Effect of ocean acidification on coastal phytoplankton composition and accompanying organic nitrogen production

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Abstract The effect of ocean acidification, caused by the increase in pCO$_2$ in seawater, on phytoplankton population and on related organic nitrogen production was experimentally examined by use of a natural coastal microbial population. pCO$_2$ and pH were controlled by aeration with air in which pCO$_2$ was at the current level (control), for which ambient air was used, and with air in which pCO$_2$ was "800" and "1200" ppm, in 500-L culture vessels. The experiment was continued for 15 days after addition of the inorganic nutrients such as nitrate, phosphate, and silicate. During most of the experimental period, a minor increase in phytoplankton biomass was noted, probably because of low irradiance, an increase in phytoplankton biomass was observed at the end of the experiment. Flow cytometric and microscopic observations revealed that this increase was because of *Chrysochromulina* sp. (Haptophyceae). The growth of *Chrysochromulina* sp. was most obvious in the control vessel, and tended to be obscured by increasing pCO$_2$ (decrease in pH), indicating the possibility that ocean acidification inhibits the growth of specific phytoplankton groups, for example *Chrysochromulina* sp. Production of particulate organic nitrogen (PON), determined by the ^{15}N tracer method, also diminished under acidified conditions compared with that at the current level.

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1 Introduction

The ocean is the earth's most significant sink of anthropogenic carbon dioxide, absorbing approximately 2.2 GtC year^{-1} from the atmosphere, and accounts for approximately one third of the carbon dioxide emitted by the combustion of fossil fuels (Denman et al. 2007). It is believed that uptake of atmospheric CO$_2$ by the ocean has delayed the progress of climate change, because the atmospheric partial pressure of CO$_2$ (pCO$_2$) would currently be 450 ppm without ocean absorption (Doney et al. 2009). However, uptake of CO$_2$ by the ocean poses a new problem, i.e., "ocean acidification," a phenomenon caused by release of hydrogen ions when carbonic acid dissociates

first to bicarbonate then to carbonate ions. The pH of ocean surface water is believed to have decreased by 0.1 U since the beginning of the industrial revolution (Riebesell et al. 2000; Doney et al. 2009). Considering that atmospheric CO_2 is predicted to increase further to 700 ppm by the end of this century (IS92a scenario, Meehl et al. 2007), the pH of the surface ocean will drop further by 0.3–0.4 U (Meehl et al. 2007). In the last decade, concern about the effect of acidification on marine organisms has increased rapidly.

The most frequently predicated effect of ocean acidification on marine organisms is that on the formation of the calcareous skeleton. The decrease in pH accompanies a concomitant decrease in the concentration of carbonate ions, causing, in turn, a drop in the saturation state of calcium carbonate (Feely et al. 2004; Fabry et al. 2008). This might inhibit healthy production of calcareous skeletons of the kind observed in the pteropod *Clio pyramidata* (Orr et al. 2005; Fabry et al. 2008) and the coccolithophorids *Emiliania huxleyi* and *Gephyrocapsa oceanica* (Riebesell et al. 2000; Engel et al. 2005).

A second possible effect is that of the increase in CO_2 in seawater on the rate of photosynthesis. Most photosynthesizing microorganisms in oceanic environments fix CO_2 via the carboxylating enzyme, ribulose-1,5-bisphosphate carboxylase/oxygenase (RUBISCO). Although this enzyme requires 25–35 μM CO_2 to become saturated (Raven 1991), free CO_2 is only ~ 10 μM in ambient seawater. To compensate for this difference, some phytoplankton groups activate a carbon concentration mechanism (CCM) to maintain a sufficient intracellular concentration of CO_2 (Giordano et al. 2005). Such an increase in the concentration of free CO_2 in seawater caused by the ocean's uptake of atmospheric CO_2 is expected to have little effect on the rate of photosynthesis of phytoplankton such as diatoms, which have an advanced CCM (Tortell et al. 1997; Beardall and Raven 2004). On the other hand, phytoplankton groups such as coccolithophorids, with a low affinity for inorganic carbon, are able to increase their rate of photosynthesis in response to an increase in CO_2 (Rost et al. 2008). However, experiments using natural phytoplankton populations have sometimes noted an increase in the growth of diatoms at higher CO_2 concentrations (Chen and Durbin 1994; Tortell et al. 2002). This implies the possibility that factor(s) other than CCM may be involved in the effect of CO_2 concentration on phytoplankton growth. Recent experiments under high-CO_2 conditions indicate that other factors, for example iron availability, irradiance, and water temperature, are inversely related to the rate of photosynthesis (Feng et al. 2008, 2010; Hurd et al. 2009; Shi et al. 2010). Although experimental studies using natural microbial populations under different CO_2 conditions have greatly increased during this decade (Tortell et al. 2002; Kim et al. 2006; Riebesell et al. 2007;

Hendriks et al. 2010; Yoshimura et al. 2010), further accumulations of data concerning the response of natural microbial populations will be needed to accurately analyze changes in phytoplankton populations and subsequent biogeochemical cycles.

In this study we conducted an incubation experiment at 3 different concentrations of CO_2 ($\times 1$, $\times 2$, and $\times 3$ of the current level), in the winter season, using large (500-L) outdoor culture vessels to assess changes in the composition of phytoplankton populations and changes in nitrogen cycles. Flow cytometry (FCM) and the ^{15}N tracer method were used to achieve these objectives.

2 Materials and methods

2.1 Experimental system

Our culture experiment under three pCO_2 conditions was carried out at Shimoda Marine Station, University of Tsukuba, Shimoda, Shizuoka, Japan. Three cylindrical acrylic vessels (800 mm ϕ, 1000 mm in height, ca. 500 L in volume) were used for the experiment. The vessels were set in an outdoor pool 1 m deep with the water temperature controlled by the flow of coastal water through the pool; water temperature was measured by use of a temperature logger (MDS-MkV/T; JFE Advantech). On 22 January 2009, 400 L of sample sea water was slowly pumped up from a nearby coastal site and used to fill the vessel while being filtered through a 100-μm mesh to remove macrozooplankton. The culture vessel was kept under an acrylic cover except during water sampling. Light intensity was reduced to 50% by covering the vessels with black mesh throughout the experiment, and photon flux was measured every 3 h during the experiment (LI-250; LI-COR).

Air with three levels of pCO_2, prepared by mixing ambient air supplied by an oil-less air compressor and 3% CO_2 with pure air (N_2 and O_2), was introduced into the cultured water from the bottom of the vessel through two airstones (300 mm in length and 20 μm in pore size). We calculated the mixing ratio of the ambient air and 3% CO_2 on the assumption that the pCO_2 in the ambient air was 400 ppm. We prepared control (ambient air only), 800, and 1200 ppm air and introduced them to vessels (these are henceforth referred to as control, "800" and "1200" ppm vessels, respectively). The air sample supplied to each vessel was corrected from the outlet of the gas mixer, and pCO_2 was measured by a CO_2 analyzer (LI-6262; LI-COR). Air was released from the vessel through an outlet (1/4 inch ϕ) in the upper part of the vessel during aeration. The preparative aeration was started at 1400 hours on 22 January 2009 local time and continued until 0900 hours on 24 January at a flow rate of 10 L min^{-1} in each tank; a

total of 25,800 L of air was introduced into each vessel. During the experiment, aeration was conducted for 6 h from 1000 to 1600 hours every day to maintain the pCO$_2$ in each vessel.

Inorganic nutrients comprising KNO$_3$ (12.6 μmol L^{-1}), KH$_2$PO$_4$ (0.77 μmol L^{-1}), and Na$_2$SiO$_3$ (12.4 μmol L^{-1}) were added at 0900 hours on 24 Jan (day 1) to increase their concentrations, and mixed by aeration for 1 h (actual nutrient concentrations were measured as described below).

2.2 Sample collection

Water samples on day 1 were collected from each culture vessel 1 h after addition of the nutrients. Samples for analysis of pH and dissolved inorganic carbon (DIC) were collected in 500 ml glass bottles by use of an air-tight sampling system, and HgCl$_2$ was added. Polycarbonate tubing 52 mm ϕ and 1000 mm in length was inserted into the culture solution and the tube was filled with water by submerging it to the bottom. The upper end of the tubing was then stoppered. The tube was then raised and the lower end was stoppered. In this way the water was sampled. This was repeated 5 times, and a total of approximately 8 L of the water was sampled from each vessel. Fractions of the raw water samples were divided for measurement of phytoplankton composition by both FCM and light microscopy. Water samples for FCM were filtered through a 59-μm mesh to remove large particles that might obstruct the sampling nozzle. The concentrations of chlorophyll a (Chl. a) for the non-filtered (all size) and filtered (<59 μm) samples was no different throughout the experiment, indicating that pretreatment with the 59-μm mesh caused only a minor compositional and quantitative bias in the phytoplankton population. Other water samples were filtered through precombusted (450°C, 4 h) glass fiber filters (Whatman GF/F). The filters and filtrates were stored at −20°C until analysis.

2.3 Sample analysis

The concentrations of nitrate, nitrite, and phosphate were determined by use of an autoanalyzer (AACSII, BRAN + LUEBBE) whereas those of ammonium and silicate were determined colorimetrically by the methods described in Parsons et al. (1984).

We measured total inorganic carbon and pH to monitor the CO$_2$ system in seawater. Total inorganic carbon (TCO$_2$) was analyzed coulometrically (Johnson et al. 1985) using an automated CO$_2$ extraction unit equipped with a carbon coulometer (Nippon ANS). It was calibrated with a series of sodium carbonate solutions prepared from primary standard-grade anhydrous sodium carbonate (99.98%;

Asahi Glass), dried at 600°C for 1 h, and with purified water provided by Milli-Q SP TOC (Ishii et al. 2000). Analysis of pH was conducted spectrophotometrically on a total hydrogen ion concentration scale using m-cresol purple as indicator dye at 25°C (Clayton and Byrne 1993) with an automated flow-cell system that we have developed for discrete bottle samples (Saito et al. 2008). Batches of certified reference material (CRM) provided by Dr A.G. Dickson (http://www-mpl.ucsd.edu/people/adickson/CO2 QC/index.html) were also used for quality control of these analyses. Partial pressure of CO$_2$ in seawater and pH at the in-situ temperature were computed from TCO$_2$, pH at 25°C, temperature, and salinity, by use of the dissociation constants of carbonic acid given by Lueker et al. (2000).

The concentration of Chl. a was measured by fluorometry (10-005; Turner Design) after extraction with N,N-dimethylformamide (Suzuki and Ishimaru 1990). Concentrations of particulate organic carbon (POC) and nitrogen (PON) were determined by use of an elemental analyzer (EA1108; Carlo Erba). The ^{15}N atom% of PON was measured by an isotope-ratio mass spectrometer (Delta Plus; Finnigan MAT) combined with EA.

The production of PON was calculated by use of Eq. 1 (Hama et al. 1983, 1993):

$$P\text{-}PON = [(a_{is} - a_{ns})/(a_{in} - a_{ns})] \times [ON] \qquad (1)$$

where, a_{is} is ^{15}N atom% of the incubated sample, a_{ns} ^{15}N atom% of the non-incubated sample, a_{in} ^{15}N atom% of the dissolved inorganic nitrogen, and [ON] concentration of PON (μM N).

In the experimental design of this study, tracer material (K^{15}NO$_3$) was added on day 1, and the organic nitrogen containing ^{15}N was followed throughout the experiment. Thus, the calculated value corresponds to the concentration of photosynthetically produced PON from the start of the experiment to a given date (Hama et al. 2004), rather than to the rate at a date with a unit of μM N day^{-1}. The values will be referred as the concentrations of photosynthetically produced PON (P-PON) with units of μM N. Because dissolved inorganic nitrogen was mostly composed of ^{15}N-KNO$_3$ at the start of the experiment, due to the low concentration of nitrate and ammonium, we used 99.5 atom%, which is the value of ^{15}N-KNO$_3$ added, as a_{in} in this calculation.

The single filter sample was used for determination of POC, PON including ^{15}N atom%, and P-PON; the coefficients of variation of the concentrations of these variables by use of our system are usually less than 5% (Hama and Yanagi 2001). Determination of Chl. a was performed by use of two filters.

Flow-cytometric analysis was performed with a FACS Calibur (4-color type; Becton–Dickinson) equipped with an argon ion laser (488 nm) and a red semi-conductor laser

(635 nm). Emitted fluorescence was monitored at FL1 (bandpass 530 ± 15 nm: green), FL2 (585 ± 21 nm: orange), FL3 (>670 nm: long-pass red), and FL4 (661 ± 8 nm: narrow red), and at a forward-light scatter (SFC) and a side-light scatter (SSC). FACSFlow sheath fluid (Becton–Dickinson) was forced by a pressure of 4.5 psi through a 100-μm nozzle, with 60 μL of sample. Fluoresbrite microspheres (6 and 10 μm; Polysciences) were used to adjust the instrumental conditions. The measurements and data analysis were controlled by CELLQuest software. The FCM analysis was complete within 12 h of collection of the sample. In addition to samples collected from culture experiments using the natural population, a cultured strain of *Chrysochromulina* sp. (Haptophyceae) (isolated by micropipetting from Uno Port, Okayama Prefecture, Japan, on 15 October 2008 and cultured by Dr Nakayama) was also measured by FCM under the same analytical conditions. Phytoplankton was identified by optical microscopy, but detailed quantitative determination was not carried out.

3 Results

3.1 Physicochemical data

The temperature of the water flowing through the outdoor pool ranged from 11.7 to 13.8°C and the photon flux on the culture vessel varied with the range 50–129, 133–235, and 13–99 μmol m^{-2} s^{-1} at 0900, 1200, and 1500 hours, respectively, during the experiment. Figure 1 shows changes in the concentrations of DIC, pCO_2, and pH in the three vessels. The concentrations of DIC changed within the ranges of 2063–2103, 2168–2205, and 2222–2260 μmol kg^{-1} in control, "800", and "1200" ppm vessels, respectively (Fig. 1a). The DIC concentrations were higher in the 800 and 1200 ppm-treated vessels, reflecting the higher concentration of CO_2. The pCO_2 of the aeration gas collected just after the gas mixer was measured as 444–514 (mean 473; σ 38.4, n 4), 803–909 (mean 865; σ 47.4, n 4), and 1285–1300 ppm (mean 1290; σ 11.2, n 4) in the control, "800", and "1200" ppm gas. The variation of actual pCO_2 in the treatment air was likely to be because of fluctuation of pCO_2 in ambient air. The pCO_2 in water vessels treated with control, "800", and "1200" ppm air varied within the ranges 345–431, 666–839, and 977–1221 ppm, respectively (Fig. 1b). pCO_2 tended to gradually increase from days 1 to 4 in all vessels, but was subsequently relatively constant until the end of the experiment. The pH in the culture vessels reflected the different pCO_2 of the treatment air (Fig. 1c). The highest values were observed in the control, ranging from 8.02 to 8.10, followed by the "800" ppm vessel, from 7.76 to 7.85. The most acidic values were observed in the "1200" ppm vessel,
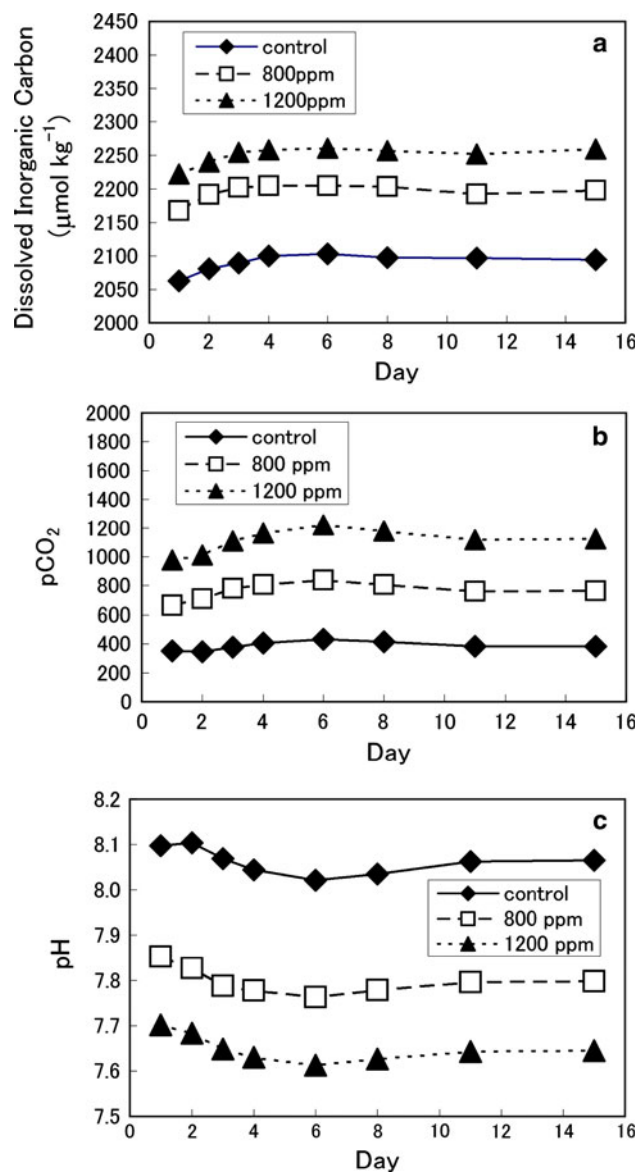

Fig. 1 Changes in: **a** the concentration of dissolved inorganic carbon (DIC), **b** the partial pressure of CO_2 (pCO_2), and **c** the pH in the culture vessels of control, "800", and "1200" ppm CO_2

in which the pH changed from 7.61 to 7.70. pH tended to decrease in the first 4 days concomitant with the increase in pCO_2. Differences in pH among the treatments were obvious throughout the experiment.

The concentrations of inorganic nutrients of the original sea water on 11 January were 0.93, 0.18 and 15.2 μM for nitrate, phosphorus, and silicate, respectively, and they increased to 13.3, 1.4 and 28–30 μM after addition of nutrients on day 1. The concentration of nutrients showed only slight variations throughout the experiment (Fig. 2). However, it is worth noting that a more than 1 μM N decrease in nitrate was found from days 11 to 15 in the control vessel. The decreases in the "800" and "1200"
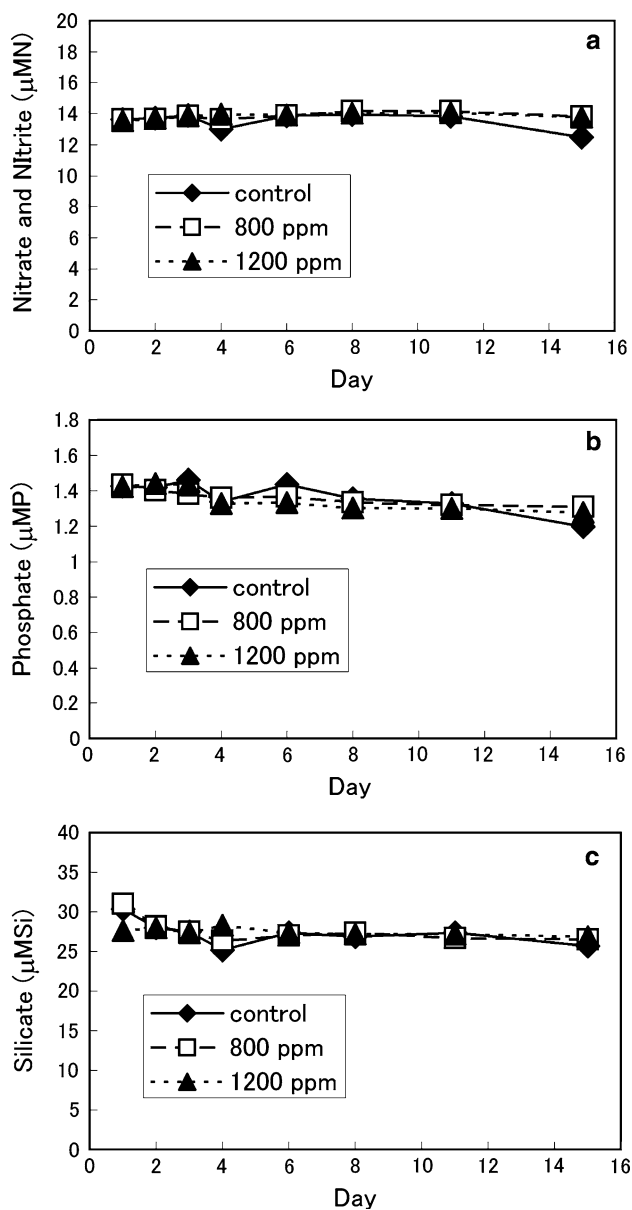
Fig. 2 Changes in the concentrations of: **a** nitrate and nitrite, **b** phosphate, and **c** silicate in the filtrate

Fig. 3 Changes in the concentration of chlorophyll *a* (Chl. *a*). The *bars* show the ranges of two determinations

ppm-treated vessels were not obvious, although minor decreases of less than 0.4 μM were found. Changes in the concentrations of phosphate and silicate were almost the same as those of nitrate.

3.2 Chl. *a*, phytoplankton and flow cytometry

The concentration of Chl. *a* of the original sea water collected on 22 January was 0.06 μg L^{-1} and varied from 0.07 to 0.3 μg L^{-1} in every vessel from days 1 to 8 (Fig. 3); little increase was found even though inorganic nutrients had been added on day 1. This finding agrees with the fact that only a slight decrease in nutrient con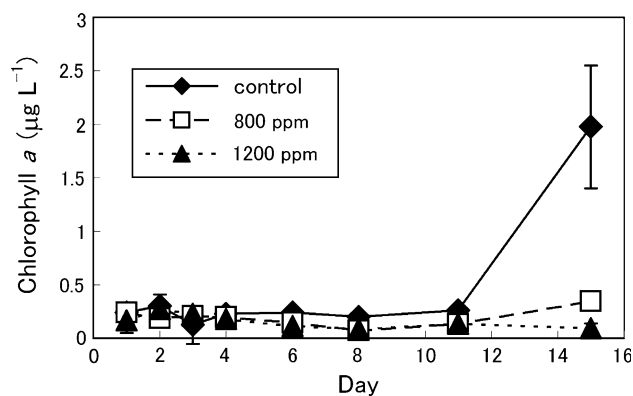centrations was found until day 8 in every vessel. Although no difference in the Chl. *a* concentration among 3 vessels was obvious on day 11, the concentration rapidly rose to 1.98 μg L^{-1} in the control vessel on day 15. The increases in Chl. *a* concentrations were also found in the "800" ppm vessel although it became less obvious as the pCO$_2$ increased (decrease in pH); the concentrations of Chl. *a* in the "800" and "1200" ppm vessels on day 15 were 0.17 and 0.05, respectively, compared to that in the control.

Bacillariophyceae (total number of genera identified: 12), including *Chaetoceros* spp., *Skeletonema* sp., and *Thalassiosira* spp., and Cryptophyceae (1), Haptophyceae (1), Dinophyceae (4), and Prasinophyceae (3) were found to be constituents of the phytoplankton population throughout the experiment (Table 1). Bacillariophyceae and Dinophyceae were mainly observed in the early stage of the experiments, but *Chrysochromulina* sp. (Haptophyceae) and *Tetraselmis* sp. (Prasinophyceae) were found mainly in the latter experimental stage from days 8 to 15. Although detailed quantitative microscopic analysis was not conducted in this study, the rapid increase in *Chrysochromulina* sp. (Fig. 4) on day 15 was quite obvious. This strongly suggested that the increase in Chl. *a* on day 15 was because of the growth of this species.

The development of particular phytoplankton groups from days 11 to 15 was clearly revealed by FCM analysis. Figure 5 shows the results from FCM for the samples collected from the three vessels on days 1, 8, 11, and 15; the fluorescent intensities of FL4 and FL2 are shown. Although the gross distribution patterns of particles in the 3 culture vessels were similar through days 1 to 8, the particles with intensities of 10^2–10^3 of FL4 and 1–5 × 10 of FL2 (group 1) became evident in the control and "800" ppm vessels on day 11. A further increase in these particles was noticed from days 11 to 15, especially in the control vessel. The results of FCM analysis strongly suggested that the increase in Chl. *a* was caused by the growth of the phytoplankton included in group 1.

Table 1 Phytoplankton species found on each sampling day by optical microscopic observation

	Day 1	Day 2	Day 3	Day 4	Day 6	Day 8	Day 11	Day 15
Cryptophyceae								
Plagioselmis spp.	X							
Haptophyceae								
Chrysochromulina sp.		X				X	X	X
Bacillariophyceae								
Centrales								
Actinoptycus senarius								X
Chaetoceros spp.				X	X	X		
Coscinodiscus sp.				X				
Dactyliosolen phuketensis					X			
Skeletonema sp.	X	X	X					
Thalassiosira spp.			X	X	X		X	X
Pennales								
Amphora sp.	X							
Cocconeis spp.	X	X	X					
Licmophora sp.								X
Nitzschia longissima	X	X						
Plagiotropis sp.		X						
Striatella sp.			X					
Dinophyceae								
Heterocapsa sp.	X	X	X					
Prorocentrum triestinum		X	X	X				
Protoperidinium sp.			X					
Scrippsiella sp.	X	X	X	X	X		X	X
Prasinophyceae								
Mamiella sp.	X							
Pyramimonas sp.	X			X				
Tetraselmis sp.			X			X	X	X

X shows the presence of the species

Fig. 4 Microscopic observation of *Chrysochromulina* sp. (Haptophyceae), collected from the control vessel on day 15. *Bar* shows 10 μm

To confirm the FCM analysis, we measured the cultured *Chrysochromulina* sp. isolated from Uno Bay, Okayama Prefecture, whose diagnostic features were found to be almost comparable with those in this study. The result of the FCM analysis (Fig. 6) clearly showed that the position of *Chrysochromulina* sp. cultured in the laboratory was identical with that in group 1, confirming that group 1 was composed of *Chrysochromulina* sp. Thus, our results concerning the composition of phytoplankton population suggested that growth of *Chrysochromulina* sp., a noncalcifying haptophyte, was inhibited by the increase in $p\mathrm{CO_2}$ (decrease in pH). Other groups with intensities of 5×10^1 to 2×10^2 of FL4 and 2×10^0 to 1×10^1 of FL2 (group 2) also showed the highest counts in the control vessel, though differences from the "800" and "1200" ppm vessel were much less than for group 1. We compared the position of this group with those of isolated species of various phytoplankton and it superimposed on that of haptophyte other than *Chrysochromulina* sp.. However, microscopic
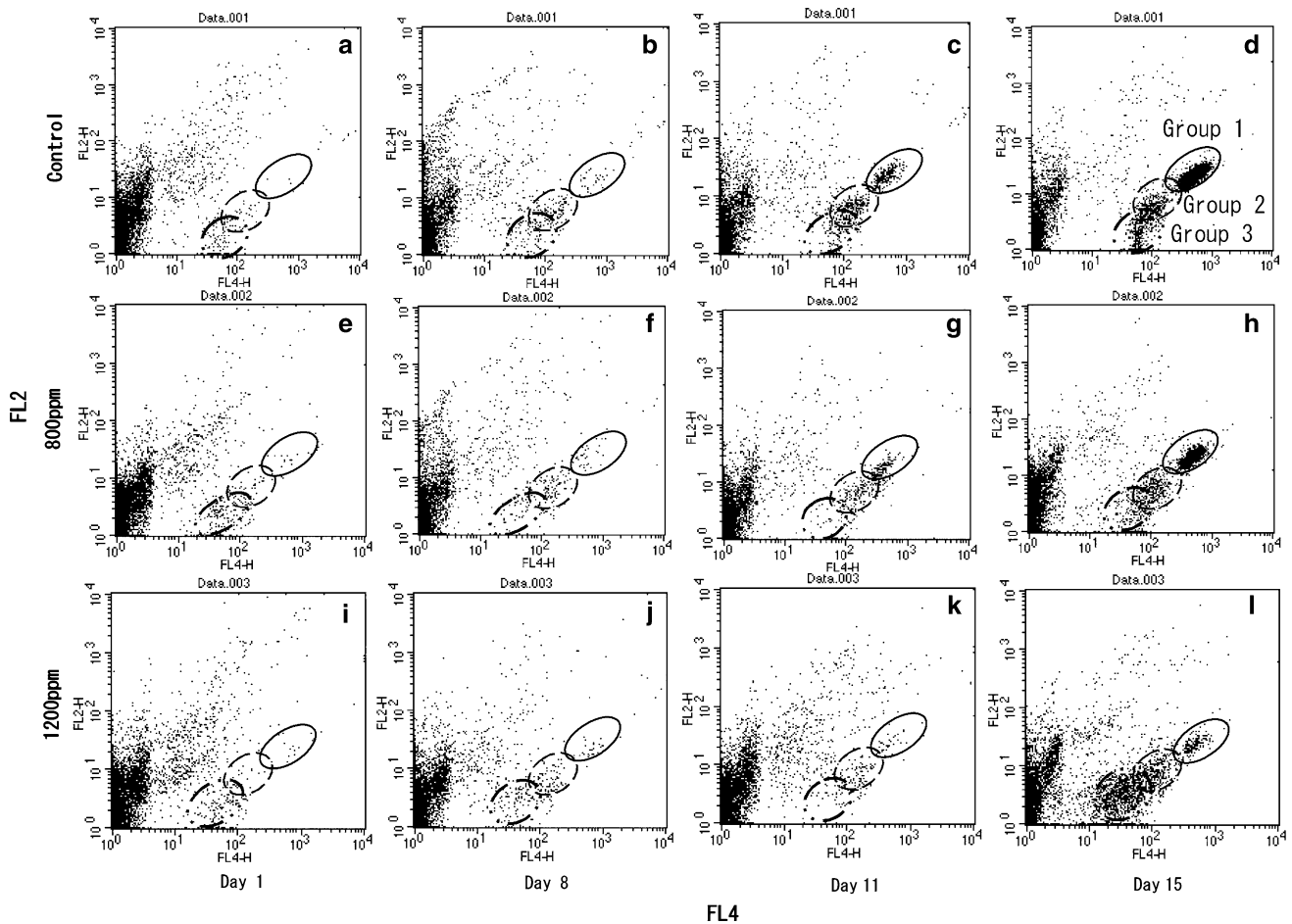
Fig. 5 Results from flow cytometry of samples collected from control, "800", and "1200" ppm vessels on days 1, 8, 11, and 15. *Circles* depict phytoplankton groups 1–3 (see text). The relationship between fluorescence of FL4 (band pass 661 ± 8 nm) and FL2 (band pass 585 ± 21 nm). **a–d** control; **e–h** "800" ppm; **i–l** "1200" ppm
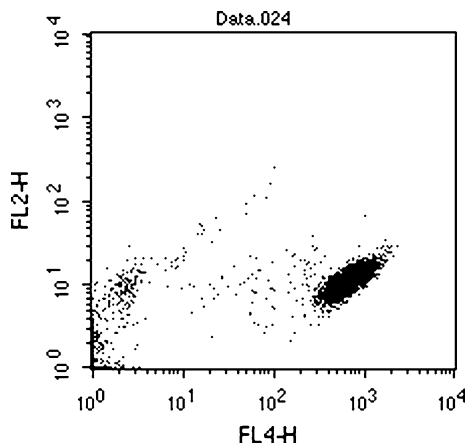

Fig. 6 Results from flow cytometry of *Chrysochromulina* sp. cultured by Dr Nakayama (see text). The relationship between fluorescence of FL4 and FL2 is shown

analysis observed *Chrysochromulina* sp. as the sole constituent of Haptophyceae on day 15. Thus, a phytoplankton genus corresponding to group 2 was not identified in this study. The same is true for that in group 3 (1×10^1 to 5×10^1 of FL4 and 1×10^0 to 7×10^1 of FL2) which was most abundant in the "1200" ppm vessel on day 15 (Fig. 5).

3.3 POC and PON

The measured concentration of POC was within the range 7.4–9.8 μM C in every vessel up to day 11 with no systematic variation (Fig. 7a). A rapid increase in that concentration was observed from days 11 to 15 in the control vessel, though the values in the other vessels remained constant. The concentrations of PON were 0.88–0.96 μM N in the three vessels at the start of the experiment (Fig. 7b). It tended to increase from day 11 in the control and "800" ppm vessels and a distinctly higher value (2.3 μM N), as well as those of Chl. *a* and POC, was observed on day 15 in the control vessel. Although high C/N ratios (9–11 molar ratio) were observed for POM at the start of the experiment, values around the Redfield ratio (6.6) were obtained on the other days.
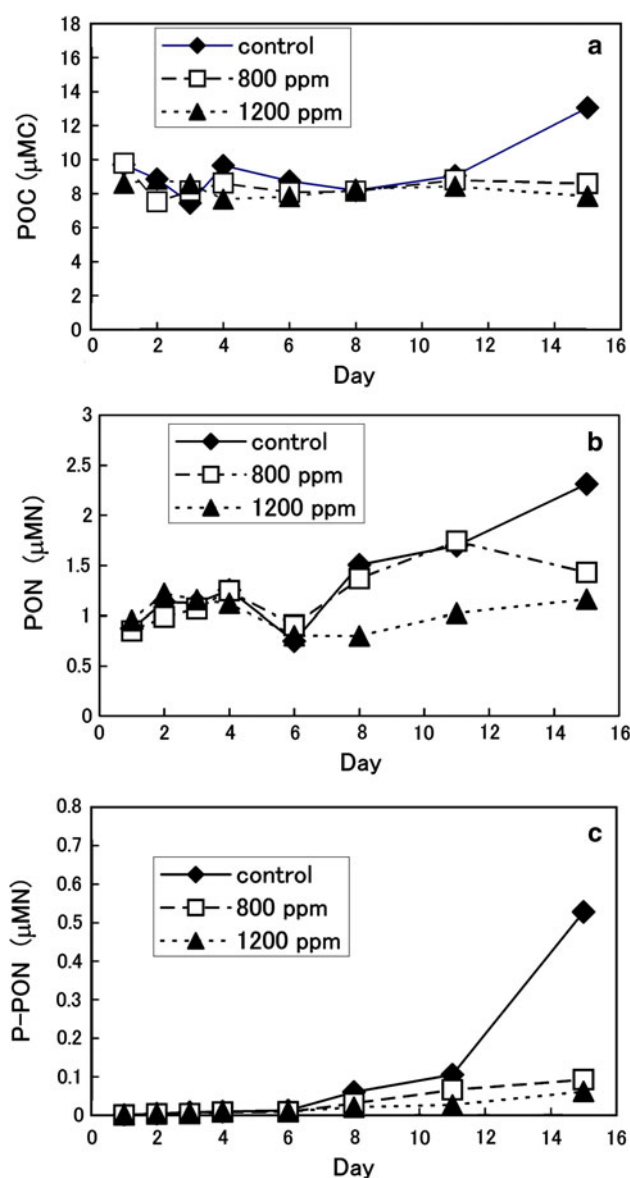
Fig. 7 Changes in the concentrations of **a** particulate organic carbon (POC), **b** particulate organic nitrogen (PON), and **c** photosynthetically produced PON (P-PON)

3.4 Concentration of P-PON

The ^{15}N tracer method was used to measure the concentration of P-PON in this study. Because ^{15}N-KNO$_3$ was added at the start of the experiment, the concentration of P-PON is comparable with the accumulation of PON produced by phytoplankton throughout the experiment; note that the value does not represent the daily production rate. The concentration of P-PON was low (less than 0.01 μM N up to day 6) with a little increase in every vessel (Fig. 7c). The concentration tended to increase from day 8 in every vessel, and a rapid increase was observed from days 11 to 15 in the control vessel. Although such increases were also

measurable in the "800" and "1200" ppm vessels, they were far less obvious than those in the control vessel (17 and 12% of that of control vessel, respectively).

4 Discussion

In this decade, many studies have been conducted on the effect of ocean acidification on phytoplankton composition (Hendriks et al. 2010). The culture volume used for experiments on natural phytoplankton populations, with the carbonate system controlled by manipulation of pCO$_2$, varies from 4 L (Tortell et al. 2002) to 27 m^3 (Riebesell et al. 2007). It is relatively easy to maintain the carbonate system in a small-volume culture, but difficult to establish and maintain specific pCO$_2$ in a large-volume culture. In an experiment using an extra-large culture vessel, aeration of the water column with air of specific pCO$_2$ has been applied only before the start of the experiments (Engel et al. 2005; Kim et al. 2006; Riebesell et al. 2007), after which the upper air portion only was maintained by special pCO$_2$. In our study, aeration for 43 h before addition of the nutrients was used, and it is conceivable that the carbonate system of the water samples in each vessel was approximately equilibrated with the air introduced. The total air volume used to aerate each vessel before day 1 (25,800 L) amounted to 65 times the water sample volume. Although a slight increase in pCO$_2$ was noticed from days 1 to 4 in each vessel, this result indicates that an aeration volume of more than 65 times the water volume would almost suffice to equilibrate the carbonate system of seawater.

In this study, the pH decreased from ca. 8.05 in the control vessel to 7.65 in the "1200" ppm vessel. A decrease of 0.4 U is well within the range expected to occur until the end of this century (Meehl et al. 2007). Thus, the conditions established in this study would occur not in the far distant future but over the next 50–100 years. The variability of pH in each vessel changed little throughout the experiment (0.08–0.09 U), while the differences between the treatments (approx. 0.15 U) were kept constant. Thus, it can be concluded that control of the carbonate system proceeded successfully.

In this study, we clearly observed that an increase in pCO$_2$ (decrease in pH) prevented the growth of Haptophyceae *Chrysochromulina* sp. The increase in free CO$_2$ concentration accompanied by ocean acidification has been generally believed to increase the rate of photosynthesis of phytoplankton, though to what extent varies depending on the efficiency of CCM (Riebesell 2004; Rost et al. 2008). Experimental analysis under higher-CO$_2$ conditions, in fact, generally produced an increase in phytoplankton productivity (Kim et al. 2006; Ramos et al. 2007; Tortell et al. 2008). An inhibitory effect had been reported for

formation of the calcareous plankton skeleton, for example that of coccolithophorids (Riebesell et al. 2000; Delille et al. 2005; Hopkins et al. 2010). Recently, however, a negative effect on non-calcified phytoplankton has been reported (Feng et al. 2010; Hopkins et al. 2010; Yoshimura et al. 2010).

Feng et al. (2010) examined the response of the phytoplankton population in Ross Sea under conditions with different iron concentrations, irradiance, and pCO_2. Under low irradiance they found that the concentration of 19′-hexanoyloxyfucoxanthin, the index pigment for haptophytes, was lower under high-pCO_2 (736–776 ppm) conditions than under low conditions (363–385 ppm). This result suggests the dominant haptophyte, *Phaeocystis antarctica,* was negatively affected by the increase in pCO_2. A comparable result has been obtained for the Equatorial Pacific phytoplankton assemblage (Tortell et al. 2002)—*Phaeocystis* sp. decreased by 60% under high-pCO_2 conditions (750 ppm) but not under low pCO_2 conditions(150 ppm). Although the species in these experiments were different from that of our experiment, it is noteworthy that the growth of haptophyte decreased under high-pCO_2 and low-pH conditions. However, experimental results obtained so far indicate that the responses of phytoplankton to the increase in pCO_2 (decrease in pH) did not show the same tendency despite being the same species (Hendriks et al. 2010; Paulino et al. 2008; Yoshimura et al. 2010). Factors other than pCO_2 and pH, for example irradiance, macro nutrients, and iron are likely to affect the physiological response of phytoplankton (Feng et al. 2008; Hurd et al. 2009; Yoshimura et al. 2010). Thus, additional experiments using isolated *Chrysochromulina* sp. under different condition are desirable to elucidate whether the negative effect on growth under high pCO_2 environments can be generalized.

The genus *Chrysochromulina* comprises diverse species of noncalcifying haptophytes, and is known to be widely distributed from coastal waters (Kaas et al. 1991; Hajdu et al. 2007) to the open ocean (Not et al. 2008; Liu et al. 2009). Recently, Liu et al. (2009) using genetic pigment and microscopy data, examined the distribution and abundance of photosynthetic picoplanktonic protists belonging to the Haptophyta in subtropical and subpolar oceanic waters. They discovered that most of the hundreds of haptophyte taxa belong to the same clade comprising the genus *Chrysochromulina*. Furthermore, they reported the novel finding that these haptophytes are responsible for 30–50% of the total standing stock of haptophytes, diatoms, and prokaryotes throughout the world's oceans. The standing stock of haptophytes (2.5×10^9 kg as Chl. *a*) was found to be higher than those of either diatoms or prokaryotes, i.e., 1.3×10^9 and 1.1×10^9 kg as Chl. *a*, respectively (Liu et al. 2009). Although it is possible that the physiological response to ocean acidification is variable

even in this genus, the high susceptibility to acidification of *Chrysochromulina* sp. observed in our study implies that ocean acidification caused substantial damage to significant components of photosynthetic eukaryotes.

The ^{15}N tracer method clearly demonstrated the production processes of organic nitrogen. The selective growth of *Chrysochromulina* sp. in the control vessel was accompanied by an increase in the concentration of P-PON. The concentration of P-PON in acidified vessels accounted only for 17 and 11% of that under current pH conditions in the "800" and "1200" ppm vessels, respectively. This result suggests the possibility that ocean acidification reduces primary productivity by inhibiting the growth of specific phytoplankton groups. This finding does not agree with most previous experimental studies, which generally observed an increase in the rate of photosynthesis under increased CO_2 conditions (Riebesell et al. 2000; Hein and Sand-Jensen, 1997; Czerny et al. 2009) assuming that ocean acidification enhances primary production (Hein and Sand-Jensen, 1997; Rost et al. 2008). The decrease in pH examined in this study (0.2–0.4 U) is most likely to be realized until the end of this century. The major contribution of noncalcified haptophytes composed mainly of the genus *Chrysochromulina* (Liu et al. 2009) implies the possibility of a substantial decline in oceanic primary production of these algal groups.

Indirect effects, for example grazing by zooplankton or infection by viruses, are comparable with the different biomass of *Chrysochromulina* sp. caused by different pH. Although water samples were prefiltered through 98-μm mesh before filling of the culture vessels, to remove macrozooplankton, some microzooplankton were possibly included in the experimental system. When microzooplankton is sensitive to changes in pCO_2 or pH, the grazing pressure was possibly different among the treatments, resulting in change in the biomass of *Chrysochromulina* sp. However, any effect of grazing on our results is difficult to evaluate, because the effect of ocean acidification on noncalcifying zooplankton has not yet been elucidated (Fabry et al. 2008). On the other hand, it is known that *Chrysochromulina* species can be infected by viruses (Suttle and Chan 1995; Sandaa et al. 2001). Larsen et al. (2008) observed that the activity of some virus groups changes, depending on pH. Their result suggests the possibility that the difference in the *Chrysochromulina* sp. biomass among the vessels was caused by differences between viral populations. These "indirect" effects have not yet been assessed, but measurements of zooplankton or virioplankton during the experiments will afford significant information about the effect of ocean acidification on the food web and the flow of energy in microbial ecosystems.

Apart from the effect on the growth of haptophytes, addition of inorganic nutrients including silicate usually

resulted in increased phytoplankton biomass mainly composed of diatoms in mesocosm experiments (Hama et al. 1988; Engel et al. 2002). However, a little increase in phytoplankton biomass was found in this experiment. Measured macronutrients, including silicate, were at high concentrations throughout the experiment, implying that macronutrients did not limit the growth of diatoms. Low light intensity is the most possible reason why dense phytoplankton bloom did not occur in this experiment. The photon flux at the culture vessel varied with the ranges 50–129, 133–235 and 13–99 $\mu mol\ m^{-2}\ s^{-1}$ at 0900, 1200 and 1500 hours, respectively during the experiment. Referring to the reported saturation photon flux for phytoplankton photosynthesis including diatoms (100–500 $\mu mol\ m^{-2}\ s^{-1}$; Sarthou et al. 2005; Lefebvre et al. 2007; Grangeré et al. 2009), the values observed in this study strongly suggest that light intensity was not enough to enable rapid accumulation of diatoms.

It is possible that the aeration to maintain the corresponding pCO$_2$ and pH throughout the experiment resulted in a slight increase in phytoplankton bloom because of the turbulence (Thomas and Gibson 1990; Hurd et al. 2009). Although it is difficult to evaluate the effect quantitatively, we observed a dense bloom of phytoplankton mainly composed of diatoms in same experimental system in other experiments conducted from spring to autumn (unpublished data). This indicates that the slight increase in phytoplankton biomass in this study was not because of aeration.

In this study, one vessel was arranged for each CO$_2$ treatment because of the difficulty of handling large vessels. More than two vessels for each experimental condition, certainly, are desirable to increase the reliability of the results. In this study, the stepwise decreasing trend in the standing stock of *Chrysochromulina* sp. with pCO$_2$ is obvious though control, "800", and "1200"ppm. From these results, we believe that the inhibitory effect of the increase in pCO$_2$ on the growth of *Chrysochromulina* sp. is a dependable result, but that it is necessary to conduct the additional mesocosm experiments with more than duplicate vessels to confirm the inhibitory effect of acidification to *Chrysochromulina* sp. Further, culture experiments using isolated *Chrysochromulina* sp. at different pH will furnish valuable information enabling elucidation of how reduction of pH inhibits the growth of this genus.

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