one. However, as mentioned above, both waters were potentially Fe-limited, inferred from the low FDFe/FNO3 ratio. Indeed, the ondeck Fe manipulation confirmed Fe limitation in the deep-mixed water, while the Fe amendment showed little changes in the abundance and photophysiology of phytoplankton in the shallow-mixed water. However, carbon fixation rates little changed in the Fe-limited community, whereas those for the Fe-replete community were enhanced, which indicates different allocative strategies of the energy produced from the improved photosynthetic machinery by the Fe augmentation: chl a or carbon fixation. This different response might be related to the size of photosynthetic antennae as an overcoming strategy against the Fe-light co-limitation. Additionally, at the shallow mixed water station, a decrease in the level of cellular chl a in Fe-starved phytoplankton may become a superior survival strategy to protect the cells from high irradiance that can cause photo-oxidative damages through photosynthesis (Sugie et al., 2011). Although Fe amendment increased large and/or chain-forming Chaetocetos species and cryptophytes, Fe depletion enhanced the abundance of heavilysilicified species (e.g., N. seminae) and resting spores observed in the +DFB bottles. This response implies that Fe depletion in the WSP during summer leads to large and heavily-silicified phytoplankton, which enhances the efficiency of the biological pump (Tréguer et al., 2017).

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Compliance and Ethical Standards

The authors declare that they have no conflicts of interest.

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Figures



Figure 1. Surface water sampling sites (red circles) for on-deck Fe manipulation incubation experiments in the western subarctic Pacific



Figure 2. Total dissolvable Fe concentrations throughout the Fe manipulation experiment at stations C5 and A5

Ini (open): Initial treatment, Cont (shaded): Control treatment (closed); +Fe: +Fe treatment (dotted)

The concentrations of TFe in the +DFB treatments were not determined at both stations.



Figure 3. A model photosynthesis–irradiance (*P*-*E*) curve fitted with Platt et al. (1980).

 $α^{\text{B}}$: Light utilization index [(mg C mg chl a^{-1} hr⁻¹) (µmol photons m⁻² s⁻¹)⁻¹]; PBmax: [mg C mg chl a^{-1} hr⁻¹]; E_k [µmol photons m⁻² s⁻¹]; $β^{\text{B}}$: Light inhibition index [(mg C mg chl a^{-1} hr⁻¹) (µmol photons m⁻² s⁻¹)⁻¹



Figure 4. Variations in chl *a* concentration during the on-deck Fe manipulation incubation experiments.

Chl a: the sum of chl a, chl a-epimer, chl a-alomer, and chlorophyllide a

Ini (open): Initial treatment, Cont (shaded): Control treatment; +Fe (closed): +Fe treatment; +DFB (dotted): +DFB treatment.



Figure 5. Variations in (A) F_v/F_m and (B) σ_{PSII} [Å² quanta⁻¹] during the on-deck Fe manipulation incubation experiments.

 $F_{\rm v}/F_{\rm m}$: Maximum quantum yield of PSII photochemistry measured with FIRe fluorometry

 σ_{PSII} : Functional absorption cross section of PSII

Ini (open): Initial treatment, Cont (shaded): Control treatment; +Fe (closed): +Fe treatment; +DFB (dotted): +DFB treatment



Figure 6. Variations in photophysiological parameters obtained from carbon-based photosynthesis–irradiance relationships; (A) α^{B} : light utilization index, (B) β^{B} : photoinhibition index, (C) P^{B}_{max} : maximum photosynthetic rate, and (D) E_{k} : Light saturation index Ini (open): Initial treatment, Cont (shaded): Control treatment; +Fe (closed): +Fe treatment; +DFB (dotted): +DFB treatment



Figure 7. Functional absorption cross-section of PSII (σ_{PSII}) for phytoplankton at the surface (5 m, open) and 5% light depth (5%, shaded) at stations C5 and A5.

The 5% light depth was calculated from the diffusive attenuation coefficient of PAR (K_d

(PAR)) (Yoshida et al., 2020).

Differences in σ_{PSII} between 5 m and 5% light depth ($\Delta \sigma_{PSII}$) were smaller ($\Delta \sigma_{PSII} = 2$) at station C5 and larger ($\Delta \sigma_{PSII} = 71$) at station A5, respectively. n = 3



Figure 8. Scanning electron micrographs of the pennate diatom *Neodenticula seminae* with elongated chains from the +DFB bottles (A) at stations C5 and (B) A5.



Figure 9. Cell abundance of ultraplankton (<10 µm) enumerated using a flow cytometer. (A) cryptophytes and (B) *Synechococcus*. Ini (open): Initial treatment, Cont (shaded): Control treatment (closed); +Fe: +Fe treatment (dotted)

Tables

Table 1. Environmental parameters during the incubation experiments. (A) Physical and chemical properties of surface waters at Stations C5 and A5. (B) optical properties during the Fe-manipulation incubation experiments.

(A)				
		Station C5	Station A5	
SST	[°C]	6.05	6.80	
SSS	-	32.77	32.81	
NO ₃	[µM]	9.93	16.64	
NO_2	[µM]	0.16	0.27	
\mathbf{NH}_4	[µM]	0.39	0.3	
PO_4	[µM]	1.09	1.53	
SiO ₄	[µM]	12.91	14.88	
DFe	[nM]	0.05	0.06	
MLD	[m]	28.5	12.8	
$Z_{ m eu}$	[m]	40.3	36.2	
N^2	$[s^{-1}]$	$9.82 imes10^{-2}$	$9.82 imes10^{-2}$	
<i>К</i> _{Т (10-50 m)}	$[m^2 s^{-1}]$	$1.88 imes 10^{-4}$	0.267×10^{-4}	
$\partial NO_3/\partial z$ (10-50 m)	$[mmol m^{-4}]$	173	129	
$F_{ m NO3}$	$[\rm mmol \ m^{-2} \ day^{-1}]$	2.82×10^3	297	
$\partial DFe/\partial z$ (10-50 m)	$[\mu mol m^{-4}]$	2.02	1.35	
$F_{ m DFe}$	$[\mu mol \ m^{-2} \ day^{-1}]$	32.7	3.11	
$F_{\rm DFe}/F_{ m NO3}$	$[nM \ \mu M^{-1}]$	0.0116	0.0105	

SST: sea surface temperature; SSS: sea surface salinity; NO₃: nitrate concentration; NO₂: nitrite concentration; NH₄: ammonium concentration; PO₄; phosphate concentration; SiO₄: silicate concentration; DFe: dissolved iron concentration; MLD: mixed layer depth; Z_{eu} : euphotic zone depth; N^2 : 3-m averaged Brunt–Väisälä frequency across the MLD; F_{NO3} , F_{DFe} : upward fluxes of nitrate and DFe, respectively; F_{DFe}/F_{NO3} : ratio of upward fluxes of DFe to NO₃ The surface data and vertical profiles were adapted from Nishioka et al. (2020, 2021) and Yoshida et al. (2020).

Station	Treatment	$E_0(PAR)$ max	$E_0(PAR)Ave$ [µmol photons m ⁻² s ⁻¹]		
	Ireatment	$[\mu mol photons m^{-2} s^{-1}]$			
C5	Initial	1111*	_		
	Control	1256 ± 358	331 ± 234		
	+Fe	1219 ± 341	251 ± 77		
	+DFB	1281 ± 309	279 ± 89		
A5	Initial	632*	_		
	Control	1246 ± 558	341 ± 238		
	+Fe	1246 ± 558	304 ± 257		
	+DFB	1334 ± 453	313 ± 224		

(B)

 $E_0(PAR)$ ave: daily average incident PAR at the surface during each treatment of the Femanipulation incubation experiments; an average value with standard deviation throughout the incubations of each treatment.

Treatment		C5_Ini	C5_Cont	C5_+Fe	C5_+DFB	A5_Ini	A5_Cont	A5_+Fe	A5_+DFB
Unidentified		0.7%	1.8%	3.0%	11.3%	9.5%	4.5%	17.7%	20.2%
Coccollithophores		3.2%	0.6%	0.2%					
Parmales		44.4%	5.2%	5.6%	0.1%	0.4%	2.1%	0.0%	0.3%
Silicoflagellate		0.6%	0.3%	0.3%	0.6%	0.2%	0.0%	0.1%	0.3%
Dinoflagellates			0.1%		2.8%	2.4%	0.2%	0.7%	3.2%
Flagellates			0.5%	0.6%	9.0%	0.5%	0.1%		1.1%
Diatoms		51.0%	91.4%	89.6%	76.2%	87.0%	93.0%	81.5%	75.0%
Centric diatoms	Size (µm)								
Chaetoceros		++++	++++	++++	++	++++	++++	++++	++
C .atlanticus		*	*			**	*	*	**
C. concavicornis	$29.3\pm8.3a^{\rm Y}$	*	*	*	**	*	*	*	**
C. convolutus	$31.1 \pm 4.8_{a}^{\ Y}$					*	*	*	**
C. deblis	$11.1\pm1.7_a{}^Y$	***	**	**		**	**	*	*
C. diadema	$23.7\pm3.2_a{}^Y$	***	***	**		***	***	**	*
C. furcellatus	$10.3\pm1.8_a{}^Y$	**	**	***		**	**	**	*
Chaetoceros resting spores	-		*	*	***	*		*	*
Other Chaetoceros	-	*	**	**	****	**	***	****	****
Thalassiosira		+	+	+	++	++	+	+	+
T. anguste-lineata	$14-78_{v}^{T}$	**				*			
T. bulbosa	$2 - 16_v^T$	**	**	**	***	*	**	**	
T. nordenskioeldii	$15.0-2.8_{v}$	**	**	**	**		*		**
T. oceanica	~3 _v	****	****	****	*	****	****	****	**
Thalassiosira spp.	-	**	**	*	****	**	*	*	****
Other centric diatoms	-	+	++	++	+++	++	+	+	++
Pennate diatoms									
Cylindrotheca closterium	$\sim 1.7^{bY}_{a}$		+	+	+	+	+	++	++
Fragilariopsis spp.	$12.5\pm7.8_a{}^Y$	++	++	++	+++	++	++	+	+
Neodenticula seminae	$21.4\pm3.7_a^{Y}$	+	+	+	++	+	+	+	++
Pseudo-nitzschia spp.	$10.3\pm1.8_a{}^Y$	+	+	++	+	+	++	++	++
Other pennate diatoms	_	+	+	+	++	+	+	+	++

Table 2. Contributions of armored protistan plankton to the total plankton in each bottle enumerated with a scanning electron microscope

Ini: Initial treatment; Control treatment; +Fe: +Fe treatment; +DFB: +DFB treatment

The contributions of each species were cell-number-based. The microscopic data of the initial bottles were modified from Table 3 of Yoshida et al. (2020).

Contributions of diatoms were indicated with the number of plus (+) symbols: +: >0–5%, ++: >5–25%, +++: >25–50%, ++++: >50%. The contributions of each *Chaetoceros* and *Thalassiosira* species were indicated with the number of asterisks (*) with the same criteria as those of diatoms described above. The contributions of diatoms were independently calculated within diatoms (i.e., excluding other planktonic species; coccolithophores, Parmales, silicoflagellates, dinoflagellates, and flagellates). a apical length; v valve diameter; Y and T cell size referenced from Yoshida et al. (2020) and Tomas (1997), respectively. ^b The approximate apical length of the diatom *Cylindrotheca closterium* that it was difficult to precisely measure due to their weak silicification and twisted body on the filter for SEM. For more details, see Table S1.