

# Response of a phytoplankton community to nutrient addition under different CO<sub>2</sub> and pH conditions

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**Abstract** Effects of nutrient enrichment on 400-L coastal phytoplankton community cultures were examined under 3 pCO<sub>2</sub> levels [ambient (400), 800 and 1200 μatm]. Three days after addition of the nutrients, rapid increases in phytoplankton pigments and cell numbers were noticed. Relative growth rates of diatoms and dinoflagellates were higher than prasinophytes and haptophytes during early stages of the culture experiment, and only limited effects of increased CO<sub>2</sub> were observed on nutrient consumption, biomass and cell numbers. Ocean acidification showed significant effects on phytoplankton composition during the post-blooming period with negligible dissolved nutrients; up to 70 % of total cells were picoplankton in the 1200-μatm condition as compared to 20 % in the ambient condition. An increase in chlorophyll *b* and a flow cytometry analysis of the cultured strains strongly suggested *Micromonas*-like (Prasinophyceae) picoplankton dominated in the acidified conditions. It is likely that the effects of ocean acidification are significant in low-nutrient conditions such as during the post-blooming period.

**Keywords** Ocean acidification · Nutrient addition · Phytoplankton · Picoplankton · Pigment · Flow cytometry

## 1 Introduction

The ocean is the most important sink of anthropogenic CO<sub>2</sub>, and thus plays a crucial role in delaying increases in atmospheric CO<sub>2</sub> concentrations and associated procession towards global warming. CO<sub>2</sub> dissolved into seawater alters the inorganic carbon-buffering system by increasing hydrogen ions when carbonic acid dissociates to bicarbonate and carbonate ions. This causes a decrease in seawater pH and, ocean acidification; the pH of surface seawater has reportedly decreased by 0.1 since the time of the industrial revolution (Midorikawa et al. 2010; Orr 2011). A further decrease by 0.3–0.4 units is expected by the end of this century (Riebesell et al. 2000; Meehl et al. 2007; Doney et al. 2009).

The increase in pCO<sub>2</sub> and the decrease in pH change the carbonate system in seawater. The concentration of CO<sub>3</sub><sup>2-</sup> will decrease by ca. 50 %, whereas CO<sub>2</sub> will increase three-fold when atmospheric CO<sub>2</sub> reaches 700 μatm (Rost et al. 2008). The possible effects of these changes on marine microorganisms have been actively examined during this decade (Riebesell and Tortell 2011; Weinbauer et al. 2011). The decrease in CO<sub>3</sub><sup>2-</sup> will affect the formation of calcium carbonate; thus, experimental studies on marine organisms having calcareous skeletons such as corals, pteropods, molluscs and echinoderms have been carried out (Knoll and Fischer 2011). As planktonic autotrophs, coccolith formations of coccolithophores such as *Emiliania huxleyi* and *Gephyrocapsa oceanica* have drawn attention (Riebesell et al. 2000; Engel et al. 2005).

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Increased CO<sub>2</sub> concentration in seawater is considered to influence all autotrophic plankton, which is likely to be favorable because CO<sub>2</sub> is the sole inorganic carbon substrate of photosynthetic carbon fixation. The response of phytoplankton, however, will vary depending on the efficiency of the carbon concentration mechanism (CCM), which increases the CO<sub>2</sub> concentration at the carboxylation site in the cell (Riebesell 2004). Diatoms have been considered to have an effective CCM, and the additional CO<sub>2</sub> is expected to cause a slight increase in their photosynthetic rate (Beardall and Raven 2004; Riebesell 2004). Although an increase in pCO<sub>2</sub> has a small effect on the growth rate or photosynthetic rate, even in natural environments and under culture conditions (e.g., Hare et al. 2007; Tatters et al. 2015), contradictory results have been also reported, which have observed enhancements of growth rate or photosynthetic rate under higher pCO<sub>2</sub> conditions (e.g., Wu et al. 2010; Kim et al. 2013). Some haptophytes such as *Emiliania huxleyi*, on the other hand, will have increased photosynthetic activity due to their less mature CCM, with a low affinity for inorganic carbon (Rost et al. 2008). Dinoflagellates, which are one of the dominant groups in coastal areas, also reportedly possess an immature CCM (Reinfelder 2011) and are expected to have a positive response to increased CO<sub>2</sub> concentration. However, experimental results on the effect of ocean acidification sometimes cannot be interpreted by the difference in CCM maturity. These inconsistencies between information accumulated regarding the CCM and the experimental response of a natural community pose a question about the application of laboratory data to natural ecosystems (Mercado and Gordillo 2011), and imply that factors other than the development of the CCM are concerned with determining the response of the phytoplankton group to acidification.

Recent studies reported that specific phytoplankton groups respond to ocean acidification (Meakin and Wyman 2011; Hama et al. 2012; Newbold et al. 2012). For example, *Chrysochromulina* spp., Haptophyceae, had decreased growth under an acidified condition (Hama et al. 2012), whereas the contribution of picoplankton, *Micromonas* spp. (Prasinophyceae), was found to increase growth by high CO<sub>2</sub>/low pH treatments (Meakin and Wyman 2011; Newbold et al. 2012). These compositional changes sometimes resulted in a shift in the size composition of phytoplankton; the predominance of picoplankton was noticed at high CO<sub>2</sub> levels (Meakin and Wyman 2011; Brussaard et al. 2013).

The nutrient levels in coastal regions change temporarily, mainly due to variable continental runoff. These pulsed nutrient inputs induce the phytoplankton community to make short-term blooms with subsequent return to baseline levels concomitant with exhaustion of the additional nutrients (Spatharis et al. 2007; Arndt et al. 2011). Coastal phytoplankton communities will be subject to the effect of

pulsed nutrient inputs under increased pCO<sub>2</sub> and decreased pH conditions. Since the coastal region plays a significant role in the global biogeochemical cycle of bioelements such as carbon, nitrogen and phosphorus, it is necessary to examine the response of the phytoplankton community to acidification. Large-scale mesocosm experiments have been carried out in the Arctic region and substantial information has been accumulated (e.g., Riebesell et al. 2007, 2013; Engel et al. 2013). In the temperate region, however, limited study has been applied (Kim et al. 2006, 2013; Yoshimura et al. 2010; Hama et al. 2012; Endo et al. 2013).

The change in phytoplankton composition due to ocean acidification likely affects the structure of the food web, including the number of trophic levels (Sommer et al. 2002; Hilligsøe et al. 2011). These variations, in turn, will cause changes in the biogeochemical cycle, such as the export flux of organic matter and the sea-air CO<sub>2</sub> flux (Cullen et al. 2002; Hilligsøe et al. 2011). Information on the response of the natural microbial community serves to predict future possible changes in natural ecosystems under acidified conditions.

In the present study, we conducted a nutrient-addition experiment with large-volume outdoor culture vessels (400 L) using a coastal phytoplankton community under varying CO<sub>2</sub> levels (ambient, 800 and 1200 μatm). Flow cytometry (FCM) and pigment analysis by high-performance liquid chromatography (HPLC) were applied to determine the composition of the phytoplankton community, including size. The photosynthetic production rate was measured by the carbon-13 (<sup>13</sup>C) tracer experiment to compare the relation between CO<sub>2</sub> levels and the phytoplankton response to nutrient addition.

## 2 Methods

### 2.1 Experimental design

The acidification experiment was carried out at Shimoda Marine Station, University of Tsukuba, Shimoda, Shizuoka, Japan, from Nov. 8 to Dec. 9, 2011. Six 500-L cylindrical acrylic vessels were used for the culture experiments. Three vessels were set up in a large water tank (outer tank; 4000 L) filled with running coastal seawater to control the water temperature and three other vessels were also set up in another tank. Water samples (400 L for each vessel) were slowly pumped from the coastal site on Nov. 8 with a 100-μm mesh filter to remove macrozooplankton. Control of the three levels of pCO<sub>2</sub> (ambient, 800 μatm, and 1200 μatm) and sampling procedure have been shown elsewhere (Hama et al. 2012). Briefly, air with three levels of pCO<sub>2</sub> was prepared by mixing ambient air and CO<sub>2</sub> gas [including 3 % in a volume of pure air (N<sub>2</sub> and O<sub>2</sub>)], and introduced from the

bottom of the vessel. Bubbling of purge air was applied for 2 days after the samples were introduced, the purpose of which was to condition the experimental waters by adjusting the pCO<sub>2</sub> and pH. After that, the purge was carried out for 6 h every day (from 10:00 to 16:00) until the end of the experiments. The inorganic nutrients KNO<sub>3</sub> (1.60 mmol per one vessel), KH<sub>2</sub>PO<sub>4</sub> (0.100 mmol) and Na<sub>2</sub>SiO<sub>3</sub>·H<sub>2</sub>O (1.60 mmol) were added to the water sample on Nov. 11 (day 1), following the standard N:P:Si (16:1:16) ratio in marine environments (Redfield 1963). These amounts correspond to the concentrations of 4.00, 0.250 and 4.00 μM for N, P and Si, respectively, in a culture vessel.

Water samples were collected on days 1, 2, 3, 5, 7, 10, 13, 17, 23, 25 and 29 using polycarbonate tubing from 9:00 to 10:00 am local time. Samples for pH, dissolved inorganic carbon (DIC) and inorganic nutrients were prepared as described in Hama et al. (2012). In addition to the above items, phytoplankton pigments were measured in the present study. After being placed onto glass fiber filters (Whatman GF/F), particulate matter was immediately stored at -80 °C until analysis.

Photosynthetic photon flux density (PPFD) was monitored by a quantum sensor (SQ-110, Apogee) throughout the experiment.

## 2.2 Photosynthetic production rate

To an aliquot of the sample water, two acid-cleaned polycarbonate bottles were introduced (500 mL) for each vessel, and <sup>13</sup>C-NaHCO<sub>3</sub> was added with the <sup>13</sup>C atom % of 15 %. The bottles were suspended in a large water tank and incubated for 5 h under natural light intensity. After samples were recovered, they were filtered onto pre-combusted glass fiber filters (Whatman GF/F) and stored at -20 °C until analysis.

## 2.3 Analysis

The procedure for determining the CO<sub>2</sub> system, including pH, has been shown elsewhere (Hama et al. 2012); total inorganic carbon (TCO<sub>2</sub>) was analyzed coulometrically (Johnson et al. 1985) and calibrated with a series of sodium carbonate solutions. The pH value was measured spectrophotometrically on a total hydrogen ion concentration scale at 25 °C (Clayton and Byrne 1993), with an automated flow-cell system (Saito et al. 2008). Partial pressure of CO<sub>2</sub> (pCO<sub>2</sub>) and pH at the in situ temperature, measured by a temperature logger (MDS-MkV/T, JFE Advantech), was calculated from TCO<sub>2</sub>, pH at 25 °C, temperature, and salinity, by use of the dissociation constants of carbonic acid according to Lueker et al. (2000).

The concentration of inorganic nutrients such as nitrate, nitrite, ammonium, phosphorus and silicate was determined

by colorimetry (Hansen and Koroleff 2007) using an auto-analyzer (AACSII, BRAN + LUEBBE).

The concentration of particulate organic carbon (POC) was measured by an elemental analyzer (EA1108, Carlo Erba) and <sup>13</sup>C atom % was measured by an isotope-ratio mass spectrometer (Delta Plus; Finnigan MAT) as shown elsewhere (Hama et al. 1993).

Production of POC during the incubation (5 h) was calculated by Eq. (1) (Hama et al. 1983, 1993).

$$\begin{aligned} \text{Production} \left( \mu\text{M}\cdot\text{CL}^{-1}\cdot\text{incubation}^{-1} \right) \\ = (\text{ais} - \text{ans}) / (\text{aic} - \text{ans}) \times \text{POC} \end{aligned} \quad (1)$$

where ais is the <sup>13</sup>C atom % of the incubated sample, ans is the <sup>13</sup>C atom % of the non-incubated sample, aic is the <sup>13</sup>C atom % of the inorganic carbon, and POC is the concentration of POC of the incubated sample (μM·C). The daily production rate was estimated assuming that the ratio of photosynthetic production from the incubation (5 h) to daily production is equivalent to the ratio of PPFD during the incubation to that of the whole day.

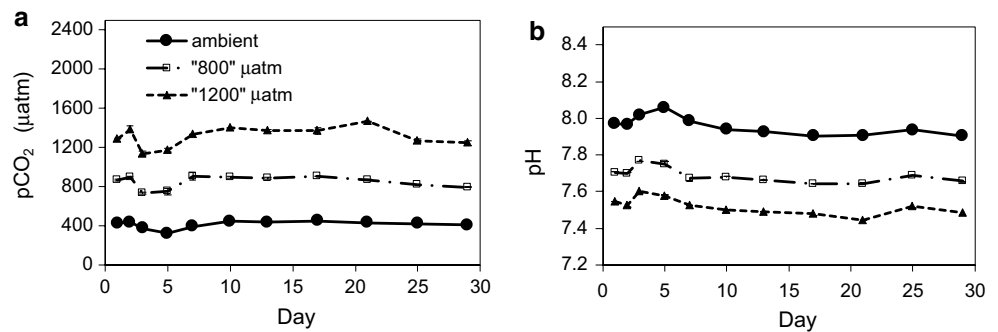
Pigment concentration was determined by HPLC (Heuvelink and Thomas 2001; Jeffrey et al. 2005). Filter samples were soaked with *N,N*-dimethyl formamide (DMF) containing canthaxanthin as an internal standard, and pigments were extracted with a probe sonicator for 30 s at 50 W. After the extract was filtered through a polytetrafluoroethylene (PTFE) filter (0.45-μm pore size), the same volume of tetrabutyl ammonium acetate (TBAA, 28 mM) with DMF was added. Pigment concentration was determined with an HPLC system (Agilent 1100 system controlled by ChemStation) equipped with a diode array detector (450 and 665 nm) and a fluorescent detector (excitation: 440 and fluorescence: 660 nm). A 100-μL sample was injected into the column (Agilent Eclipse XDB-C8), and separation of the pigments was achieved with a gradient between solution A (28 mM TBAA) and solution B (100-% methanol). Three determinations were carried out for each sample and the ratio of the standard deviation (SD) to the average value was generally less than 7 %. The specific growth rate of each phytoplankton taxon from days 1 to 3 was calculated using the concentration of pigments by Eq. (2).

$$\text{Specific growth rate} \left( \text{d}^{-1} \right) = 1/2 \times \ln(\text{C3}/\text{C1}), \quad (2)$$

where C1 and C3 are the pigment concentrations on days 1 and 3, respectively.

For FCM analysis, sample water was filtered through a 59-μm mesh to avoid obstructing the sampling nozzle; it was ascertained that this treatment caused a slight deviation in the chlorophyll concentration from the non-filtered sample. However, it is possible that a portion of the

**Fig. 1** Changes in the partial pressure of CO<sub>2</sub> (pCO<sub>2</sub>) (a), and the pH in the total hydrogen ion scale (b), in the ambient, 800- and 1200- $\mu$ atm CO<sub>2</sub> culture vessels. The mean value of the two vessels is shown with the bar representing the range (the range was too little to identify the bars in the figures)



of chain-forming diatoms such as *Chaetoceros* sp. and *Skeletonema* sp., which are usually found in this region (Hama et al. 2012), were removed during this process. FCM analysis was performed with a FACSCalibur system (four-color type: Becton–Dickinson). Fluorescences emitted were monitored at FL 1 (bandpass  $530 \pm 15$  nm), FL 2 ( $585 \pm 21$  nm), FL 3 ( $>670$  nm) and FL 4 ( $661 \pm 8$  nm). A FACSFlow sheath fluid (Becton–Dickinson) was forced through a 100- $\mu$ m nozzle with the pressure of 4.5 psi, with a 60- $\mu$ L min<sup>-1</sup> sample flow rate. Fluoresbrite microspheres (2 and 6  $\mu$ m, Polysciences) were used to estimate the phytoplankton size, and "Trucount Tubes" (Becton–Dickinson) were used to evaluate the real flow rate. FCM analysis was carried out as soon as possible after collection of the samples (usually finished within 5 h). Analyses of samples were not carried out on days 5 and 7 due to malfunction of the apparatus.

## 2.4 Statistical analysis

The effect of the change in the CO<sub>2</sub> treatments on the biological parameters was tested on each day by means of one-way analysis of variance (ANOVA). When the one-way ANOVA was significant, post hoc multiple comparisons were made using Tukey's test between the treatments.

## 3 Results

### 3.1 Physico-chemical conditions

Changes in the pCO<sub>2</sub> in the three treatments are shown in Fig. 1a. The initial values, which were determined after the introduction of sample waters to the vessel, were the same in all six vessels. The values on day 1 varied, depending on the treatments of the purged gas; 423, 861 and 1285 for the ambient, 800- and 1200- $\mu$ atm vessels, respectively. All pCO<sub>2</sub> values tended to decrease from days 2 to 3, probably due to the increase in phytoplankton biomass induced by the addition of the nutrients. Relatively constant pCO<sub>2</sub> values were maintained in every vessel from day 10 to the

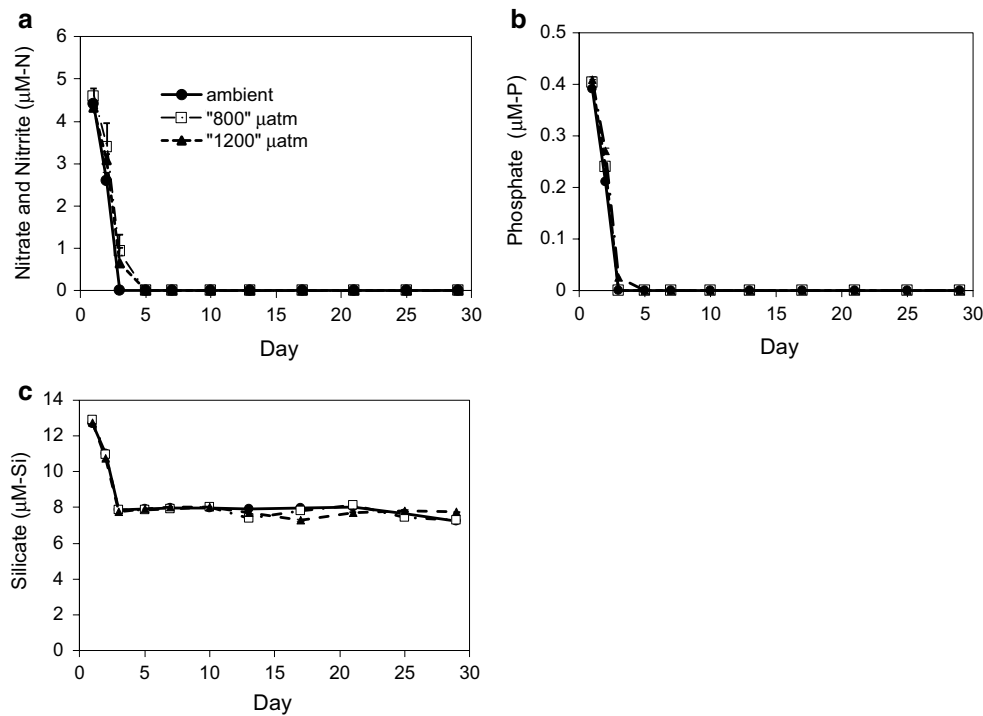
end of the experiments. Variation between the two vessels with the same treatment was relatively low; the ratio of the deviation from the mean value of the two vessels was usually within 1 %. The pH value varied, reflecting a change in the pCO<sub>2</sub>, showing a range of 7.90–8.06, 7.64–7.77 and 7.48–7.60 in the ambient, 800- and 1200- $\mu$ atm vessels, respectively (Fig. 1b). An increase in pH values was noted in all vessels from days 3 to 7, concomitant with the decrease in pCO<sub>2</sub>. The pCO<sub>2</sub> and pH results demonstrated that the carbonate system in each treatment was well controlled throughout the experiments by the pre-conditioning for 2 days and the 6-h purge every day.

Nutrient concentrations of the original sample water introduced to the experimental vessels were 1.56, 0.18 and 7.83  $\mu$ M for nitrate, phosphate and silicate, respectively (Fig. 2a, b). The concentrations increased to 4.4–4.5, 0.40 and 12.8–12.9  $\mu$ M for nitrate, phosphate and silicate, respectively, after addition of nutrients on day 1. A rapid decrease in the concentration of nitrate and phosphorus was found from the start of the experiment, and they were almost depleted on day 3. Both concentrations were lower than the detection limit after day 5. The concentration of silicate decreased in the first 2 days and less than 8  $\mu$ M remained on day 3 (Fig. 2c). Comparable values were measured through the rest of the experiments, but a small difference among the treatments was found.

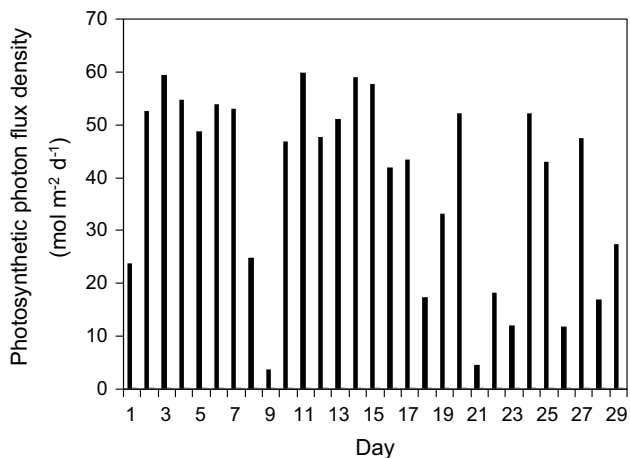
PPFD varied from 3.8 (day 9) to 60.0 (day 3) mmol photons m<sup>-2</sup> d<sup>-1</sup> during the experiment (Fig. 3). It was generally fine weather during the former period (days 1–17) of the experiment, and high daily PPFD, more than 40 mmol photons m<sup>-2</sup> d<sup>-1</sup>, was measured for 14 days. In the latter period (days 18–29), on the other hand, high values with  $>40$  mmol photons m<sup>-2</sup> d<sup>-1</sup> were noticed only on 5 out of 12 days.

### 3.2 Phytoplankton pigments

Rapid increases in the concentration of chlorophyll (chl.) *a* were found from days 1 to 3 in every vessel (Fig. 4a). The maximum concentration of chl. *a* varied from 4.16 to 4.56  $\mu$ g L<sup>-1</sup> on day 3; they showed drastic decreases



**Fig. 2** Changes in the concentration of nitrate and nitrite (a), phosphate (b) and silicate (c) in the filtrate. The mean value of two vessels is shown with the bar representing the range



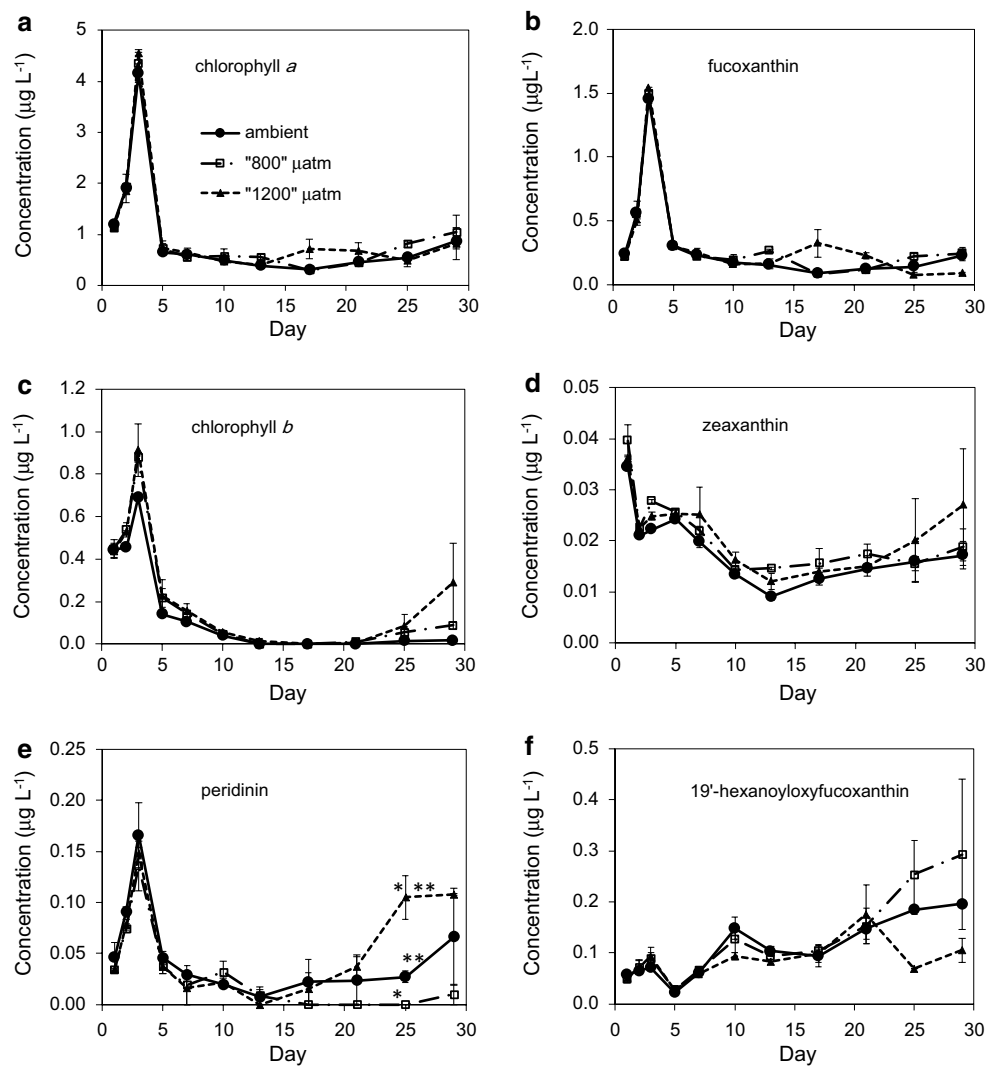
**Fig. 3** Daily photosynthetic photon flux density (PPFD) measured at the experimental site throughout the experiment

until day 5 of around  $0.7 \mu\text{g L}^{-1}$ . The concentrations varied from  $0.3$  to  $0.7 \mu\text{g L}^{-1}$  in the post-blooming period, and increases were observed for all treatments from days 25 to 29. A rather higher concentration was found in the  $1200\text{-}\mu\text{atm}$  sample during days 17 and 21 compared to other treatments. Almost comparable day-to-day changes with chl. *a* were noticed for fucoxanthin (Fig. 4b), with the highest concentrations on day 3 ( $1.45\text{--}1.54 \mu\text{g L}^{-1}$ ), and relatively low concentrations of less than  $0.3 \mu\text{g L}^{-1}$  from

day 5. Maximum chl. *b* was also found on day 3 (Fig. 4c); higher concentrations were noticed for the  $800\text{-}$  and  $1200\text{-}\mu\text{atm}$  treatments than for the ambient condition. In the acidified condition, a considerable increase in chl. *b* on days 21–29 was obvious, and on day 29, the mean concentrations of chl. *b* in the  $800$  and  $1200 \mu\text{atm}$  were higher than that of the ambient treatment, though the concentrations between two vessels in the  $1200\text{-}\mu\text{atm}$  treatment showed a significant difference. The concentration of zeaxanthin showed a trend to decrease from the start of the experiment to day 13 (Fig. 4d) being different from the other pigments. A slight increase was noticed in the  $1200\text{-}\mu\text{atm}$  treatment on day 29 for zeaxanthin. The rapid increases in the concentration of peridinin (Fig. 4e) in the first 3 days were almost comparable with chl. *a* and fucoxanthin. Increase in the latter period was obvious for the highest CO<sub>2</sub> condition from day 25. With time, a different pattern was found for 19'-hexanoyloxyfucoxanthin (Fig. 4f), which showed only small maxima on day 3, and concentrations of 19'-hexanoyloxyfucoxanthin gradually increased later. Although no difference in the concentration among treatments was obvious until day 21, the differences became definite after day 25. The concentration in the  $1200\text{-}\mu\text{atm}$  condition was the lowest on day 29, being about one third and half of those in the  $800 \mu\text{atm}$  and ambient treatments, respectively.

Statistical analysis was applied to the concentration of pigments to evaluate the difference in the CO<sub>2</sub> treatments.

**Fig. 4** Changes in the concentration of chlorophyll *a* (a), fucoxanthin (b), chlorophyll *b* (c), zeaxanthin (d), peridinin (e) and 19'-hexanoyloxyfucoxanthin (f). The mean value of two vessels is shown with the bar representing the range of the two vessels. When the difference between the treatments was statistically significant ( $p < 0.05$ ) with Tukey's test, \* or \*\* are shown



**Table 1** Specific growth rate ( $d^{-1}$ ) estimated by the increases in the pigment concentrations from day 1 to day 3

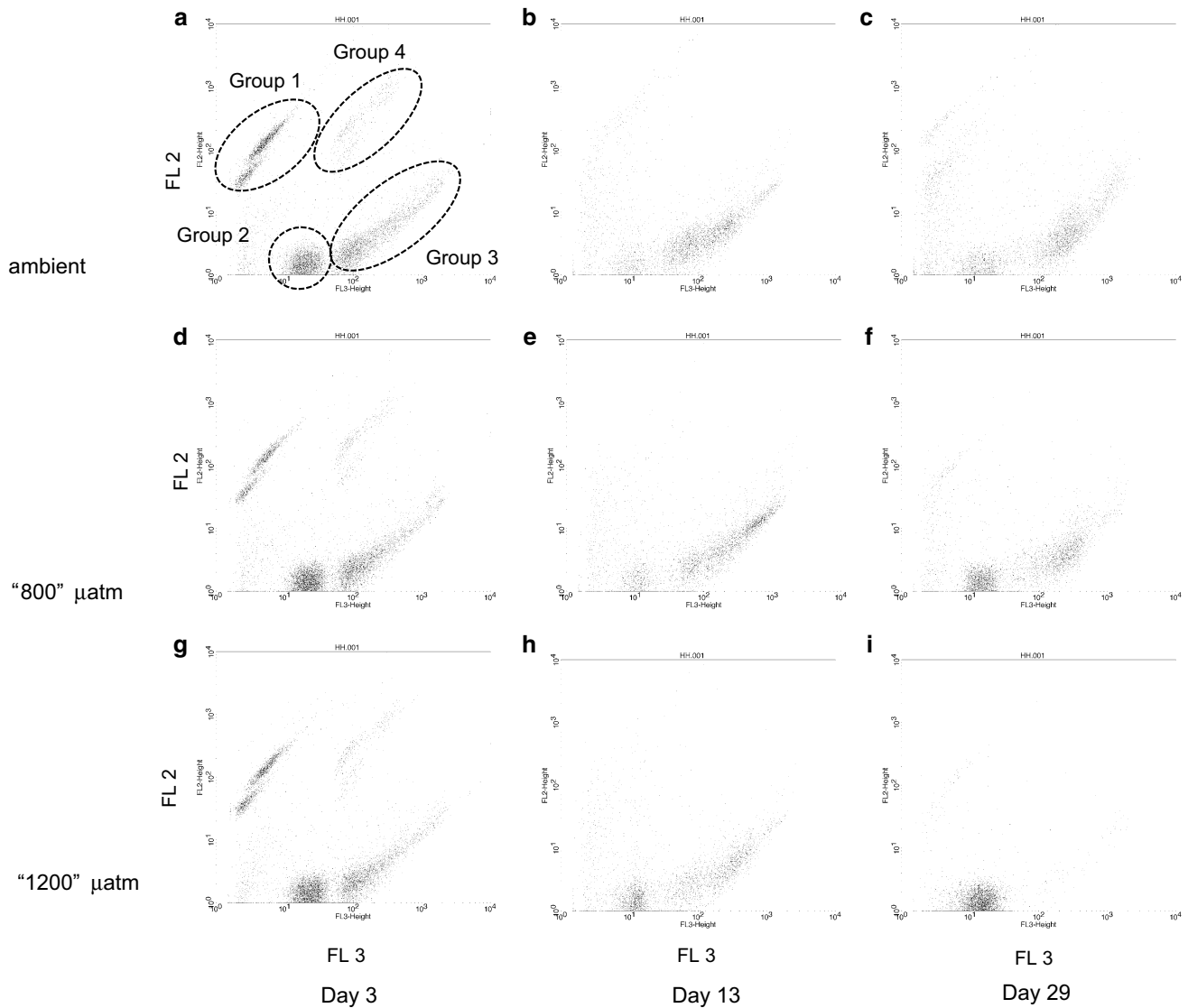
	Pigment				
	Chlorophyll <i>a</i>	Fucoxanthin	Chlorophyll <i>b</i>	Peridinin	19'-Hexanoyloxyfucoxanthin
Ambient	0.620 (0.041)	0.911 (0.025)	0.307 (0.194)	0.728 (0.134)	0.105 (0.081)
800 $\mu\text{atm}$	0.672 (0.006)	0.963 (0.004)	0.362 (0.009)	0.684 (0.116)	0.233 (0.131)
1200 $\mu\text{atm}$	0.699 (0.024)	0.991 (0.017)	0.409 (0.047)	0.657 (0.087)	0.261 (0.049)

Deviation from the mean value is shown in parentheses

The difference in the concentration of peridinin among the treatments (ambient—800 and ambient—1200  $\mu\text{atm}$ ) on day 25 was confirmed statistically ( $p < 0.05$ ). Although the mean concentrations of chl. *b* and 19'-hexanoyloxyfucoxanthin seemed to be different among the treatments on days 25 and 29, Tukey's test did not show the statistically significant difference in the concentration.

The relative growth rates of phytoplankton responding to the nutrient addition were estimated by the increase rates of the chl. *a* concentrations from days 1 to 3 (Table 1). The

rates varied from 0.620 (ambient) to 0.699  $d^{-1}$  (1200  $\mu\text{atm}$ ) among the treatments. Pigments including fucoxanthin, chl. *b*, peridinin and 19'-hexanoyloxyfucoxanthin, which showed increases during days 1–3, were provided to estimate the specific growth rates of phytoplankton taxonomic groups separately. The variations among the  $\text{CO}_2$  treatments were not obvious for every pigment and no statistical difference was found, indicating only a minor effect of  $\text{CO}_2$  and pH on the growth of these four taxonomic groups in this stage. When we compare the rates among the pigments



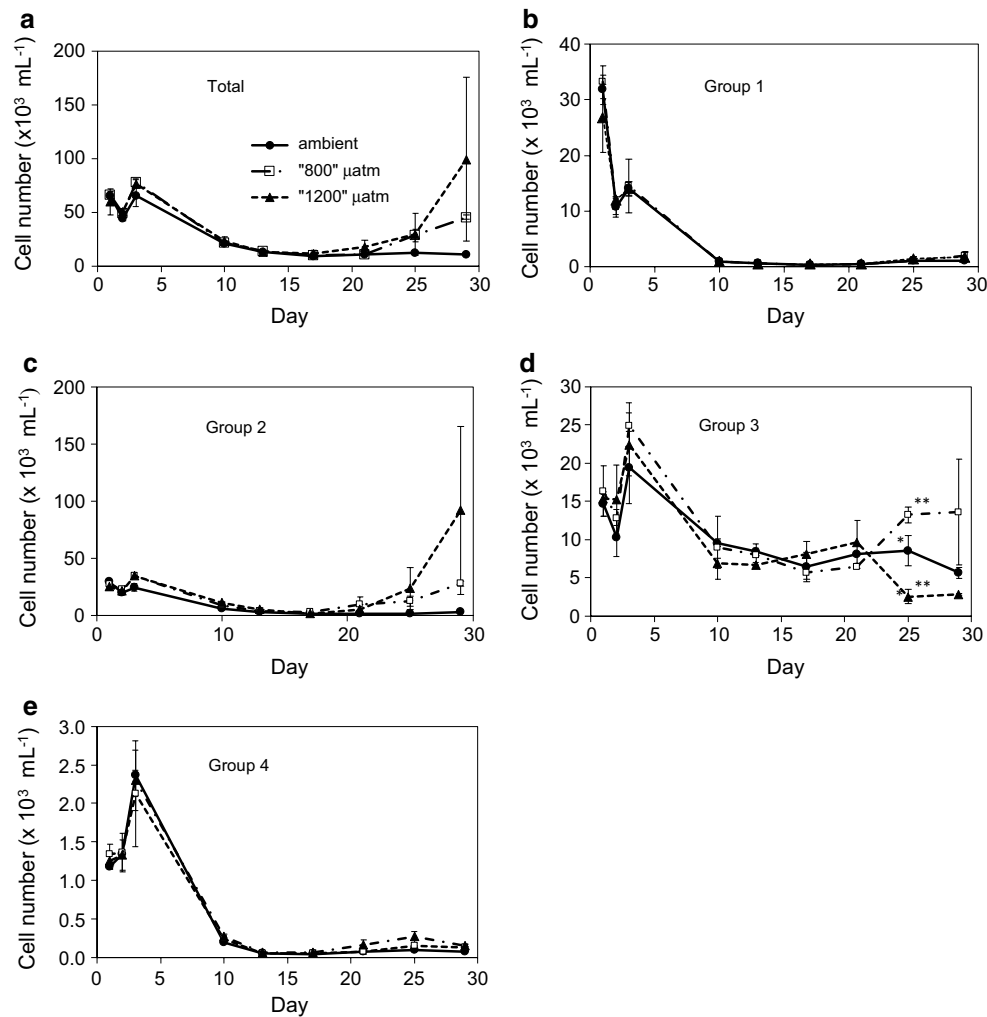
**Fig. 5** Flow cytometry of the samples collected from the ambient, 800- and 1200-μatm vessels on days 3, 13 and 29. Circles depict phytoplankton groups 1–4. The relationship between FL3 (>670 nm) and FL2 (585 ± 21 nm) are shown. **a–c** ambient, **d–f** 800 μatm, **g–i** 1200 μatm

in the same treatment, they varied considerably. The rate of fucoxanthin was higher than that of chl. *a* in every treatment. Peridinin showed an almost comparable rate with chl. *a*, whereas rates of chl. *b* and 19'-hexanoylfucoxanthin were considerably low compared with those of chl. *a*.

### 3.3 Flow cytometry (FCM)

The results of the FCM analysis of the phytoplankton community are shown in Fig. 5; the plot of FL 2 versus FL 3 was found to be the most suitable for grasping the phytoplankton composition. Four groups were gated depending on the analysis. Total cell number showed a slight decrease from day 1 to 2 and then increased from 44.5–50.8 × 10<sup>3</sup> cell mL<sup>-1</sup> on day 2 to

66.5–78.3 × 10<sup>3</sup> cells mL<sup>-1</sup> on day 3 (Fig. 6a). The cell number on day 10 was significantly low compared with those on day 3 and little temporal change was noticed up to day 17. Although little change was found in the ambient condition, the cell numbers increased in the acidified condition after day 21. Both incubation vessels with 800 μatm showed increases on days 25 and 29 with the average values of 28.8 × 10<sup>3</sup> and 45.8 × 10<sup>3</sup> cells mL<sup>-1</sup>, respectively. A distinctive increase in the average cell number of the 1200-μatm treatment was found as 29.6 × 10<sup>3</sup> and 99.5 × 10<sup>3</sup> on days 25 and 29, respectively. However, this increase was mainly due to one vessel, and the increase in the other vessel was not distinct, resulting in the statistical insignificance of the difference between the 1200-μatm treatment and other treatments.



**Fig. 6** Total changes in cell number (a), in group 1 (b), in group 2 (c), in group 3 (d) and in group 4 (e). The mean value of the two vessels is shown with the range. \* and \*\* show the statistical significance ( $p < 0.05$ ) between the treatments. Measurement was not done

on days 5 and 7 due to malfunction of the apparatus. The mean value of the two vessels is shown with the *bar*, representing the range for the two vessels

Cell numbers of group 1 (G1) which is likely composed of *Synechococcus* (cyanobacteria) showed a drastic decrease from day 1 to 2, suggesting that the decrease in total cell number in this period was primarily due to this group (Fig. 6b). The cell numbers were less than  $1 \times 10^3 \text{ mL}^{-1}$  after day 10 in all treatments and little difference was noticed among the treatments. The contribution of G1 accounted for about 40 % of the total phytoplankton cells on day 1 (Table 2; note that since not all cells were fractionated to four groups, the sum of the contribution of four groups in each treatment was less than 100 %, accounting for 81.2–97.2 % of total cell number with 91.6 % as a mean). It showed a rapid decrease with time in the bloom period in every treatment to about 5 % on day 10. The low contribution continued throughout the experiment. The results clearly showed that a little difference was noticed among the treatments for G1.

The cell number of group 2 (G2) having low FL 2 and medium FL3 intensities (phytoplankton composing this group will be discussed in detail later) at the start of the experiment was almost comparable with those of G1 and they were kept low till day 21 in all treatments (Fig. 6c). Although the number in the ambient treatment was low through the post-blooming period of the experiment, those in the 800- and 1200- $\mu\text{atm}$  vessels increased from day 21. The cell number of one 1200- $\mu\text{atm}$  vessel increased more than  $160 \times 10^3 \text{ cells mL}^{-1}$  on day 29, but a slight increase was found in the other 1200- $\mu\text{atm}$  vessel. G2 accounted for 33.7–37.2 % on day 1 being lesser than those of G1. In the ambient condition, the contribution of G2 decreased from day 3 to a minimum (13.6 %) on day 21, with a small increase from days 25 to 29. A drastic increase was found in the 1200- $\mu\text{atm}$  vessel from 13.7 % on day 17 to 86.7 %



**Table 2** Contribution of cell number in each group to total cell number

	Day 1	Day 2	Day 3	Day 10	Day 13	Day 17	Day 21	Day 25	Day 29
<b>Group 1</b>									
Ambient	39.9 (0.5)	24.3 (3.7)	21.5 (1.9)	4.6 (1.4)	3.9 (0.6)	2.4 (0.5)	3.2 (1.1)	8.7 (1.0)	8.6 (1.0)
800 $\mu\text{atm}$	41.5 (1.3)	21.5 (3.0)	18.0 (3.0)	3.9 (1.0)	3.9 (1.6)	2.9 (0.4)	3.1 (1.7)	3.5 (0.1)	4.2 (1.5)
1200 $\mu\text{atm}$	37.0 (3.2)	23.2 (0.3)	18.3 (2.0)	3.9 (0.3)	3.2 (0.6)	3.8 (2.2)	2.6 (0.3)	6.7 (3.2)	2.9 (1.7)
<b>Group 2</b>									
Ambient	37.2 (2.0)	43.8 (1.8)	37.2 (0.6)	*28.8 (3.0)	21.4 (4.5)	16.9 (2.8)	13.6 (1.7)	14.9 (6.1)	*27.3 (1.7)
800 $\mu\text{atm}$	33.7 (0.5)	45.0 (1.9)	43.5 (1.7)	39.9 (0.3)	27.5 (1.9)	30.4 (3.5)	48.1 (18.7)	42.9 (7.6)	61.2 (8.2)
1200 $\mu\text{atm}$	35.1 (1.5)	41.2 (3.7)	45.6 (1.3)	*46.3 (1.3)	35.7 (10.6)	13.7 (5.9)	26.0 (13.2)	67.5 (17.6)	*86.7 (7.8)
<b>Group 3</b>									
Ambient	18.5 (2.3)	23.0 (2.6)	29.4 (0.5)	47.3 (2.4)	65.1 (4.0)	*72.1 (0.9)	73.1 (4.1)	*67.7 (6.3)	*51.0 (3.2)
800 $\mu\text{atm}$	20.3 (1.0)	25.8 (0.8)	31.4 (0.3)	39.5 (4.6)	58.5 (4.4)	*56.2 (3.1)	40.9 (13.6)	47.5 (5.9)	29.2 (9.1)
1200 $\mu\text{atm}$	21.6 (2.2)	29.4 (4.8)	29.2 (2.4)	29.8 (0.4)	51.5 (8.0)	70.6 (3.4)	59.8 (11.4)	*12.9 (6.4)	*6.6 (5.0)
<b>Group 4</b>									
Ambient	1.5 (0.0)	3.0 (0.3)	3.7 (0.7)	1.0 (0.0)	0.4 (0.1)	0.6 (0.1)	0.7 (0.3)	0.8 (0.2)	0.7 (0.1)
800 $\mu\text{atm}$	1.7 (0.0)	2.8 (0.4)	2.8 (1.1)	1.1 (0.1)	0.4 (0.0)	0.5 (0.0)	0.5 (0.2)	0.6 (0.2)	0.3 (0.1)
1200 $\mu\text{atm}$	1.8 (0.0)	2.6 (0.5)	3.0 (0.7)	1.2 (0.2)	0.6 (0.2)	0.5 (0.2)	1.0 (0.0)	1.3 (0.6)	0.4 (0.3)

The deviation from the mean value is shown in parentheses

\* Statistical significance between the treatments ( $p < 0.05$ ). Note that the sum of each treatment is less than 100, since total cell number includes cells from other than the four groups

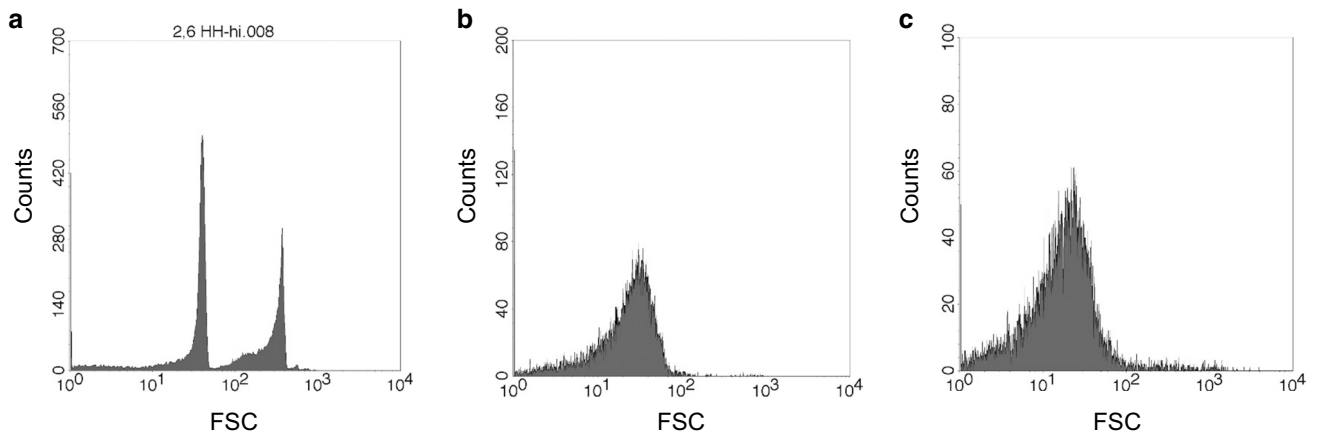
on day 29 as average. An intermediate G2 contribution between the ambient and the 1200  $\mu\text{atm}$  was measured for the 800- $\mu\text{atm}$  treatment on days 25 and 29. Statistical significance ( $p < 0.05$ ) among the treatments was not found for cell number but noticed for the contribution of G2 between the ambient and 1200- $\mu\text{atm}$  treatments on day 29.

Various phytoplankton groups, including haptophytes and diatoms, likely constitute group 3 (G3; Hama et al. 2012). The cell numbers in G3 varied from 10–25  $\times 10^3 \text{ mL}^{-1}$  during days 1 to 3 and showed relatively constant values from days 10 to 21 between 5–10  $\times 10^3 \text{ cells mL}^{-1}$  (Fig. 6d). On days 25 and 29, the cell number of the 800- $\mu\text{atm}$  treatments increased more than 10  $\times 10^3 \text{ cells mL}^{-1}$ , but that of the 1200 vessel decreased to about 2  $\times 10^3 \text{ cells mL}^{-1}$ . The differences between the ambient–1200- $\mu\text{atm}$  and the 800–1200- $\mu\text{atm}$  vessels on day 25 were statistically significant ( $p < 0.05$ ). Although the contribution of G3 was not significant on day 1 (18.5–21.6 %), the contribution increased as the experiment proceeded in every treatment. In the 1200- $\mu\text{atm}$  condition, a rapid decrease was obvious after the maximum level (70.6 %) on day 17. In the ambient treatment, on the other hand, a high contribution with a range of 51.0–73.1 % was maintained between days 13 and 29. On day 29, the contribution of G3 decreased with the degree of acidification as 51.0, 29.2 and 6.6 % in the ambient, 800- and 1200- $\mu\text{atm}$  vessels, respectively. The low contributions in the 1200 vessel on days 25 and 29 were statistically confirmed compared with the ambient treatment ( $p < 0.05$ ).

The cell numbers in G4 were lowest among the four groups, less than 3  $\times 10^3 \text{ cells mL}^{-1}$  (Fig. 6e); they showed a distinct maxima on day 3 and showed a rapid decrease until day 10, and then the cell numbers remained low throughout the rest of the experiment. No significant difference was found among the treatments, except on day 29. Analysis of the cultured phytoplankton indicates that cryptophytes are one of the main components of this group. G4 was not an important group in terms of cell number throughout the experiment and its contribution was less than 4 %, with little difference among treatments.

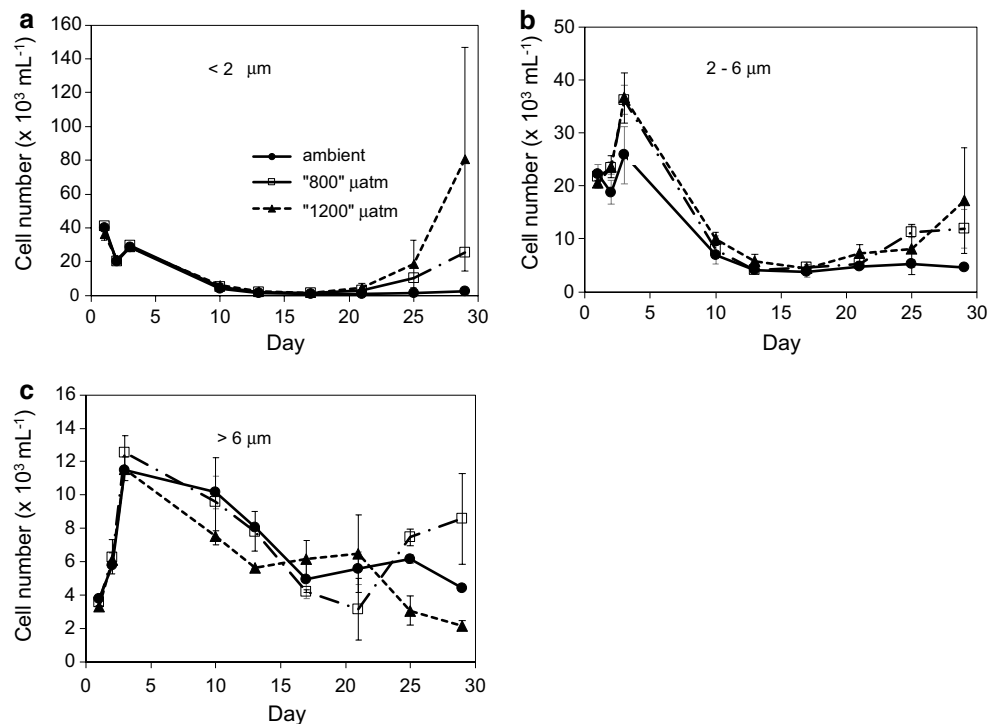
### 3.4 Size composition

The phytoplankton community was divided into three size categories, <2, 2–6, and >6  $\mu\text{m}$ , by measuring the standard beads with 2- and 6- $\mu\text{m}$  diameters (Fig. 7a). The cell number of the <2- $\mu\text{m}$  group varied from 20.0–21.2  $\times 10^3$  to 36.3–41.1  $\times 10^3 \text{ cells mL}^{-1}$  during days 1–3 without significant differences among the treatments (Fig. 8a). The number in the ambient treatment varied with low values in the range of 0.7–2.4  $\times 10^3 \text{ mL}^{-1}$  from days 13 to 29, but substantial increases were found in the acidified conditions. In the 1200- $\mu\text{atm}$  treatments, especially, the average value of the 2 vessels on day 29 was more than 20 times higher than that on day 21. However, the difference between 2 vessels of the 1200 treatment was high and the statistical significance between the CO<sub>2</sub> treatments was not confirmed in absolute cell number. The contribution of cell



**Fig. 7** Forward scatter (FSC) intensity histogram of standard beads with diameters of 2 and 6  $\mu\text{m}$  (a), of the 1200- $\mu\text{atm}$  sample on day 29 (b), and of *Micromonas pusilla* (NIES-1411) from the microbial

culture collection at the National Institute of Environmental Sciences (<http://mcc.nies.go.jp/localeAction.do?lang=en>) (c)



**Fig. 8** Cell number change in the  $<2\text{-}\mu\text{m}$  (a), the  $2\text{-}6\text{-}\mu\text{m}$  (b), and the  $>6\text{-}\mu\text{m}$  (c) size fractions through the experiment. The mean values of two vessels are shown with the range. The mean value of two vessels is shown with the bar representing the range

number of the  $<2\text{-}\mu\text{m}$  fraction accounted for about 60 % at the start of the experiment (Table 3). The contributions of this fraction showed a decrease until day 17, with values of 7.4, 15.4 and 9.9 % in the ambient, 800- and 1200- $\mu\text{atm}$  vessels, respectively. Substantial increases were noticed for the 1200- and 800- $\mu\text{atm}$  vessels after day 21, and values on day 29 were almost comparable with those of day 1 in

both treatments; 72.1 and 55.6 % in 1200- and 800- $\mu\text{atm}$  treatments, respectively. However, only a small increase to 20.6 % was found in the ambient vessel; the difference between 1200- $\mu\text{atm}$  and the ambient treatments on day 29 was statistically significant ( $p < 0.05$ ).

Phytoplankton 2–6  $\mu\text{m}$  in diameter were the secondary important size fraction, ranging from 17.8–36.6  $\times 10^3 \text{ mL}^{-1}$

**Table 3** Contribution of cell number in each size fraction to total cell number

	Day 1	Day 2	Day 3	Day 10	Day 13	Day 17	Day 21	Day 25	Day 29
<b>&lt;2 μm</b>									
Ambient	60.3 (0.6)	44.8 (2.3)	42.5 (0.2)	18.4 (3.2)	9.6 (0.6)	7.4 (1.3)	9.3 (0.8)	9.4 (1.8)	*20.6 (0.2)
800 μatm	61.7 (0.5)	40.3 (3.1)	37.6 (1.7)	23.0 (5.0)	14.5 (4.3)	15.4 (3.1)	26.1 (9.1)	32.7 (11.1)	55.6 (12.1)
1200 μatm	60.1 (2.0)	41.4 (0.5)	37.3 (2.0)	26.1 (0.3)	16.2 (2.1)	9.9 (1.7)	21.0 (7.6)	55.5 (10.5)	*72.1 (10.9)
<b>2–6 μm</b>									
Ambient	33.9 (0.5)	42.1 (2.9)	39.6 (2.4)	33.1 (2.0)	30.2 (3.1)	39.6 (0.8)	41.6 (5.6)	41.7 (3.0)	*40.3 (1.2)
800 μatm	32.8 (0.6)	47.0 (1.3)	46.2 (0.4)	34.4 (3.8)	29.7 (0.6)	42.8 (3.1)	46.4 (6.3)	39.9 (4.0)	25.8 (6.9)
1200 μatm	34.3 (1.6)	46.4 (1.8)	47.5 (3.7)	41.9 (1.3)	41.8 (3.8)	37.4 (1.3)	42.4 (6.7)	29.5 (2.6)	*23.1 (7.6)
<b>&gt;6 μm</b>									
Ambient	5.8 (0.1)	13.1 (0.6)	17.9 (2.5)	48.5 (5.0)	60.2 (2.5)	53.0 (0.5)	49.1 (6.6)	*48.9 (1.2)	*39.1 (0.9)
800 μatm	5.5 (0.2)	12.7 (1.8)	16.2 (2.4)	42.6 (1.4)	55.8 (4.8)	41.8 (6.2)	27.5 (15.4)	27.4 (7.2)	18.6 (5.2)
1200 μatm	5.6 (0.4)	12.2 (1.4)	15.2 (1.7)	32.0 (1.7)	42.0 (6.0)	52.7 (0.5)	36.6 (0.9)	*15.0 (6.9)	*4.8 (3.4)

The deviation from the mean value is shown in parentheses

\* Statistical significance between the treatments ( $p < 0.05$ )

in the first 3 days (Fig. 8b). The average numbers in the acidified condition were higher than those in the ambient condition on days 2 and 3, but they were not statistically significant. The number in the ambient condition was kept constant with  $3.7\text{--}5.3 \times 10^3 \text{ mL}^{-1}$  from day 13 to 29, whereas tendencies to increase in the 800- and 1200-μatm conditions were noticed on days 25 and 29. The contribution of this group to total cell number showed moderate changes with time in every treatment throughout the experiment, ranging from 25.8 (800 μatm on day 29) to 47.0 % (800 μatm on day 2). High contributions of more than 40 % were found on days 2 and 3, coinciding with increases in chl. *a*. Difference among treatments were noticed between the ambient and the 1200-μatm treatment on day 29.

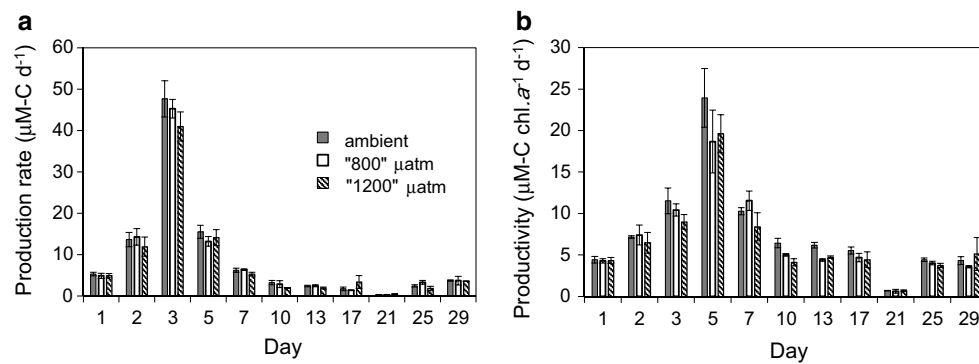
The cell number in the >6-μm size fraction was low compared with the other two fractions by one order of magnitude at the start of the experiment ( $3.3\text{--}3.8 \times 10^3 \text{ mL}^{-1}$ , Fig. 8c). The number showed an increase by day 3, with a maxima on day 3 in all treatments ( $11.5\text{--}12.6 \times 10^3 \text{ mL}^{-1}$ ), with a little variability among the acidification. They showed a slight variation from day 13 to 29 compared with the other fractions and the variation was not systematic among the treatments. The contribution of the largest group of more than 6 μm was quite low (about 5 %) at the start of the experiment, but gradually increased to day 13 for the ambient and 800-μatm vessels, and to day 17 for the 1200-μatm vessels. The highest contribution was greater than 50 % for every treatment. Although the largest fraction tended to decrease considerably towards the end of the experiment in the acidified environments, i.e., 18.6 and 4.8 % in 800- and 1200-μatm vessels on day 29, respectively, a small decrease was recorded in the ambient condition, maintaining a high value of about

40 %. Statistical analysis shows that differences in the contribution of the >6-μm fraction between the ambient and 1200-μatm treatments were significant ( $p < 0.05$ ) on days 25 and 29.

### 3.5 Photosynthetic production

The daily photosynthetic production rates (Fig. 9a) were comparable among the three treatments on day 1 (5.32, 4.90 and 4.92 μM-C d<sup>-1</sup> for ambient, 800 and 1200 μatm, respectively), and rapidly increased to the maximum on day 3 (47.7, 45.3 and 41.0 μM-C d<sup>-1</sup> for ambient, 800 and 1200 μatm, respectively). On day 5, the production rate decreased to about one third of the maximum values displayed on day 3. The rate gradually decreased from days 5 to 21, and then increased on days 25 and 29. The extremely low values of all treatments on day 21 were probably due to the low photon flux; the values were lower by one order of magnitude than the other days (Fig. 3). Although the average rate tended to be higher under the ambient condition than the higher CO<sub>2</sub> conditions, the difference among three CO<sub>2</sub> treatments was not statistically significant according to Tukey's test.

The chlorophyll *a*-specific production rates (Fig. 9b) increased from 4.3–4.4 μM-C μgchl. *a*<sup>-1</sup> d<sup>-1</sup> on day 1 to the maximum on day 5 (18.7–23.9 μM-C μgchl. *a*<sup>-1</sup> d<sup>-1</sup>); they were not comparable with the rate per water volume in which the maximum rates were observed on day 3. The rates decreased until day 10 and a definite time change was not observed until the end of the experiment, with the exception of day 21. No statistically significant difference was found between the treatments throughout the experiment ( $p < 0.05$ ).



**Fig. 9** Photosynthetic production rate (a), chlorophyll *a*-specific production rate (b). The mean value of the two vessels is shown with the *bar* representing the range

## 4 Discussion

### 4.1 Compositional change in phytoplankton community

#### 4.1.1 Blooming period

Phytoplankton biomass rapidly increased in the first three days irrespective of the degree of pCO<sub>2</sub>. The growth rates of the pigments determined by the difference in the concentrations on day 1 and 3 and fucoxanthin showed the highest rate, exceeding that of chl. *a*. This strongly suggests that diatoms exhibited the highest growth rate among the phytoplankton, in agreement with previous results (Hama et al. 1988; Pehler et al. 2004). The increase in the concentration of peridinin indicates that dinoflagellates also exhibited a high rate during the blooming period, though their growth rate was lower than the diatoms. The response of dinoflagellates to the nutrient addition was also observed in the coastal phytoplankton community (Schlüter 1998; Kremp et al. 2008). Both diatoms and dinoflagellates are major constituents of microplankton in coastal regions, including at our research site (Baek et al. 2007; Kremp et al. 2008; Hama et al. 2012). The increase in the fraction >6 μm in the blooming period observed in this study, being likely due to diatoms and dinoflagellates, revealed that the nutrient addition to the coastal area results in a change in the size fraction of the phytoplankton community with a higher contribution of larger cells.

All results concerning the phytoplankton community, pigment concentrations, group cell numbers and size, and photosynthetic rate showed little variation with CO<sub>2</sub> treatments in the blooming period; the difference in these parameters among the CO<sub>2</sub> level was not statistically significant. The results obtained in the blooming period indicate that pCO<sub>2</sub> and the concomitant difference in pH has little

effect on the growth rate of phytoplankton, irrespective of the phytoplankton group.

The results strongly suggest that the uptake rates of nutrients likely determine the growth rate in all CO<sub>2</sub> treatments in the present experiment. A minor effect of pCO<sub>2</sub> and pH on the growth of phytoplankton under nutrient-replete conditions was also found in the experiments using a natural phytoplankton community (Engel et al. 2005; Hare et al. 2007; Riebesell et al. 2007; Hopkinson et al. 2010). The small effect of increased CO<sub>2</sub> on the growth of phytoplankton has generally been considered due to their effective CCM (Riebesell 2004; Hopkinson et al. 2011), which can elevate the CO<sub>2</sub> concentration in the chloroplast even under the low ambient CO<sub>2</sub> condition. Since diatoms reportedly have the most mature CCM in phytoplankton (Riebesell 2004; Reinfelder 2011), the small response of diatoms to CO<sub>2</sub> enrichment is probably due to their effective CCM mechanism (Shultz et al. 2013).

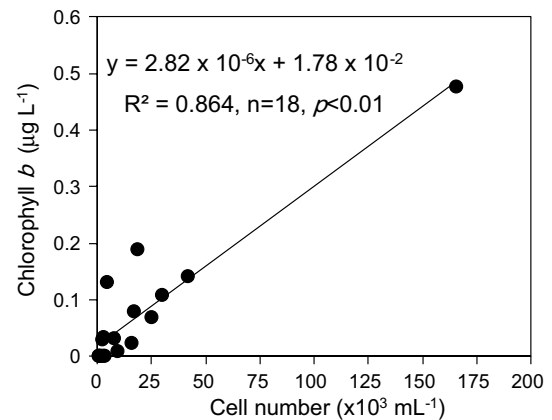
In a recent mesocosm study in the Arctic region, Shultz et al. (2013) observed the positive response of dinoflagellates, prasinophytes, chrysophytes and haptophytes to increases in CO<sub>2</sub> levels. Among these taxa, dinoflagellates are known to possess an immature CCM (Reinfelder 2011). When it would be a common property for all species of this taxon, it would be expected that dinoflagellates show a positive response to a higher CO<sub>2</sub> level as observed by Shultz et al. (2013). In the present study, however, little difference in the growth rate was found among different CO<sub>2</sub> treatments for dinoflagellates. The effect of the CO<sub>2</sub> level on two bloom-forming species of dinoflagellates was recently examined (Eberlein et al. 2014) and the growth of both species showed little change through the pCO<sub>2</sub> range applied (180–1200 μatm); moreover, both species are expected to possess an efficient CCM, which contradicts the previous information. This implies that the CCM and the resultant response to increased CO<sub>2</sub> differs among species within

the same taxon (Kim et al. 2006; Hurd et al. 2009), making it difficult to evaluate the experimental results at the taxon level; research at the species level will be important. Furthermore, experiments combining other factors such as light (Gao et al. 2012a, b), temperature (Hare et al. 2007; Feng et al. 2009) and iron (Fe; Hopkinson et al. 2010; Endo et al. 2013) will offer significant information on the diatoms of the future oceanic environments.

FCM results of the samples on day 1 strongly suggest that *Synechococcus* sp., which was fractionated into G1, was one of the important members of the phytoplankton community in the study field. The analysis of the sample just after it was introduced into the experimental vessels (2 days before the nutrient addition) revealed that the contribution of G1 was 66.5 %, substantially higher than that on day 1 (37.1–41.5 %), showing that the contribution largely decreased during the “conditioning period” of pCO<sub>2</sub> and pH by bubbling the air with the specific pCO<sub>2</sub>. This phenomenon suggests that *Synechococcus* sp. is sensitive to the bubbling of the air from the bottom of the culture vessel. Thus, the contribution of G1 and cyanobacteria through the present experiment could be reduced by the experimental system. The bubbling of the air with specific CO<sub>2</sub> level has been applied as one of the major methods to adjust pCO<sub>2</sub> and pH (Rost et al. 2008; Hurd et al. 2009); we should evaluate the results with care the effect to compositional change which may have resulted due to reasons other than the carbonate system.

#### 4.1.2 Post-blooming period

The size composition of the phytoplankton at the latter period of the experiment was different among the CO<sub>2</sub> treatments. The contribution of the smallest group to the total cell number was higher in the acidified conditions, both in the 800- and 1200- $\mu$ atm treatments, than in the ambient condition; the smallest fraction accounted for 55.6 and 72.1 % of the total cell numbers in the 800- and 1200- $\mu$ atm treatments, respectively, on day 29, but 20.6 % in the ambient one (Table 3); the difference between the ambient and the 1200- $\mu$ atm treatments was statistically significant ( $p < 0.05$ ). The difference in the phytoplankton biomass in the 1200- $\mu$ atm treatment was significantly different between the two vessels, as shown by the results of pigment concentration and cell number. However, the contributions of the <2- $\mu$ m fraction between the two vessels with the 1200  $\mu$ atm were calculated to be 61.2 and 83.0 %; the deviation from the average value was 10.9 %. Although the reason for the difference in the absolute phytoplankton biomass between two vessels was not elucidated, the trend in the predominance of picoplankton was similar in the two vessels. The predominance of picophytoplankton in the increased pCO<sub>2</sub> was comparable with the recent results



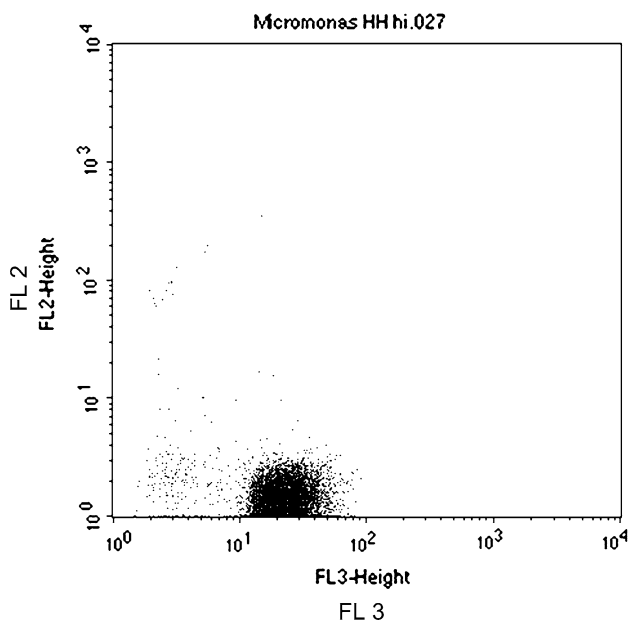
**Fig. 10** Relation between the cell number in Group 2 and the concentration of chlorophyll *b* in each vessel from days 21 to 29. The results of all vessels are plotted ( $n = 18$ )

obtained in the mesocosm experiments (Paulino et al. 2008; Brussaard et al. 2013; Shultz et al. 2013).

A substantial difference was also found in the phytoplankton group’s composition determined by FCM during days 21–29, as well as the size composition; the relative abundance of G2 was higher as pCO<sub>2</sub> increased and pH decreased (Table 2). The cell number of G2 accounted for 86.7 % of the 1200- $\mu$ atm treatment on day 29 on average (the difference in the contribution between the ambient and the 1200- $\mu$ atm treatment was significant ( $p < 0.05$ )), implying that the shift to the small-sized community in the acidified condition was primarily due to the increase in the cell number of this group.

Among the index pigments analyzed in the present study, three pigments (chl. *b*, peridinin and zeaxanthin) showed an increase in 1200  $\mu$ atm from day 21 to 29 (Fig. 4). The relationships between the cell number of the G2 and the concentration of these three pigments on days 21, 25 and 29 were analyzed to estimate the constituents of this group. A definite positive relationship was found for chl. *b* ( $R^2 = 0.864$ ,  $n = 18$ ,  $p < 0.01$ , Fig. 10), but a less significant relation was obtained for peridinin and zeaxanthin. This result strongly suggested that pico-sized plankton included in the Chlorophyta increased in the acidified condition during days 21–29.

In a recent study using quantitative PCR analysis, Meakin and Wyman (2011) found that prasinophytes related to *Micromonas pusilla* increased 20-fold more in the elevated CO<sub>2</sub> condition than in the ambient condition. The predominance of *Micromonas*-like picophytoplankton in the acidified condition has also been observed (Paulino et al. 2008; Newbold et al. 2012; Shultz et al. 2013). To compare the position of *Micromonas* in the FCM analysis in the present study, *M. pusilla* (NIES-1411) from the microbial culture collection at the National Institute of



**Fig. 11** Flow cytometry of *Micromonas pusilla* (NIES-1411) obtained from the microbial culture collection at the National Institute of Environmental Sciences (<http://mcc.nies.go.jp/localeAction.do?lang=en>) and the sample obtained from the 1200- $\mu$ atm treatment on day 29. The relationship between FL3 (>670 nm; long pass red) and FL2 (585  $\pm$  21 nm) of *M. pusilla* (NIES-1411)

Environmental Sciences (<http://mcc.nies.go.jp/localeAction.do?lang=en>) was analyzed under the same analytical conditions. The position of *M. pusilla* overlapped the G2 plot (Fig. 11). FSC histograms, which reflect the size of the particles of the sample obtained from the 1200- $\mu$ atm treatment on day 29 (Fig. 7b) were also comparable with *M. pusilla* (NIES-1411; Fig. 7c). These results suggest that the picoplankton that increased under the acidified condition in this study was a *Micromonas*-like species. Our study suggests that the predominance of *Micromonas* species under the increased CO<sub>2</sub> and the decreased pH condition is likely a common phenomenon.

One of the likely advantages that small cell phytoplankton have in high CO<sub>2</sub> conditions is their ability to readily diffuse inorganic carbon within the cell; the energy cost to transport inorganic carbon in the cell decreases as the cell size decreases (Raven 1991; Shultz et al. 2013). Thus, an increase in the ambient CO<sub>2</sub> is likely favorable to smaller-cell phytoplankton. However, recent studies, including the present one, strongly suggest that the predominance of picoeukaryotes in high CO<sub>2</sub> conditions is primarily brought about by *Micromonas*-like species, and that is unlikely for various groups of picoplankton. Hence, the physiological processes ubiquitous to pico-sized phytoplankton are not likely to be the main reason for the predominance of picoeukaryotes, but rather the specific processes of a *Micromonas*-like species which resulted in the shift to

picoplankton at the high CO<sub>2</sub> level. *Micromonas* has been reported to have an inefficient CCM (Engel et al. 2008) and another study indicated that this group possesses a C-4-like carbon fixation pathway (Worden et al. 2009). These photosynthetic properties have likely resulted in its increased photosynthetic rate under elevated CO<sub>2</sub> conditions, compared with the ambient one (Meakin and Wyman 2011; Newbold et al. 2012; Shultz et al. 2013). Repeated observation of the predominance of the *Micromonas*-like species under elevated CO<sub>2</sub> and decreased pH conditions led us to conduct a study evaluating the physiological basis which makes it possible to grow it at a higher rate when not in ambient conditions.

FCM analysis showed that the cell number of G3 in the 1200- $\mu$ atm vessel was considerably lower than that in the ambient vessel on days 25 and 29 ( $p < 0.05$ ). Considering that the concentration of 19'-hexanoyloxyfucoxanthin was lower in the 1200- $\mu$ atm treatment than other treatments on day 29 ( $p < 0.05$ ), the growth of haptophycean alga(e) was negatively affected by acidification. Hama et al. (2012) demonstrated that the growth of *Chrysochromulina* spp. may be inhibited under acidified conditions. The results obtained in the present experiment support their findings, although the current experiment was performed during a different season, and the extent of inhibition in 1200  $\mu$ atm was not serious compared with the observations made during winter (Hama et al. 2012). The importance of this genus, both in coastal and pelagic ocean environments, has been revealed (e.g., Liu et al. 2009). The repeated findings of *Chrysochromulina*'s vulnerability to ocean acidification indicate that substantial change will occur as acidification proceeds, considering that this genus is a major constituent of haptophytes.

The size composition of phytoplankton is possibly affected by composition of zooplankton through the food web. Reul et al. (2014) observed the decrease in picoplankton in a microcosm experiment using a coastal plankton community, and this was noticed irrespective of the CO<sub>2</sub>, nutrients and light levels. The same as with our experiment, mesozooplankton was removed by screening prior to their experiment (Reul et al. 2014). They speculated that the decrease in picophytoplankton was due to the increase in microzooplankton due to less grazing pressure of mesozooplankton. Although the effect of removing mesozooplankton on the decrease in *Synechococcus* sp. in the first three days was not quantitatively evaluated, the increase in picoplankton in the post-blooming period of the experiment implies that the effect of mesozooplankton removal is unlikely important in this study. On the other hand, Park et al. (2014) evaluated the direct effect of acidification on the grazing rate of microzooplankton, and reported a decreased rate under increased pCO<sub>2</sub>. When the vulnerability of microzooplankton to acidification may be

generalized, it is possible that the lower grazing effect in the acidified condition resulted in the increase in *Micromonas*-like picoplankton in the present study. To evaluate the effect of acidification on the composition of the phytoplankton community, information on the other constituents of the microbial food web, including meso- and microzooplankton and bacteria, is likely efficacious.

In the present study, the difference in the phytoplankton composition was obvious in the post-blooming period with a little concentration of nutrients, being generally comparable with the recent mesocosm experiment (Shultz et al. 2013). This indicates that the response of coastal phytoplankton communities to pulsed addition of nutrients under more acidified conditions will not change significantly in the blooming period. Considering the comparable growth rate among CO<sub>2</sub> treatments, the present results imply that phytoplankton growth rate in the bloom-forming period mainly reflects the nutrient uptake capacity of phytoplankton rather than the availability of CO<sub>2</sub>. After the nutrients have almost been consumed, on the other hand, the CO<sub>2</sub> availability for photosynthesis seems to determine the growth rate; it is possible that the composition of the phytoplankton community under a higher CO<sub>2</sub> condition likely changes from the present condition, reflecting the difference in their physiological properties, such as their CCMs.

## 4.2 Relation to the biogeochemical cycle

The photosynthetic production rate responded to the nutrient addition and increase from day 1 to day 3 by a factor of 8.2–9.2 in three CO<sub>2</sub> treatments. Although the photosynthetic production rate showed maxima on day 3, the maxima of chl. *a*-specific production rate were observed on day 5 in every treatment. A high chl. *a*-specific rate just after the maximum concentration of chl. *a* was also observed in our mesocosm experiment with a pulsed input of nutrients (Hama et al. 1988). Hama et al. (1988) reported that the contribution of carbohydrates in photosynthetic products increased after nutrient depletion, and indicated that it suppresses the production of nitrogen-containing compounds such as proteins. The elevated chl. *a*-specific production rate on day 5 likely reflects that the change in photosynthetic products shifted to a higher contribution of non-N-containing compounds such as carbohydrates.

Carbon–nitrogen stoichiometry is one of the main interests in the study on ocean acidification (Riebesell et al. 2007; Hama et al. 2012; Bellerby et al. 2008; Silyakova et al. 2013). The present study suggests that ocean acidification has a minor effect on the elemental composition of organic matter produced, since the primary production rate and the nutrient consumption rate showed almost comparable values among the CO<sub>2</sub> treatments. Large-scale mesocosm studies in an Arctic fjord showed evidence that an

increase in CO<sub>2</sub> concentration enhances the uptake of DIC and resulted in an increase in the C/N value of organic matter (Riebesell et al. 2007). The enhanced photosynthetic carbon uptake rate under a high CO<sub>2</sub> condition was also noticed in a recent experiment (Engel et al. 2013), but the response varied with the experimental phases. Recent studies imply that the response in fluxes of bioelements through the change in phytoplankton community to increasing CO<sub>2</sub> is more complex compared with the consideration in previous studies, as discussed by Silyakova et al. (2013).

A possible shift to a smaller community in acidified conditions implies that the related biogeochemical processes will be affected, such as the food web structure and vertical transport of organic matter. Since these processes are closely linked with oceanic and global change, continuous examination of the effect of ocean acidification on microbial communities and related biogeochemical cycles would aid in predicting the feedback of ocean acidification to global change.

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