Effects of CO₂ and iron availability on phytoplankton and eubacterial community compositions in the northwest subarctic Pacific

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Abstract

On-deck CO$_2$-Fe-manipulated incubation experiments were conducted using surface seawater collected from the Western Subarctic Gyre of the NW Pacific in the summer of 2008 to elucidate the impacts of ocean acidification and Fe enrichment on the abundance and community composition of phytoplankton and eubacteria in the study area. During the incubation, excluding the initial period, the mean partial pressures of CO$_2$ in non-Fe-added bottles were 230, 419, 843, and 1124 μatm, whereas those in Fe-added treatments were 152, 394, 791, and 1008 μatm. Changes in the abundance and community composition of phytoplankton were estimated using HPLC pigment signatures with the program CHEMTAX and flow cytometry. A DGGE fingerprint technique targeting 16S rRNA gene fragments was also used to estimate changes in eubacterial phylotypes during incubation. The Fe addition induced diatom blooms, and subsequently stimulated the growth of heterotrophic bacteria such as *Roseobacter*, *Phaeobacter*, and *Alteromonas* in the post-bloom phase. In both the Fe-limited and Fe-replete treatments, concentrations of 19’-hexanoyloxyfucoxanthin, a haptophyte marker, and the cell abundance of coccolithophores decreased at higher CO$_2$ levels (750 and 1000 ppm), whereas diatoms exhibited little response to the changes in CO$_2$ availability. The abundances of *Synechococcus* and small eukaryotic phytoplankton (< 10 μm) increased at the higher CO$_2$ levels. DGGE band positions revealed that *Methylobacterium* of Alphaproteobacteria occurred solely at lower CO$_2$ levels (180 and 380 ppm) during the post-bloom phase. These results suggest that increases in CO$_2$ level could affect not only the community composition of phytoplankton but also that of eubacteria. As these microorganisms play critical roles in the biological carbon pump and microbial loop, our results indicate that the progression of ocean acidification can alter the biogeochemical processes in the study area.

Keywords:
Ocean acidification; Iron enrichment; Phytoplankton; Eubacteria; Pacific Ocean
1. Introduction

An increase in atmospheric CO$_2$ concentration due primarily to the burning of fossil fuels is causing a rapid increase in the CO$_2$ level of seawater CO$_2$ level, resulting in decreases in the carbonic ion concentration and seawater pH (i.e., ocean acidification). It is predicted that the atmospheric CO$_2$ concentration could reach more than 700 ppm by the end of the 21st century (Meehl et al., 2007), driving a decrease in sea surface water pH of 0.3–0.4 (Orr et al., 2005). Such a rapid decrease in sea surface pH has most likely not occurred for millions of years of the earth’s history (Pearson and Palmer, 2000). Hence, elucidating the responses of marine organisms to ocean acidification and predicting the feedback processes initiated by climate change are major challenges for scientists. Previous ocean acidification studies for microorganisms have primarily focused on the decrease in the carbonate ion concentration and an increase in dissolved CO$_2$ in seawater, both of which could affect the calcification and photosynthetic processes of organisms. More recently, interactions between CO$_2$ and other factors such as temperature and nutrients have been reported (e.g., Feng et al., 2009; Shi et al., 2010; Sun et al., 2011). For example, Shi et al. (2010) noted that ocean acidification could decrease Fe availability in seawater for phytoplankton.

Eukaryotic phytoplankton and cyanobacteria utilize inorganic carbon for their photosynthesis, and a significant portion of the resulting photosynthates can be exported to the ocean interior (Field et al., 1998). Thus, organic matter production and the subsequent export processes can drive CO$_2$ sequestration in the ocean, and that feeds back into the atmospheric CO$_2$ concentration and global climate change. In terms of carbon fixation metabolisms in phytoplankton cells, the catalytic efficiency of the CO$_2$-fixing enzyme Ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) can differ among algal taxa (Badger et al., 1998). Carbon concentrating mechanisms (CCMs) also vary among phytoplankton clades. For example, diatoms and cyanobacteria are considered to possess efficient and highly regulated CCMs, and therefore they may rarely be subjected to CO$_2$ limitation (Badger and Price, 2003; Rost et al., 2003; Fu et al., 2007; Trimborn et al., 2009). On the other hand, coccolithophores such as *Emiliania huxleyi* have less efficient CCMs, and their photosynthesis is far below saturation under the present CO$_2$ level of ca. 380 ppm (Rost et al., 2003). Hence, an elevated CO$_2$ concentration might drive a shift in phytoplankton community structure.
due to differences in the ability of taxa to physiologically adapt to a changing CO₂ level.

In terms of particulate inorganic carbon (PIC) deposition in the sea, calcareous organisms such as coccolithophores play an important role in the global carbon cycle by producing calcium carbonate shells or skeletons (e.g., Chikamoto et al., 2009). Recent investigation has indicated that CaCO₃ acts as ballast and increases the sinking velocity of aggregates to the ocean interior (Iversen and Ploug, 2010). As the formation of CaCO₃ in the upper layer and its dissolution in the deep ocean redistribute the total alkalinity (TA) in the water column, a CaCO₃ flux would alter the calcium carbonate compensation depth (CCD) in the water column and the rate of CaCO₃ sedimentation on the seafloor (Sarmiento and Gruber, 2006a). Hence, the CaCO₃ cycle in the ocean plays an important role in controlling the atmospheric CO₂ concentration on timescales of a few hundred years. On a shorter timescale, however, the ratio of vertical fluxes of PIC to particulate organic carbon (POC), the so-called the rain ratio, is important for regulating the atmospheric CO₂ concentration (Archer and Maier-Reimer, 1994). Recently, it has been noted that the cellular ratio of PIC to POC in coccolithophores could decrease in response to rising pCO₂ in seawater (Riebesell et al., 2000; Müller et al., 2010). Hence, changes in the calcification process and/or abundance of coccolithophores would also feed back into the atmospheric CO₂ concentration.

Previous studies have reported significant changes in primary productivity and/or algal community structure in response to increased CO₂ (e.g., Tortell et al., 2002; Hare et al., 2007). However, the effects of an elevated CO₂ level on natural phytoplankton populations have differed among experiments in terms of the magnitude and/or direction of the results; those differences are likely to be due to differences in initial algal assemblages and/or environmental conditions. Therefore, the influences of ocean acidification on natural phytoplankton communities are still unclear.

Less is known about the effects of elevated CO₂ on the heterotrophic bacterial community. Heterotrophic bacteria (eubacteria and archaea) are also recognized as important organisms in marine ecosystems and biogeochemical cycles through the microbial loop (Azam, 1998) and their carbon pump (Jiao et al., 2010). Heterotrophic bacteria efficiently generate refractory dissolved organic matter (DOM) from labile DOM (Ogawa et al., 2001), and therefore they could also contribute to oceanic carbon storage (the so-called microbial carbon pump; Jiao et al., 2010). They also compete with
phytoplankton for nutrients (Joint et al., 2002; Meseck et al., 2007). On the other hand, heterotrophic bacteria support phytoplankton production by recycling nutrients, and phytoplankton are important sources of both the particulate organic matter (POM) and DOM required for the growth of heterotrophic bacteria. For instance, Riemann et al. (2000) demonstrated that bacterial community structures varied in response to changes in algal community composition in a mesocosm. More recently, Allgaier et al. (2008) found significant changes in the bacterial community composition with CO2 levels in a mesocosm, and concluded that the community changes were possibly caused by changes in the phytoplankton community composition. At present, it is unclear whether the projected change in CO2 concentration could directly affect heterotrophic bacteria. Moreover, there is still a very poor understanding of the response of bacterial communities to elevated CO2 in the open ocean.

In this study, we conducted CO2 and Fe manipulated bottle incubation experiments using natural seawater collected from the Western Subarctic Gyre (WSG) of the northwest Pacific in the summer of 2008 as part of the Plankton Ecosystem Response to CO2 Manipulation Study (PERCOM). Previously, two in situ Fe enrichment experiments (SEEDS and SEEDS-II) were conducted in the WSG, which is known to be a high-nitrate, low-chlorophyll (HNLC) regions. The Fe additions had significant impacts on the community compositions of not only phytoplankton (Suzuki et al., 2005; 2009) but also heterotrophic bacteria (Kataoka et al., 2009). However, no study has ever reported on the effects of CO2 availability on the algal and heterotrophic bacterial communities in the WSG. Recently, Hopkinson et al. (2010) hypothesized that elevated CO2 could be beneficial to phytoplankton under Fe-limited conditions because the increased CO2 levels might reduce their cellular Fe demands. However, such knowledge is still very scarce. The aim of this study is to determine whether CO2 levels could affect the abundance and community composition of phytoplankton and/or heterotrophic bacteria with or without Fe addition in the study area.
2. Materials and Methods

2.1 Experimental setup

An on-deck incubation experiment was conducted aboard the R/V *Hakuho Maru* (JAMSTEC) in August 2008. Water samples were collected from a 10 m depth at a station (46°N, 160°E) in the WSG of the western North Pacific on 5 August (day 0) with acid-cleaned Niskin-X bottles attached to a CTD–CMS system. A total of 400 L of seawater was poured into eight 50 L polypropylene carboys through silicon tubing with a 197 µm mesh Teflon net to remove large plankton. Subsamples were taken from each carboy and poured into triplicate acid-cleaned 12 L polycarbonate bottles (total of 24 bottles) for incubation. Similarly, initial samples were also collected from each 50 L carboy. All of the sampling was conducted using a trace metal clean technique to avoid any trace metal contamination. Prior to incubation, an FeCl$_3$ solution (5 nM in final concentration) was added to 12 bottles to examine the concomitant effects of Fe and CO$_2$ enrichments on the phytoplankton populations.

Incubation was conducted on deck in temperature-controlled water circulating tanks for 14 days at an in situ temperature of 14 °C and 50% surface irradiance, which was adjusted using a neutral density screen. To control the CO$_2$ concentrations in all incubation bottles, air mixtures containing 180, 380, 750, and 1000 ppm CO$_2$, purchased from a commercial gas supply company (Nissan-Tanaka Co., Japan), were bubbled into the incubation bottles. The flow rate of the gases was set at 200 mL min$^{-1}$ for the first 24 h and thereafter maintained at 50 mL min$^{-1}$.

2.2 Carbonate chemistry

Subsamples from the incubation bottles for dissolved inorganic carbon (DIC) and total alkalinity (TA) were collected in 120 mL glass vials, and HgCl$_2$ was added as a biocide. DIC and TA concentrations were measured using coulometric titration (Johnson et al., 1985) and single point titration (Culberson et al., 1970), respectively. The precisions of both DIC and TA were within less than 0.1% from replicate determinations. The certified reference for DIC, equivalent to Dickson’s certified reference materials (Dickson et al., 2007), was obtained from General Environmental Technos CO., Ltd. The levels of $p$CO$_2$ and pH in the seawater were calculated using the CO2SYS Excel
Macro (Pierrot et al., 2006), for which the input parameters were set at as follows: the set of CO$_2$ constant of “K1 and K2 from Mehrbach et al. (1973) refitted by Dickson and Millero (1987)”, the KHSO$_4$ of “Dickson”, and the pH scale of “Total scale”.

2.3 Nutrients

Subsamples were poured into 10 mL plastic tubes and stored at −20 °C until they could be analyzed on land. Nitrate plus nitrite (NO$_3$+NO$_2$), nitrite (NO$_2$), phosphate (PO$_4$), and silicate (Si(OH)$_4$) concentrations were determined with a segmented continuous flow-analyzer (QuAAtro, Bran+Luebbe). Drawdown ratios of Si/N and P/N were calculated from changes in Si(OH)$_4$, NO$_3$+NO$_2$, and PO$_4$ concentrations between two successive sampling periods (i.e., days 0–3, 3–6, 6–9, 9–11, and 11–13 in the non-Fe-added bottles, and days 0–2, 2–4, and 4–5 in the Fe-added bottles).

2.4 PAM fluorometry

To obtain the maximum photochemical quantum efficiency ($F_v/F_m$) of photosystem II (PSII) for phytoplankton, pulse amplitude-modulated (PAM) fluorometry was used following the method of Liu et al. (2009). Triplicate samples (50 mL) were transferred to acid-cleaned amber high density polyethylene (HDPE) bottles. Then, the bottles were placed in an incubator adjusted to the surface water temperature for 30 min to open the reaction centers of PSII for phytoplankton. The water samples were transferred to a quartz cell under low light conditions and analyzed in the dark using a PAM fluorometer (WATER-PAM, Heinz Waltz GmbH) with red LEDs.

2.5 Phytoplankton biomass and community composition

2.5.1 Chlorophyll $a$

Triplicate seawater samples for size-fractioned chlorophyll $a$ (Chl-$a$) were filtered through 10 μm pore size 47 mm polycarbonate filters, followed by filtration onto 25 mm Whatman GF/F filters under a gentle vacuum (< 0.013 MPa). Pigments were extracted with $N,N$-dimethylformamide (DMF) at −20°C in the dark for 24 h (Suzuki and Ishimaru, 1990). Chl-$a$ concentrations were measured using a Turner Design fluorometer (model 10-AU) with the non-acidification method of Welschmeyer (1994).
2.5.2 HPLC pigment analysis

Triplicate water samples (300 mL per sample except for 1000 mL on day 0) were filtered onto Whatman GF/F filters (25 mm in diameter) under a gentle vacuum (< 0.013 MPa). The filters were folded once, blotted with a filter paper, and stored in liquid nitrogen or a deep freezer (–80 °C) until analysis. The frozen filter was broken into small pieces, and soaked in 3 mL DMF containing a known amount of canthaxanthin as an internal standard. Then, the samples were sonicated with a Branson SONIFIER model 250 to break the cell walls. The extract was filtered through 0.45 μm PTFE filters to remove fine particles. All procedures for the extraction were conducted under subdued light to prevent photo-degradation. The 250 μL of supernatant was mixed with 250 μL of 28 mM tetrabutylammonium acetate (TBAA) aqueous solution and analyzed using high-performance liquid chromatography (HPLC; CLASS-VP system, Shimadzu) incorporating an Agilent Eclipse XDB C₈ column (3.5 μm particle size, 4.6 × 150 mm) following the analysis method of Van Heukelem and Thomas (2001). The HPLC solvent system was as follows: solvent A was 70% methanol and 30% 28 mM TBAA aqueous solution by volume and solvent B was 100% methanol. The linear gradients used for pigment separation were as follows: 0 min 95% A/5%B, 22 min 5%A/95%B, and 22–30 min 5%A/95%B. The flow rate was set at 1.2 mL min⁻¹. The temperature of the column was set at 60 °C.

2.5.3 CHEMTAX analysis

To estimate the temporal changes in phytoplankton community structure during the incubation, the CHEMTAX program (MacKey et al., 1996) was used. Because the initial pigment/Chl-a ratios are influential factors for determining community structure, optimal initial ratios were obtained by following the method of Latasa (2007). Matrix A (Table 1) was obtained from Suzuki et al. (2002), who investigated phytoplankton community compositions in the subarctic Pacific during the summer. Matrixes B, C and D (Table 1) were also prepared in order to find the optimal pigment/Chl-a ratios. The pigment ratios of Matrixes B and C were double and half the Matrix A ratios, respectively. As for Matrix D, values of 0.75, 0.5, and 0.25 for dominant (rank in high pigment/Chl-a ratio: 1–5), secondary (rank: 6–10), and minor (rank: 11–16) pigments,
respectively, were multiplied by each pigment ratio of Matrix A.

The variables required by CHEMTAX for the calculations for day 0 and non-Fe-added samples were set as follows; ratio limit = 500, weighting was ‘bounded relative error by pigment’, iteration limit = 5000, epsilon limit = 0.0001, initial step size = 25, step ratio = 2, cutoff step = 30000, subiterations = 1, and weight bound = 5. As for Fe-added samples, the ratio limit values of fucoxanthin (Fucox) and Chl-a were set to 10 and 100, respectively. We averaged the successive convergent ratios after the 10 runs among the 4 matrices to obtain the most promising initial pigment ratios. The final ratios obtained by CHEMTAX are shown in Table 2.

2.5.4 Light microscopy

Seawater samples collected on days 0 and 5 were preserved with neutral buffered formalin (2% in final concentration). The samples were concentrated by precipitation and analyzed with an upright microscope (BX50, Olympus). Identifications of phytoplankton were made following Cupp (1943) and Thomas (1997).

2.6 Cell densities of ultraphytoplankton and heterotrophic bacteria

To estimate the ultraphytoplankton (eukaryotic ultraphytoplankton and Synechococcus spp.) and heterotrophic bacterial cell densities, flow cytometry was used. Duplicate samples (2 mL) were preserved with paraformaldehyde (0.2% final concentration) and stored in liquid nitrogen or a deep freezer at -80 °C until analysis. Ultraphytoplankton and heterotrophic bacteria counts were performed with an EPICS flow cytometer (XL ADC system, Beckman Coulter) equipped with a 15 mW air-cooled laser exciting at 488 nm. The standard filter setup was used to enumerate ultraphytoplankton (< 10 μm in size) and heterotrophic bacteria. Prior to analysis, samples were thawed and then filtered through a 35 μm nylon-mesh-capped Falcon cell strainer (Becton-Dickinson) to remove larger cells. For the enumeration of heterotrophic bacteria, cells were stained with the nucleic acid stain SYBR Gold (Invitrogen) and incubated in the dark at room temperature (25 °C) for 30 min before analysis. Each sample was analyzed with EXPO32 software (Beckman Coulter). The details of the procedure are described in Suzuki et al. (2005).
2.7 Bacterial community composition

2.7.1 DNA extraction

Seawater samples were filtered onto 0.2 μm pore size polycarbonate Nuclepore filters (Whatman) with gentle vacuum pressure (< 0.013 MPa). Then, the filters were stored in liquid nitrogen or a deep freezer at -80°C until analysis. DNA extraction was performed following the method of Short and Zehr (2005). Briefly, 600 μL of XS buffer (1% potassium ethyl xanthogenate; 100 mM Tris HCl, pH 7.4; 1 mM EDTA, pH 8.0; 1% sodium dodecylsulfate; 800 mM ammonium acetate) and ca. 0.2 g of 0.1 mm glass beads were added to the vials containing the filters. The vials are agitated at 4,800 rpm for 50 s three times by a Biospec bead beater (Mini-Beadbeater) at intervals of 5 min. Then, samples were incubated at 70 °C for 60 min. Following incubation, the liquid phase was transferred to a 1.5 mL centrifuge tube, vortexed for 10 s, and placed on ice for 30 min. Cell debris was precipitated by centrifugation at 10,000 g for 12 min and the supernatant was transferred into 1.5 mL tube containing 600 μL of isopropanol and incubated at room temperature for 10 min, then centrifuged at 10,000 g for 16 min. After the liquid phase was removed, the DNA pellets were washed with 600 μL of 70% ethanol, dried in a vacuum, and resuspended in 100 μL of Tris-EDTA (TE) buffer. The concentration of DNA in extracts was quantified using a Thermo NanoDrop spectrophotometer (ND-1000) and adjusted to 0.01 ng μL⁻¹ in TE buffer for PCR.

2.7.2 PCR for DGGE

PCR amplification and denaturing gradient gel electrophoresis (DGGE) were generally performed following the method of Kataoka et al. (2009). Briefly, the 550 bp fragment of the 16S rRNA gene was amplified using a universal primer complementary to positions 907–926 Escherichia coli numbering; (5’-CCGTCAATTCMTTTGAGTTT-3’) and complementary to positions 341–357 plus a GC clamp (5’-CGCCCCGCCCAGGGCCCGTGCCGCCGCCGGCCCCGCTACGGGA GGCAG-3’). Triplicate PCR products were mixed to avoid PCR bias, and the concentrations of amplicons were estimated by agarose gel electrophoresis using molecular weight markers.

PCR products (ca. 300 ng of DNA) were loaded on 16 × 16 cm², 1 mm thick,
6% polyacrylamide (acrylamide:bis = 37.5:1) containing a denaturing gradient of 30–60% from top to bottom (7 M urea and 40% formamide were considered 100% denaturant). Electrophoresis was performed with a hot bath DGGE unit (Bio-Rad Laboratories) using 1 × TAE running buffer (20 mM Tris, 10 mM acetic acid, 0.5 mM EDTA, pH 8.0) at 60 °C for 16 h at 85 V. The gel was stained for 30 min in 1/10,000 SYBR Gold (Invitrogen) and destained for 10 min in distilled water. The DGGE gel was visualized using an Invitrogen Safe Imager and photographed; a high-resolution image was stored.

2.7.3 Analysis of DGGE patterns

DGGE images were analyzed with the one-dimensional gel analysis software Gel-Pro Analyzer (Media Cybernetics). To detect DGGE bands, a density profile was obtained to estimate the ratio of each band’s intensity to the total. Bands with relative intensities of ≥1.0% were examined for further phylogenetic analysis. The Jaccard coefficient was calculated for the cluster analysis of DGGE profiles, and the distance matrix was analyzed using Ward’s method. Statistical calculations were performed in the software program R (version 2.10.1).

2.7.4 Sequencing and Phylogenetic analysis

To obtain sequence information from the DGGE bands, all visualized band positions were excised using a clean razor blade. The cut bands were then washed twice using 2 mL of sterilized Milli-Q water and frozen until re-amplification with PCR. Approximately 1 mm³ of each band was directly re-amplified. PCR conditions and purification of PCR products followed Kataoka et al. (2009). The purified amplified products were sequenced on an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems) with both the 907–926 and 341–357 primers without the GC-clamp (Kataoka et al., 2009) using a Big Dye v1.0 Terminator cycle sequencing kit (Applied Biosystems). Then, 16S rRNA gene sequences were analyzed for each sample. Obtained sequences were compared to known sequences using a basic local alignment search tool, BLAST (http://www.ddbj.nig.ac.jp/search/blast-j.html), and examined for chimeras. Non-chimeric sequences were assigned to each phylogenetic group based on the most similar sequence in the DNA Data Bank of Japan (DDBJ). Bacterial sequences were
aligned using CLUSTAL W and corrected manually to delete ambiguous base positions
with MEGA 4 (Tamura et al., 2007). A distance tree was constructed using the
neighbor-joining (NJ) method based on the Jukes and Cantor (1969) distance model
with a bootstrap test based on 1,000 resamplings.

2.7.5 Sequence deposition

The 16S rRNA gene sequences of the DGGE bands obtained in the present
study were deposited in the DDBJ database, and the GenBank accession numbers were
assigned from AB714947 to AB714964.

2.8 Statistical analysis

Differences in nutrient concentrations among CO₂ treatments were evaluated
with a one-way analysis of variance (ANOVA). Tukey’s post-hoc test was also used to
identify the source of the variance. To assess the statistically significant differences in
the biological data (F₉/F₈, Chl-α, increased ratio of phytoplankton pigments (final
day/day 0), contributions of each phytoplankton group to Chl-α, and cell abundance)
among CO₂ treatments, non-parametric Kruskal-Wallis and subsequent Steel-Dwass
tests were used. The confidence level for all analyses was set at 95% (p < 0.05).
3. Results

3.1 Temporal changes in carbonate chemistry and nutrients

The levels of TA and DIC in the initial seawater were 2199.91 ± 3.36 and 2032.96 ± 8.13 μmol kg⁻¹ (mean ± 1 standard deviation), respectively. The variations in TA among all treatments were within ± 22.90 μmol kg⁻¹ throughout the incubations (Figs. 1A and 1B). The aeration with four different concentrations of CO₂-enriched air succeeded in creating significant gradients in DIC (Figs. 1C and 1D) and pH levels (Figs. 1E and 1F). However, the DIC and pH values in the Fe-enriched bottles were relatively variable compared to those in the non-Fe-added bottles. Because the TA and DIC data were obtained from a single bottle in each treatment, the significant differences for these values could not be evaluated. The calculated pCO₂ value of the initial seawater was 467.45 ± 3.02 μatm. In the non-Fe-added bottles, between days 3 and 13, the pCO₂ values of the 180, 380, 750, and 1000 ppm CO₂ treatments were calculated as 229.62 ± 46.27, 418.73 ± 26.97, 843.45 ± 35.58, and 1123.59 ± 59.53 μatm, respectively. On the other hand, in the Fe-added bottles between days 2 and 10, the pCO₂ values of 180, 380, 750, and 1000 ppm CO₂ treatments were calculated as 152.14 ± 68.54, 393.95 ± 126.54, 791.25 ± 145.08, and 1008.19 ± 229.93 μatm, respectively.

The initial concentrations of NO₃+NO₂, PO₄ and Si(OH)₄ were 13.41 ± 0.26, 1.21 ± 0.25, and 13.41 ± 0.03 μmol L⁻¹, respectively (Figs. 2A–F). In the non-Fe-added bottles, these nutrients gradually decreased over time, and no significant differences in nutrient concentration among the CO₂ treatments were detected. On the other hand, in the Fe-added bottles, macronutrient concentrations decreased rapidly until day 5, and thereafter NO₃+NO₂ and PO₄ levels remained low, whereas Si(OH)₄ concentrations increased after day 10. The decreases in the nutrient concentrations in the Fe-added treatment were not significantly different among the CO₂ treatments throughout the experiment except on day 4, when the NO₃+NO₂ and PO₄ concentrations with the lower CO₂ levels (180 and 380 ppm) were lower than those with higher CO₂ levels (750 and 1000 ppm) (ANOVA, p < 0.05, Tukey’s post-hoc test, p < 0.05).

In the non-Fe-added bottles, the Si/N drawdown ratio ranged from 0.66 to 2.14 with an average value of 1.46 ± 0.49 (Fig. 3A). The values reached maxima between...
days 3 and 6 for the 750 ppm CO₂ level and between days 6 and 9 for the 180, 380, and 1000 ppm CO₂ levels. The mean N/P drawdown ratio in the non-Fe-added bottle was 13.0 ± 5.1 between days 0 and 11 and increased to 32.5 ± 20.5 between days 11 and 14 (Fig. 3B). In the non-Fe-added bottles, no significant difference in the Si/N and N/P drawdown ratios on each sampling day was detected among CO₂ levels (ANOVA, p > 0.05).

In the Fe-added bottles, the mean Si/N drawdown ratio was 0.60 ± 0.23 between days 0 and 4 and increased to 2.86 ± 1.04 between days 4 and 5 (Fig. 3C). In addition, during days 4 and 5, the values of the Si/N drawdown ratio were significantly higher in the low CO₂ level treatments (180 and 380 ppm) than at higher CO₂ levels (750 and 1000 ppm) (ANOVA, p < 0.05, Tukey’s post-hoc test, p < 0.05). The mean N/P drawdown ratio in the Fe-added bottles was 13.1 ± 5.1, and no significant difference was observed among CO₂ levels throughout the experiment (Fig. 3D; ANOVA, p > 0.05).

3.2 Chl-α and \( F_v/F_m \)

The initial Chl-α concentration was 0.34 ± 0.06 µg L⁻¹, composed of 0.29 ± 0.03 µg L⁻¹ from small-sized (< 10 µm) and 0.05 ± 0.06 µgL⁻¹ from large-sized (≥10 µm) cells. In the non-Fe-added bottles, total Chl-α concentrations gradually increased toward the end of the experiment and reached maxima of between 2.61–3.41 µg L⁻¹ in each CO₂ treatment (Fig. 4A). In the Fe-added bottles, total Chl-α concentrations increased exponentially and showed maxima between 16.4–18.7 µg L⁻¹ for each CO₂ treatment on day 5 (Fig. 4B). Most of these increases on day 5 were attributed to large-sized (≥10 µm) cells (Fig. 4D). No significant differences in Chl-α concentrations among the CO₂ treatments were found in the non-Fe-added or the Fe-added bottles (Figs. 4A–F).

\( F_v/F_m \) of algal PSII on day 0 was 0.25 ± 0.04. In the non-Fe-added treatments, \( F_v/F_m \) values remained low (0.25–0.31) throughout the experiment (Fig. 5A). In the Fe-added bottles, in contrast, \( F_v/F_m \) values increased until day 5, when the maxima were 0.65 ± 0.02 (Fig. 5B). With respect to CO₂ treatment, no significant differences in \( F_v/F_m \) levels were observed.
3.3 Phytoplankton community composition

3.3.1 Phytoplankton pigments

The carotenoids Fucox, generally a biomarker for diatoms (Ondrusek, et al., 1991) in oceanic waters, and 19’-hexanoyloxyfucoxanthin (19’-Hex), a chemotaxonomic maker for haptophytes (Jeffrey and Wright, 1994), demonstrated taxon-specific responses to Fe and CO$_2$ additions. In the non-Fe-added bottles, concentrations of Fucox increased more than 23-fold by the end of the experiment (Fig. 6A). As for 19’-Hex in the non-Fe-added bottles, the pigment concentration changed little compared to Fucox (Fig. 6B). No significant difference in the ratios of Fucox and 19’-Hex concentration on the final day relative to day 0 among the CO$_2$ treatments was found in the non-Fe-added bottles (Kruskal-Wallis ANOVA, $p > 0.05$). In the Fe-added bottles, the ratios of Fucox concentration between the final and initial days were 2–3 times higher than those of the non-Fe-added bottles (Fig. 6A). In addition, the ratios of 19’-Hex concentration between the final and initial days in the Fe-added bottles differed significantly among the CO$_2$ levels (Fig. 6B; Kruskal-Wallis ANOVA, $p < 0.05$). Compared to the non-Fe-added treatments, the changes in the ratio of Fucox levels were significantly higher in the Fe-added bottles across all CO$_2$ treatments (Kruskal-Wallis ANOVA, $p < 0.05$). On the other hand, the ratios of 19’-Hex concentration between the final and initial sampling days were significantly higher in the non-Fe-added bottles except for the 180 ppm CO$_2$ treatment (Kruskal-Wallis ANOVA, $p < 0.05$).

3.3.2 CHEMTAX outputs

All of the calculated pigment ratios (Table 2) were within the range of values reported by Mackey et al. (1996), Wright and van den Enden (2000), and Suzuki et al. (2002). The rates for pigments other than Fucox, 19’-Hex, Diadinox, Chl-b and Zeax in the non-Fe-added bottles and Fucox, Diadinox, Violax, Chl-b, Zeax and Peri in the Fe-added bottles were not significantly changed from their initial ratios by CHEMTAX.

The initial phytoplankton community was dominated primarily by cyanobacteria and cryptophytes (Figs. 7A and 7B; contributions of 45% and 27% to the Chl-a concentration, respectively). Prasinophytes, pelagophytes and haptophytes were also important phytoplankton groups (contributions of 7%, 11% and 11% to the Chl-a concentration, respectively). On the other hand, diatoms initially contributed < 2% of
the Chl-a biomass in the phytoplankton assemblage. In the non-Fe-added bottles, the contributions of diatoms to the Chl-a biomass increased over time, and they became predominant in the phytoplankton assemblages by the end of experiment, contributing 75–79% of Chl-a. In the Fe-added bottles, on the other hand, phytoplankton community structures changed more rapidly in terms of increases in the relative abundance of diatoms. Contributions of chlorophytes also increased over time and they became a secondary dominant group after day 6 in the non-Fe-added and after day 5 in the Fe-added treatments. On the other hand, the contributions of cyanobacteria decreased after days 6 and 5 (< 13% of total Chl-a) in the non-Fe-added and Fe-added treatments, respectively. Haptophytes were also of relatively low relative abundance (< 5%) after day 5 or 6. However, their contribution to Chl-a biomass varied significantly among CO2 levels (Kruskal-Wallis ANOVA, p < 0.05) and was higher at the 180 ppm CO2 level than at 1000 ppm (Steel-Dwass test, p < 0.05) in both the Fe-added and non-Fe-added treatments. No significant differences were found among the CO2 levels for the relative contributions of the other phytoplankton groups to Chl-a biomass (Kruskal-Wallis ANOVA, p > 0.05).

3.3.3 Light microscopy

The initial major phytoplankton groups determined by microscopy were haptophytes, cryptophytes, and diatoms (153, 27, and 26 cells mL\(^{-1}\), respectively). Among the haptophytes, Prymnesiales were predominant on day 0 (84% of cell density), although their abundances declined in all Fe and CO2 treatments on day 5. On the other hand, coccolithophores became the main group among the haptophyte assemblages in both the non-Fe-added and Fe-added treatments on day 5, except at the high CO2 treatments (750 and 1000 ppm) in which coccolithophore abundances were very low (Table 3). Diatoms were the most abundant group among the phytoplankton assemblages in terms of cell density in both the non-Fe-added and Fe-added bottles on day 5. Among the diatom assemblages, the large pennate *Pseudo-nitzschia* spp. (> 30 μm in size) cells were predominant in all bottles. In addition, chrysophytes such as *Dinobryon* spp. were abundant (144–562 cells mL\(^{-1}\)) in the non-Fe-added and Fe-added bottles on day 5. The ratio of diatoms to coccolithophores (D/C) was 1.1 on day 0, and the values increased in all bottles on day 5 (Table 3). Moreover, the ratios increased
with CO₂ concentration on day 5; that was primarily due to the decrease in the cell densities of coccolithophores.

3.4 Ultraphytoplankton abundance

The cell density of eukaryotic ultraphytoplankton (< 10 μm) was (1.7 ± 0.2) × 10⁴ cells mL⁻¹ on day 0. In the non-Fe-added bottles, the abundance of eukaryotic ultraphytoplankton increased over time and reached a maximum (5.7–8.9 × 10⁴ cells mL⁻¹) in the final days of the experiment in all CO₂ treatments (Fig. 8A). After day 10, the differences in the cell densities of eukaryotic ultraphytoplankton in the non-Fe-added bottles were significant among CO₂ treatments (Kruskal-Wallis ANOVA, \( p < 0.05 \)). Those were confirmed by subsequent multiple comparisons among the 180, 380 and 1000 ppm CO₂ treatments (Steel-Dwass test 180 or 380 ppm < 1000 ppm, \( p < 0.05 \)). In the Fe-added bottles, the cell densities of eukaryotic ultraphytoplankton (Fig. 8B) reached maxima (9.7–12.7 × 10⁴ cells mL⁻¹) on day 5 and then slightly decreased on day 10 (7.7–9.1 × 10⁴ cells mL⁻¹). Significant differences in the eukaryotic ultraphytoplankton among CO₂ levels were also found in the Fe-added bottles on day 5 (Kruskal-Wallis ANOVA, \( p < 0.05 \)) and in the subsequent multiple comparisons between 380 and 1000 ppm CO₂ (Steel-Dwass test 380 ppm < 1000 ppm, \( p < 0.05 \)).

The initial abundance of *Synechococcus* was (8.2 ± 0.8) × 10⁴ cells mL⁻¹. On the final sampling day, its cell density dropped to ca. 1.0 × 10⁴ cells mL⁻¹ in the non-Fe-added bottles and to 0.5 × 10⁴ cells mL⁻¹ in the Fe-added bottles (Figs. 8C and 8D). The abundance of *Synechococcus* in the non-Fe-added bottles differed significantly among CO₂ levels on day 6 (Kruskal-Wallis ANOVA, \( p < 0.05 \)) and was lower at 380 ppm than at the other concentrations (Steel-Dwass test, \( p < 0.05 \)). Significant differences in *Synechococcus* abundance were also detected in the Fe-added bottles on day 5 (Kruskal-Wallis ANOVA, \( p < 0.05 \)) and the abundance at 380 ppm was lower than at 750 and 1000 ppm CO₂ levels (Steel-Dwass test, \( p < 0.05 \)).

3.5 Heterotrophic bacteria

3.5.1 Cell abundance

The abundance of heterotrophic bacteria was (3.4 ± 0.2) × 10⁵ cells mL⁻¹ on day 0. In the non-Fe-added bottles, the cell densities ranged between 1.2 and 2.8 × 10⁵.
cells mL⁻¹ throughout the experiment (Fig. 8E). Meanwhile, bacterial abundances increased over time in the Fe-added bottles and reached maxima of \((1.5 \pm 0.2) \times 10^6\) cells mL⁻¹ on day 10 (Fig. 8F). In both the Fe-limited and Fe-replete conditions, significant differences in bacterial abundance among CO₂ levels were not detected by the Kruskal-Wallis ANOVA \((p > 0.05)\) on each sampling day.

3.5.2 Phylogenetic analysis of 16S rRNA gene fragments

Forty-nine unique band positions were detected in our DGGE, and the number of bands in each lane ranged from 10 to 34 except day 0 in the non-Fe-added treatment (Fig. 9). At the beginning of the experiment (i.e., day 0), 21 bands were detected, and 24–34 bands were detected among the CO₂ treatments on day 10 in the Fe-added bottles. On the other hand, only 10–18 bands were detected in the non-Fe-added bottles on day 11. The cluster analysis of DGGE profiles distinguished the bacterial community structures in the non-Fe-added bottles from those in Fe-added conditions, except for the 180 ppm CO₂ treatment in the non-Fe-added bottles (Fig. 10). In the Fe-added treatment, the DGGE profile on day 5 was clearly separated from those on day 10.

We succeeded in identifying 18 unique sequences out of the 49 band positions (1–18 in Fig. 9). Based on the band positions identified (see Fig. 9), the sequences obtained were named “PE08-xx” (Table 4), in which “xx” corresponded to the band positions (see Fig. 9). Except for PE08-02 and PE08-08, all sequences identified were more than 98% similar to the sequences that had been deposited in the DDBJ (Table 4).

In the NJ tree (Fig. 11), 17 eubacteria derived from the sequences were clustered into six groups: Alphaproteobacteria (7 Rhodobacteraeae and 2 Methylobacteriaceae), Gammaproteobacteria, Actinobacteria, Bacteroidetes, cyanobacteria and others. A plastid group was also clustered. Seven sequences of Rhodobacteraeae consisted of 4 Roseobacter (PE08-06, PE08-09, PE08-10 and PE08-11), 1 Ruegeria (PE08-07), 1 Sulfitobacter (PE08-08), and 1 Phaeobacter (PE08-12). The sequence PE08-02 was assigned to others. In the Fe-added bottles, PE08-12 and PE08-13 were unique, and PE08-09, PE08-10, and PE08-11 became intense on day 10. The band positions of PE08-14 and PE08-16, which clustered into Methylobacterium, were detected only in lower CO₂ bottles (180 and 380 ppm) in the Fe-added treatment on day 10.
4. Discussion

4.1 Carbonate chemistry and nutrient dynamics

We successfully created significant gradients in DIC and pH without any artificial change in TA among the four CO$_2$ treatments by bubbling CO$_2$ mixed air into the incubation seawater (Fig. 1). Although DIC and pH were stabilized in the non-Fe added bottles throughout the incubation period, the levels of these CO$_2$ parameters in the Fe-added bottles departed from our intended values after day 5. The latter was most likely due to the abrupt drawdown of DIC by algal photosynthesis during the bloom. Phytoplankton blooms formed in response to the Fe infusion, indicating that the growth of phytoplankton assemblages in this area was limited by the availability of Fe. This view is also supported by previous in situ Fe enrichment experiments in the WSG of the Pacific Ocean in summer (Tsuda et al., 2005; 2007).

Macronutrient assimilations by phytoplankton were promoted by the addition of Fe, especially between days 2 and 5 along with the abrupt increases in the Chl-$a$ concentration (Figs. 2B, 2D, and 2F). Our CHEMTAX estimates indicated that diatoms were the principal contributor to the Chl-$a$ increases (Fig. 7B). Therefore, increases in Si/N drawdown in the Fe-added bottles between days 4 and 5 were primarily attributable to diatoms. According to Sarthou et al. (2005), the Si/N elemental ratios in diatoms average 1.0 ± 1.0, although the ratios are highly variable according to species, ranging from 0.17 to 7.24. Our results in the Fe-added bottles during the bloom were higher than the average value reported by Sarthou et al. (2005). The Si/N drawdown ratios between days 0 and 5 in the non-Fe-added bottles were significantly higher than those in the Fe-added bottles between days 0 and 4 (ANOVA, $p < 0.05$). Our results were consistent with Takeda (1998), who demonstrated decreases in Si/N consumption ratios in response to Fe addition in the subarctic North Pacific and other HNLC areas. Takeda (1998) interpreted that these results were caused by the preferential drawdown of Si(OH)$_4$ compared to NO$_3$ for diatoms in Fe-deficient conditions. In our study, nutrient drawdown ratios might be controlled not only by shift in community composition but also by physiological adaptation. Interestingly, Si/N drawdown ratios showed higher values in the low-CO$_2$ bottles relative to high-CO$_2$ between days 4 and 5 (Fig. 3B). This result is consistent with Sun et al. (2011), who demonstrated increases in
the Si/C ratio without a significant change in the C/N ratio in the diatom
*Pseudo-nitzschia multiseries* with increased pCO$_2$. A decrease in Si quotas in response
to elevated CO$_2$ levels was also demonstrated in the diatom *Thalassiosira weissflogii*
(Milligan et al., 2004). They indicated that the lower Si quota was most likely
concomitant with an increase in the efflux and dissolution of Si into seawater. Moreover,
this might be associated with the decrease in carbonic anhydrase (CA) activity:
extracellular CA formation requires a pH buffer, and diatoms’ silica frustules can
become one of the principal buffers for CA (Milligan and Morel, 2002). Milligan et al.
(2004) presumed that diatoms control the Si efflux by regulating silicon metabolism.

4.2 Phytoplankton abundance and community structure

The ratio of 19'-Hex concentration between the initial and final days showed
decreased trend in response to elevated CO$_2$ in the Fe-added bottles (Fig. 6B). In
addition, our microscopic data also showed a decrease in the abundance of
coccolithophores and an increase in the D/C ratio with increases in CO$_2$ concentration
on day 5 (Table 3). These results indicate that coccolithophores were sensitive to
increases in the CO$_2$ concentration in this area. Decreases in the relative abundance of
coccolithophores might reduce the ratio of CaCO$_3$ to organic carbon (i.e., rain ratio) in
settling particles. The precipitation of 1 mol CaCO$_3$ can reduce TA by 2 mol and
produce 1 mol CO$_2$ (Zeebe and Wolf-Gladrow, 2001). Hence, a decrease in calcification
in seawater would affect the sequestration of atmospheric CO$_2$ (i.e., carbonate pump;
Sarmiento and Gruber, 2006b). On the other hand, diatoms form silica frustules and
their aggregates can also contribute to the vertical transportation of organic materials
(Smetacek, 2000). In the summer, diatoms in the WSG of the NW Pacific are more
abundant than in the Alaskan Gyre of the northeast Pacific (Suzuki et al., 2002), and it is
reported that the rain ratio in the WSG is relatively low (ca. 0.25; Tsunogai and Noriki,
1991). Therefore, our results suggest that a rise in the CO$_2$ concentration might further
reduce the rain ratio by decreasing the biomass of coccolithophores. In addition, several
studies have shown that the abundance of haptophytes estimated by 19'-Hex and/or cell
densities decreases with increasing CO$_2$ levels in natural phytoplankton assemblages
(Table 5). However, it should be noted that a decrease in 19'-Hex and/or
coccolithophore abundance does not always reflect a decrease in CaCO$_3$ production. For
example, Feng et al. (2009) reported that PIC production decreased in response to simultaneous increases in CO$_2$ level and temperature, although 19’-Hex concentrations were insensitive to those during a shipboard incubation experiment using a natural phytoplankton community in the North Atlantic. In addition, coccolithophore abundance increased in the high CO$_2$ and temperature treatment in their experiment. Further studies are needed for a better understanding of the response of PIC production to changes in CO$_2$ level in the WSG.

Previous studies have shown both positive and negative impacts on diatom abundance with increasing CO$_2$ (Table 5). In this study, diatoms were insensitive to CO$_2$ levels under both non-Fe-added and Fe-added conditions as estimated from Fucox concentration (Figs. 6 and 7). Light microscopy indicated that diatom assemblages primarily consisted of large pennate *Pseudo-nitzschia* spp. in both the non-Fe-added and Fe-added bottles. Based on a theoretical model of reaction-diffusion CO$_2$ supply for algal carbon fixation, larger phytoplankton cells are more sensitive to CO$_2$(aq) concentration in seawater (Reinfelder, 2010), whereas some diatoms have highly efficient CCMs (Badger et al., 1998; Roberts et al., 2007) to overcome the low catalytic efficiency of RuBisCO.

Remarkable increases in both the Chl-a concentration for > 10 μm cells and the relative abundance of diatoms were observed in the Fe-added bottles on day 5 (Figs. 4D and 7B). In addition, $F_v/F_m$ values also clearly increased after the Fe addition (Fig. 5B). These results indicate that the growth of the initial phytoplankton, especially diatoms, was limited by Fe availability. Significant increases in Chl-a concentrations and/or the relative abundance of diatoms among the total phytoplankton were also observed during an in situ Fe fertilization experiment in the WSG (Suzuki et al., 2005). On the other hand, we found that CO$_2$ availability did not affect the $F_v/F_m$ values in either the non-Fe-added or Fe-added bottles (Fig.5), suggesting that the activity of PSII for the phytoplankton assemblages was influenced little by the CO$_2$ level of seawater. Shi et al. (2010) reported that an increase in CO$_2$ level could down-regulate Fe availability for phytoplankton. In our study, however, the effects of a rise in CO$_2$ levels on $F_v/F_m$ and Chl-a concentration were unclear in the non-Fe-added bottles (Figs. 5A and 4A).

Comparing the changes in the pigment ratios between non-Fe-added and Fe-added treatments (Fig. 6), Fucox showed higher values in the Fe-added bottles,
whereas the ratios of 19'-Hex became higher in the non-Fe-added bottles. The results show that Fe addition induced the rapid growth of diatoms, and the haptophytes were ultimately outcompeted. This view can be supported by their different traits for succession: diatoms tend to dominate in nutrient-replete conditions where they can grow faster (i.e., r-strategy), whereas coccolithophores have high affinities for resources and they tend to be a major group in rather low-nutrient conditions (i.e., K-strategy) (Margalef, 1978).

The effects of CO₂ level on the cell abundance of eukaryotic ultraphytoplankton were distinctive when cell abundance was highest in the non-Fe-added and Fe-added bottles (Figs. 8A and 8B). Higher CO₂ levels induced relatively high abundances of eukaryotic ultraphytoplankton. Rost et al. (2003) reported that CO₂ availability might become a limiting factor for algal growth during blooms. Similar trends were also reported by Engel et al. (2008), who conducted a CO₂-manipulated mesocosm experiment in Raunefjord, Norway (PeECE II) and demonstrated that small-sized phytoplankton were more sensitive to CO₂ elevation and the CO₂ effects were more obvious during the bloom phase. Paulino et al. (2008) also showed similar trends in another mesocosm experiment (PeECE III). These results, including ours, suggest that ocean acidification might increase the abundance of small-sized eukaryotic phytoplankton, especially in their growth phases.

*Synechococcus* abundance was higher at high CO₂ levels (750 and 1000 ppm) than at the present atmospheric CO₂ level (380 ppm) on day 6 in the non-Fe-added bottles and on day 5 in the Fe-added bottles (Figs. 8C and 8D). This may be partly attributable to their properties of inorganic carbon acquisition. From the standpoint of CO₂ assimilation, it is known that *Synechococcus* species have rather inefficient RuBisCO but extremely high CCM activity (Badger et al., 1998; 2006). Accordingly, they might use a relatively high energy to take up inorganic carbon (Raven, 1991). Ocean acidification could be advantageous for cyanobacteria by reducing the energetic cost for CCMs. According to Fu et al. (2007), the growth rates of *Synechococcus* became higher at elevated CO₂ concentrations during a laboratory culture experiment. However, the opposite trend was observed in a mesocosm experiment where the cell densities of *Synechococcus* were higher in the 350 μatm CO₂ than in the 700 and 1050 μatm CO₂ levels (Paulino et al., 2008). In the experiment of Paulino et al. (2008),
Synechococcus abundance began to increase and showed clear CO₂ effects after day 16 when nutrients were almost depleted. Therefore, one possible explanation for the discrepancy between our results or those of Fu et al. (2007) and Paulino et al. (2008) is the difference in nutrient availability for the growth of Synechococcus.

4.3 Heterotrophic bacterial abundance and community structure

The initial abundance of heterotrophic bacteria was roughly equal that reported by previous studies conducted in the North Pacific during the summer (Kataoka et al., 2009; Suzuki et al., 2005; Taniguchi and Hamasaki, 2008). The growth of heterotrophic bacteria in this study was stimulated by Fe addition (Figs. 8E and 8F), but their maximum abundance was far above the values measured in previous in situ Fe enrichment experiments (8 × 10⁵ and 4 × 10⁵ cells mL⁻¹ during SEEDS and SEEDS-II, respectively) in the WSG (Suzuki et al., 2005; Kataoka et al., 2009). Because heterotrophic bacteria have a higher Fe/C ratio for growth than phytoplankton (Maldonado and Price, 1999), Fe availability could limit the growth of heterotrophic bacteria in HNLC regions. In addition, drastic increases in heterotrophic bacterial abundance in the Fe-added bottles could have been induced by an increase in DOM released from the phytoplankton assemblages.

Bacterial community composition also differed between the non-Fe-added and Fe-added bottles (Fig. 10), indicating that Fe enrichment had a significant impact on bacterial community structure. The unique DGGE bands that occurred in the Fe-added bottles on day 10 were derived from Phaeobacter sp., Alteromonas sp., and Roseobacter spp. The Phaeobacter clade is closely related to the Roseobacter clade within the Alphaproteobacteria (Gupta, 2005). Alphaproteobacteria are thought to often dominate the bacterioplankton community in marine environments and to play a key role in the microbial loop (Yokokawa and Nagata, 2010). The predominance of Alphaproteobacteria is also sometimes associated with phytoplankton blooms (e.g., Riemann et al., 2000; Grossart et al., 2005). Kataoka et al. (2009) reported that Alphaproteobacteria including Roseobacter appeared in the Fe-enriched patch during SEEDS-II. The phylotypes detected in the Fe-enriched seawater during SEEDS-II are closely related to those in our experiment (Fig. 11). The genus Alteromonas, a member of the Gammaproteobacteria, and their close relatives are known to have a rapid
potential growth rate and geographical diversity (e.g., García-Martínez et al., 2002). Alteromonas is known to possess chitinases, which catalyze the degradation of chitin, a major source of carbon in the ocean (Orikoshi et al., 2005). Because chitin occurs in the extracellular fibers of diatoms (Souza et al., 2011), the presence of Alteromonas sp. in the post diatom bloom phase in this study might be due to the preferential use of chitin derived from the diatoms.

In our DGGE analysis, PE08-14 and PE08-16, classified into Methylobacterium in Alphaproteobacteria, appeared only at low CO$_2$ levels in the Fe-added bottles. It is known that Methylobacterium can use reduced carbon compounds such as methane, methanolamines, and methylated sulfur compounds for their growth (i.e., methylotroph; Giovannoni and Rappé, 2000). It is also reported that many forms of single-carbon (C$_1$) compounds are found in the marine environment, and the growth of marine methylotrophs including Methylobacterium can regulated by the availability of C$_1$ compounds (Neufeld et al., 2007). Hence, one possible explanation for the disappearance of Methylobacterium from elevated CO$_2$ level treatments in our study is the changes in DOM composition induced by the phytoplankton assemblages. Yoshimura et al. (2010) demonstrated a decrease in DOC accumulation in response to increased CO$_2$ level, although minor effects of CO$_2$ enrichment on DOM concentration have also been reported (Engel et al., 2004; Schulz et al., 2008).

Another important aspect of ocean acidification for the growth of marine bacteria is related to change in the activity of enzymes such as glucosidase and protease. In general, bacterial enzymatic activity depends on cellular or ambient pH, and plays an important role in the degradation of organic matter in the ocean (Grossart et al., 2006; Yamada and Suzumura, 2010). For example, Witt et al. (2011) reported that increased $p$CO$_2$ could change bacterial community composition in biofilms from the Great Barrier Reef and that this was possibly due to changes in enzymatic activities. Although we did not estimate the enzymatic activity of bacteria in this study, further study is needed for a better understanding of the effects of ocean acidification on bacterial assemblages in the WSG, because the annual POC flux and export ratio ($e$-ratio) are much higher in the study area than in other oceans (Kawakami and Honda, 2007) due to the higher production and transport of carbon by the diatoms that proliferate in the WSG every spring (Imai et al., 2002).
5. Conclusion

The present study examined, for the first time, changes in the community composition of phytoplankton and eubacteria in response to increased CO$_2$ and Fe levels in the WSG of the northwest Pacific. The Fe infusion induced diatom blooms, demonstrating that those algal cells were limited by low Fe availability in seawater. Although the diatoms were not influenced by elevated CO$_2$ levels, some phytoplankton groups such as haptophytes and Synechococcus were sensitive to an increase in CO$_2$ concentration regardless of the Fe level. On the other hand, both Fe and CO$_2$ infusions altered the community composition of eubacteria, although the effects of CO$_2$ enrichment on the bacterial assemblages were small in the non-Fe-added bottles. As further work, physiological study is most likely needed to confirm the sensitivity and the metabolic mechanisms of the phytoplankton and eubacteria groups regarding CO$_2$ availability. In this study, no significant differences in $F_v/F_m$ values were found among different CO$_2$ levels. However, Wu et al. (2010) demonstrated that increased CO$_2$ levels in seawater could change the rates of photosynthesis and respiration of the diatom Phaeodactylum tricornutum. Additionally, the activity of predators of phytoplankton and eubacteria (i.e., zooplankton and heterotrophic nanoflagellates, respectively) under a variety of CO$_2$ levels has been less well studied. Recently, Li and Gao (2012) showed that increased $p$CO$_2$ and acidity in seawater could enhance the feeding rates of the copepod Centropages tenuiremis. Such experimental work would be useful toward a better understanding of the responses of phytoplankton and eubacteria assemblages to Fe and CO$_2$ enrichments, which could occur in the near future.
Acknowledgments

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References


Table 1. Initial pigment : Chl-\(a\) ratios for CHEMTAX analysis: (A) True ratio matrix of Suzuki et al. (2002); (B) double and (C) half the ratios of (A); (D) assigned ratios of 0.75, 0.50 and 0.25 to each element following the method of Latasa (2007).

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| (B)   |       |         |         |      |          |      |        |          |          |      |          |
| Diatoms | 1.5   | 0       | 0       | 0    | 0.48     | 0    | 0      | 0        | 0        | 0    | 0        |
| Hapto  | 0     | 0       | 2.8     | 0    | 0.32     | 0    | 0      | 0        | 0        | 0    | 0        |
| Pelago | 1.24  | 1.86    | 0       | 0    | 0.88     | 0    | 0      | 0        | 0        | 0    | 0        |
| Chloro | 0     | 0       | 0       | 0    | 0.06     | 0    | 0.56   | 0.12     | 0        |      | 1        |
| Prasino| 0     | 0       | 0       | 0    | 0.17     | 0    | 0.36   | 0.89     | 0        |      | 1        |
| Crypto | 0     | 0       | 0       | 0    | 0.28     | 0    | 0      | 0        | 0        | 0    | 1        |
| Dino   | 0     | 0       | 0       | 1.06 | 0        | 0    | 0      | 0        | 0        | 0    | 1        |
| Cyanobacteria | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0.66 | 1 |

| (C)   |       |         |         |      |          |      |        |          |          |      |          |
| Diatoms | 0.375 | 0       | 0       | 0    | 0.12     | 0    | 0      | 0        | 0        | 0    | 0        |
| Hapto  | 0     | 0       | 0.7     | 0    | 0.08     | 0    | 0      | 0        | 0        | 0    | 0        |
| Pelago | 0.31  | 0.465   | 0       | 0    | 0.22     | 0    | 0      | 0        | 0        | 0    | 0        |
| Chloro | 0     | 0       | 0       | 0    | 0        | 0    | 0.15   | 0.14     | 0.03     |      | 1        |
| Prasino| 0     | 0       | 0       | 0    | 0.055    | 0    | 0.18   | 0.445    | 0        |      | 1        |
| Crypto | 0     | 0       | 0       | 0    | 0.07     | 0    | 0      | 0        | 0        | 0    | 1        |
| Dino   | 0     | 0       | 0       | 0.265| 0        | 0    | 0      | 0        | 0        | 0    | 1        |
| Cyanobacteria | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0.165 | 1 |

| (D)   |       |         |         |      |          |      |        |          |          |      |          |
| Diatoms | 0.75  | 0       | 0       | 0    | 0.25     | 0    | 0      | 0        | 0        | 0    | 0        |
| Hapto  | 0     | 0       | 0.75    | 0    | 0.25     | 0    | 0      | 0        | 0        | 0    | 0        |
| Pelago | 0.75  | 0.75    | 0       | 0    | 0.5      | 0    | 0      | 0        | 0        | 0    | 0        |
| Chloro | 0     | 0       | 0       | 0    | 0.25     | 0    | 0.5    | 0.25     | 1        |      | 1        |
| Prasino| 0     | 0       | 0       | 0    | 0.25     | 0    | 0.5    | 0.25     | 1        |      | 1        |
| Crypto | 0     | 0       | 0       | 0    | 0.25     | 0    | 0      | 0        | 0        | 0    | 1        |
| Dino   | 0     | 0       | 0       | 0.5  | 0        | 0    | 0      | 0        | 0        | 0    | 1        |
| Cyanobacteria | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0.5 | 1 |

Abbreviations: Hapto, Haptophytes; Pelago, Pelagophytes; Chloro, Chlorophytes; Crypto, Cryptophytes; Dino, Dinoflagellates; Cyanobacteria, Cyanobacteria; Fucox, Fucoxanthin; 19'-But, 19'-Butanoyloxyfucoxanthin; 19'-Hex, 19'-Hexanoyloxyfucoxanthin; Peri, Peridinin, Diadinox, Diadinoxanthin; Allox, Alloxanthin; Violax, Violaxanthin; Prasinox, Prasinoxanthin; Chl-\(b\), Chlorophyll \(b\); Zeax, Zeaxanthin; Chl-\(a\), Chlorophyll \(a\).
Table 2. Final pigment:Chl-\textit{a} ratio matrices obtained by CHEMTAX program: (A) non-Fe-added and (B) Fe-added samples.

<table>
<thead>
<tr>
<th></th>
<th>Fucox</th>
<th>19'-But</th>
<th>19'-Hex</th>
<th>Peri</th>
<th>Diadinox</th>
<th>Allox</th>
<th>Violax</th>
<th>Prasinox</th>
<th>Chl-b</th>
<th>Zeax</th>
<th>Chl-a</th>
</tr>
</thead>
<tbody>
<tr>
<td>(A)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diatoms</td>
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<td>0</td>
<td>0</td>
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<td>0</td>
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</tr>
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<td>Hapto</td>
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<tr>
<td>Pelago</td>
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<td>0.67</td>
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<td>0</td>
<td>0.46</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
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</tr>
<tr>
<td>Chloro</td>
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<td>0</td>
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<td>Prasino</td>
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<td>0</td>
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<tr>
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<td>0</td>
<td>0</td>
<td>0</td>
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<td>0</td>
<td>0.37</td>
<td>0.08</td>
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<td>(B)</td>
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<td></td>
<td></td>
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<tr>
<td>Diatoms</td>
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<td>0.46</td>
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<td>0</td>
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<td>1</td>
</tr>
<tr>
<td>Chloro</td>
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<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0.09</td>
<td>0</td>
<td>0.30</td>
<td>0.09</td>
<td>0.09</td>
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<td>Prasino</td>
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<td>0.67</td>
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<td>1</td>
</tr>
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<td>0</td>
<td>0</td>
<td>0.16</td>
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<td>1</td>
</tr>
<tr>
<td>Dino</td>
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<td>0.92</td>
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<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Cyano</td>
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<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
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<td>0.39</td>
<td>0.39</td>
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<td>1</td>
</tr>
</tbody>
</table>

Abbreviations: as in Table 1.
Table 3. Abundances (cells mL\(^{-1}\)) of diatoms and coccolithophores in the different CO\(_2\) and Fe treatments on days 0 and 5. D/C denotes the ratio of diatoms (D) to coccolithophores (C) in terms of abundance.

<table>
<thead>
<tr>
<th></th>
<th>Diatoms</th>
<th>Coccolithophores</th>
<th>D/C</th>
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</thead>
<tbody>
<tr>
<td><strong>Initial (day 0)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>non-Fe-added on day 6</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>180 ppm</td>
<td>25.7</td>
<td>23.7</td>
<td>1.1</td>
</tr>
<tr>
<td>380 ppm</td>
<td>34.8</td>
<td>8.0</td>
<td>4.8</td>
</tr>
<tr>
<td>750 ppm</td>
<td>94.7</td>
<td>21.6</td>
<td>4.4</td>
</tr>
<tr>
<td>1000 ppm</td>
<td>85.2</td>
<td>4.2</td>
<td>20.3</td>
</tr>
<tr>
<td><strong>Fe-added on day 5</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>180 ppm</td>
<td>117.8</td>
<td>0.0</td>
<td>-</td>
</tr>
<tr>
<td>380 ppm</td>
<td>12941.1</td>
<td>540.4</td>
<td>23.9</td>
</tr>
<tr>
<td>750 ppm</td>
<td>3570.6</td>
<td>36.0</td>
<td>99.2</td>
</tr>
<tr>
<td>1000 ppm</td>
<td>9055.6</td>
<td>0.4</td>
<td>25873.0</td>
</tr>
</tbody>
</table>
Table 4. Affiliation of excised DGGE band sequences with other sequences in the DNA database.

<table>
<thead>
<tr>
<th>Name⁽ᵃ⁾</th>
<th>Group⁽ᵇ⁾</th>
<th>Closest relative’s form name (Accession no.)</th>
<th>Sequence length</th>
<th>Similarity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PE08-01</td>
<td>Bact</td>
<td>Tenacibaculum sp. MED341 (EU253574)</td>
<td>537</td>
<td>99.8</td>
</tr>
<tr>
<td>PE08-02</td>
<td>Unknown</td>
<td>Clone JL-BS-K49 (AY664338)</td>
<td>541</td>
<td>95.2</td>
</tr>
<tr>
<td>PE08-03</td>
<td>Bact</td>
<td>Flexibacter aurantiaus subsp. (AB078044)</td>
<td>539</td>
<td>99.4</td>
</tr>
<tr>
<td>PE08-04</td>
<td>Bact</td>
<td>Flavobacteriaceae bacterium G1112S4A (AY353814)</td>
<td>539</td>
<td>98.9</td>
</tr>
<tr>
<td>PE08-05</td>
<td>Plastide</td>
<td>DGGE band: KH040536 (AB307995)</td>
<td>521</td>
<td>100</td>
</tr>
<tr>
<td>PE08-06</td>
<td>α, Rose</td>
<td>Clone JL-BS-K54 (AY664344)</td>
<td>523</td>
<td>98.5</td>
</tr>
<tr>
<td>PE08-07</td>
<td>α, Rueg</td>
<td>Clone SGST647 (GQ348093)</td>
<td>523</td>
<td>100</td>
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<tr>
<td>PE08-08</td>
<td>α, Sulf</td>
<td>Sulfitobacter sp. NF4-11 (FJ196047)</td>
<td>510</td>
<td>94.5</td>
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<tr>
<td>PE08-09</td>
<td>α, Rose</td>
<td>Roseobacter sp. RED1 (AY136122)</td>
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<td>100</td>
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<tr>
<td>PE08-10</td>
<td>α, Rose</td>
<td>Roseobacter sp. RED1 (AY136122)</td>
<td>523</td>
<td>99</td>
</tr>
<tr>
<td>PE08-11</td>
<td>α, Rose</td>
<td>Clone BG29-9 (AY904508)</td>
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<td>100</td>
</tr>
<tr>
<td>PE08-12</td>
<td>α, Phae</td>
<td>Phaeobacter sp. ZXM232 (FJ436729)</td>
<td>523</td>
<td>100</td>
</tr>
<tr>
<td>PE08-13</td>
<td>γ</td>
<td>Alteromonas sp. HB1 (FJ968735)</td>
<td>549</td>
<td>99.8</td>
</tr>
<tr>
<td>PE08-14</td>
<td>α, Meth</td>
<td>Methylobacterium sp. 1c.14 (FJ157964)</td>
<td>523</td>
<td>100</td>
</tr>
<tr>
<td>PE08-15</td>
<td>Cyano</td>
<td>Synechococcus sp. CC9902 (CP000097)</td>
<td>527</td>
<td>100</td>
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<tr>
<td>PE08-16</td>
<td>α, Meth</td>
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<td>99.8</td>
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<tr>
<td>PE08-17</td>
<td>Actino</td>
<td>Propionibacterium acnes W1392 (AY642051)</td>
<td>531</td>
<td>100</td>
</tr>
<tr>
<td>PE08-18</td>
<td>Actino</td>
<td>Propionibacterium acnes WD1(AY642054)</td>
<td>531</td>
<td>99.8</td>
</tr>
</tbody>
</table>

a Sequence names correspond to band positions (cf. Fig. 9)
b α, γ, Actino, Unknown, Bact and Cyano indicate Alpha-, Gammaproteobacteria, unknown group of bacteria, Bacteroidetes and Cyanobacteria, respectively; Rose, Roseobacter spp.; Rueg, Ruegeria spp.; Phae, Phaeobacter spp.; Meth, Methylobacterium spp.
Table 5. Effects of increased $p$CO$_2$ on the abundance of phytoplankton groups estimated from the field incubation experiments.

<table>
<thead>
<tr>
<th>Region/Month/Year</th>
<th>Temp (°C)</th>
<th>Diatoms</th>
<th>Haptophytes</th>
<th>Cyano-bacteria</th>
<th>Other phytoplankton</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Raunefjord/May/2001</td>
<td>10 - 13</td>
<td>–</td>
<td>0</td>
<td>–</td>
<td>–</td>
<td>Engel et al. (2005); Delille et al. (2005)</td>
</tr>
<tr>
<td>Raunefjord/May/2003</td>
<td>7.9 - 10.0</td>
<td>0</td>
<td>N</td>
<td>0</td>
<td>P</td>
<td>Engel et al. (2008); Schulz et al. (2008)</td>
</tr>
<tr>
<td>Bering Sea shelf/August/2003</td>
<td>10.4</td>
<td>N</td>
<td>0</td>
<td>–</td>
<td>N</td>
<td>Hare et al. (2007)</td>
</tr>
<tr>
<td>Bering Sea offshore/August/2003</td>
<td>10.4</td>
<td>N</td>
<td>0</td>
<td>–</td>
<td>0</td>
<td>Hare et al. (2007)</td>
</tr>
<tr>
<td>Raunefjord/May/2005</td>
<td>9 - 12</td>
<td>0</td>
<td>P</td>
<td>N</td>
<td>P</td>
<td>Paulino et al. (2008)</td>
</tr>
<tr>
<td>North Atlantic Ocean/June/2005</td>
<td>12</td>
<td>P</td>
<td>0</td>
<td>–</td>
<td>N</td>
<td>Feng et al. (2009)</td>
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<tr>
<td>Ross Sea/December/2005</td>
<td>0</td>
<td>P</td>
<td>N</td>
<td>–</td>
<td>–</td>
<td>Feng et al. (2010)</td>
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<tr>
<td>Gulf of Alaska/August/2007</td>
<td>11 - 14</td>
<td>0</td>
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<td>Hopkinson et al. (2010)</td>
</tr>
<tr>
<td>Sea of Okhotsk/August/2006</td>
<td>13.5</td>
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<td>–</td>
<td>0</td>
<td>0</td>
<td>Yoshimura et al. (2010)</td>
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<tr>
<td>North Pacific/August/2008</td>
<td>14</td>
<td>0</td>
<td>N</td>
<td>P</td>
<td>P</td>
<td>This study</td>
</tr>
</tbody>
</table>

P: positive; N: negative; 0: no response; –: not available
Figure captions

Fig. 1. Temporal variations in TA (A and B), DIC (C and D), and calculated pH (E and F) with the CO2SYS program (Pierrot et al., 2006) under an in situ temperature of 14.0°C and salinity of 32.70. Left (A, C and E) and right (B, D and F) graphs indicate data from the non-Fe-added and Fe-added bottles, respectively.

Fig. 2. Temporal changes in the concentrations of nitrate (A and B), phosphate (C and D), and silicate (E and F). Left (A, C and E) and right (B, D and F) graphs indicate data from the non-Fe-added and Fe-added bottles, respectively. Error bars denote ± 1 SD (n = 3).

Fig. 3. Temporal changes in the drawdown ratios of Si/N (A and B) and N/P (C and D). Left (A and C) and right (B and D) graphs indicate data from the non-Fe-added and Fe-added bottles, respectively. Error bars denote ± 1 SD (n = 3).

Fig. 4. Temporal changes in the concentrations of total Chl-a (A and B), large-sized Chl-a (L-Chl-a, ≥ 10 µm, C and D), and small-sized Chl-a (S-Chl-a, < 10 µm, E and F). Left (A, C and E) and right (B, D and F) graphs indicate data from the non-Fe-added and Fe-added bottles, respectively. Error bars denote ± 1 SD (n = 3).

Fig. 5. Temporal changes in $F_v/F_m$ in the (A) non-Fe-added and (B) Fe-added bottles. Error bars denote ± 1 SD (n = 3).

Fig. 6. Ratios of (A) fucoxanthin (Fucox) or (B) 19’-hexanoyloxyfucoxanthin (19’-Hex) concentrations between day 0 and the final sampling day (final day/day 0). Open bars and closed bars denote non-Fe-added and Fe-added treatments, respectively. Error bars indicate ± 1 SD (n = 3).

Fig. 7. Mean contributions of each phytoplankton group to total Chl-a biomass as estimated by CHEMTAX and temporal changes in Chl-a concentration in the
(A) non-Fe-added and (B) Fe-added bottles at 180, 380, 750 and 1000 ppm CO₂. Error bars denote ± 1 SD (n = 3).

Fig. 8. Temporal changes in the cell densities of eukaryotic ultraphytoplankton (A and B), cyanobacteria *Synechococcus* spp. (C and D) and heterotrophic bacteria (E and F). Left (A, C and E) and right (B, D and F) graphs indicate data from the non-Fe-added and Fe-added bottles, respectively. Error bars denote ± 1 SD (n = 6).

Fig. 9. DGGE images targeting the 16S rRNA genes in non-Fe-added and Fe-added bottles.

Fig. 10. Cluster analysis of DGGE data on bacterial community compositions. The labels –Fe and +Fe represent non-Fe-added and Fe-added treatments, respectively. The label D represents the day of sampling.

Fig. 11. A neighbor-joining tree of 16S rRNA gene sequences of eubacteria. The scale bar indicates 0.05 substitutions per nucleotide position. PE08-xx represents the sequences obtained in this study. CZII_q1, CZII_q2, CZII_l1, CZII_za and CZII_zb indicate sequences detected specifically inside the iron-enriched patch during an in situ iron enrichment (SEEDS-II) in the WSG (Kataoka et al., 2009).
Fig. 1
Fig. 2
Fig. 3
Fig. 4
Fig. 5
Fig. 7
Fig. 8
Fig. 10
Fig. 11