Dear Dr. Emilio Marañón,

We would like to thank you and the three reviewers for careful examination of our manuscript (bg-2014-554) entitled "Effects of CO_2 and iron availability on *rbcL* gene expression in Bering Sea diatoms". Attached is the track changes version of revised manuscript combined with the supplement and author's response. In the revised manuscript and supplement, the modified places were shown in blue.
We hope that our manuscript will be acceptable for publication in Biogeosciences.

Yours sincerely,

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Abstract

Iron (Fe) can limit phytoplankton productivity in approximately 40% of the global ocean, including high-nutrient, low-chlorophyll (HNLC) waters. However, there is little information available on the impact of CO_2-induced seawater acidification on natural phytoplankton assemblages in HNLC regions. We therefore conducted an on-deck experiment manipulating CO_2 and Fe using Fe-deficient Bering Sea waters during the summer of 2009. The concentrations of CO_2 in the incubation bottles were set at 380 and 600 ppm in the non-Fe-added (control) bottles and 180, 380, 600, and 1000 ppm in the Fe-added bottles. The phytoplankton assemblages were primarily composed of diatoms followed by haptophytes in all incubation bottles as estimated by pigment signatures throughout the 5 (controls) or 6 (Fe-added treatments) days incubation period. At the end of incubation, the relative contribution of diatoms to chlorophyll *a* biomass was significantly higher in the 380 ppm CO_2 treatment than in the 600 ppm treatment in the controls, whereas minimal changes were found in the Fe-added treatments. These results indicate that, under Fe-deficient conditions, the growth of diatoms could be negatively affected by the increase in CO_2 availability. To further support this finding, we estimated the expression and phylogeny of *rbcL* (which encodes the large subunit of RubisCO) mRNA in diatoms by quantitative reverse transcription PCR and clone library techniques, respectively. Interestingly, regardless of Fe availability, the expression of *rbcL* decreased in the high CO_2 treatments (600 and 1000 ppm). The present study suggests that the projected future increase in seawater pCO_2 could reduce the RubisCO transcription of diatoms, resulting in a decrease in primary productivity and a shift in the food web structure of the Bering Sea.

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1. Introduction

The atmospheric CO_2 concentration has risen from a pre-industrial level of approximately 280 ppm to the present level of approximately 400 ppm (WMO, 2013). Since the industrial revolution, the ocean has absorbed about one-third of CO_2 emitted by human activity (Sabine et al., 2004). 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 surface seawater pH decrease of 0.3–0.4, the so-called "ocean

43 acidification” (Caldeira and Wickett, 2003). Such a rapid decrease in seawater pH has
44 most likely not occurred for at least millions of years in the earth’s history (Pearson and
45 Palmer, 2000). Therefore, it has been suggested that these predicted changes in seawater
46 carbonate chemistry would have enormous impacts on the health and function of marine
47 organisms (Raven et al., 2005).

48 In the last decade, numerous studies have been performed to evaluate the impacts of
49 ocean acidification on marine phytoplankton. In laboratory incubation experiments
50 using individual species (a single strain), the response of phytoplankton to increased
51 CO_2 levels differed among phytoplankton species, possibly depending on their ability to
52 assimilate carbon (Riebesell and Tortell, 2011; Collins et al., 2014). In the natural
53 environment, these taxon-specific differences in CO_2 response can cause a shift in the
54 phytoplankton community composition (Engel et al., 2008; Meakin and Wyman, 2011;
55 Endo et al., 2013) and subsequent changes in ocean trophic structures and
56 biogeochemical cycles (Riebesell et al., 2007; Yoshimura et al., 2013). However, the
57 current understanding of the effects of elevated CO_2 on marine phytoplankton is still
58 insufficient at the community level.

59 In terms of physiology, CO_2 is fixed by the carboxylation enzyme ribulose
60 bisphosphate carboxylase/oxygenase (RubisCO) in the Calvin-Benson-Bassham (CBB)
61 cycle. In general, the half-saturation constant of the enzyme ranges between 20 and 70
62 μmol kg^{-1} CO_2 (Badger et al., 1998), whereas the ambient seawater CO_2 levels are
63 between 10 and 25 μmol kg^{-1}. Therefore, the present CO_2 concentration could be
64 insufficient to ensure effective RubisCO carboxylation. The progression of ocean
65 acidification could enhance photosynthetic carbon fixation in marine phytoplankton by
66 increasing CO_2 availability.

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削除: Consequently, algae have mechanisms to actively increase the CO_2 concentration at the site of carboxylation (i.e., carbon-concentrating mechanisms, CCMs) to overcome the low RibisCO CO_2 affinity (e.g., Raven, 2010; Reinfelder, 2011). Because the operation of CCMs requires nutrients and energy (Beardall and Giordano, 2002), additional materials would be needed for photosynthetic carbon assimilation under CO_2-limited conditions. Therefore, the progression of ocean acidification could enhance photosynthetic carbon fixation in marine phytoplankton by reducing the investment in CCMs. Until now, most physiological work has been performed in laboratory incubation experiments using particular organisms, but there are few reports on natural phytoplankton assemblages.

67 Recent advances in molecular biology techniques have enabled us to examine the
68 taxon-specific responses to environmental changes by quantifying functional gene
69 expression in natural phytoplankton assemblages. For example, John et al. (2007a)
70 developed a suite of quantitative reverse transcription PCR (qRT-PCR) assays to
71 quantify *rbcL* (gene encoding the large subunit of RubisCO) mRNA in *Synechococcus*,
72 haptophytes, and heterokonts including diatoms. John et al. (2007b) demonstrated a
73 strong negative correlation between diatom-specific *rbcL* mRNA abundance and

93 seawater pCO_2 in the Mississippi River plume, suggesting that diatoms were
94 responsible for the greatest drawdown in seawater pCO_2. In addition, positive
95 correlations between diatom-specific *rbcL* transcripts and light-saturated photosynthetic
96 rates (P_{max}) in seawater were reported (Corredor et al., 2004; John et al., 2007b). These
97 results suggest that *rbcL* expression in diatoms could be used to estimate the
98 photosynthetic carbon-fixation capacity of natural phytoplankton assemblages.
99 Therefore, quantification of clade-specific *rbcL* transcripts can be used to assess the
100 physiological photosynthetic responses of individual phytoplankton taxa to
101 environmental changes.

102 The oceanic Bering Sea investigated in this study is an HNLC region (Banse and
103 English, 1999), where low iron (Fe) availability limits phytoplankton growth and nitrate
104 utilization, so surface chlorophyll *a* (Chl-*a*) concentrations usually remain low in the
105 summer (Suzuki et al., 2002). Despite the low phytoplankton biomass, the oceanic
106 domain has the greatest amount of total primary and secondary production in the Bering
107 Sea (Springer et al., 1996). Suzuki et al. (2002) reported that diatoms were the dominant
108 phytoplankton group in the oceanic regions of the Bering Sea in the summer. In
109 addition, Takahashi et al. (2002) showed that diatoms had the greatest contribution in
110 the sinking particles in the area. However, less is known about the combined effects of
111 ocean acidification and Fe enrichment on diatoms in such HNLC regions. In addition,
112 there are no reports on the effects of CO_2 and Fe availability on *rbcL* transcription of
113 natural diatom community in HNLC regions.

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削除: Additionally, whether CO_2 level and/or Fe availability could affect *rbcL* transcription in diatoms is completely unknown.

114 The purpose of this study is to clarify the responses of phytoplankton, especially
115 diatoms, to CO_2 enrichment under Fe-depleted and Fe-replete conditions in the Bering
116 Sea basin using on-deck bottle incubation. Recently, Sugie et al. (2013) reported
117 changes in phytoplankton biomass and nutrient stoichiometry in this experiment. They
118 showed that Chl-*a* biomass decreased with increased CO_2 levels only in Fe-depleted
119 treatments, suggesting that Fe deficiency and increased CO_2 synergistically reduced the
120 growth of phytoplankton in the study area. In addition, Yoshimura et al. (2014)
121 demonstrated that the net production of particulate organic carbon (POC) and total
122 organic carbon (TOC) decreased under high CO_2 levels only in the Fe-limited
123 treatments, whereas those in the Fe-replete treatments were insignificantly different.

127 These studies suggest that the increase in CO_2 could have negative impacts on
128 phytoplankton growth and/or organic-matter production especially under Fe-depleted
129 conditions. However, the molecular mechanisms of photosynthetic carbon assimilation
130 in phytoplankton assemblages were not mentioned in the previous studies. Therefore, in
131 the present paper, we primarily focused on changes in *rbcL* transcription in diatoms
132 with different CO_2 and/or Fe availability.
133

134 **2. Materials and Methods**

135 **2.1 Experimental setup**

136 The study was carried out aboard the R/V *Hakuho Maru* (JAMSTEC) during the
137 KH-09-4 cruise in September 2009. The water samples for incubation were collected
138 from 10 m depth at a station (53° 05’ N, 177° 00’ W) in the Bering Sea on 9 September
139 with acid-cleaned Niskin-X bottles attached to a CTD-CMS system. A total of 300 L of
140 seawater was poured into six 50 L polypropylene carboys through acid-clean silicon
141 tubing with a 197 μm mesh Teflon net to remove large particles. Subsamples were taken
142 from each carboy and poured into triplicate acid-cleaned 12 L polycarbonate bottles
143 (total 18 bottles) for incubation. Initial samples were collected from each carboy. All
144 sampling was carried out using a trace-metal clean technique to avoid any trace metal
145 contamination. Prior to incubation, $FeCl_3$ solutions (5 nmol L^{-1} in final concentration)
146 were added to 12 bottles in order to reduce Fe limitation for the phytoplankton
147 communities. The CO_2 levels in the incubation bottles were manipulated by injecting
148 CO_2 controlled dry air purchased from a commercial gas supply company
149 (Nissan-Tanaka Co., Japan). The air mixtures were passed through 47 mm PTFE filters
150 (0.2 μm pore size, Millipore) before being added to the incubation bottles. The detailed
151 procedures for trace metal clean techniques were described in Yoshimura et al. (2013).
152 The CO_2 concentrations were set at 380 and 600 ppm for the non-Fe-added (control)
153 bottles (hereafter referred to as ‘C-380’ and ‘C-600’, respectively), and 180, 380, 600,
154 and 1000 ppm for the Fe-added bottles (hereafter referred to as ‘Fe-180’, ‘Fe-380’,
155 ‘Fe-600’, and ‘Fe-1000’, respectively). Incubation was performed on deck in
156 temperature-controlled water-circulating tanks for 5 (controls) or 6 (Fe-added
157 treatments) days at the in situ temperature (8.2°C) and 50% surface irradiance adjusted

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159 by natural density screens. The sampling opportunities for each parameter are shown in
160 Table S1.
161
162 **2.2 Carbonate chemistry, nutrients, and Chl-*a***
163 The detailed methodology and basic chemical and biological parameters were
164 reported in Sugie et al. (2013). In brief, during the incubation experiment, samples were
165 collected from the incubation bottles for dissolved inorganic carbon (DIC), total
166 alkalinity (TA), nutrients, and Chl-*a* determination. DIC and TA concentrations were
167 measured with a total alkalinity analyzer using the potentiometric Gran plot method
168 (Kimoto Electric) following Edmond (1970). The levels of pCO_2 and pH were
169 calculated from the DIC and TA using the CO2SYS program (Lewis and Wallance,
170 1998). Concentrations of nitrate plus nitrite, nitrite, phosphate, and silicic acid were
171 measured using a QuAATro-2 continuous-flow analyzer (Bran+Luebbe). The
172 concentration of total dissolved Fe (TD-Fe) was determined by a flow-injection method
173 with chemiluminescence detection (Obata et al., 1993). Chl-*a* concentrations were
174 determined with a Turner Design fluorometer (model 10-AU) with the non-acidification
175 method (Welschmeyer, 1994).
176
177 **2.3 HPLC and CHEMTAX analyses**
178 Samples for high-performance liquid chromatography (HPLC) pigment analysis
179 were collected on days 3 and 5 for the control treatments and on days 2, 4, and 6 for the
180 Fe-added treatments. Water samples (400–1000 mL) were filtered onto GF/F filters
181 under gentle vacuum (< 0.013 MPa) and stored in liquid nitrogen or a deep freezer
182 (−80°C) until analysis. HPLC pigment analysis was performed following the method of
183 Endo et al. (2013).
184 To estimate the temporal changes in phytoplankton community structure during
185 incubation, the CHEMTAX program (MacKey et al., 1996) was used following Endo et
186 al. (2013). Briefly, optimal initial ratios were obtained following the method of Latasa
187 (2007). Matrix A was obtained from Suzuki et al. (2002) (Table S1), who examined
188 phytoplankton community compositions in the Bering Sea. Matrices B, C, and D were
189 also prepared to determine the optimal pigment/Chl-*a* ratios (Table S1). The pigment

190 ratios of Matrices B and C were double and half the Matrix A ratio, respectively. For
191 Matrix D, values of 0.75, 0.5, and 0.25 for dominant (rank in high pigment/Chl-*a* ratio:
192 1–5), secondary (rank: 6–10), and minor (rank: 11–15) pigments, respectively, were
193 multiplied by each pigment ratio of Matrix A. We averaged the successive convergent
194 ratios after the 10 runs among the 4 matrices to identify the most promising initial
195 pigment ratios. The calculated final pigment/Chl-*a* ratios in both the control and
196 Fe-added treatments (Table S2) were within the range of values reported in Mackey et
197 al. (1996), Wright and van den Enden (2000), and Suzuki et al. (2002).
198

199 **2.4 qPCR and qRT-PCR**

200 Water samples for DNA and RNA analyses were collected on days 3 and 5 for the
201 control treatments and on days 2, 4, and 6 for the Fe-added treatments. DNA samples
202 (400–500 mL) were collected onto 25 mm, 0.2 μm pore size polycarbonate Nuclepore
203 filters (Whatman) with gentle vacuum (< 0.013 MPa) and stored in liquid nitrogen or a
204 deep freezer at −80°C until analysis. DNA extraction was performed following the
205 method of Endo et al. (2013). Extracted DNA pellets were resuspended in 100 μL of 10
206 mM Tris-HCl buffer (pH 8.5).
207 For RNA analysis, seawater samples (400–500 mL) were filtered onto 25 mm, 0.2
208 μm pore size polycarbonate Nuclepore filters (Whatman) with gentle vacuum (< 0.013
209 MPa) and stored in 1.5 mL cryotubes previously filled with 0.2 g of muffled 0.1 mm
210 glass beads and 600 μL RLT buffer (Qiagen) with 10 μL mL^{-1} β-mercaptoethanol
211 (Sigma, St Louis, USA). RNA samples were stored in liquid nitrogen or a deep freezer
212 at −80°C until analysis. Extraction and purification of RNA samples were performed
213 using the RNeasy extraction kit (Qiagen) on a vacuum manifold with on-column DNA
214 digestion using RNase-free DNase (Qiagen) according to the manufacturer's protocol.
215 RNA was eluted using 50 μL of RNase-free H_2O. Total RNA was then reverse
216 transcribed into complementary DNA (cDNA) using the PrimeScript^{TM} RT regent Kit
217 with gDNA Eraser (TaKaRa) following the manufacturer's specifications.
218 Following Smith et al. (2006), we used double-stranded DNA and single-stranded
219 cDNA standards for DNA and cDNA quantification, respectively. Standard curves for
220 *rbcL* DNA were generated from plasmid DNA (pUC18, TaKaRa) containing an
221 artificial gene fragment (113 bp in size) of *rbcL* from the diatom *Thalassiosira*

222 *weissflogii* (CCMP1336). The plasmid DNA was linearized with *Hind*III (TaKaRa) and
223 quantified using a Thermo NanoDrop spectrophotometer (ND-1000). On the other hand,
224 to produce a cDNA standard, a PCR-amplified *rbcL* gene fragment of *T. weissflogii*
225 (CCMP1336) was inserted into a plasmid DNA (pCR2.1, Invitrogen). The plasmid
226 DNA was purified using the Plasmid maxi kit (Qiagen) and linearized with *Bam*HI
227 (TaKaRa), and in vitro transcription was performed using T7 RNA polymerase
228 (Invitrogen) for 2 hours at 37°C with Recombinant RNase Inhibitor (TaKaRa). To
229 eliminate DNA contamination, RNA was digested for 2 min at 42°C using gDNA
230 Eraser (TaKaRa). RNA was purified using an RNeasy column (Qiagen) following the
231 manufacturer's instructions and quantified with a Ribogreen RNA quantification kit
232 (Molecular Probes) using the manufacturer's standard. RNA was reverse transcribed
233 into cDNA using the PrimeScript^{TM} RT regent Kit with gDNA Eraser (TaKaRa).
234 Copy numbers of DNA and cDNA standards were calculated using the equation of
235 Smith et al. (2006), where the molecular mass of each nucleotide (or nucleotide pair) in
236 double- and single-stranded DNA is assumed to be 660 and 330 Da, respectively. Serial
237 dilutions of DNA and cDNA standards were prepared using sterilized Milli-Q water.
238 To amplify the *rbcL* gene and cDNA fragments from diatoms, the following specific
239 primer set designed by John et al. (2007a) was used: forward primer:
240 5'-GATGATGARAAYATTAACTC-3', reverse primer:
241 5'-TAWGAACCTTTWACTTCWCC-3'. Real-time PCR amplification was performed
242 using SYBR Premix Ex Taq II (Perfect Real Time, TaKaRa) with primer concentrations
243 of 0.4 µM each and a Thermal Cycler Dice Real Time System (TP800, TaKaRa).
244 Diluted nucleic acid standards were then added to the PCR mixture. The thermal
245 cycling conditions were 95°C for 60 s, then 40 cycles of 95°C 5 s and 52°C 60 s. The
246 fluorescence intensity of the complex formed by SYBR green and the double-stranded
247 PCR product was continuously monitored from cycle 1 to 40. Quantification was
248 achieved by the second-derivative maximum method (Luu-The et al., 2005), and the
249 copy number for each sample was determined by the standard curves generated by serial
250 dilutions of the standards.
251
252 **2.5 Clone libraries**

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255 Clone libraries of *rbcL* cDNA were constructed for the C-380 and C-600 samples on
256 day 3, and Fe-380 and Fe-600 samples on day 2. The cDNA samples were PCR
257 amplified with the diatom-specific primer set and thermal cycling condition described
258 above using the TaKaRa Ex Taq Hot Start Version (TaKaRa). Triplicate PCR products
259 were mixed and then purified with agarose gel electrophoresis and the PureLink Quick
260 Gel Extraction Kit (Invitrogen). Purified amplicons from cDNA samples were then
261 cloned into the pCR2.1 vector using the TOPO TA cloning kit (Invitrogen) following
262 the manufacturer's instructions. Thirty-five to 50 colonies were randomly picked from
263 each clone library. Correct cDNA insertions were identified by PCR amplification using
264 the M13 forward (5'-GTAAAACGACGGCCAG-3') and reverse
265 (5'-CAGGAAACAGCTATGA-3') primers flanking the cloning site. Plasmid DNA
266 containing the inserts was cycle-sequenced using the Big Dye Terminator v3.1 Kit
267 (Applied Biosystems) with the M13 forward primer. The cycle sequencing products
268 were cleaned by isopropanol precipitation. Sequencing was performed with a 3130
269 Genetic Analyzer (Life Technologies). The obtained sequences were compared with
270 *rbcL* sequences deposited in GenBank database (http://www.ncbi.nlm.nih.gov) using
271 the BLAST query engine. Our *rbcL* cDNA sequences were deposited in the DDBJ
272 database with the following accession numbers: AB985799–AB986033.
273
274 **2.6 Phylogenetic and diversity analyses**

275 The *rbcL* sequences obtained were assembled into operational taxonomic units
276 (OTUs) with > 95% sequence identity, and rarefaction curves were plotted for each
277 clone library with the software mothur v. 1.27 (Schloss et al., 2009). To estimate OTU
278 richness, chao1 index (Chao, 1984) values were calculated using the number of
279 singleton sequences obtained in this study. Genetic diversity was assessed based on the
280 Shannon-Wiener index (*H'*, Shannon, 1948) and Simpson's index (1-*D*, Simpson, 1949).
281 The statistical significance of differences in the compositions of pairs of *rbcL* sequences
282 in the libraries was tested using LIBSHUFF (Singleton et al., 2001). The LIBSHUFF
283 program determined the integral form of the Cramer-von Mises statistic for each pair of
284 communities using 10,000 randomizations. Any two libraries were considered to be
285 significantly different from each other if the lower of the significance values generated

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削除: Genetic diversity was assessed based on the number of OTUs, the Shannon-Wiener index (*H'*, Shannon, 1948), and Simpson's index (1-*D*, Simpson 1949).

290 by the software was < 0.025 ($p < 0.05$).

291

292 **2.7 Statistical analysis**

293 Statistical analyses were performed with the program R (http://www.r-project.org).
294 To assess the statistically significant differences between pCO_2 levels in the control
295 treatments or between control and Fe treatments, Welch's *t*-test was used. Differences
296 among pCO_2 levels in the Fe-added treatments were evaluated with Kruskal-Wallis
297 one-way analysis of variance (ANOVA). Holm's test for multiple comparisons was
298 used to identify the source of the variance. For all of the analyses, the confidence level
299 was set at 95% ($p < 0.05$).

300

301 **3 Results**

302 **3.1 Experimental conditions**

303 The bubbling of CO_2-controlled air succeeded in creating significant gradients in
304 pCO_2, pH, and DIC in the different CO_2 treatments except on day 4 in the Fe-added
305 treatments, when those values did not significantly differ between Fe-380 and Fe-600
306 (Table 1; Fig. S1). The initial concentrations of nitrate, phosphate, and silicic acid were
307 18.06 ± 0.10, 1.47 ± 0.01, and 16.90 ± 0.12 μmol L^{-1}, respectively (Table 1). In the
308 control bottles, these macronutrients remained until the end of the incubation in both
309 CO_2 treatments except for silicic acid, which was almost depleted on day 5 in the C-380
310 treatment (Fig. S2). In the Fe-added bottles, macronutrients were depleted on days 4 or
311 6 in all CO_2 treatments (Fig. S2). The TD-Fe concentration was 1.35 nmol L^{-1} in the
312 initial seawater, and it remained low throughout the experiment in the control treatments
313 (Table 1). In the Fe-added treatments, the TD-Fe concentrations were 5.50 ± 0.10 nmol
314 L^{-1} in the initial bottles and remained above 4 nmol L^{-1} until the end of incubation
315 (Table 1). The initial Chl-*a* concentration was 1.96 ± 0.14 μg L^{-1} (Table 1). In the
316 control bottles, the Chl-*a* concentration increased until the end of the incubation and
317 reached 10.22 ± 0.89 μg L^{-1} in the C-380 and 6.28 ± 0.64 μg L^{-1} in the C-600
318 treatments (Fig. S3). In the Fe-added bottles, the Chl-*a* concentration increased rapidly
319 and reached the maximum on day 4 in the Fe-180 and Fe-380 treatments (27.51 ± 0.71
320 μg L^{-1} and 28.45 ± 3.40 μg L^{-1}, respectively) and on day 5 in the Fe-600 and Fe-1000

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削除: Carbonate chemistry, macronutrients, and Fe parameters (value ± 1 standard deviation, n = 3) during the incubation experiment are shown in Table 1, and the complete data set was reported in Sugie et al. (2013).

327 treatments (27.68 ± 0.44 μg L^{-1} and 27.32 ± 3.05 μg L^{-1}, respectively), then declined
328 toward the end of the incubation (Fig. S3).

329

330 **3.2 Phytoplankton pigments**

331 Throughout the experiment, the concentrations of fucoxanthin (Fucox), mainly a
332 biomarker for diatoms (Ondrusek et al., 1991; Suzuki et al., 2011), and
333 19'-hexanoyloxyfucoxanthin (19'-Hex), an indicator of haptophytes (Jeffrey and Wright,
334 1994), were relatively high among the phytoplankton pigments. In the control bottles,
335 the concentrations of Fuco and 19'-Hex increased over time and reached the maximum
336 values on day 5 in both the C-380 and C-600 treatments (Figs. 1a and c). After day 3,
337 the concentrations of Fuco and 19'-Hex were higher in the C-380 treatment than in the
338 C-600 treatment (day 5: Welch's *t*-test C-380 > C-600, $p < 0.05$), although no statistical
339 significance was assessed on day 3 because samples were collected from each single
340 bottle. In the Fe-added bottles, Fuco concentrations increased throughout the incubation
341 and reached the maximum values on day 6, whereas 19'-Hex concentrations decreased
342 after day 4 (Figs. 1b and d). The concentrations of Fuco were significantly different
343 among CO_2 treatments on day 6 (Kruskal-Wallis ANOVA, $p < 0.05$), although these
344 differences were not supported by multiple comparisons (Holm's test, $p > 0.05$).
345 Significant differences among CO_2 treatments were also found for the 19'-Hex
346 concentration on day 6 (Kruskal-Wallis ANOVA, $p < 0.05$), and the values in the
347 Fe-180 treatment was significantly higher than those in the Fe-1000 treatment (Holm's
348 test, $p < 0.05$).

349

350 **3.3 CHEMTAX outputs**

351 In the initial phytoplankton community, diatoms and haptophytes were the
352 predominant numbers of the phytoplankton groups (i.e., they contributed 45% and 17%
353 of the Chl-*a* concentration, respectively). The initial contributions of chlorophytes,
354 cryptophytes, peridinin-containing dinoflagellates, pelagophytes, prasinophytes, and
355 cyanobacteria to the Chl-*a* biomass were 10%, 9%, 8%, 5%, 4%, and 2%, respectively.
356 In the control bottles, the contributions of diatoms to the Chl-*a* biomass increased with
357 time, and their contributions reached the maximum (70% at the C-380 and 60% at the

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388 C-600 treatments) on day 5 (Fig. 2a). On day 5, the contribution of diatoms in the
389 C-380 treatment was significantly higher than that in the C-600 treatment (Welch's
390 *t*-test, $p < 0.05$). However, the contribution of haptophytes to the Chl-*a* biomass was
391 higher in the C-600 treatment (21%) than in the C-380 treatment (14%) on day 5
392 (Welch's *t*-test, $p < 0.05$). Increases in the contributions of diatoms were also observed
393 in the Fe-added treatment, and the contributions reached the maximum (82–85%) on
394 day 4 in all CO_2 treatments (Fig. 2b). In terms of diatom contribution, a significant
395 difference among CO_2 treatments was not detected with Kruskal-Wallis ANOVA ($p >$
396 0.05) in the Fe-added bottles. The contributions of haptophytes to Chl-*a* biomass did not
397 differ significantly among CO_2 levels in the Fe-added bottles (Kruskal-Wallis ANOVA,
398 $p > 0.05$).
399

400 **3.4 Expression of diatom *rbcL* gene**

401 A significant linear relationship between the Fuco concentration and the
402 diatom-specific *rbcL* gene copy number was found (regression analysis: $r^2 = 0.677$, $p <$
403 0.001, n = 28) in our experiment (Fig. 3). In the control bottles, the transcript abundance
404 normalized to gene abundance (i.e., cDNA/DNA) of the diatom-specific *rbcL* gene
405 fragment for the C-380 treatment was significantly higher than that of the C-600
406 treatment on day 3 (Fig. 4; Welch's *t*-test, $p < 0.05$). In the Fe-added bottles, the
407 cDNA/DNA ratio of the diatom *rbcL* fragment in the lower CO_2 treatments (Fe-180 and
408 Fe-380) was higher than that in the Fe-600 treatment on day 2 (Fig. 4; Holm's test, $p <$
409 0.05).
410

411 **3.5 Clone libraries of diatom *rbcL* cDNA**

412 Rarefaction curves were plotted for the *rbcL* cDNA libraries (Fig. 5). In terms of
413 unique taxa, the highest number of OTUs was found in the C-380 treatment (Table 2).
414 The highest chao1value was found in the C-600 treatment, whereas the lowest value
415 was found in the Fe-600 treatment. Shannon-Wiener and Simpson diversity indices
416 revealed that the cDNA libraries in the 380 ppm CO_2 bottles were more diverse than
417 those in the 600 ppm CO_2 bottles in both the control and Fe-added treatments, although
418 the values were not statistically significant between CO_2 treatments (*t*-test, $p > 0.05$)

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420 (Table 2).

421 All sequences obtained from the cDNA libraries were more than 95% similar to
422 sequences deposited in the GenBank. These sequences could be classified into the
423 following 11 phylogenetic groups: Chaetocerotaceae, Coscinodiscaceae,
424 Cymatosiraceae, Stephanodiscaceae, Thalassiosiraceae, unidentified centrics,
425 Bacillariaceae, Naviculaceae, Fragillariaceae, unidentified pennates, and other
426 eukaryotes by comparison with known *rbcL* sequences from GenBank. Sequences that
427 could not classified into a specific diatom family (e.g., closely related to two or more
428 diatom families with same similarity score) were assigned as unidentified centrics or
429 unidentified pennates. Other eukaryotes consisted of haptophytes, pelagophytes,
430 dictyochophytes, dinoflagellates, and diatoms which could be assigned to centrics and
431 pennates. For all of the cDNA libraries, more than 88% of *rbcL* sequences were most
432 closely affiliated with those of cultured diatoms. In the initial cDNA library, the most
433 abundant sequences were closely affiliated with the diatom family Bacillariaceae (46%),
434 followed by other eukaryotes and Cymatosiraceae (17% and 14%, respectively) (Fig. 6).
435 The contributions of other diatom groups were less than 6% in the initial clone library.
436 In the control bottles, the contributions of Coscinodiscaseae increased to 12–14%,
437 whereas those of Cymatosiraceae decreased to 4%. In the Fe-added bottles, the
438 contributions of Chaetocerotaceae and unidentified centrics to the total increased to
439 more than 8% and 20%, respectively. In contrast, the contributions of Bacillariaceae
440 decreased below 24% in both the Fe-380 and Fe-600 treatments.

441 Statistic analysis using LIBSHUFF revealed that the cDNA libraries in the control
442 treatments were not significantly different from the initial sample regardless of the CO_2
443 level, whereas those in the Fe-added bottles differed significantly from the initial
444 assemblage (LIBSHUFF, $p < 0.05$) (Table 3). No significant difference in the cDNA
445 library was found between C-380 and C-600 treatments in the control bottles
446 (LIBSHUFF, $p > 0.05$). However, a significant difference between the Fe-380 and
447 Fe-600 treatments was detected in the Fe-added bottles (LIBSHUFF, $p < 0.05$). In
448 addition, cDNA libraries in the Fe-added bottles differed significantly from those of the
449 control bottles in both the Fe-380 and Fe-600 treatments (LIBSHUFF, $p < 0.05$).
450

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削除: In addition, in both the control and Fe-added bottles, the numbers of OTUs in the 380 ppm CO_2 treatments was higher than that in the 600 ppm. The Shannon-Wiener and Simpson diversity indices also showed that the cDNA libraries in the 380 ppm CO_2 bottles were more diverse than those in the 600 ppm CO_2 bottles in both the control and Fe-added treatments (Table 2).

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削除: that were neither centrics nor pennates.

461 **4 Discussion**

462 **4.1 Changes in phytoplankton community structure during incubation**

463 Our CHEMTAX analysis suggested that the diatoms were the principal contributors
464 to the Chl-*a* biomass in the initial phytoplankton community, followed by haptophytes
465 (Fig. 2). The results were consistent with those reported by Suzuki et al. (2002), who
466 examined the community structure in the Bering Sea during early summer of 1999.
467 These results suggest that diatoms and haptophytes are ecologically important
468 phytoplankton groups in the study area during the summer. Compared with previous
469 reports in the area (Suzuki et al., 2002; Yoshimura et al., 2013), a relatively high initial
470 Chl-*a* concentration was observed in our experiment, possibly due to an intrusion of the
471 coastal seawater mass from the Aleutian trenches (Sugie et al., 2013). However, the Fe
472 infusion induced significant increases in Chl-*a* biomass and concomitant rapid
473 drawdowns of macronutrients in our incubation bottles (Fig. S2). This indicates that the
474 seawater used for the incubation was Fe-limited for phytoplankton assemblages. Our
475 HPLC and CHEMTAX results suggested that the increase in phytoplankton biomass
476 was mainly due to an increase in diatoms (Figs. 1a and 2b).

477 We found that the growth of Fuco was less in the high CO_2 bottles in the control
478 treatments (Fig. 1a), suggesting that the elevated CO_2 levels could have a negative
479 impact on the diatom biomass in the study area. Negative effects on diatoms induced by
480 an increase in CO_2 availability were also reported in field incubation experiments
481 conducted in the Bering Sea and the Okhotsk Sea (Hare et al., 2007 and Yoshimura et
482 al., 2010, respectively). However, such trends have rarely been observed in other
483 regions of the world's oceans (e.g., Tortell et al., 2002; Kim et al., 2006; Feng et al.,
484 2009; Hoppe et al., 2013; Endo et al., 2013). Therefore, the responses of phytoplankton
485 assemblages to ocean acidification can differ among geographic locations due to the
486 differences in the biogeography of phytoplankton and/or environmental conditions.

487 One possible cause of the geographic specificity in the open Bering Sea is the
488 differences in the species composition of diatoms. Our microscope data showed that
489 centric diatoms such as Chaetocerataceae and Rhizosoreniaceae were predominant at
490 the beginning of the incubation in terms of carbon biomass, and the coastal diatom
491 species *Chaetoceros* spp. became predominant in all incubation bottles after day 2
492 (Sugie et al., 2013). Therefore, the relative decrease in Fuco biomass with increased

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削除: Sugie et al., 2013

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削除: We found that the ratios of Fucox concentration between initial and the final days decreased with increased CO_2 levels in both the control and Fe-added treatments (Fig. 1a), suggesting that the elevated CO_2 levels had a negative impact on the diatom biomass in the study area.

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削除: Our study indicates that the decrease in diatom biomass given elevated CO_2 levels was unique to the Bering Sea basin.

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505 CO_2 levels might be partially explained by the decrease in *Chaetoceros* spp. A previous
506 field incubation experiment conducted in the Bering Sea also showed that the carbon
507 biomass of the *Chaetoceros* spp. decreased at higher CO_2 levels (600–960 µatm CO_2),
508 although it increased at 1190 µatm CO_2 (Yoshimura et al., 2013). However, Tortell et al.
509 (2008) demonstrated that relative abundance of *Chaetoceros* spp. increased under
510 elevated CO_2 levels in the Ross Sea. In the previous laboratory culture experiments, the
511 effects of increased CO_2 on the growth and/or photosynthesis of *Chaetoceros* spp. were
512 also inconsistent. For example, Ihnken et al. (2011) demonstrated that the growth of
513 diatom *Chaetoceros muelleri* decreased with elevated CO_2 and decreased pH levels
514 although their photosynthetic capacity increased. In contrast, Trimborn et al. (2013)
515 showed a significant increase in the growth rate of *Chaetoceros debilis* under high CO_2
516 condition. In addition, no CO_2-related change in the growth and photosynthetic
517 physiology of *Chaetoceros brevis* was found (Boelen et al., 2011). These results suggest
518 that the responses to elevated CO_2 differ among *Chaetoceros* species.
519 The concentrations of 19'-Hex were significantly lower in the C-600 treatment than
520 those in the C-380 treatment (Fig. 1c), suggesting that the ocean acidification could
521 induce negative effects not only on the biomass of diatoms, but also on that of
522 haptophytes in the study area. Similar results were obtained from the previous field
523 studies in other regions (e.g., Feng et al., 2010; Endo et al., 2013). One possible factor
524 underlying these decreases is that the reduced carbonate-saturation states under high
525 CO_2 conditions. The energetic cost of calcification in coccolithophores will increase
526 with a decrease in pH (Mackinder et al., 2010). Therefore, additional energy might be
527 needed for cell growth in seawater with high CO_2 levels. In addition, non-calcifying
528 haptophytes such as *Phaeocystis* spp. often dominate among haptophytes in the natural
529 phytoplankton community (Schoemann et al., 2005), although the effects of ocean
530 acidification on them are still not well understood. Therefore, additional study using a
531 wide range of haptophyte species would be required for a detailed understanding of the
532 responses of the haptophyte community to CO_2-induced ocean acidification.
533 Our CHEMTAX outputs showed that the relative contributions of diatoms decreased
534 with increased CO_2 levels, whereas the contributions of haptophytes increased in both
535 the control and Fe-added bottles (Fig. 2). This indicates that the negative impacts of
536 increased CO_2 on diatoms were greater than those on haptophytes and other

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削除: however,

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削除: The decrease in diatom biomass at elevated CO_2 levels may have been induced by decreased CCM activity of diatom cells. A recent study demonstrated that elevated CO_2 levels could decrease the growth of diatoms under high-light conditions, likely due to an increase in photoinhibition (Gao et al., 2012). The authors suggested that CCMs act as a diffusive process for photoinhibition in high-light conditions, and therefore, diatoms tend to be more vulnerable to light stress due to the downregulation of CCM activities at higher CO_2 levels.

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削除: The ratios of 19'-Hex concentration between initial and the final days decreased in response to increased CO_2 levels regardless of Fe availability (Fig. 1b), suggesting that ocean acidification can induce negative effects on not only the biomass of diatoms but also that of haptophytes in the study area.

558 phytoplankton groups. Another possibility is that the competitions between diatoms and
559 other phytoplankton taxa could occur. For example, diatoms could become less
560 competitive when silicic acid is exhausted, because Si-depletion significantly depressed
561 the growth and could induce their cell death (Harrison et al., 1977; Jiang et al. 2014).
562 However, concentrations of silicic acid were not significantly different among CO_2
563 levels in the Fe-added treatments (Fig. S2f). Moreover, in the control treatments, silicic
564 acid was almost depleted in the low CO_2 treatment after day 5 but not in the high CO_2
565 treatment (Fig. S2e). These results suggest that availability of silicic acid little affected
566 the decreases in relative diatom contribution to Chl *a* biomass. Larger diatoms can
567 contribute to efficient transfer of energy and organic compounds to higher trophic levels
568 because they would create a shorter food chain compared with nano- and pico-sized
569 phytoplankton (Michaels and Silver, 1988). Because diatoms form a large part of
570 phytoplankton biomass in the Bering Sea basin (Suzuki et al., 2002; Takahashi et al.,
571 2002), the decrease in the relative contribution of diatoms with increasing CO_2 could
572 reduce the energy transferred from the primary producers to the higher trophic levels.
573 The decreases in Fuco growth ratio and relative contribution of diatoms were larger
574 in the control bottles than those in the Fe-added treatments (Figs. 1a and 2), suggesting
575 that the negative effect of CO_2 enrichment was greater in the Fe-limited conditions.
576 These results are consistent with Sugie et al. (2013) and Yoshimura et al. (2014), who
577 observed significant decreases in diatom carbon biomass and particulate organic carbon
578 (POC) production under high CO_2 levels in the control treatments, whereas those were
579 insignificantly changed in the Fe-added treatments. Sugie et al. (2013) indicated that the
580 Fe limitations for phytoplankton in the control bottles were enhanced at high CO_2 levels,
581 likely due to the reduction of Fe bioavailability as reported in Shi et al. (2010). The
582 combined effects of CO_2 and Fe availability were also tested in a diatom-dominated
583 phytoplankton community in the Southern Ocean (Hoppe et al., 2013). In their study,
584 net primary productivity in seawater decreased with increased pCO_2 levels in the
585 Fe-depleted treatments but not in the Fe-enriched treatments. These studies indicate that
586 an interactive effect of CO_2 enrichment and Fe limitation could occur in the
587 diatom-dominated natural phytoplankton assemblages in the HNLC region.
588
589 **4.2 *rbcL* expression in diatom**

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591 A significant correlation between diatom *rbcL* copies per liter and Fuco
592 concentration was found in this study (Fig. 3), suggesting the usefulness of the *rbcL*
593 gene fragment as a proxy for diatoms. In addition, the cDNA sequences obtained from
594 cloning were dominated by the diatom-derived *rbcL* gene (Fig. 6). These results
595 indicate that the *rbcL* primers used successfully and selectively amplified the *rbcL* gene
596 of diatoms. Suzuki et al. (2011) showed that Fuco concentration significantly correlated
597 with diatom carbon biomass in the subarctic Pacific. Furthermore, Matsuda et al. (2011)
598 showed that the number of *rbcL* gene per cell varies among diatom species, and it was
599 positively correlated with cell size. Therefore, we concluded that the *rbcL* gene could
600 serve as a potential molecular marker for diatom biomass.

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削除: A significant correlation between *rbcL* copy number in diatoms and Fucox concentration was found in this study (Fig. 3), suggesting the usefulness of the *rbcL* gene fragment as a proxy for diatoms as well as Fucox.

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601 The transcription of the diatom-specific *rbcL* gene decreased with elevated CO_2
602 levels in both the control and Fe-added treatments (Fig. 4). Because RubisCO
603 expression is primarily controlled at the transcriptional level in the natural
604 phytoplankton community (Xu and Tabita, 1996; Wawrik et al., 2002), our results
605 suggest that increased CO_2 levels could reduce the amount of RubisCO in diatoms. It
606 should be noted that significant decreases in *rbcL* expression with increased CO_2 levels
607 were observed on days 2 or 3, when macronutrients still remained (Fig. S2). This
608 indicates that the downregulation of *rbcL* expression in diatoms was probably caused by
609 the increase in CO_2 availability. It has been shown that some land plants can increase
610 their nitrogen utilization efficiency under elevated CO_2 levels by reducing the
611 investment of nitrogen in RubisCO (Curtis et al., 1989; Makino et al., 2003). Losh et al.
612 (2012; 2013) also demonstrated a decreased RubisCO contribution to the total protein in
613 the California Current phytoplankton community with an increase in CO_2 level.
614 Because a decrease in the expression of RubisCO can result in a reduction of the
615 potential capacity for carbon fixation in the natural environment (John et al., 2007b),
616 our results indicate that an increase in CO_2 levels could have a negative impact on
617 photosynthetic carbon fixation for diatoms in the study area. Recently, Gontero and
618 Salvucci (2014) pointed out that RubisCO activase plays a key role in the modification
619 of RubisCO activity, and consequently in the capacity of carbon fixation, although the
620 occurrence of RubisCO activase in diatoms is not well understood. Further studies must
621 be needed for better understanding of the impacts of elevated CO_2 on photosynthetic

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削除: Sugie et al., 2013

630 physiology in diatoms.

631 The negative effects of increasing CO_2 on diatom biomass were not severe in the
632 Fe-added bottles relative to Fe-limited control bottles (Figs. 1a and b), whereas *rbcL*
633 transcription decreased with increased CO_2 regardless of Fe availability (Fig. 4). This
634 suggests that the diatoms could overcome the decrease in RubisCO activity in the
635 Fe-added treatments. According to our cloning data (Fig. 6), a shift in phylogenetic
636 composition of the diatoms actively transcribed *rbcL* was observed in the Fe-added
637 bottles. In addition, F_v/F_m values increased significantly with Fe enrichment in our
638 incubation experiments (Sugie et al., 2013), indicating an increase in the photochemical
639 quantum efficiency of photosystem II for the diatoms. Therefore, the photosystem II
640 activity might compensate for the decrease in RubisCO expression under Fe-replete
641 conditions.

642 It is generally recognized that phytoplankton autonomously regulate the transcription
643 of the *rbcL* gene in response to environmental conditions such as light and nutrient
644 availability (Pichard et al., 1996; Granum et al., 2009; John et al., 2010). However, the
645 mechanisms controlling the transcription of RubisCO operon in diatoms are largely
646 unknown. Recently, Minoda et al. (2010) showed that the red alga *Cyanidioschyzon*
647 *merolae* increased *rbcL* transcription at high levels of NADPH, 3-phoshoglyceric acid
648 (3-PGA), or ribulose-1,5-bisphosphate (RuBP) under the influence of the transcription
649 factor Ycf30. In addition, it has been reported that regeneration of RuBP could be a
650 limiting factor for the CBB cycle in high CO_2 conditions (von Caemmerer and Farquhar,
651 1981; Stitt, 1991; Onoda et al., 2005). Thus, one possible mechanism underlying the
652 reduction of diatom *rbcL* transcription observed in our study is related to a decrease in
653 RuBP concentration in the chloroplasts due to the increase in CO_2 availability for
654 diatoms. Because diatoms possess the same type of RubisCO (Form ID) and gene
655 homologs encoding the Ycf30 protein (i.e., *ycf30*) (Kowallik et al., 1995), they could
656 control *rbcL* gene expression using the same mechanisms as *C. merolae*. Further studies
657 using marine diatom cultures are required to obtain a better understanding of the
658 physiological mechanisms controlling the expression of RubisCO.

659 In our experiment, the rarefaction curves plateaued to some extent in all treatments
660 (Fig. 5), indicating that the clone numbers screened from each library were statistically

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削除: potentially by induction of CCMs given that diatoms can upregulate CCM activity at elevated CO_2 levels (e.g., Trimborn et al., 2009). In the Fe-added treatments, photosynthetic carbon fixation in diatoms could not be limited by CO_2 availability as a consequence of the CCMs. This speculation is supported by the values of the maximum photochemical quantum yield (F_v/F_m) of photosystem II (PSII). In our incubation experiments, F_v/F_m values increased significantly with Fe enrichment (Sugie et al., 2013), suggesting that phytoplankton could produce sufficient energy for CCMs in the Fe-added bottles. However, we speculate that CCMs in the diatoms might not be active in the control treatments because Fe deficiency could reduce the functionality of algal CCMs due to a reduction in their light energy-harvesting ability (Giordano et al., 2005).

682 sufficient for further diversity analysis. Taxonomic compositions in the cDNA library
683 were considerably different from those in the diatom carbon biomass revealed by
684 microscopic analysis by Sugie et al. (2013), which were composed primarily by
685 Chaetocerataceae. This implies that the predominant diatoms did not necessarily
686 become transcriptionally active *rbcL* phylotypes in our experiment. In addition, because
687 16–42% of the sequences were classified as unidentified diatoms or other eukaryotes,
688 the primer set used in this study might be insufficient to estimate diatom composition at
689 the family level.

690 The *rbcL* cDNA libraries in the Fe-added treatments differed significantly from the
691 initial library, whereas those in the control treatments were not significantly different
692 (Table 3), suggesting that the diatom blooms induced by Fe infusion were associated
693 with the change in the relative contribution of *rbcL* expression in diatoms. For example,
694 compared to the initial seawater, the relative contributions of Chaetocerataceae and
695 unidentified centrics to the *rbcL* cDNA library increased markedly in the Fe-added
696 bottles whereas they remained minor components in the control bottles (Fig. 6). This
697 indicates that the relative significance of the RubisCO activity of these phylotypes could
698 be increased by Fe enrichment. In addition, cDNA libraries were significantly different
699 from each other at different CO_2 levels in the Fe-added bottles (Table 3). This indicates
700 that the transcriptionally active phylotypes in diatoms could shift in response to an
701 increase in the CO_2 level. On the other hand, the diversity indices for the
702 diatom-specific *rbcL* cDNA sequences were not affected by CO_2 availability (Table 2).
703 In addition, the highest chao1 (richness) value was observed in C-600 treatment. These
704 results suggest that the richness and/or diversity of diatom phylotypes actively
705 transcribing *rbcL* gene could remain under elevated CO_2 levels.
706

707 **5 Conclusion**

708 The present study showed that an increase in CO_2 levels could have negative impacts
709 on diatom biomass in the Bering Sea, especially under Fe-limited conditions. Because
710 diatoms play pivotal roles in carbon sequestration and food webs in the Bering Sea
711 (Springer et al., 1996; Takahashi et al., 2002), our results indicate that ocean
712 acidification might alter the biogeochemical processes and ecological dynamics in the

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削除: The diversity of the diatom-specific *rbcL* cDNA sequences decreased with increased CO_2 levels in terms of number of OTUs and the Shannon-Wiener and Simpson's indices (Table 2). These results suggest that the number of diatom phylotypes actively transcribing the *rbcL* gene decreased with increased CO_2 level. Because the different types of diatoms could have different functions in relation to sinking flux and trophic position in the ecosystem (e.g., Michaels and Silver, 1988), a decrease in diatom biodiversity could cause a decrease in the functional diversity of marine ecosystems. Furthermore, a decrease in biodiversity may weaken the ecological stability of phytoplankton ecosystems and make them more vulnerable to other environmental changes such as an increase in sea surface temperature (Overland and Stabeno, 2004).

733 study area. Although the present results cannot be extrapolated to other HNLC
734 ecosystems due to differences in other environmental conditions, our findings suggest
735 that the combined effects of CO_2 and other environmental factors such as Fe availability
736 need to be examined for a better understanding of the potential impacts of ocean
737 acidification on marine ecosystems.

738 We examined, for the first time, the relationships between CO_2 levels or Fe
739 availability and RubisCO expression of diatoms in the Bering Sea. Significant decreases
740 in the *rbcL* expression of diatoms were observed at elevated CO_2 levels in both the
741 Fe-limited and Fe-enriched treatments, suggesting that ocean acidification could reduce
742 the primary productivity in the study area. Our results indicate that the amount of *rbcL*
743 transcripts could be an important indicator to assess the physiological responses of
744 RubisCO activity in diatoms to environmental drivers. However, photosynthetic carbon
745 fixation in diatoms can be controlled not only by RubisCO activity, but also other
746 processes such as carbon concentrating mechanisms (CCMs) and/or RuBP regeneration
747 (Rost et al., 2003; Onoda et al., 2005). More detailed studies on molecular mechanisms
748 are required to clarify the physiological responses of the diatom community to CO_2 and
749 Fe availability.

750

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759

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削除: However, because carbon fixation in diatoms is controlled not only by RubisCO activity but also by CCMs (Rost et al., 2003), more detailed studies of molecular mechanisms are required to clarify the physiological responses of the diatom community to CO_2 and Fe enrichments.

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1040

1041 **Table 1.** Carbonate chemistry, nutrients, and Fe parameters (value ± 1 standard
1042 deviation, n = 3) during the incubation experiment. Carbonate parameters are the initial
1043 and mean values throughout the incubation. Macronutrients and Fe parameters are the
1044 values at the initial or final sampling days (i.e., day 5 for the control and day 6 for the
1045 Fe-added treatments). Standard deviation was not assessed for initial TD-Fe
1046 concentration because samples were collected from single source. See figures S1 and S2
1047 for the complete data set.
1048

	DIC (μmol kg^{-1})	TA (μmol kg^{-1})	pCO_2 (μatm)	CO_2 (μmol kg^{-1})	pH (Total scale)
C-Initial	2086.4 ± 2.8	2249.1 ± 5.0	388.4 ± 18.1	18.4 ± 0.9	8.05 ± 0.02
C-380	2075.5 ± 8.1	2252.9 ± 10.8	355.7 ± 34.7	16.8 ± 1.6	8.09 ± 0.04
C-600	2151.6 ± 7.8	2250.9 ± 4.7	604.1 ± 36.2	28.5 ± 1.7	7.88 ± 0.02
Fe-Initial	2085.3 ± 0.8	2250.0 ± 4.9	383.4 ± 12.6	18.1 ± 0.6	8.06 ± 0.01
Fe-180	1959.9 ± 62.0	2244.1 ± 16.0	202.0 ± 50.9	9.5 ± 2.4	8.21 ± 0.10
Fe-380	2068.5 ± 27.7	2235.7 ± 14.9	375.9 ± 47.9	17.8 ± 2.3	8.01 ± 0.05
Fe-600	2120.6 ± 33.5	2248.5 ± 12.0	512.6 ± 135.5	24.2 ± 6.4	7.96 ± 0.11
Fe-1000	2200.2 ± 12.6	2248.4 ± 9.8	913.8 ± 159.8	43.2 ± 7.6	7.72 ± 0.07

1049
1050 **Table 1.** (Continued)
1051

	Nitrate (μmol L^{-1})	Phosphate (μmol L^{-1})	Silicic acid (μmol L^{-1})	TD-Fe (nmol L^{-1})
C-Initial	18.06 ± 0.10	1.47 ± 0.01	16.95 ± 0.12	1.35
C-380	7.09 ± 0.27	0.65 ± 0.02	0.28 ± 0.05	0.27 ± 0.03
C-600	12.01 ± 0.27	0.98 ± 0.02	3.04 ± 0.32	0.29 ± 0.04
Fe-Initial	18.09 ± 0.11	1.47 ± 0.01	16.90 ± 0.12	5.50 ± 0.10
Fe-180	0.13 ± 0.04	0.10 ± 0.01	0.66 ± 0.09	4.60 ± 0.19
Fe-380	0.09 ± 0.00	0.12 ± 0.04	0.50 ± 0.01	4.48 ± 0.12
Fe-600	0.08 ± 0.00	0.10 ± 0.00	0.50 ± 0.01	4.34 ± 0.08
Fe-1000	0.08 ± 0.00	0.08 ± 0.02	0.47 ± 0.02	4.18 ± 0.24

1052

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削除: Macronutrients and Fe parameters are the values at the initial or final sampling days. See Sugie et al. (2013) for the complete data set.

1057 **Table 2.** Number of OTUs, richness index, and diversity indices (value ± 95%
1058 confidence interval) for *rbcL* cDNA libraries obtained from the initial seawater and the
1059 incubation bottles on day 2 (Fe-380 and Fe-600) and day 3 (C-380 and C-600).
1060

Library	No. of sequences	No. of OTUs	Chao1	*H*'	1−*D*
Initial	35	10	25.0	1.81 ± 0.32	0.197 ± 0.086
C-380	50	15	20.0	1.98 ± 0.36	0.232 ± 0.110
C-600	50	14	29.0	1.60 ± 0.41	0.369 ± 0.148
Fe-380	50	13	23.0	2.24 ± 0.23	0.116 ± 0.042
Fe-600	50	12	19.5	2.01 ± 0.26	0.158 ± 0.053

1062

Endo Hisashi 2015/2/26 16:43
削除: **Table 2.** Number of OTUs and diversity indices for *rbcL* cDNA libraries obtained from the initial seawater and the incubation bottles on day 2 (Fe-380 and Fe-600) and day 3 (C-380 and C-600).
No. of sequences ... [4]

1069 **Table 3.** Significance levels for differences among *rbcL* cDNA libraries as calculated
1070 with LIBSHUFF. *p* values < 0.05 are bolded.
1071

	Library (Y)				
	Initial	C-380	C-600	Fe-380	Fe-600
Library (X)					
Initial	—	0.434	0.573	0.383	0.587
C-380	0.153	—	0.086	0.101	0.898
C-600	0.523	0.500	—	**0.004**	0.033
Fe-380	**<0.001**	**<0.001**	**<0.001**	—	**0.002**
Fe-600	**0.009**	**0.004**	**<0.001**	0.030	—

1072

1073 **Figure captions**

1074

1075 **Figure 1.** Temporal changes in fucoxanthin (a and b) and 19'-hexanoyloxyfucoxanthin
1076 (c and d) concentrations. Left (a and c) and right graphs indicate data from
1077 the control and Fe-added treatments, respectively. Error bars denote ± 1
1078 standard deviation (SD, n = 3). Standard deviations were not assessed on
1079 days 2 (Fe-added treatmets) and 3 (control treatments) because samples were
1080 collected from each single bottle.
1081

1082 **Figure 2.** Mean contributions of each phytoplankton group to total Chl-*a* biomass
1083 estimated by CHEMTAX in the (A) control bottles at 380 and 600 ppm CO_2,
1084 and (B) Fe-added bottles at 180, 380, 600 and 1000 ppm CO_2 (n = 2 or 3).
1085

1086 **Figure 3.** Relationship between fucoxanthin (Fucox) concentration and diatom-specific
1087 *rbcL* copy number ($y = 7.62\times10^8x+1.90\times10^8$, $r^2 = 0.677$, $p < 0.001$, n = 28).
1088

1089 **Figure 4.** Abundances of *rbcL* mRNA (cDNA) normalized to *rbcL* gene copy number
1090 (*rbcL* cDNA/DNA) in the control bottles on day 3 and the Fe-added bottles
1091 on day 2. Open bars and closed bars denote control and Fe-added treatments,
1092 respectively. Error bars indicate ± 1 SD (n = 3).
1093

1094 **Figure 5.** Rarefaction analysis of the diatom-specific *rbcL* clone libraries. The
1095 rarefaction curves, plotting the number of operational taxonomic units
1096 (OTUs) as a function of the number of sequences, were computed by the
1097 software mothur. C and Fe indicate the control and Fe-added treatments,
1098 respectively.
1099

1100 **Figure 6.** Relative phylotype contributions in the *rbcL* cDNA libraries obtained from
1101 the initial seawater and the incubation bottles at day 2 (Fe-380 and Fe-600)
1102 and day 3 (C-380 and C-600).

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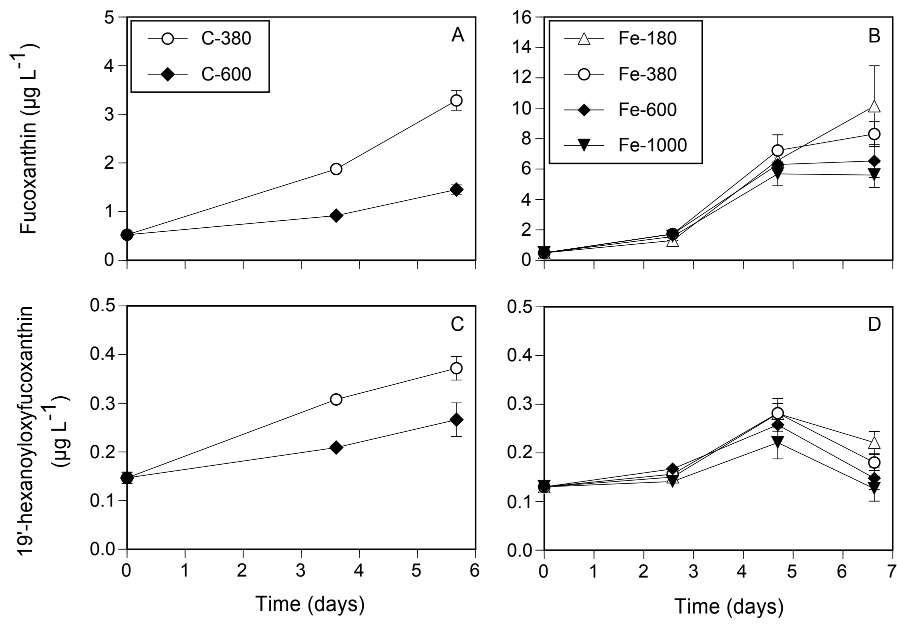
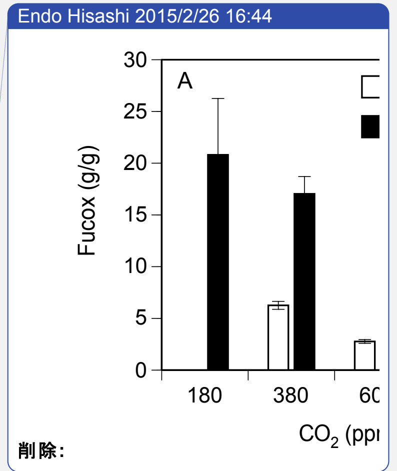
削除: **Figure 1.** Ratios of (A) fucoxanthin (Fucox) or (B) 19'-hexanoyloxyfucoxanthin (19'-Hex) concentrations at the initial and final sampling day (final day/initial). Open bars and closed bars denote control and Fe-added treatments, respectively. Error bars denote ± 1 standard deviation (SD, n = 3).

1116

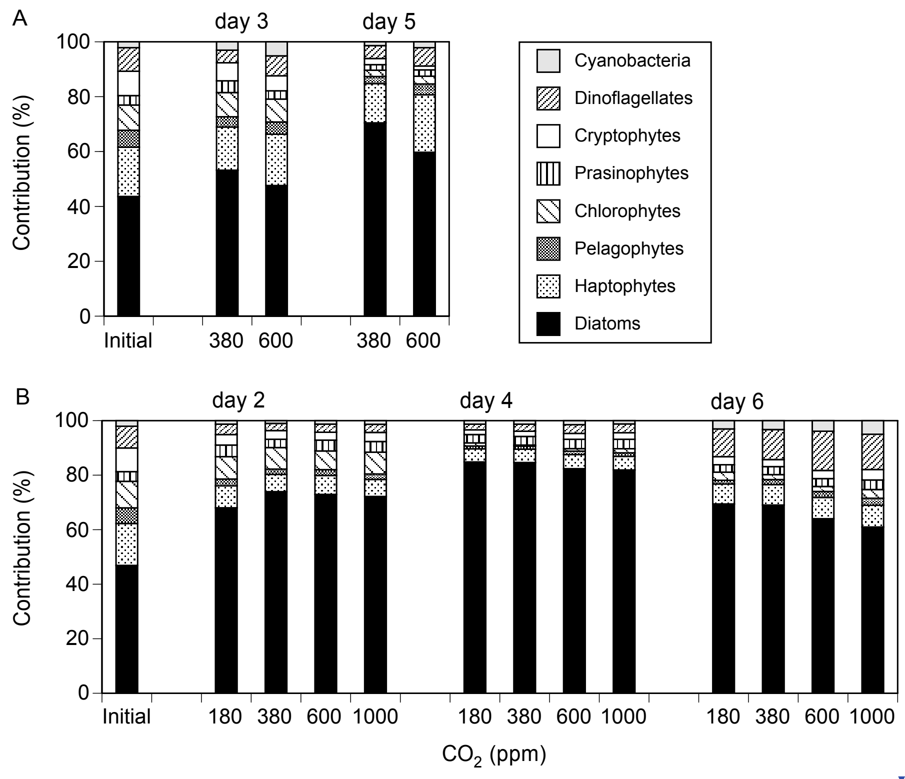


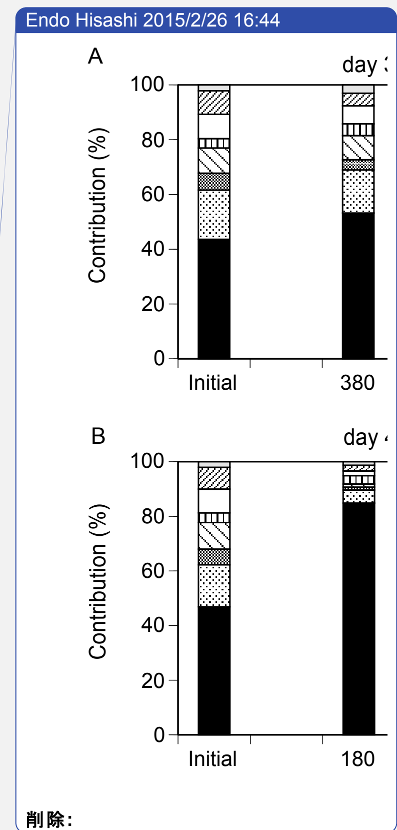


1117 **Figure 1**

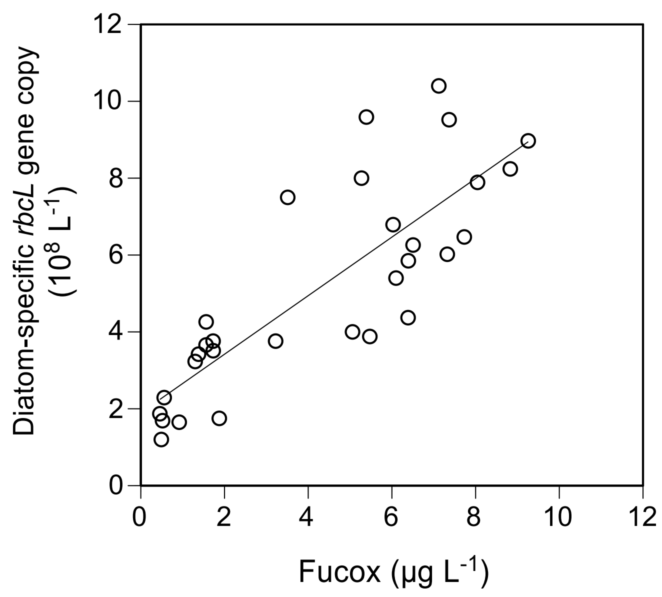
1118

1120





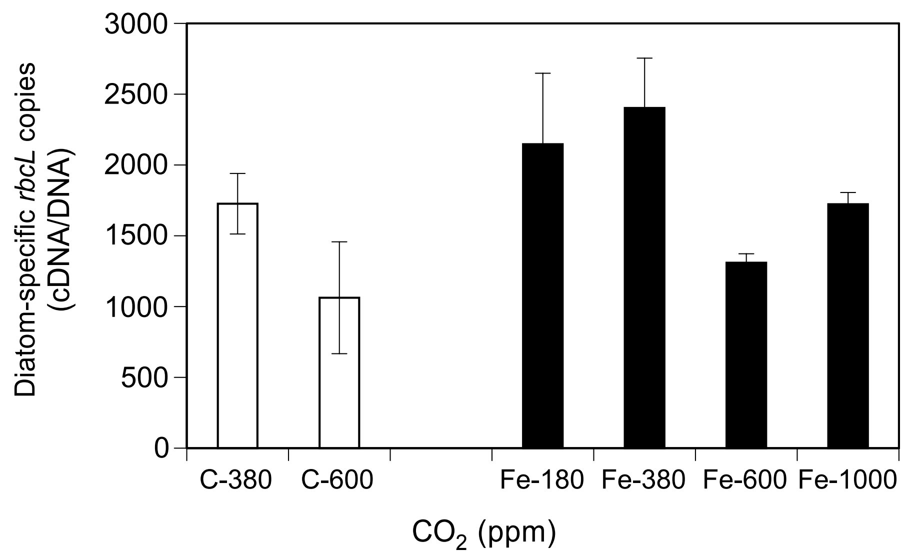
1121 **Figure 2**



1123

1124 **Figure 3**

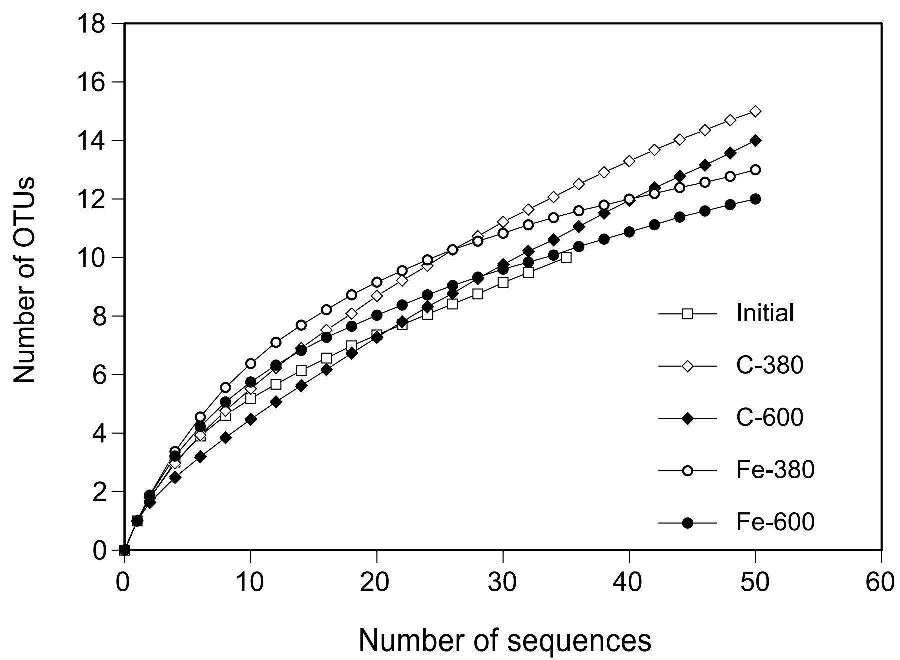
1125



1126

1127 **Figure 4**

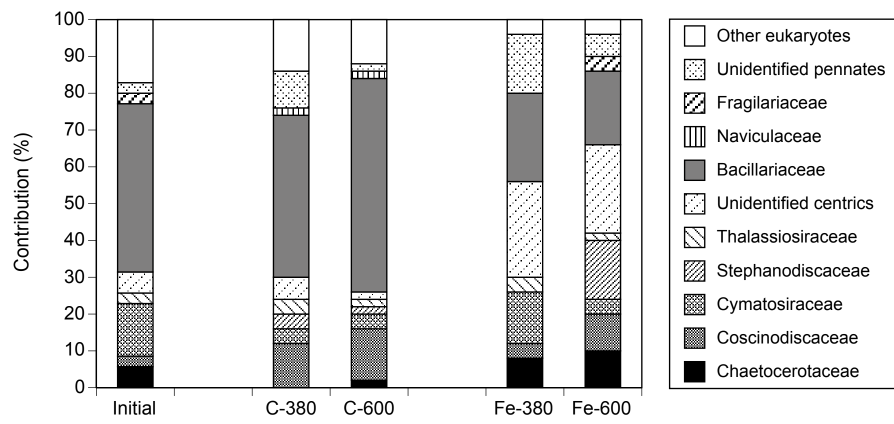
1128



1129

1130 **Figure 5**

1131



1132

1133 **Figure 6**

1134

1135 # Supplementary Material
1136
1137

1138 ## Effects of CO_2 and iron availability on *rbcL* gene expression in
1139 ## Bering Sea diatoms

1140

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1148 270-1194, Japan}
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1150 Technology (JAMSTEC), 3173-25 Showa-machi, Kawasaki-ku, Yokohama, Kanagawa
1151 236-0001, Japan}
1152 Correspondence to: H. Endo (endo@ees.hokudai.ac.jp)
1153

1154 **Table S1.** Sampling opportunities for each parameter during the incubation period.
1155

		Incubation time (day)						
		0	1	2	3	4	5	6
Control	TA	○	○		○		○	
	DIC	○	○		○		○	
	Nutrients	○	○	○	○	○	○	○
	TD-Fe	○						○
	Chl *a*	○	○	○	○	○	○	○
	HPLC	○			○		○	
	DNA	○			○		○	
	RNA	○			○		○	
Fe-added	TA	○	○	○		○		○
	DIC	○	○	○		○		○
	Nutrients	○	○	○	○	○	○	○
	TD-Fe	○						○
	Chl *a*	○	○	○	○	○	○	○
	HPLC	○		○		○		○
	DNA	○		○		○		○
	RNA	○		○		○		○

1156

Table S1. 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).

	Fuco	19'-But	19'-Hex	Peri	Diadinox	Allox	Violax	Prasinox	Chl-*b*	Zeax	Chl-*a*
(A)											
Diatoms	0.75	0	0	0	0.24	0	0	0	0	0	1
Hapto	0	0	1.4	0	0.16	0	0	0	0	0	1
Pelago	0.62	0.93	0	0	0.44	0	0	0	0	0	1
Chloro	0	0	0	0	0	0	0.03	0	0.28	0.06	1
Prasino	0	0	0	0	0	0	0.11	0.36	0.89	0	1
Crypto	0	0	0	0	0	0.14	0	0	0	0	1
Dino	0	0	0	0.53	0	0	0	0	0	0	1
Cyano	0	0	0	0	0	0	0	0	0	0.33	1
(B)											
Diatoms	1.5	0	0	0	0.48	0	0	0	0	0	1
Hapto	0	0	2.8	0	0.32	0	0	0	0	0	1
Pelago	1.24	1.86	0	0	0.88	0	0	0	0	0	1
Chloro	0	0	0	0	0	0	0.06	0	0.56	0.12	1
Prasino	0	0	0	0	0	0	0.22	0.72	1.78	0	1
Crypto	0	0	0	0	0	0.28	0	0	0	0	1
Dino	0	0	0	1.06	0	0	0	0	0	0	1
Cyano	0	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	1
Hapto	0	0	0.7	0	0.08	0	0	0	0	0	1
Pelago	0.31	0.465	0	0	0.22	0	0	0	0	0	1
Chloro	0	0	0	0	0	0	0.015	0	0.14	0.03	1
Prasino	0	0	0	0	0	0	0.055	0.18	0.445	0	1
Crypto	0	0	0	0	0	0.07	0	0	0	0	1
Dino	0	0	0	0.265	0	0	0	0	0	0	1
Cyano	0	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	1
Hapto	0	0	0.75	0	0.25	0	0	0	0	0	1
Pelago	0.75	0.75	0	0	0.5	0	0	0	0	0	1
Chloro	0	0	0	0	0	0	0.25	0	0.5	0.25	1
Prasino	0	0	0	0	0	0	0.25	0.5	0.75	0	1
Crypto	0	0	0	0	0	0.25	0	0	0	0	1
Dino	0	0	0	0.5	0	0	0	0	0	0	1
Cyano	0	0	0	0	0	0	0	0	0	0.5	1

Abbreviations: Hapto, Haptophytes; Pelago, Pelagophytes; Chloro, Chlorophytes; Crypto, Cryptophytes; Dino, Dinoflagellates; Cyano, Cyanobacteria; Fuco, 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*.

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削除: Fucox

1171 **Table S2.** Final pigment:Chl-*a* ratio matrices obtained by the CHEMTAX program. (A)
1172 Control and (B) Fe-added treatments.
1173

	Fuco	19'-But	19'-Hex	Peri	Diadinox	Allox	Violax	Prasinox	Chl-*b*	Zeax	Chl-*a*
(A)											
Diatoms	1.6	0	0	0	0.27	0	0	0	0	0	1
Hapto	0	0	1.1	0	0.16	0	0	0	0	0	1
Pelago	0.56	0.72	0	0	0.37	0	0	0	0	0	1
Chloro	0	0	0	0	0	0	0.06	0	0.17	0.08	1
Prasino	0	0	0	0	0	0	0.03	0.28	1.2	0	1
Crypto	0	0	0	0	0	0.11	0	0	0	0	1
Dino	0	0	0	0.42	0	0	0	0	0	0	1
Cyano	0	0	0	0	0	0	0	0	0	0.35	1
(B)											
Diatoms	1.9	0	0	0	0.29	0	0	0	0	0	1
Hapto	0	0	0.86	0	0.18	0	0	0	0	0	1
Pelago	0.69	0.84	0	0	0.44	0	0	0	0	0	1
Chloro	0	0	0	0	0	0	0.01	0	0.24	0.03	1
Prasino	0	0	0	0	0	0	0.16	0.42	1.1	0	1
Crypto	0	0	0	0	0	0.14	0	0	0	0	1
Dino	0	0	0	0.64	0	0	0	0	0	0	1
Cyano	0	0	0	0	0	0	0	0	0	0.50	1

1174 Abbreviations: as in Table S1.
1175

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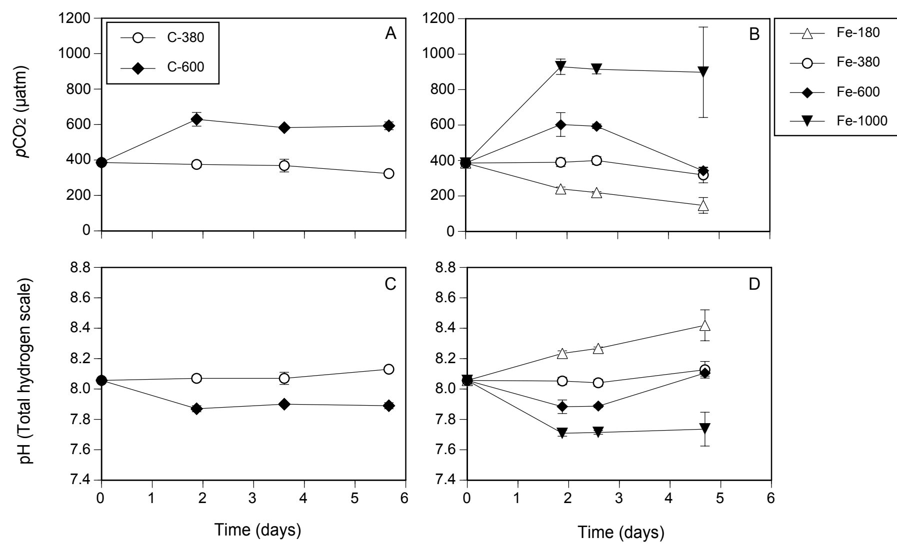
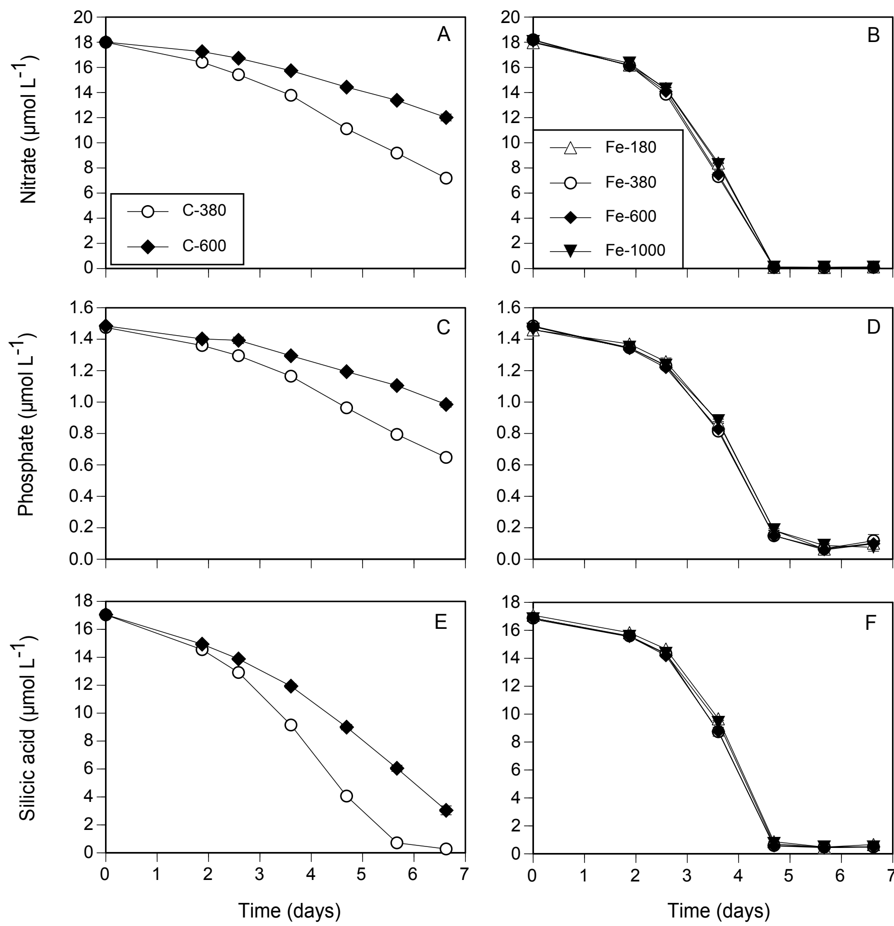
1177



1178

1179 **Figure S1.** Time course of pCO_2 (a and b) and pH (c and d) calculated from TA and
1180 DIC. Left (a and c) and right (b and d) graphs indicate data from the control and
1181 Fe-added treatments, respectively (redrawn from Sugie et al., 2013). Error bars denote ±
1182 1 SD (n = 3).

1183



1184

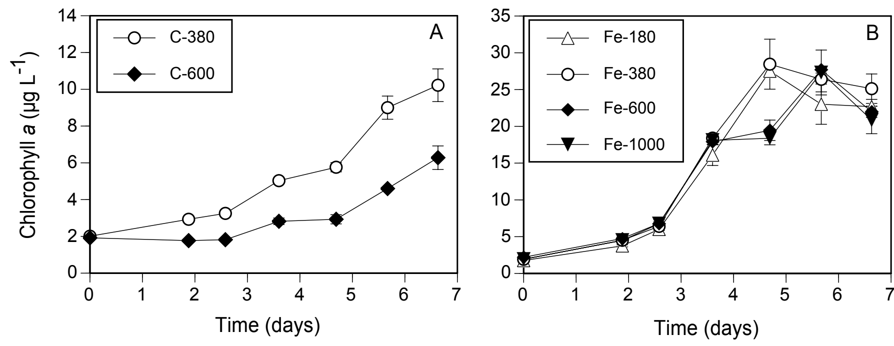
1185

1186 **Figure S2.** Time course of nitrate (a and b), phosphate (c and d), and silicic acid (e and
1187 f). Left (a, c, and e) and right (b, d, and f) graphs indicate data from the control and
1188 Fe-added treatments, respectively (redrawn from Sugie et al., 2013). Error bars denote ±
1189 1 SD (n = 3).

1190

1191

1192



1193

1194 **Figure S3.** Temporal changes in chlorophyll *a* concentration. Left and right graphs
1195 indicate data from the control and Fe-added treatments, respectively (redrawn from
1196 Sugie et al., 2013). Error bars denote ± 1 SD (n = 3).

1197

1198

1199 Reply to Referee #1 Dr. D. Campbell,

1200

1201 Thank you very much for your positive and constructive comments on our discussion paper.
1202 Below are our point-by-point responses to your comments.

1203

1204 1. *Materials & Methods: Given the importance to the findings, I think the authors should*
1205 *include a diagramatic figure of the standards, the amplification primers, and the*
1206 *amplicons used for the DNA and cDNA quantitations. From the text, I infer that the*
1207 *standard is only 113 bp long, for the DNA quantitations, but that a different standard was*
1208 *used for cDNA (length?). The primers 5'- GATGATGARAAYATTAACTC-3', reverse*
1209 *primer: 5'-TAWGAACCTTTWACTTCWCC-3'. are 19-20 bases long, leaving an amplified*
1210 *region of only 60 bp between the primers. It appears (but I am not sure) that the same*
1211 *primers are used for both DNA and cDNA quantitation. If so, why would you use two*
1212 *different quantitation standards?*
1213 We used the same region (same length and same sequence) for both DNA and cDNA
1214 quantifications. However, double stranded DNA and single stranded cDNA standards were
1215 used for DNA and cDNA samples, respectively, because these samples should be
1216 quantified as copy numbers. According to Smith et al. (2006), standard curves must be
1217 constructed from single stranded cDNA for the accurate determination of RNA transcript
1218 numbers, because cDNA exists as a single stranded form in the samples. We consider that
1219 the diagrammatic figure is not necessarily to explain our qPCR method, since we followed
1220 the general procedures described in Smith et al. (2006) and John et al. (2007).
1221 Alternatively, we have added the following sentence to the revised manuscript (see Lines
1222 218–219 in the track changes version):
1223 "Following Smith et al. (2006), we used double-stranded DNA and single-stranded cDNA
1224 standards for DNA and cDNA quantification, respectively."

1225

1226 2. *Discussion: "Our study indicates that the decrease in diatom biomass given elevated CO2*
1227 *levels was unique to the Bering Sea basin." No. Unique would mean that this response is*
1228 *only present in the Bering Sea, and we do not know that yet. In fact a preceding sentence*
1229 *mentions similar responses in the Okhotsk Sea.*
1230 This sentence has been deleted (see Line 486 in the track changes version).

1231

1232 3. *"However, we speculate that CCMs in the diatoms might not be active in the control*
1233 *treatments because Fe deficiency could reduce the functionality of algal CCMs due to a*

1234 *reduction in their light energy-harvesting ability (Giordano et al., 2005)." This needs to*
1235 *be better explained. It is unlikely that Fe deficiency would limit CCM simply through a*
1236 *limitation on light energy harvesting.*
1237 According to the valuable comments from you and other reviewers, we have excluded the
1238 discussion on CCMs and reconstructed the corresponding paragraph as follows (see Lines
1239 631−641 in the track changes version):
1240 "The negative effects of increasing CO_2 on diatom biomass were not severe in the
1241 Fe-added bottles relative to Fe-limited control bottles (Figs. 1a and b), whereas *rbcL*
1242 transcription decreased with increased CO_2 regardless of Fe availability (Fig. 4). This
1243 suggests that the diatoms could overcome the decrease in RubisCO activity in the
1244 Fe-added treatments. According to our cloning data (Fig. 6), a shift in phylogenetic
1245 composition of the diatoms actively transcribed *rbcL* was observed in the Fe-added bottles.
1246 In addition, F_v/F_m values increased significantly with Fe enrichment in our incubation
1247 experiments (Sugie et al., 2013), indicating an increase in photochemical quantum
1248 efficiency of photosystem II for the diatoms. Therefore, the photosystem II activity might
1249 compensate for the decrease in RubisCO expression under Fe-replete conditions."
1250
1251 4. *"However, because carbon fixation in diatoms is controlled not only by RubisCO activity*
1252 *but also by CCMs (Rost 20 et al., 2003)," Actually, in the discussion you raised the issue*
1253 *of RuBP regeneration as a limiting factor under elevated CO2 as well.*
1254 We have amended the sentence as follows (see Lines 744−749 in the track changes
1255 version):
1256 "However, photosynthetic carbon fixation in diatoms can be controlled not only by
1257 RubisCO activity, but also other processes such as carbon concentration mechanisms
1258 (CCMs) and/or RuBP regeneration (Rost et al., 2003; Onoda et al., 2005). More detailed
1259 studies on molecular mechanisms are required to clarify the physiological responses of the
1260 diatom community to CO_2 and Fe enrichments."
1261
1262 5. *Technical corrections: Table 1: "Macronutrients and Fe parameters are the values at the*
1263 *initial or final sampling days." Is the final sampling day 4? or day 6? Or either depending*
1264 *upon the particular treatment? I think this needs to be defined.*
1265 We have modified the caption of Table 1 as follows (see Lines 1049−1051 in the track
1266 changes version):
1267 "Macronutrients and Fe parameters are the values at the initial or final sampling days (i.e.,
1268 day 5 for the control and day 6 for the Fe-added treatments)."

1269 In addition, we have added a supplemental table (Table S1) showing the sampling times
1270 for each parameter (see Lines 1161−1163 in the track changes version).
1271
1272 *6. Given the large drops in NO3-, PO43- and silicic acid, what is the time course? By the*
1273 *final sampling points the cells were likely limited by macronutrients.*
1274 Macronutrients were depleted after days 4 or 5 in the Fe-added treatments (see Lines 310–
1275 311 in the track changes version), suggesting that the phytoplankton cells were limited by
1276 nutrient availability at the final sampling day. We have added the time course of
1277 macronutrients in the supplementary (Fig. S2; see Lines 1191−1198 in the track changes
1278 version).
1279
1280 *7. Figure 1 legend: define the basis of the normalization (g pigment/g chlorophyll a, I think).*
1281 In the previous manuscript, the pigment concentration on the final sampling days was
1282 divided by initial concentration of the same pigment. However, in the revised manuscript,
1283 figure 1 has been replaced by the graphs showing temporal changes in the concentrations
1284 of fucoxanthin and 19'-hexanoyloxyfucoxanthin, following the suggestions from the other
1285 reviewers.
1286
1287 *8. Figure 3 & Results: Fucox This is not a standard abbreviation. Why use it? Why not just*
1288 *write Fucoxanthin? Fucox also has an unfortunate pronounciation in English. Discussing*
1289 *the 'Fucox' graph is going to make people think of rude behaviour with neutered male*
1290 *cattle ;).*
1291 Following the kind suggestions from you and the reviewer #2, we have amended the
1292 abbreviation "Fucox" to "Fuco".
1293
1294 *9. Dinoflagellates, not dinoflagillates*
1295 Corrected (see Line 354 in the track changes version).
1296
1297 *10. "and diatoms that were neither centrics nor pennates" Do you mean diatom sequences*
1298 *that could be assigned to centrics or pennates? Or diatoms that are actually something*
1299 *other than centric or pennate? I did not know about any.*
1300 We intended that "diatoms which could be assigned to centrics and pennates". We have
1301 amended the sentence (see Lines 430−431 in the track changes version).
1302

1303 *11. "A significant correlation between rbcL copy number in diatoms and Fucox concentration*
1304 *was found in this study (Fig. 3), suggesting the usefulness of the rbcL gene fragment as a*
1305 *proxy for diatoms as well as Fucox." I think, rather: "A significant correlation between*
1306 *diatom rbcL copies per litre and Fucox concentration was found in this study (Fig. 3),*
1307 *suggesting the usefulness of the rbcL gene fragment as a proxy for diatom biomass."*
1308 Corrected (see Lines 591−593 in the track changes version). Thank you for your kind
1309 suggestion.
1310
1311 References:

1312 1. Smith, C. J., Nedwell, D. B., Dong, L. F., and Osborn, A. M.: Evaluation of quantitative
1313 polymerase chain reaction-based approaches for determining gene copy and gene transcript
1314 number in environmental samples, Environ. Microbiol, 8, 804–815, 2006.
1315 2. John, D. E., Patterson, S. S., and Paul, J. H.: Phytoplankton group specific quantitative
1316 polymerase chain reaction assays for RuBisCO mRNA transcripts in seawater, Mar.
1317 Biotechnol., 9, 747–759, 2007.
1318
1319

1320 Reply to Referee #2

1321

1322 Thank you very much for your helpful suggestions and constructive comments. Below are our
1323 point-by-point responses to your comments.

1324

1325 1. *The abstract states: "At the end of the incubation, the relative contributions of diatoms to*
1326 *chl a biomass decreased significantly with increased CO2 levels in the controls". This is*
1327 *misleading as the contribution of diatoms to chl a biomass increased over the course of*
1328 *the incubation in all bottles; it is the extent of this increase that is less at high CO2.*

1329 Thank you for pointing it out. We have amended the sentence as follows (see Lines 12−14
1330 in the track changes version):

1331 "At the end of incubation, the relative contribution of diatoms to chlorophyll *a* biomass
1332 was significantly higher in the 380 ppm CO_2 treatment than in the 600 ppm treatment in
1333 the controls, whereas minimal changes were found in the Fe-added treatments."

1334

1335 2. *In addition, the sentence starts with "At the end of the incubation. . ..". This would be*
1336 *after 7 days when the bottles were clearly depleted of nutrients. Table 2 gives insufficient*
1337 *information to know when nutrient limitation occurred and I would also like information*
1338 *on how long it took the bottles to equilibrate with CO2 (this information is given in Sugie*
1339 *et al, 2013 but is not sufficiently discussed in this manuscript). In addition, it is confusing*
1340 *to know when the data points were collected. Table 2 and Figure 1 show data from the*
1341 *final day (7?) whereas Figure 2 shows data from days 3 – 6. This lack of clarification*
1342 *makes it difficult to draw conclusions to what is happening and raises question to whether*
1343 *the results are purely due to CO2 manipulation and not due to nutrient limitation.*

1344 Thank you for pointing them out. We have added the Figs. 1S and 2S showing the time
1345 courses of carbonate chemistry and macronutrients, respectively. In addition, temporal
1346 changes in fucoxanthin and 19'-hexanoyloxyfucoxanthin concentrations have been shown
1347 in Fig. 1 instead of the growth ratios of these pigments. We have also reconstructed the
1348 results and discussion in the revised manuscript in accordance with the new figures (see
1349 Lines 334–348, 477–479, and 519–522 in the track changes version).

1350

1351 3. *The abstract further states "These results indicate that under Fe-deficient conditions, the*
1352 *growth of diatoms was negatively affected by the increase in CO2 availability". I would be*
1353 *careful with this statement. I would say their ability to compete is better at high CO2. I am*
1354 *interested in what is happening with the haptophytes. Like diatoms they also increase in*

1355 *abundance over the course of the incubation but this increase is less under high CO2.*
1356 *However, in Fig 2 it looks like they increase their contribution to total chl a at high CO2*
1357 *in control bottles. Perhaps the story is more about the competition between diatoms and*
1358 *haptophytes under different CO2 rather than just interpreting everything in terms of*
1359 *diatom growth.*
1360 Following your kind suggestion, we have amended the sentence as follows (see Lines 14−
1361 16 in the track changes version):
1362 "These results indicate that, under Fe-deficient conditions, the growth of diatoms could be
1363 negatively affected by the increase in CO_2 availability."
1364 Furthermore, we have added the following sentences to the discussion (see Lines 558−566
1365 in the track changes version):
1366 "Another possibility is that the competitions between diatoms and other phytoplankton
1367 taxa could occur. For example, diatoms could become less competitive when silicic acid is
1368 exhausted, because Si-depletion significantly depressed the growth and could induce their
1369 cell death (Harrison et al., 1977; Jiang et al. 2014). However, concentrations of silicic acid
1370 were not significantly different among CO_2 levels in the Fe-added treatments (Fig. S2f).
1371 Moreover, in the control treatments, silicic acid was almost depleted in the low CO_2
1372 treatment after day 5 but not in the high CO_2 treatment (Fig. S2e). These results suggest
1373 that availability of silicic acid little affected the decreases in relative diatom contribution
1374 to Chl *a* biomass."
1375
1376 *4. A lower expression of diatom rbcL normalized to rbcL gene number certainly implies the*
1377 *diatoms are less active. This finding supports other studies that show Rubisco is regulated*
1378 *at the expression level in diatoms. However, this has not been absolutely proven yet, and*
1379 *given the tight regulation of Rubisco protein activation in plants, it is hard to accept that*
1380 *rbcL expression equals photosynthetic rates in this paper without more study.*
1381 We have added the following sentences to the end of the paragraph (see Lines 617−630 in
1382 the track changes version):
1383 "Recently, Gontero and Salvucci (2014) pointed out that RubisCO activase plays a key
1384 role in the modification of RubisCO activity, and consequently in the capacity of carbon
1385 fixation, although the occurrence of RubisCO activase in diatoms is not well understood.
1386 Further studies must be needed for better understanding of the impacts of elevated CO_2 on
1387 photosynthetic physiology in diatoms."
1388

1389 5. *How do the authors reconcile that rbcL expression is lower in both Fe-added and Fe*
1390 *limited incubations whereas fucoxanthin concentrations are only lower in Fe-limited*
1391 *cultures?*
1392 We have explained this in the chapter of discussion as follows (see Lines 635−641 in the
1393 track changes version):
1394 "According to our cloning data (Fig. 6), a shift in phylogenetic composition of the diatoms
1395 actively transcribed *rbcL* was observed in the Fe-added bottles. In addition, F_v/F_m values
1396 increased significantly with Fe enrichment in our incubation experiments (Sugie et al.,
1397 2013), indicating an increase in the photochemical quantum efficiency of photosystem II
1398 for the diatoms. Therefore, the photosystem II activity might compensate for the decrease
1399 in RubisCO expression under Fe-replete conditions."
1400
1401 6. *It is difficult to tell from the rarefraction curves whether they are approaching saturation.*
1402 *As such, it is difficult to say whether the number of OTUs are different between the*
1403 *treatments. I do not have a good understanding on whether the differences found in the*
1404 *Shannon Index and Simpson diversity are significant. More details would be appreciated.*
1405 The difference between samples cannot be determined from the number of OTUs, because
1406 the rarefaction curves were not completely saturated. Alternatively, we have added the
1407 chao1 index as an indicator of OTU richness (Chao, 1984) (Table 2). This index was
1408 calculated based on the number of singleton OTUs (OTUs with only one sequence
1409 obtained) in the clone library. In addition, we showed the 95% confidence intervals (CI)
1410 for the Shannon and Simpson indices (Table 2) to clarify the statistical significance among
1411 treatments. According to these results, we have reconstructed the results and discussion on
1412 the manuscript (Lines 414–420 and 701–705 in the track changes version).
1413
1414 7. *Significant differences were found in the cDNA libraries under different CO2 within the*
1415 *Fe-treated incubations. Are the authors certain that this is due to a change in diatom rbcL*
1416 *sequences rather than a change in the non-diatom rbcL sequences that were detected? (in*
1417 *the initial treatment it seems that _ 17 % of the rbcL cDNA library comes from other*
1418 *eukaryotes).*
1419 As mentioned in the results on the revised manuscript, other eukaryotes contain diatoms
1420 that could be assigned to centrics and pennates. Actually, initial treatment contains only
1421 11% of the sequence derived from eukaryotes other than diatoms. In addition, the other
1422 libraries consist of ≥92% sequences from diatoms. Therefore, we considered that the

1423 differences between CO_2 treatments were primarily due to the changes in diatom *rbcL*
1424 sequences.
1425
1426 *8. The authors discuss the influence of Fe and CO2 on the CCM. However, I feel that their*
1427 *link between Fe and the CCM is tenuous. Fe is important for PSII, and Sugie et al (2013)*
1428 *found increased Fv/Fm with increased Fe, which is to be expected. However, speculating*
1429 *that the Fe limitation down-regulates the CCM through lack of energy provided by PSII*
1430 *seems tenuous. Without any further measurements it is difficult to draw any conclusions*
1431 *about the role of the CCM in this paper.*
1432 In the revised manuscript, the discussion on CCMs has been excluded.
1433
1434 *9. In the discussion about Chaetoceros, it should be noted that Trimborn et al, 2013 Limnol.*
1435 *Oceanogr., 58(3), 2013, 997-1007 | DOI: 10.4319/lo.2013.58.3.0997, found that*
1436 *Chaetoceros debilis increased growth rates under high CO2 and that Tortell et al 2008*
1437 *GRL, 35 (4) DOI: 10.1029/2007GL032583 found Chaetoceros spp. to dominate shipboard*
1438 *incubations from the Ross Sea under high CO2.*
1439 According to your kind suggestion, the following two sentences have been added to the
1440 chapter of discussion (see Lines 508−510 and 514−516 in the track changes version):
1441 “However, Tortell et al. (2008) demonstrated that relative abundance of *Chaetoceros* spp.
1442 increased under elevated CO_2 levels in the Ross Sea.”…….“In contrast, Trimborn et al.
1443 (2013) showed a significant increase in the growth rate of *Chaetoceros debilis* under high
1444 CO_2 condition.”
1445
1446 *10. I presume figure 1 is the concentration of fucoxanthin (μg/L) in the final sampling day*
1447 *(day 7?) divided by the fucoxanthin concentration (μg/L) in the initial bottle? This should*
1448 *be clearer.*
1449 We have revised figure 1 as temporal changes in phytoplankton pigment concentrations.
1450
1451 *11. Fucox is a strange abbreviation and I would keep the full word fucoxanthin.*
1452 We have changed the abbreviation “Fucox” to “Fuco”.
1453
1454 *12. In abstract “To confirm this. . .” (that diatom growth is negatively affected by high CO2)*
1455 *seems to be too strong a statement as mRNA of rbcL is not a direct measurement of growth.*
1456 *“To further support this finding. . .” would be better.*

1457 Following your kind suggestion, we have corrected this sentence in the revised manuscript
1458 (see Line 16 in the track changes version).
1459
1460 *13. In the supplementary I would appreciate more information about how the conditions*
1461 *changed in the bottles over time. Perhaps a Table showing the measured values from*
1462 *every day so the readers can see when nutrient limitation occurs, how long it takes CO2 to*
1463 *equilibrate, the increase of total phytoplankton biomass (POC or Chla) over time and*
1464 *when different samples were collected.*
1465 Following your kind suggestion, we have added the time courses of carbonate chemistry,
1466 macronutrients, and chl *a* in the supplementary materials (Figs. S1-S3). In this manuscript,
1467 we have dismissed POC, because Yoshimura et al. (2014) discussed this matter.
1468
1469
1470 References

1471 Yoshimura, T., Sugie, K., Endo, H., Suzuki, K., Nishioka, J., and Ono, T.: Organic matter
1472 production response to CO_2 increase in open subarctic plankton communities: Comparison
1473 of six microcosm experiments under iron-limited and-enriched bloom conditions,
1474 Deep-Sea Res. I, 94, 1–14, 2014.
1475 Chao, A.: Nonparametric estimation of the number of classes in a population. Scand. J.
1476 Stat., 265–270, 1984.

1477
1478

1479 Reply to Referee #3

1480

1481 We are very grateful for your constructive comments to our manuscript. Following the helpful
1482 suggestions from you and the other reviewers, we believe that our manuscript has been
1483 modified significantly. Below are our point-by-point replies to your comments given in italics.

1484

1485 *1. Page 18106, lines 11-15. This is misleading! 1) "At the END of incubation, the relative*
1486 *contributions of diatoms to. . .". According to Materials and Methods, the incubation last*
1487 *for 7 days and therefore day 7 should be the END; however, Fig. 2A. only shows data*
1488 *collected on days 3 and 5.*
1489 In the revised manuscript, we have amended the incubation periods as 5 day for the control
1490 and 6 day for the Fe-added treatments (see Lines 156−157 in the track changes version).

1491

1492 *2. 2) The contribution of diatom to total Chla biomass actually increased over the course of*
1493 *the experiment regardless of CO2 or Fe treatments (Fig. 2). It is the extent of this increase*
1494 *that was less at high CO2.*
1495 We have amended the sentence as follows (see Lines 12−14 in the track changes version):
1496 "At the end of incubation, the relative contribution of diatoms to chlorophyll *a* biomass
1497 was significantly higher in the 380 ppm CO_2 treatment than in the 600 ppm treatment in
1498 the controls, whereas minimal changes were found in the Fe-added treatments."

1499

1500 *3. Page 18106, line 21. No, it is not the "activity" – there is no RubisCO activity*
1501 *measurement in this study – it should be RubisCO transcription.*
1502 According to your kind suggestion, we have corrected the word (see Line 22 in the track
1503 changes version).

1504

1505 *4. Page 18109, lines 2-4. Is the effect of CO2 and/or Fe availability on rbcL transcription in*
1506 *diatoms really COMPLETELY unknown? Here I just give two examples: Granum et al.*
1507 *2009 J Phycol; Shi et al. 2013 Appl Environ Microb.*
1508 Thank you for your valuable comments. As you kindly suggested, the effects of iron
1509 and/or CO_2 (including low CO_2) on the diatom *Thalassiosira pseudonana* CCMP1335
1510 were investigated. However, the diatom strain was isolated from Moriches Bay (New York,
1511 USA) and the cell size is rather small (4−6 μm: http://ncma.bigelow.org/ccmp1335) as
1512 compared with those observed in our experiments (see Sugie et al., 2013). We consider
1513 that it is difficult to apply the results from laboratory experiments using the diatom strain

1514 to our study conducted in the oceanic Bering Sea. In the former manuscript, we intended
1515 that the effects of increased CO_2 and Fe levels were little known on natural diatom
1516 assemblages in HNLC waters. Therefore, we have amended the sentence as follows (see
1517 Lines 111−113 in the track changes version):
1518 "In addition, there are no reports on the effects of CO_2 and Fe availability on *rbcL*
1519 transcription of natural diatom community in HNLC regions."
1520
1521 *5. Pages 18109-18110, "Experimental setup". More details on how trace metal clean*
1522 *techniques were applied should be provided. For instance, under what conditions and how*
1523 *was the seawater poured into 50 L carboys? Did the CO2 gas pass through 0.22 filters*
1524 *before being introduced into the incubation bottles?*
1525 We have added the detailed procedures for trace metal clean technique to the revised
1526 manuscript (see Lines 140 and 149−151 in the track changes version). We followed the
1527 procedures of Yoshimura et al. (2013).
1528
1529 *6. The authors discuss the roles of the CCM in the response of diatoms to CO2 and Fe. They*
1530 *first (page 18121, lines 4-5) suggest that CCM may have been down-regulated at high*
1531 *CO2, resulting in the decrease in biomass in both Fe-deficient and Fe-added bottles;*
1532 *however, later on (page 18123, line 26 to page 18124, line 1) they suggest that "diatoms*
1533 *can upregulate CCM activity at elevated CO2. . ., photosynthetic carbon fixation in*
1534 *diatoms could not be limited by CO2 availability as a consequence of the CCMs". These*
1535 *two statements are contradictory to each other. Please clarify! Without any direct*
1536 *experimental evidence it would be impossible to evaluate the roles the CCM may play in*
1537 *this paper.*
1538 We have deleted the paragraphs in pages 18121, 18123, and 18124, because we have no
1539 experimental evidence on it. In the revised manuscript, we have minimized the description
1540 on CCMs (see Lines 744–749 in the track changes version)
1541
1542 *7. Page 18124, lines 2-8. Fv/Fm indicates the maximum photochemical quantum yield of*
1543 *PSII. An increase in Fv/Fm doesn't necessarily mean more energy for CCMs.*
1544 In the revised manuscript, this sentence has been deleted.
1545
1546 *8. Figs. 1, 2, and 4. The time points at which the data presented in these figures were*
1547 *collected are inconsistent. Fig. 1 shows pigment data from the first and the last day (day 7,*
1548 *I presume), Fig. 2 shows data from days 3 and 5 for the Fe-deficient and days 4 and 6 for*

1549 *the Fe-added treatments, and Fig. 4 shows data from day 3 for the controls and day 2 for*
1550 *the Fe-added bottles. The authors need to clarify why the samplings/measurements were*
1551 *performed in such a way, which makes it difficult to compare the results among the*
1552 *treatments to arrive at conclusions.*
1553 We have added a supplemental table (Table S1) for clarifying the sampling points. In
1554 addition, figures 1 and 2 have been replaced by new graphs showing the temporal changes
1555 in fucoxanthin, 19'-hexanoyloxyfucoxanthin, and relative phytoplankton composition to
1556 Chl *a* biomass in order to exhibit the data from all sampling days in our experiment. We
1557 have also revised the chapters of results and discussion in accordance with the new data
1558 set (see Lines 334–348, 477–479, and 519–522 in the track changes version).
1559

1560 Reference

1561 Yoshimura, T., Suzuki, K., Kiyosawa, H., Ono, T., Hattori, H., Kuma, K., and Nishioka,
1562 J.: Impacts of elevated CO_2 on particulate and dissolved organic matter production:
1563 Microcosm experiments using iron deficient plankton communities in open subarctic
1564 waters, J. Oceanogr., 69, 601–618, 2013.