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Distribution of *Synechococcus* in the dark ocean

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ABSTRACT: *Synechococcus* is widely distributed in the world's ocean surfaces, and is often found in sediment traps. However, its distribution and ecological importance have not been well studied in meso- and bathypelagic waters. We measured *Synechococcus* abundance in the Suruga Bay (central Japan) and the subtropical NW Pacific. *Synechococcus* abundance at depths of 200 m and below varied from 2.4 to 190 cells ml^{-1}, but was in proportion to the surface abundance, suggesting transport of epipelagic populations to greater depths. Surprisingly, *Synechococcus* was evenly distributed from 200 m down to 1420 m (Suruga Bay) or to 2000 m (subtropical NW Pacific), regardless of season. The contribution of deep *Synechococcus* to the total population was highest in spring in the Suruga Bay (36 to 77%), and lowest in summer in the Suruga Bay (1 to 9%) and in the subtropical NW Pacific (4 and 10%). These results suggest effective transport of *Synechococcus* cells down the water column during productive seasons by attachment to large particles and limited transport under oligotrophic and stratified conditions. Deep *Synechococcus* abundance decreased from fall to winter in the Suruga Bay, though in filtered deep seawater it did not significantly decrease for 30 d in the dark, and it increased in a light/dark cycle. Our investigations show that the standing stock of *Synechococcus* has been significantly underestimated in previous studies of epipelagic waters conducted during productive seasons and that *Synechococcus* seems to be grazed and to contribute to biogeochemical cycles in the dark ocean.

KEY WORDS: *Synechococcus* · Dark ocean · Vertical distribution · Vertical export

INTRODUCTION

Synechococcus is a unicellular photosynthetic prokaryote that is ubiquitous in the world's ocean surfaces (Johnson & Sieburth 1979, Waterbury et al. 1979). *Synechococcus* populations are responsible for a significant portion of primary production, in some cases more than half of the total (Gutiérrez-Rodríguez et al. 2010). They are removed by the grazing of microzooplankton, such as nanoflagellates and ciliates (Christaki et al. 1999, 2001) and by viral infection (Suttle & Chan 1993, 1994).

Despite the demonstrated ecological importance of *Synechococcus* in the euphotic zone and detection of the cells in the meso- and bathypelagic waters (Fuhrman et al. 1989, Yamaguchi et al. 2002, Vilibi & Šanti 2008), little attention has been paid to populations in the dark ocean (i.e. meso- and bathypelagic zones). This is understandable because *Synechococcus* cells were thought to be unlikely to grow in darkness (Six et al. 2004, Timmermans et al. 2005), and are too small to sink below the seasonal picnocline unaided (Michaels & Silver 1988). However, there are many studies which have detected *Synechococcus* cells attached to sinking particles in the meso- and bathypelagic waters (Vanucci et al. 2001 and references therein) and in photodetritus on the abyssal sediment (Lochte & Turley 1988). These studies reveal transport of *Synechococcus* cells associated with large particles. Recently, other transport processes were suggested, for example, aggregation of *Synechococcus* cells (Richardson & Jackson 2007) and winter ventilation (Vilibi & Šanti 2008). Furthermore, a growing body of evidence shows heterotrophic activity of *Synechococcus*, i.e. the

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ability to utilize organic substrates (Zubkov et al. 2003, Zubkov & Tarran 2005), suggesting that *Synechococcus* can survive in darkness (Cottrell & Kirchman 2009).

These earlier studies led us to examine how *Synechococcus* is dispersed throughout the dark ocean. The purpose of the present study is to clarify the abundance and distribution of *Synechococcus* in the dark ocean and to make inferences about the ecological and biogeochemical roles of the organism. Surprisingly, at depths of 200 m and below, *Synechococcus* was evenly distributed regardless of the season or area studied. These deep populations accounted for up to 77% of total *Synechococcus* population in the entire water column.

MATERIALS AND METHODS

Sampling. Seawater was collected in Niskin bottles mounted on a CTD-carousel sampler at Station (Stn) 2 (34° 51′ N, 138° 37′ E, Suruga Bay) and Stn C2700 (27° 00′ N, 138° 00′ E, subtropical NW Pacific), during cruises on board the RV 'Suruga-maru' (Shizuoka Prefectural Research Institute of Fisheries) and RV 'Soyomaru' (National Research Institute of Fisheries Science, Fisheries Research Agency). The sampling location and hydrological conditions are shown in Table 1. Stn 2 is located near the Suruga Trough, at the center of the Suruga Bay, and Stn C2700 is a time-series station on O-Line (Sugisaki et al. 2010). Samplings at Stn 2 were conducted in the uppermost 1000 m between February 2005 and November 2006, and full-depth profiles, from the surface to a depth of 20 m above the bottom (B – 20 m; B = 1440 m), were made between April 2007 and May 2008 (Table 2). Sample collection was always started at 10:00 h. At Stn C2700, seawater was taken from the top 2000 m in August and November 2007 (Table 2). The depth of the euphotic zone (i.e. receiving 1% of the surface photosynthetic available radiation, PAR) was determined mostly from PAR data taken from the upper 100 m (Stn 2) and 200 m (Stn C2700) on previous cruises due to insufficient data obtained in this study. Winter mixed layer depth (MLD) was calculated according to Levitus (1982), using CTD data obtained in February 2005 to 2008 (Stn 2), and January 2007 and 2008 (Stn C2700).

Measurements. ***Synechococcus abundance:*** Seawater samples of *Synechococcus* were transferred to black plastic bottles and immediately fixed with neutralized formalin (final conc. 1%), then stored in a refrigerator. The fixed samples were collected on 0.4 µm pore size polycarbonate filters (diameter, 25 mm; Advantec, Japan) under a vacuum pressure of 0.02 to 0.03 MPa within 2 wk of sample collection. The filtration volume was set at between 100 ml (≤50 m) and 1 l (≥100 m) depending on sampling depth, and it took 2 h on average to filter the latter sample types. The filters were then mounted on glass slides using non-fluorescent immersion oil and coverslips, and kept in a freezer until counting. Yellowish orange fluorescent cells with sizes smaller than 2 µm were counted in at least 10 eyepiece fields (100 × 100 µm) using an epifluorescence microscope (BX51, Olympus, Japan) under blue excitation (excitation filter, BP470-490; dichroic mirror, DM505; barrier filter, BA515; Olympus) with a 100× objective lens and 10× eyepiece (Putland & Rivkin 1999). If fewer than 300 cells were detected on a filter, cells in 1000 fields were counted. It should be noted that *Synechococcus* abundance might have been overestimated at Stn C2700 because of the frequent occurrence of other small, yellowish orange fluorescent cyanobacteria such as *Crocosphaera* and *Cyanothece*, which has been reported in the subtropical oceans (Neveux & Lantoine 1999, Church et al. 2005).

Chlorophyll a concentration and total prokaryotic abundance: Seawater samples of chlorophyll *a* (chl *a*) were placed into amber plastic bottles, and immediately collected on GF/F filters (Whatman). The volume of filtered seawater was set at between 275 ml (≤150 m) and 3270 ml (≥300 m), depending on sampling depth, and either a 25 mm or 47 mm GF/F filter was chosen, depending on filtration volume. Chl *a* concentration was measured using a spectrofluorometer (RF-5300, Shimadzu, Japan) after extraction of suspended matter on filters with N, N-dimethylformamide (Suzuki & Ishimaru 1990).

Samples of total prokaryotic abundance were placed into clear plastic tubes, and fixed with neutralized for-

Table 1. Study area. Location, bottom depth, and euphotic zone and winter mixed layer (MLD) depths

Station	Area	Location	Bottom depth (m)	Depth of euphotic zone (m)[a]	Winter MLD (m)[b]
Stn 2	Suruga Bay	34° 51′ N, 138° 37′ E	1440	31–51	86–133
Stn C2700	Subtropical NW Pacific	27° 00′ N, 138° 00′ E	5000	80–120	47, 90

[a]Depth of 1% PAR level mostly obtained in the previous cruises
[b]MLD in February 2005 to 2008 (Stn 2) and January 2007 and 2008 (Stn C2700)

Table 2. Sampling information, temperature (temp.), and nutrient concentrations at 10 m

Sampling		Data at 10 m			
Date	Depth range (m)[a]	Temp. (°C)	NO_3^- (µmol l^{-1})	NH_4^+ (µmol l^{-1})	PO_4^{3-} (µmol l^{-1})
Stn 2, Suruga Bay					
22 Feb 2005	2–1000	15.3	5.84	0.05	0.42
22 Apr 2005	2–1000	16.4	0.68	0.52	0.16
13 Jul 2005	10–1000	21.9	0.45	<0.01	0.08
28 Nov 2005	20–1000	20.8	1.42	0.09	0.23
15 Feb 2006	20–1000	13.3	11.3	<0.01	0.77
19 Apr 2006	4–1000	14.4	5.05	0.36	0.37
12 Jul 2006	2–1000	19.5	0.34	<0.01	0.09
27 Nov 2006	2–1000	19.1	3.04	0.04	0.29
24 Apr 2007	2–*B* – 20	16.8	0.62	0.43	3.16
27 Nov 2007	2–*B* – 20	20.1	2.65	0.11	0.23
20 Feb 2008	2–*B* – 20	14.0	8.34	0.09	0.65
16 May 2008	2–*B* – 20	19.6	<0.01	0.06	0.09
Stn C2700, Subtropical NW Pacific					
31 Aug 2007	5–2000	29.3	<0.01	0.14	0.05
1 Nov 2007	5–2000	26.2	0.04	0.23	0.03

[a]*B* – 20 m; depth of 20 m above the bottom, *B* (*B* ~1440 m)

malin (final conc. 2%), then preserved in a refrigerator. A volume of 10 to 30 ml of the fixed samples was stained with 4′,6-diamidino-2-phenyl indole (final conc. 0.1 µg ml^{-1}) and collected on Irgalan black-stained 0.2 µm pore size polycarbonate filters (Advantec) within 2 wk of sample collection (Porter & Feig 1980). Filters were mounted on glass slides and kept frozen until counting. Cells were counted in 10 fields under UV excitation of an epifluorescence microscope (BX51). Abundance of picophytoplankton, that is to say *Synechococcus*, *Prochlorococcus* (not detected at Stn 2) and picoeukaryotic phytoplankton, was measured using flow cytometry (see below), and was more than one order of magnitude lower than total prokaryotic abundance. Thus, total prokaryotic abundance could be used as a suitable proxy for heterotrophic prokaryotic abundance.

Methodological check. ***Comparison between Niskin sampling and siphon sampling:*** We checked our approach to determining *Synechococcus* abundance by comparing *Synechococcus* abundance between Niskin sampling and siphon sampling to determine whether surface *Synechococcus* could attach itself to the interiors of the Niskin bottles during descent to greater depths. The Suruga Bay has siphon-sampling systems, which supply seawater via polyethylene tubes directly to tanks in the underground basement of a land station, from depths of 397 m (tube length 3323 m; inside diameter 200 mm) and 687 m (tube length 7273 m; inside diameter 225 mm). In these systems, differences between tank water levels and seawater levels generate gravity-driven flow, and seawater passes through the tubes at the maximum flow rate of 2000 m^3 d^{-1} without pumping, which gives a turnover time of seawater within the systems of a few hours. We took seawater simultaneously at the land institution (Suruga-Bay Deep Sea Water Aquaculture Research Center, Shizuoka Prefectural Research Institute of Fisheries) by siphon sampling, and at the inlets of polyethylene tubes by Niskin sampling on board the RV 'Suruga-maru' in November 2005 and February 2006. *Synechococcus* was then counted by epifluorescence microscopy.

Comparison between epifluorescence microscopy and flow cytometry: Generally, *Synechococcus* cells are counted with flow cytometry, and similar measurements have been reported with flow cytometry and epifluorescence microscopy in epipelagic samples (Putland & Rivkin 1999, Havskum et al. 2004). However, a decrease in the fluorescence of phycoerythrin (PE) has been shown in subsurface samples when using flow cytometry (Olson et al. 1990), suggesting that epifluorescence microscopy might fail to detect weakly fluorescent *Synechococcus* cells in deep seawater samples. We compared *Synechococcus* abundance obtained from flow cytometry and epifluorescence microscopy in samples taken from the water column at Stn 2 in November 2007 and February and May 2008. Samples for flow cytometry measurement were fixed with 20% glutaraldehyde (electron microscopic grade; final conc. 1%) and frozen in liquid N_2 (Vaulot et al. 1989). Frozen samples were preserved in a deep freezer until analysis. *Synechococcus* cells were counted using an EPICS XL (Beckman Coulter) at a medium flow rate for 12 min (samples from <100 m) or 30 min (≥100 m). The high flow rate was not used due to the high noise level. The cells were identified within an adequate area (gate) on plots of red fluorescence (chl *a*) versus orange fluorescence (PE). A solution of fluorescent beads (Flow Count; size 10 µm; Beckman Coulter) was added at 2% vol/vol to the samples as an internal reference for the quantification of cell concentration. A vertical series of samples was analyzed and the same gate was applied on the same analysis day. The number of identified *Synechococcus* cells ranged between <10 and >7000 cells per sample. Samples collected in May 2008 were measured in triplicate to examine reproducibility (the other samples were measured only once). The reproducibility of epifluorescence microscopy was examined in seawater taken at a depth of 1000 m at Stn 2 in May 2008 by filtering the seawater in triplicate. To examine procedural blanks, 3.5% NaCl solution was processed in the same way as the deep seawater samples (≥200 m), and abundance of *Synechococcus*, total prokaryotes, and chl *a* concentration were measured. The procedural blank value was subtracted from the sample values for *Synechococcus* abundance obtained using flow cytometry.

Incubation experiments. To examine the tolerance of *Synechococcus* populations to sunlight deprivation and the response of the deep population to light, dark (Dark Expt) and light manipulation (Light/Dark Expt) incubation experiments were conducted. Seawater samples were collected from depths of 20 and 300 m at Stn 2 in November 2005 by Niskin sampling (Dark Expt) and from 397 m in October 2006 by siphon sampling (Light/Dark Expt). To remove grazers, samples were filtered through a GA-55 glass-fiber filter (nominal pore size 0.6 µm; diameter 9 cm; Advantec) into a 20 l polycarbonate spigot bottle in dim light in a laboratory. We selected GA-55 because of its lower retention efficiency for *Synechococcus* over the other glass-fiber filters, such as Whatman GF/A (nominal pore size 1.6 µm) and GF/C (1.2 µm) (data not shown). Recovery of *Synechococcus* in the filtrate in Dark Expt was 66% (20 m) and 45% (300 m). The bottles used for dark incubation were covered with aluminum foil. Filtered samples were incubated individually at 20°C (seawater temperature at 20 m at the time of sampling) in the dark for 30 d (Dark Expt), and in a 12 h light:12 h dark cycle or in continuous darkness for 14 d (Light/Dark Expt). Irradiance was set at 60 µmol photons m^{-2} s^{-1} using cool white lights, which was equivalent to the irradiance at 30 m at Stn 2. Subsamples were periodically obtained from a spigot and *Synechococcus* abundance was counted by epifluorescence microscopy.

RESULTS

Methodological considerations

Comparison between Niskin sampling and siphon sampling

Synechococcus abundance in the mesopelagic Suruga Bay seawater was 66 to 250 cells ml^{-1} for Niskin sampling and 63 to 207 cells ml^{-1} for siphon sampling. There was no significant difference between the *Synechococcus* abundance obtained with Niskin and siphon sampling (paired Student's *t*-test, n = 4, p > 0.05), and we concluded that surface *Synechococcus* cells were unlikely to be drawn down to deeper waters by Niskin sampling.

Comparison between epifluorescence microscopy and flow cytometry

Meso- and bathypelagic *Synechococcus* cells were detected as yellowish orange fluorescent cells under blue excitation by epifluorescence microscopy in a similar way to the epipelagic cells (Fig. 1). However, *Synechococcus* abundance obtained by epifluorescence microscopy was significantly lower than that obtained by flow cytometry through the water column (Fig. 1). Model II linear regression between epifluorescence microscopy (x) and flow cytometry (y) data sets was expressed as $y = (1.15 \pm 0.05)x - (408 \pm 1542)$ (r = 0.93, n = 65), where the slope was significantly higher than 1 and the y-intercept did not significantly differ from 0 (Student's *t*-test, $p < 0.001$ and $p > 0.05$, respectively). The regression slope was not significantly different between depths of <200 m (1.2 ± 0.1) and ≥200 m (1.6 ± 0.2) (Student's *t*-test, $p > 0.05$). This shows that epifluorescence microscopy underestimated *Synechococcus* abundance by 15% though the water column. However, epifluorescence microscopy demonstrated superior reproducibility (8.5%) to flow cytometry (average, 29%) for the samples collected at ≥200 m depths, with abundance in the order of tens of cells ml^{-1}, with much lower procedural blank values (0.021 cells ml^{-1}) than obtained with flow cytometry (mean = 10 cells ml^{-1}). For this reason, we used epifluorescence microscopy in preference to flow cytometry.

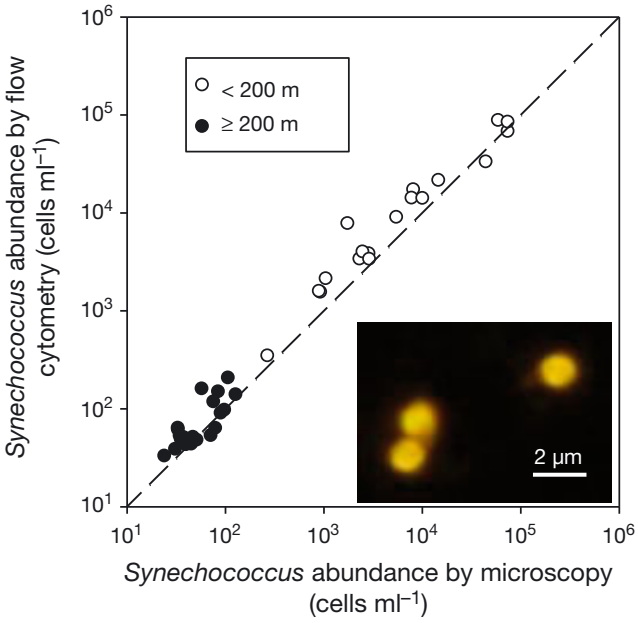

Fig. 1. Comparison of *Synechococcus* abundance measurements using epifluorescence microscopy and flow cytometry. Samples were taken from depths of 5 to B – 20 m (20 m above the bottom) at Stn 2 (Suruga Bay) in November 2007 and in February and May 2008. The broken line indicates $y = x$. The relationship over the water column is expressed as $y = (1.15 \pm 0.05)x - (408 \pm 1542)$ (r = 0.93, n = 65) using Model II linear regression. Note that samples taken in May 2008 were measured in triplicate with flow cytometry. The insert represents *Synechococcus* cells under blue-excitation of epifluorescence microscopy. The sample was taken at 1000 m at Stn 2 (Suruga Bay) in May 2008, and 1 l of seawater was filtered through a 25 mm diameter filter with a pore size of 0.4 µm

Hydrography

The depth of the euphotic zone was estimated at 31 to 51 m (Stn 2, Suruga Bay) and 80 to 120 m (Stn C2700, subtropical NW Pacific) during previous cruises (Table 1). Winter MLD was 86 to 133 m (Stn 2) and 47 and 90 m (Stn C2700). There were salinity minima at 380 to 520 m (Stn 2) and 680 m (Stn C2700), where potential density anomaly (σ_θ) was around 26.9 and 26.7, respectively (Fig. 2). These salinity minima are considered as the upper part of the North Pacific Intermediate Water (NPIW, Talley et al. 1995). Salinity of NPIW was notably low in the Suruga Bay in July 2006, suggesting an occasional intrusion of substantial volumes of NPIW. The subsurface salinity maximum was observed at around 100 m at Stn 2 and was accounted for as a mixture of coastal water and the Kuroshio Water (Iwata et al. 2005). For Stn C2700, the subsurface salinity maximum at 120 m is considered to be due to the North Pacific Tropical Water formed at 20° to 30°N latitude and 140°E to 160°W longitude (Qu & Mitsudera 1999, Suga et al. 2000). The core of the Kuroshio main stream was situated at around 32° 30′N along the meridian of Stns 2 and C2700.

Vertical profiles

Synechococcus abundance decreased exponentially from the surface (2 or 5 m) to 200 m, falling to 0.060% (May 2008) and 9.7% (April 2006) of the surface abundance at Stn 2 (Fig. 3). Surprisingly, *Synechococcus* abundance was more or less constant at depths of 200 m and below (coefficient of variation, ~30%) regardless of season, and a similar profile was also observed at Stn C2700 (Fig. 4). In contrast, total prokaryotic abundance and chl *a* concentration generally decreased with increasing depth below 200 m despite the contribution of *Synechococcus* cells.

To compare these profiles more precisely, we calculated the ratios of abundance (or concentration) between the upper end (200 m) and the lower end (1000 m) of the mesopelagic zone. Comparison of correlation coefficients was not made because of the small sample size of each series of profiles (n = 3 to 7). The ratios of *Synechococcus* at 200 m to 1000 m were not significantly different from 1 (Student's *t*-test, $p > 0.05$), while the ratios were significantly greater than 1 for total prokaryotes (Student's *t*-test, $p < 0.001$) and chl *a* (sign test, $p < 0.01$); the latter 2 measures showed a significant decrease from 200 m to 1000 m (Fig. 5A). Moreover, the ratios of abundance (or concentration) at 1000 to 1420 m (B – 20 m, Stn 2) or to 2000 m (Stn C2700) were not significantly different from 1 for *Synechococcus* or chl *a* (Student's *t*-test, $p > 0.05$), while the ratios were significantly higher than 1 for total prokaryotes (sign test, $p < 0.05$) (Fig. 5B). Thus, a vertically constant distribution was confirmed only for *Synechococcus* in the mesopelagic and the upper bathypelagic zones. It should be noted that the ratio for the upper bathypelagic chl *a* was less than 1 at Stn 2 in April 2007 and May 2008, since

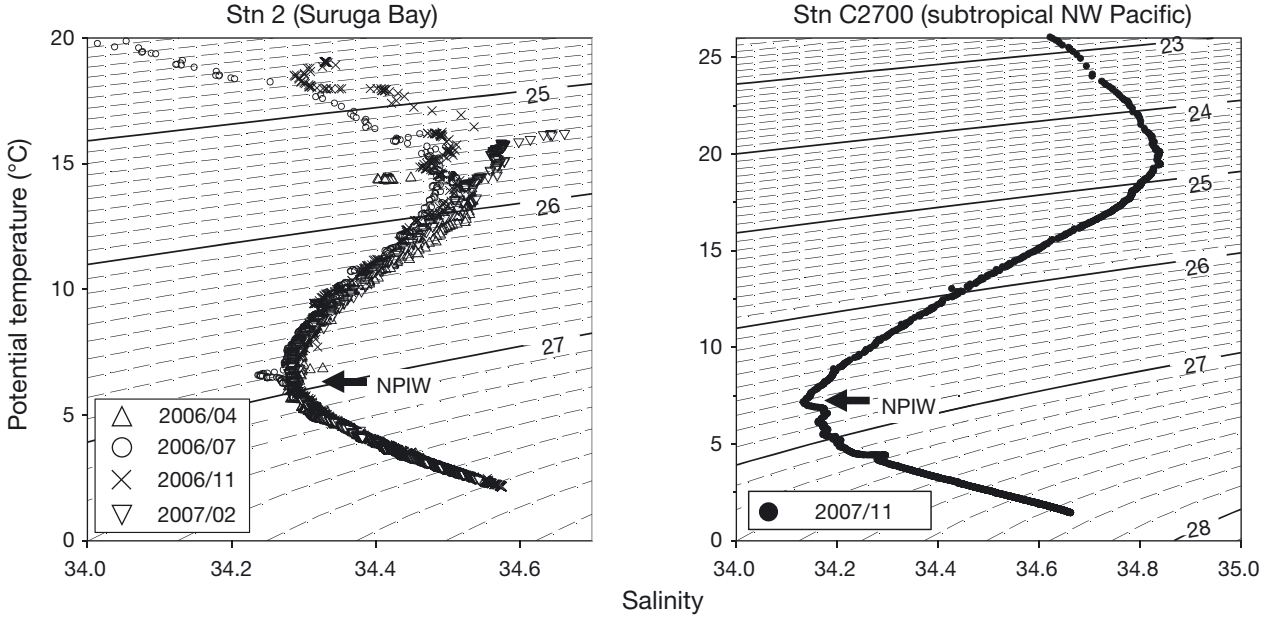

Fig. 2. Temperature–salinity diagrams at Stn 2 (Suruga Bay) and Stn C2700 (subtropical NW Pacific). Solid and broken lines show contours of potential density anomaly (σ_θ). Arrows indicate the water mass showing the signature of the North Pacific Intermediate Water (NPIW)

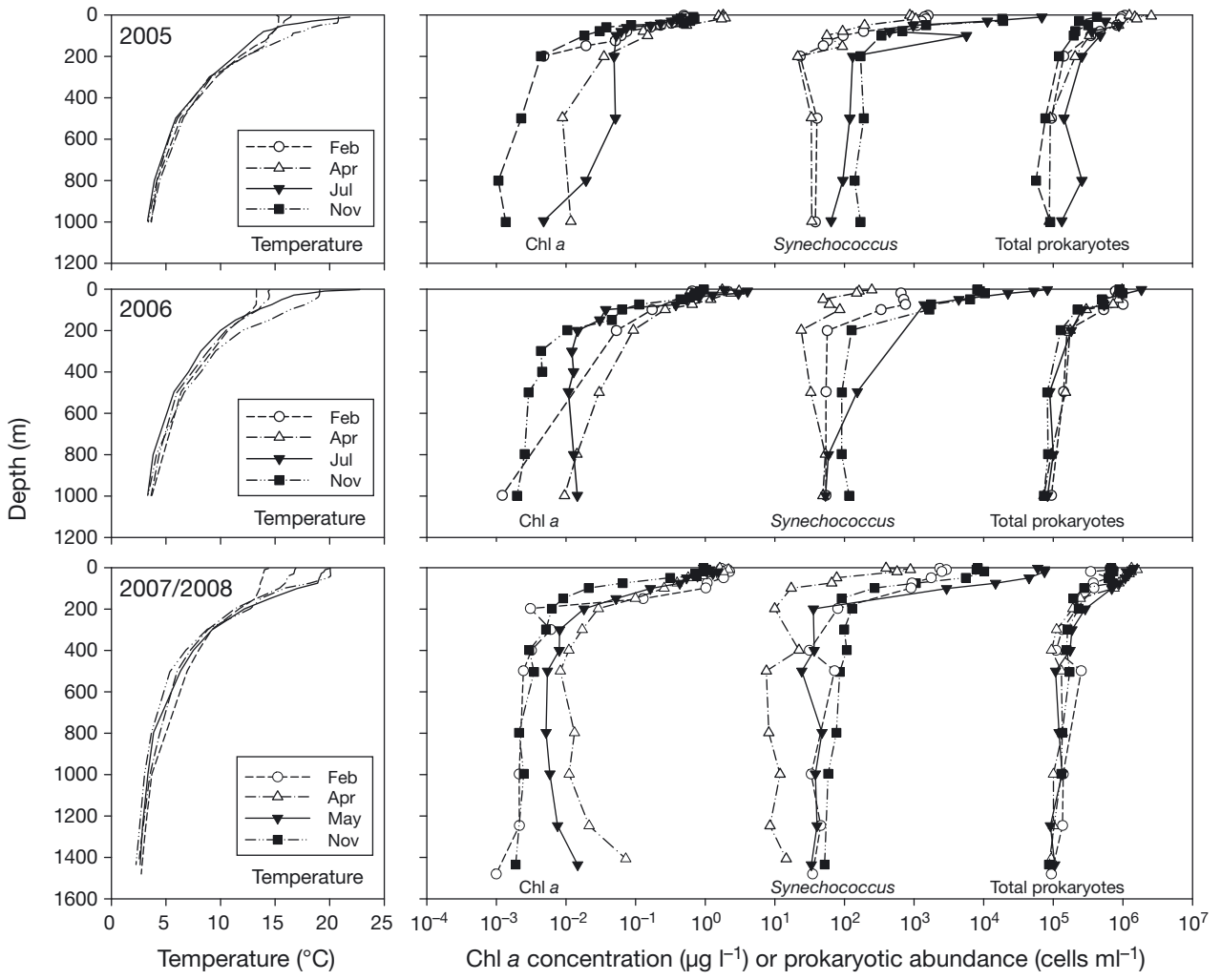

Fig. 3. The top 1000 m-depth profiles (2005, 2006) and full-depth profiles (2007/2008) of temperature, chl *a* concentration, *Synechococcus* and total prokaryote abundances at Stn 2 (Suruga Bay). Profiles of chl *a* and total prokaryotes in Feb 2005 were obtained in the top 200 m and 500 m, respectively. The procedural blank was 0.021 cells ml^{-1} (*Synechococcus*), 1.1 × 10^4 cells ml^{-1} (total prokaryotes) and 2.0 × 10^{-4} µg l^{-1} (chl *a*) at ≥200 m. Symbols are omitted for temperature

chl *a* increased with increasing depth below 1000 m (Fig. 3), suggesting a release of phytoplankton from a nepheloid layer.

Relative cellular contents of chl *a* and PE among *Synechococcus* cells, which are represented by red and orange fluorescence intensities, respectively, in flow cytometry (Six et al. 2004) were low at 2 m, increased with increasing depth down to 50 m, and then decreased down to 100 m, similarly to the results for PE in the NE Atlantic (Olson et al. 1990) (Fig. 6). The increase in PE in the upper 50 m can be explained by phytoacclimation, and the decrease below 50 m might reflect an unhealthy state caused by low light. Chl *a* and PE fluorescence varied considerably at 200 m and below but was within a similar range to the fluorescence levels seen in the top 100 m. This finding suggests that the deep *Synechococcus* population is composed of cells with a variety of pigment levels and that at least a proportion of the cells retain pigments in prolonged darkness.

Seasonal and regional differences

While *Synechococcus* abundance was constant at depths of 200 m and below (deep water), it did vary with season and study area (Figs. 3 & 4). Deep *Synechococcus* was more abundant in summer and fall (24 to 190 cells ml^{-1}) than in spring (7.6 to 49 cells ml^{-1}) in accordance with the seasonal changes in the epipelagic zone (Fig. 3). Averaged *Synechococcus* abundance at ≥200 m depths was marginally significantly correlated with *Synechococcus* abundance at 10 m (r = 0.59; Student's *t*-test, p = 0.07) (Fig. 7A). The highest

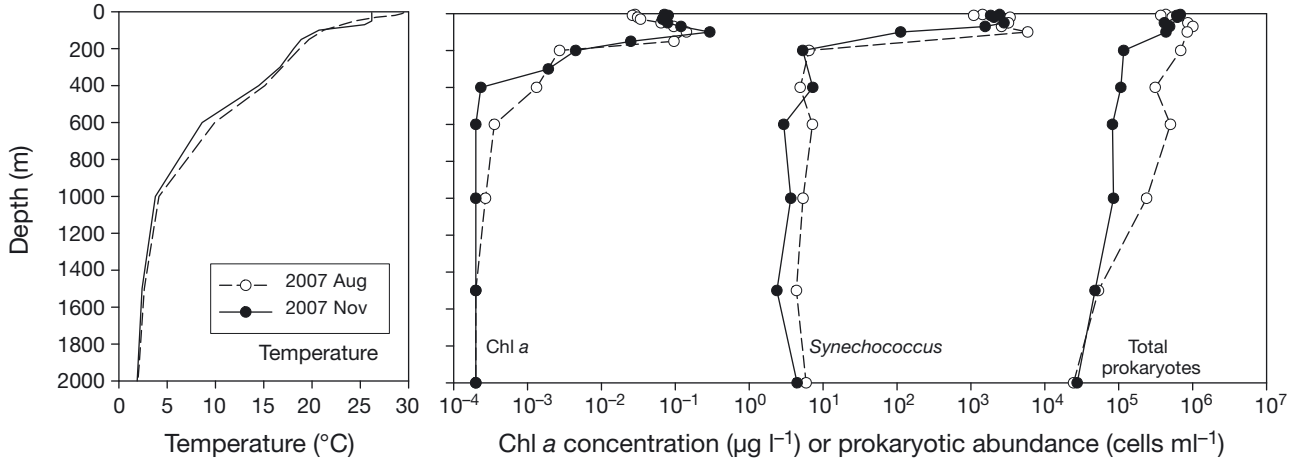

Fig. 4. Depth profiles of temperature, chl *a* concentration, *Synechococcus* and total prokaryote abundances at Stn C2700 (subtropical NW Pacific) in 2007. The procedural blank was 0.021 cells ml^{-1} (*Synechococcus*), 1.1×10^4 cells ml^{-1} (total prokaryotes) and 2.0×10^{-4} µg l^{-1} (chl *a*) at ≥200 m, and chl *a* concentration below the procedural blank was set at 2.0×10^{-4} µg l^{-1}. Symbols are omitted for temperature

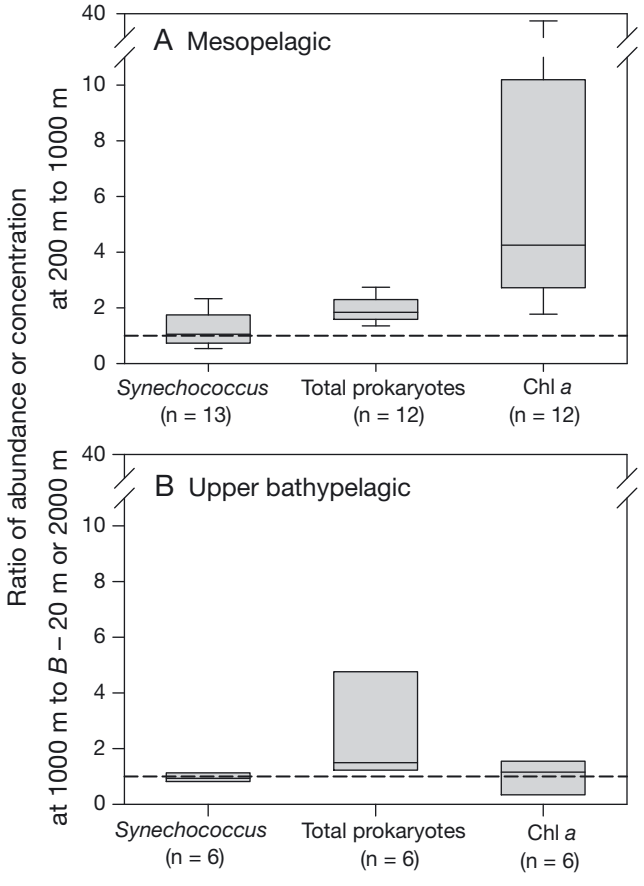

Fig. 5. Box plots of (A) the ratios of prokaryote abundance and chl *a* concentration at 200 to 1000 m and (B) at 1000 m to 20 m above the bottom (Stn 2, Suruga Bay) or to 2000 m (Stn C2700, subtropical NW Pacific). Upper and lower ends of boxes show 75 and 25% confidence intervals (CI), and upper and lower ends of error bars show 90 and 10% CI, respectively. Solid lines in boxes show medians. The broken line indicates a ratio of 1. Sample size is given in parentheses

abundance of epipelagic *Synechococcus* occurred in May and July, consistent with annual variations in *Synechococcus* abundance in La Jolla, California (Tai & Palenik, 2009), but different from that seen at oligotrophic oceanic sites (Malmstrom et al. 2010). The greater abundance in summer was related to high temperature (Table 2), as shown in many earlier studies (Agawin et al. 1998, Sommaruga et al. 2005, Jing et al. 2009). Chl *a* exhibited different seasonal changes from *Synechococcus*, with higher concentrations in spring and lower concentrations in fall throughout the water column, though the correlation was not significant between 10 m and ≥200 m ($r = 0.28$, $p > 0.3$; Fig. 7C). Seasonal variations in total prokaryotes were much smaller than those in *Synechococcus* and chl *a*, and abundance was not correlated between 10 m and ≥200 m ($r = 0.33$, $p > 0.3$; Fig. 7B). Deep *Synechococcus* abundance was roughly 10-fold lower at Stn C2700 (2.4 to 7.2 cells ml^{-1}) than at Stn 2, although the surface abundance was within a similar range. In contrast, total prokaryotic abundance at Stn 2 (0.056×10^6 to 1.2×10^6 cells ml^{-1}) and Stn C2700 (0.087×10^6 to 1.0×10^6 cells ml^{-1}) was relatively similar throughout the water column. Chl *a* concentration was roughly 10-fold lower at Stn C2700 (<0.0002 to 0.29 µg l^{-1}) than at Stn 2 (0.0011 to 4.0 µg l^{-1}), and was below the procedural blank value at ≥1500 m (August) and ≥600 m (November) at Stn C2700.

Contribution of the deep *Synechococcus* population to the total population in the water column

To evaluate the numerical importance of *Synechococcus* populations in the dark ocean, the contribu-

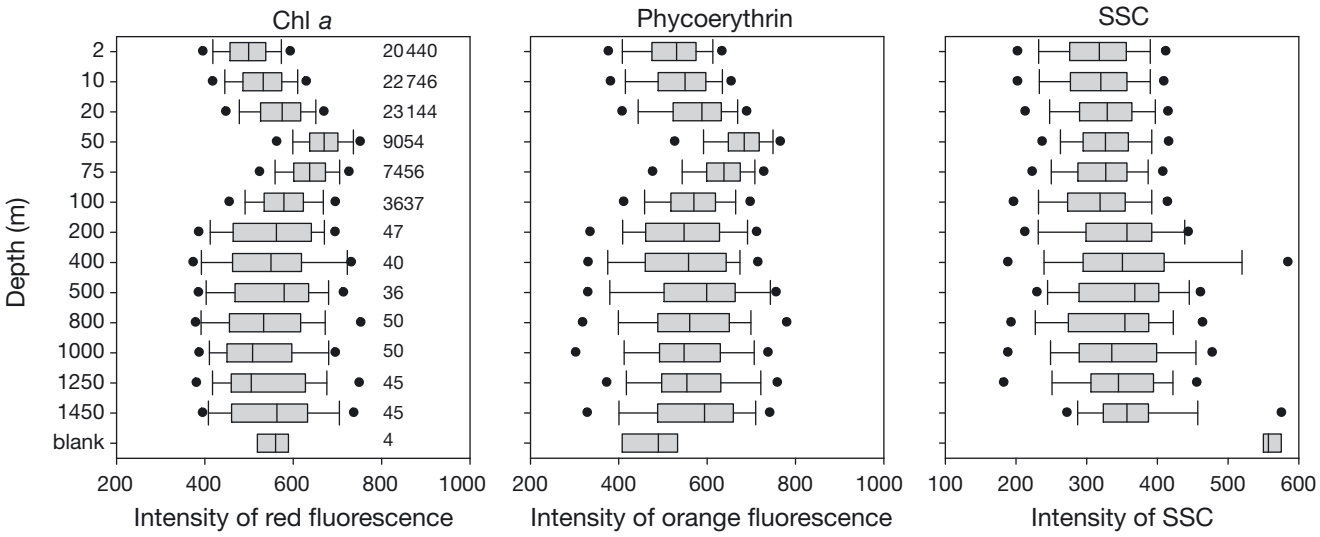

Fig. 6. Box plots of intensities of red (chl *a*) and orange fluorescence (phycoerythrin) and side scatter (SSC) in *Synechococcus* cells obtained using flow cytometry in samples collected in May 2008. Data from triplicate analyses were combined. Upper and lower ends of boxes show 75 and 25% confidence intervals (CI), and upper and lower end of error bars show 90 and 10% CI, respectively. Dots represent 5 and 95% CI, and solid lines in boxes show medians. Numbers in the plot of chl *a* show sample size

tion of *Synechococcus* at depths of ≥200 m to the total *Synechococcus* population in the water column was calculated (Fig. 8). When sampling was not conducted near the bottom, depth-integrated abundance at ≥200 m was estimated by multiplying the average abundance at ≥200 m by the water depth minus 200 m. The estimated ratios were quite similar to the estimates from the actual data obtained at Stn 2 in 2007 and 2008 (Fig. 8). The contribution of *Synechococcus* at ≥200 m ranged from 1 to 77% at Stn 2, with the highest values occurring in spring (36 to 77%) and the lowest in summer (1 to 9%). The contribution at Stn C2700 was relatively low (4 and 10%) and similar to the contribution at Stn 2 in summer. The contributions at Stn C2700 were potential maxima because *Synechococcus* abundance might well decrease below 2000 m.

Incubation experiments

Synechococcus abundance increased by 40% during the first 3 d of the dark incubation experiment (Dark Expt) for cells taken from 20 m depth (Fig. 9A). This result reveals that *Synechococcus* can grow for a few days in the dark, presumably by consuming ATP and NADPH produced during exposure to light. *Synechococcus* populations taken from 20 m declined rapidly between Day 5 and Day 10 and continued to decrease until Day 30. In contrast, *Synechococcus* populations taken from 300 m remained relatively constant over a period of 30 d, and the slope of linear regression between incubation period and abundance was not significantly different from 0 ($r = -0.44$, $p > 0.05$). Abundance on Day 30 was quite similar between the 20 m (65 cells ml^{-1}) and 300 m (60 cells ml^{-1}) populations, and accounted for 0.53% (20 m) and 77% (300 m), respectively, of the initial abundance.

In the light manipulation experiment (Light/Dark Expt), abundance of *Synechococcus* populations taken from 397 m decreased to 49% of the initial level in the first 2 d in a 12 h light and 12 h dark cycle, and then increased 220-fold from Day 2 to Day 14 (Fig. 9B). The initial decrease in abundance suggests susceptibility of the deep population to light. *Synechococcus* abundance remained relatively constant in continuous darkness.

DISCUSSION

Distribution of *Synechococcus*

The most interesting result in this study is the constant vertical abundance profiles of *Synechococcus* in deep seawater (≥200 m), which occurred regardless of season or study area (Figs. 3 & 4). In contrast, the vertical distribution of total prokaryotes or chl *a* was not constant, and a decrease in total prokaryotic abundance with increasing depth probably resulted from a decrease in the supply of organic substrates via sinking flux (Sohrin et al. 2010). *Synechococcus* strains can photosynthesize at a minimum irradiance of 2 µmol photons m^{-2} s^{-1} (Six et al. 2004, Timmermans et al. 2005), which corresponds to the PAR level at 60 m in

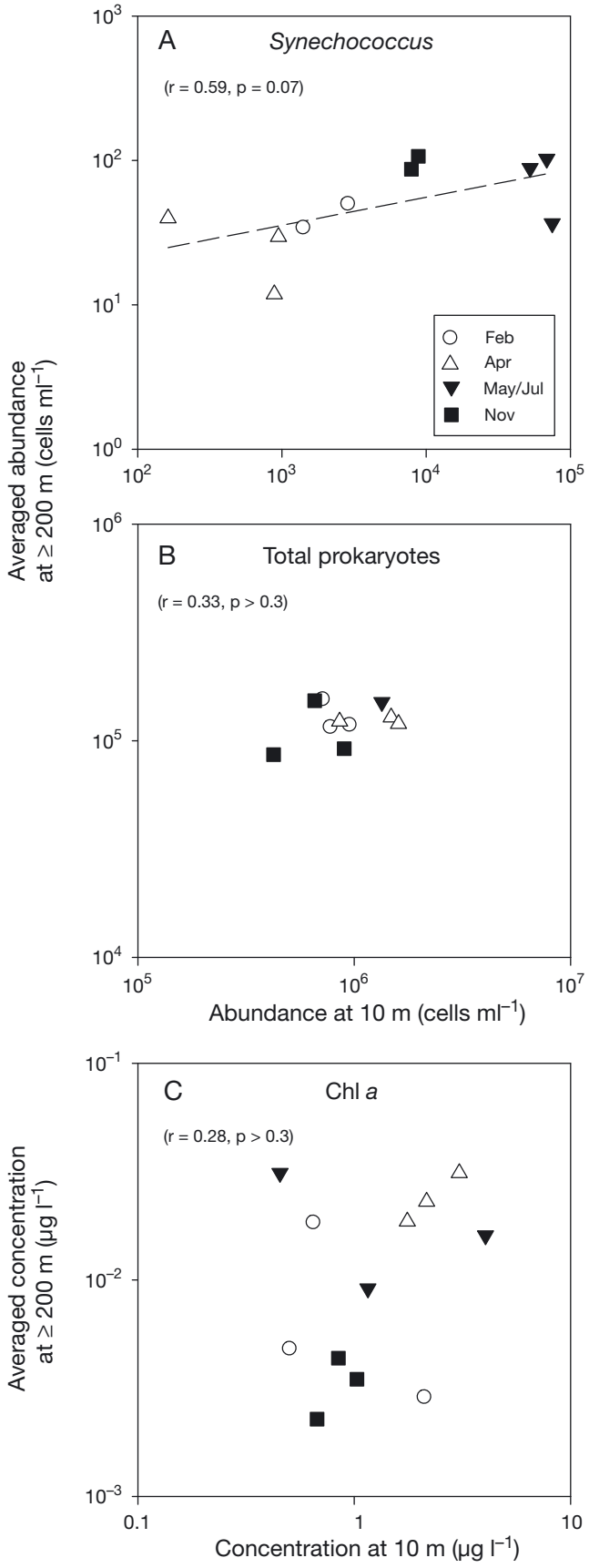

Fig. 7. Comparison of (A) *Synechococcus* and (B) total prokaryote abundance, and (C) chl *a* concentration at 10 m to the averages at depths of ≥200 m. Correlation coefficient (r) and results of Student's *t*-test (p) are shown in parentheses. The broken line was fitted by linear regression

the Suruga Bay in April 2001, and we infer the irradiance is so low at ≥200 m that the photosynthetic activity of *Synechococcus* would be negligible. Vilibi & Šanti (2008) reported a downward export of *Synechococcus* to the sea floor (1150 m) in the south Adriatic Sea through a combination of winter convection and density currents on the downslope. At the sites in the present study, winter MLD is so shallow (≤133 m, Table 1) that *Synechococcus* would be unlikely to sink as a result of winter convection. Regarding the possibility of lateral transport, NPIW intruded at around 450 m (Stn 2) and 680 m (Stn C2700) (Fig. 2). There are no available data on *Synechococcus* abundance in NPIW, but the unique formation process of NPIW explains the different *Synechococcus* abundances between NPIW and adjacent water masses. NPIW is formed in the interfrontal zone between the Oyashio Front and the Kuroshio Extension, and consists of the Kuroshio and Oyashio waters (Talley et al. 1995, Yasuda et al. 1996). NPIW did not appear to influence the levels of *Synechococcus*. This finding may be partly due to a lack of sampling in the core of NPIW, but the synchronized seasonal changes in *Synechococcus* between 10 m and ≥200m at Stn 2 (Fig. 7A) strongly suggest that the distributions of deep *Synechococcus* are influenced by vertical transport of the epipelagic populations rather than lateral transport in our study area. This hypothesis is supported by many previous studies that have detected *Synechococcus* cells on sinking particles (Vanucci et al. 2001 and references therein). Richardson & Jackson (2007) showed that picophytoplankton can sink from the epipelagic zone even without large carrier particles, by aggregating into cell clumps. However, given that the highest contribution of *Synechococcus* at ≥200 m at Stn 2 was seen in spring, it seems that transport of *Synechococcus* mainly occurred by attachment to larger particles, such as microphytoplankton, and that self-aggregation in the epipelagic zone would be less effective in spring due to the lower total abundance observed at this time. We did not find fluorescent phytodetritus in deep seawater samples with epifluorescence microscopy, and phytodetritus may in fact hydrolyze in the water column. Copepods and salps can ingest *Synechococcus* cells attached to microphytoplankton and transport them to greater depths by packing them into rapidly sinking fecal pellets (Turner 2002 and references therein). Other possible carriers are mineral particles and appendicularian houses. Appendicularians are found

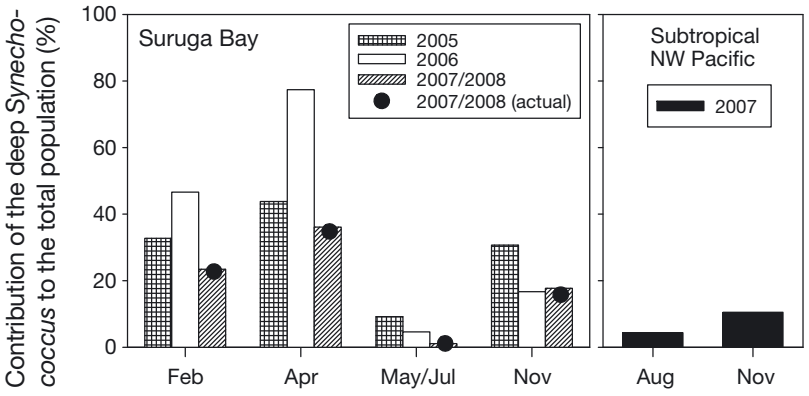

Fig. 8. Contribution of *Synechococcus* abundance in the deep seawater (200 m to bottom) to the total abundance in the water column. Values were calculated as the ratios of depth-integrated abundance. Bars indicate the ratios, in which abundance at ≥200 m was represented by averages, and dots show the ratios of actual data

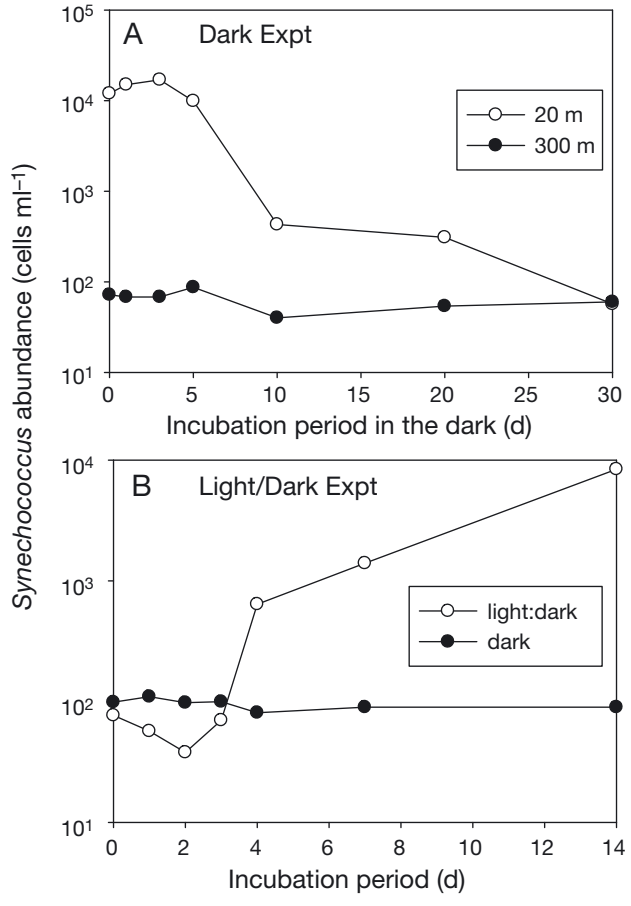

Fig. 9. Time course of *Synechococcus* abundance during incubation experiments using (A) seawater from 20 m and 300 m performed in constant darkness (Dark Expt) and (B) seawater from 397 m in a cycle of 12 h light:12 h dark and continuous darkness (Light/Dark Expt). Seawater was collected in November 2005 (Dark Expt) and October 2006 (Light/Dark Expt) in the Suruga Bay and filtered before the experiments to remove grazers

in the surface layer in the Suruga Bay throughout most of the year (Itoh 1990), and *Synechococcus* cells have been reported to adhere to their houses (Silver et al. 1998). *Synechococcus* readily adheres to mineral particles; it can attach to glass slides placed in seawater faster than other phytoplankton (Nayar et al. 2005). The contribution of *Synechococcus* at ≥200 m was extremely low at Stn 2 in summer and at Stn C2700 (Fig. 8). This result implies less effective transport of *Synechococcus* under stratified and oligotrophic conditions despite the enhanced potential for self-aggregation in nutrient-depleted cells (Klut & Stockner 1991, Table 2).

If *Synechococcus* cells are associated with sinking particles which they subsequently detach from, the supply of *Synechococcus* to the surrounding seawater would decrease exponentially with increasing depth in proportion to the exponential decrease in sinking flux (Martin et al. 1987). However, this hypothesis is not in keeping with our finding of a vertically constant abundance profile. We then applied a cell digestion assay, which enabled cells with intact membranes (live cells) to be distinguished from those with damaged membranes (dead cells) (Darzynkiewicz et al. 1994). We found an overwhelming contribution of live cells (almost 100%) to total *Synechococcus* cells with relatively constant levels of live cells through the mesopelagic zone at Stns 2 and C2700 and a rapid removal of dead *Synechococcus* cells at the beginning of dark incubation experiments, apparently due to autolysis (R. Sohrin, Y. Obara, K. Endoh, R. Handa unpubl. data). We speculate that the vertically constant *Synechococcus* abundance profiles are related to rapid removal of dead cells and of acclimation of the survivors to the dark, and illustrated our proposed model in Fig. 10. Briefly, a proportion of the dead *Synechococcus* cells is immediately eliminated from the water column, and their number decreases with increasing depth because of acclimation. The remaining cells distribute evenly as a result.

The mesopelagic *Synechococcus* abundance did not decrease in the Dark Expt though the surface *Synechococcus* abundance continued to decrease from Day 3 to Day 30 (Fig. 9A). In a different dark incubation experiment, the surface *Synechococcus* abundance decreased in the first 20 d and then remained stable until Day 40 (data not shown). These results suggest dark acclimation and/or occurrence of a population with a tolerance to darkness. The vertically constant levels of live cells cannot be explained in this study, and the downward transport of surface *Synechococcus*

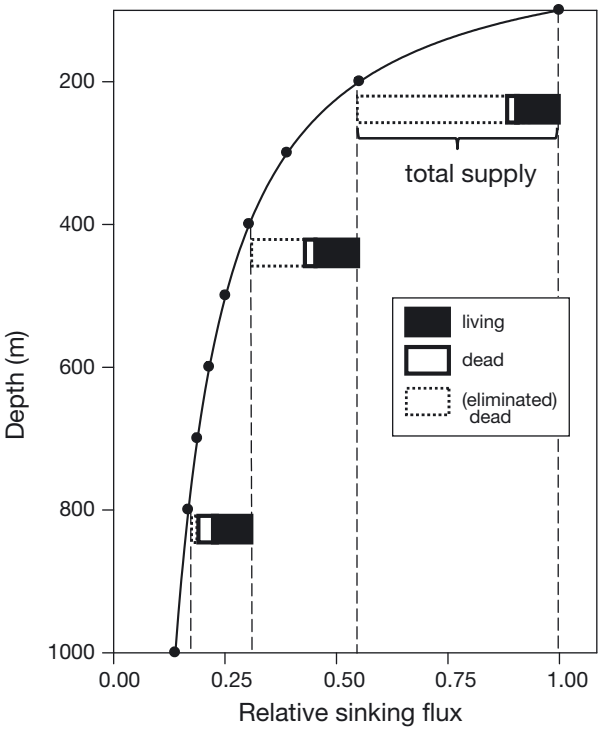

Fig. 10. Proposed *Synechococcus* transportation model associated with sinking flux. Sinking flux was expressed as ratios of the flux measured at 100 m depth and fitted to a power function described by Martin et al. (1987). Differences in sinking flux at various depths correspond to the relative amount of *Synechococcus* supplied by the sinking flux to the surrounding water in a corresponding depth stratum. Supplied *Synechococcus* includes dead cells, a part of which is immediately eliminated by autolysis, leaving behind mostly live cells

populations by sinking flux remains hypothetical. Uncovering the link between the epipelagic and deep *Synechococcus* populations using molecular genetic approaches and physiological studies of isolates might help explain the observed *Synechococcus* profiles.

Physiological state of *Synechococcus* in the dark ocean

The relative cellular contents of chl *a* and PE among *Synechococcus* cells were found to be within a similar range of between <100 m and ≥200 m for *Synechococcus* (Fig. 6), and the mesopelagic *Synechococcus* population was grown in a light/dark cycle (Fig. 9B). These results suggest that at least a proportion of the deep *Synechococcus* cells is viable and retains its pigments in prolonged darkness.

Many previous studies have shown that *Synechococcus* in epipelagic waters can incorporate exogenous amino acids but at a lower rate than *Prochlorococcus* (Zubkov et al. 2003, Zubkov & Tarran 2005). Several *Synechococcus* strains have ectoproteolytic activity (Martinez & Azam 1993); however, these rates are greatly reduced in the dark (Kramer 1990, Martinez & Azam 1993, Mary et al. 2008). Lochte & Turley (1988) incubated phytodetritus taken from the sediment surface (water depth 4500 m) with deep sea (450 atm, 2°C) or surface water (1 atm, 15°C) conditions in the dark and detected a 4-fold increase in *Synechococcus* abundance on Day 1 under both conditions. The relatively constant abundance of mesopelagic *Synechococcus* during the dark incubation suggests a limitation on *Synechococcus* growth caused by restricted availability of dissolved organic substrates.

Some genera of cyanobacteria can survive in the dark by catabolizing cellular storage molecules such as carbohydrates, lipids and proteins (Osanai et al. 2005, Montechiaro et al. 2006). In the case of *Phormidium autumnale*, about 50% of endogenous organic carbon is catabolized after 21 d and cell size is halved (Montechiaro et al. 2006). Side scatter light (SSC) of flow cytometry is related to cellular contents and cell size for prokaryotes and picophytoplankton (Wittrup et al. 1988, Jochem 2000). In the present study, SSC of *Synechococcus* was higher in the mesopelagic than in the epipelagic (Mann-Whitney *U*-test, $p < 0.001$; Fig. 6), which does not support the idea of a survival mechanism involving the catabolism of assimilated carbon in the mesopelagic. *Synechococcus* sp. WH7803 can uptake leucine and uracil in the dark for at least 24 h, though incorporation is suppressed (Kramer 1990). Further study would be required to determine whether exogenous organic compounds meet the energy demands of *Synechococcus* in deep seawater or whether the rates of metabolism of deep *Synechococcus* cells fall to lower levels, as is seen in some strains of chlorophytes and prymnesiophytes (Jochem 1999).

Ecological and biogeochemical implications for *Synechococcus* in the deep ocean

Although the mesopelagic *Synechococcus* populations collected in November 2005 did not decline significantly in the Dark Expt, the *in situ* population decreased from November to February. This difference may reflect the removal of *in situ Synechococcus* populations by grazing. Predation by cyanophages removes *Synechococcus* from epipelagic waters (Suttle & Chan 1993, 1994), but the constant abundance seen in the incubation experiments suggests this had little effect on the deep *Synechococcus* population, presumably due to the kill the winner hypothesis (Thingstad 2000), since its abundance was low at greater depths. From the differences in the average *Synechococcus*

abundance at ≥200 m at Stn 2 between November and February, grazing rates were estimated at 0.0069 to 0.014 d^{-1} (average, 0.013 d^{-1}). This is likely to be a low value, because the replenishment of *Synechococcus* cells caused by sinking flux was not taken into account. By dividing the grazing rate by the depth-weighted average of heterotrophic nanoflagellates (HNF) or ciliate abundance in the 200 to 1000 m range at Stn 2 for November and February (2.8×10^4 cells l^{-1} and 12 cells l^{-1}, respectively, Imazawa 2006), clearance rates of HNF and ciliates on the mesopelagic *Synechococcus* were roughly estimated at 8.6 to 17 nl HNF^{-1} h^{-1} and 25 to 50 µl $ciliate^{-1}$ h^{-1}, respectively. The former estimate is within the range of clearance rates of HNF on *Synechococcus* in subsurface seawater (0.5 to 25 nl HNF^{-1} h^{-1}, Christaki et al. 2001) and on prokaryotes in mesopelagic seawater (1 to 16 nl HNF^{-1} h^{-1}, Cho et al. 2000). On the other hand, the latter estimate is 1 to 3 orders of magnitude higher than the clearance rates of ciliates on *Synechococcus* in epipelagic waters (10 to 3150 nl $ciliate^{-1}$ h^{-1}; Šimek et al. 1995, Christaki et al. 1998, 1999, Tsai et al. 2007), so we contend that ciliates are not a significant *Synechococcus* predator in the mesopelagic Suruga Bay. McCarthy et al. (1998, 2004) compared D/L ratio of alanine and $\delta^{13}C$ of D-alanine between *Synechococcus bacillaris* and ultrafiltered dissolved organic matter (DOM) in the central Pacific, and they suggested that cyanobacteria are the dominant source of DOM throughout the water column. The grazing of HNF on *Synechococcus* is a mechanism that could supply cyanobacterial organic matter to the mesopelagic DOM pool. Although the abundance and ecological importance of deep *Synechococcus* populations are much lower than that of their surface counterparts and of heterotrophic prokaryotes in the deep ocean, they nonetheless contribute to the food web and biogeochemical cycles more significantly than previously hypothesized.

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