Differences in cell viabilities of phytoplankton between spring and late summer in the northwest Pacific Ocean

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Abstract

Cell viabilities of phytoplankton in the Oyashio and Kuroshio-Oyashio transition regions of the northwest Pacific Ocean were examined in September 2003 (late summer) and May 2005 (spring) using a membrane permeability test. Specific lysis rates of the phytoplankton during late summer were also assessed by an esterase activity assay. In late summer, cyanobacteria *Synechococcus* spp. were > 2 x 10^4 cells ml^{-1} and numerically dominated the phytoplankton communities. The cell viabilities of *Synechococcus* spp. and eukaryotic ultraphytoplankton (<10 μm in size) were 60-79% and 26-41% in surface waters, respectively. The specific lysis rates of the phytoplankton were 0.12-0.67 d^{-1} in late summer. By contrast, in spring, eukaryotic cells were predominant in the phytoplankton communities. The cell viabilities of surface eukaryotic ultraphytoplankton in spring were > 70% and significantly higher than those in late summer. During spring, *Synechococcus* spp. only occurred with < 1 x 10^4 cells ml^{-1} in the Kuroshio-Oyashio transition region, and their viabilities were 80%. In the Oyashio region where a spring diatom bloom developed, the viability of fucoxanthin-containing algae (mainly diatoms and prymnesiophytes) was ca. 90%. These results suggested that the cell viability of phytoplankton could vary seasonally with their community structure in the study area. The phytoplankton cell death in late summer was particularly significant for their loss process and could support the microbial food webs by supplying dissolved organic carbon (DOC) derived from the dead cells.

KEY WORDS: Phytoplankton · Cell death · Viability · Cell lysis · Oyashio · Pacific Ocean

1. Introduction

Zooplankton grazing and irreversible cell sinking were thought to be the main loss processes of phytoplankton in surface waters (Bidle and Falkowski, 2004). However, it has become evident that phytoplankton can also be lost through several factors such as viral infection (Fuhrman, 1999; Castberg et al., 2001) and programmed cell death in response to environmental stress (Dunn et al., 2002; Bidle and Falkowski, 2004; Franklin et al., 2006). For example, van Boekel et al. (1992) showed that nitrate depletion induced cell lysis of *Phaeocystis*, which led to the termination of their blooms in the North Sea. Agustí and Sánchez (2002) reported that the cell viability of each phytoplankton group varied seasonally with their class-level community compositions in the Mediterranean Sea. Thus, the cell death of phytoplankton induces changes in their biomass, composition, and net productivity, which can directly affect zooplankton grazing and carbon sequestration ability in the ocean. Moreover, such phytoplankton cell lysis results in the release of their cellular components, which can serve as an important carbon source for the microbial loop (van Boekel et al., 1992; Brussaard et al., 1995). According to Smith et al. (1995), the carbon demand of heterotrophic bacteria accounted for 40 to 60% of the total carbon fixed during a bloom. However, the source of this organic matter could not be attributed solely to the exudation of microorganisms

(Bjørnsen, 1988; van den Meersche et al., 2004) and the sloppy feeding of zooplankton (Møller et al., 2003). In fact, Brussaard et al. (1995) showed that bacterial production was positively correlated with the specific lysis rates of phytoplankton in the North Sea, and that the bacterial carbon demand could be supplied by the cell lysis of phytoplankton. Thus it is obvious that the cell death of phytoplankton is significant not only for phytoplankton population dynamics but also for marine ecosystems and biogeochemical cycles.

It should be noted that field studies of the cell death of marine phytoplankton remain limited to several regions: the North Sea (van Boekel et al., 1992; Brussaard et al., 1995; Riegman and Winter 2003), the Adriatic Sea (Baldi et al., 1997), the Mediterranean Sea (Agustí et al., 1998; Agustí and Duarte, 2000; Agustí and Sánchez, 2002; Alonso-Laita et al., 2005), and the Atlantic Ocean (Veldhuis et al., 2001; Agustí, 2004; Alonso-Laita and Agustí, 2006; Llabrés and Agustí, 2006). No information was available on the cell death of phytoplankton in the Pacific Ocean.

The northwest subarctic Pacific Ocean (especially in the Oyashio region) is known to be one of the regions where the biological drawdown effect of pCO_2 in surface waters is among the highest in the world (Takahashi et al., 2002). This is mainly due to the massive diatom blooms in and around Oyashio waters during spring (Saito et al., 2002).

On the other hand, from summer to winter, the abundance of phytoplankton was relatively low (< 1 μg l^{-1} in Chl *a*), and picophytoplankton (<2 μm in size) were predominant in the phytoplankton community in the northwest subarctic Pacific, including Oyashio waters (Liu et al., 2004). However, it has recently been pointed out from a 30-year retrospective data analyses of surface Oyashio waters that year by year the chlorophyll (Chl) *a* level, the abundance of diatoms, and net community production in spring have gradually decreased as has the phosphate concentration in winter (Ono et al., 2002; Chiba et al., 2004). In addition, the species composition of bloom-forming diatoms in spring has also been gradually altered (Chiba et al., 2004). It would be important to learn whether phytoplankton cell death could be increasing with time in the area. Shinada et al. (2001) also indicated that microbial food webs were dominant in the region from summer to winter. These results suggested that the cell death of phytoplankton could be also important for the supply of dissolved organic matter that can be utilized by heterotrophic bacteria, and that it stimulates the microbial food web. In order to evaluate the significance of phytoplankton cell death in the northwest Pacific Ocean, the cell membrane permeability test (i.e. cell digestion assay; Darzynkiewicz et al., 1994; Agustí and Sánchez, 2002) was applied in this study. This methodology is a direct estimate (and a direct identification) of the abundance of living and dying cells.

Moreover, the specific lysis rates of phytoplankton during late summer were also assessed using the dissolved esterase activity method developed by van Boekel et al. (1992) and modified by Agustí et al. (1998). The lysis rates estimated are based on an indirect methodology. Here we report, for the first time, the importance of phytoplankton cell death in the northwest Pacific Ocean in spring and late summer.

2. Materials and methods

2.1. Study area and sampling

Sampling and incubation experiments were carried out along the monitoring line (*A-line*) in the northwest Pacific Ocean (Fig. 1) in September 2003 and May 2005 on board the FR/V 'Wakataka Maru' (Tohoku National Fisheries Research Institute). Seawater samples were collected from surface waters (at a depth of 5 m in May 2005 or 10 m in September 2003) by Niskin bottles attached to a CTD carousel multi-sampler system. Hydrographic data (seawater temperature, salinity, and macronutrients) were obtained from the cruise database of the Research Institute. Water masses in the study area were classified into the Oyashio, Kuroshio-Oyashio transition, and warm-core ring regions in the following manner. The region where the temperature at 100 m depth was

less than 5˚C was defined as the Oyashio region (Kawai, 1972). The Oyashio is a cold current that forms the western boundary of the subarctic circulation flowing southwestward along the Kurile Islands (Ohtani, 1970), while the Kuroshio is a warm and western boundary current (Yasuda, 2003). Since the water temperature of Kuroshio waters is more than 14˚C at 200 m depth (Kawai, 1969), the region between the Oyashio and Kuroshio was categorized as the Kuroshio-Oyashio transition region. For the warm-core ring region, reference was made to our CTD data and the quick bulletin of ocean conditions of the Japan Coast Guard issued during the observation period.

2.2. Cell viability of phytoplankton

Cell viabilities were examined using the cell membrane permeability test (Darzynkiewicz et al., 1994; Agustí and Sánchez, 2002). That assay is based on exposing cells to deoxyribonuclease I (DNase I) and trypsin for a short time. For living cells, morphology, function, and viability are not affected by these enzymes (Darzynkiewicz et al., 1994; Agustí and Sánchez, 2002). However, the membrane permeability should be increased for dead cells, whose plasma membranes are damaged. By exposure to these enzymes, the hydrolysis of DNA and protein in the dead cells are induced by DNase I and trypsin, respectively, and these cells are digested

(Darzynkiewicz et al., 1994; Agustí and Sánchez, 2002). As a result, the dead cells can no longer be detected after the treatment.

In September 2003, the cell viabilities of ultraphytoplankton (<10 μm in size) at 10 m depth were assessed by the cell digestion assay coupled with flow cytometry, since ultraphytoplankton cells were predominant in the phytoplankton community. Flow cytometry provides a quick and accurate estimate of the abundances and optical properties of small marine prokaryotic and eukaryotic cells (Campbell, 2001). In contrast, in May 2005, the cell viabilities of such communities at 5 m depth were estimated by the cell digestion assay together with flow cytometry and HPLC pigment analysis in order to elucidate the significance of the cell death of small- and large-sized phytoplankton, respectively, during spring.

After sampling, 200 μl of DNase I solution in the Hanks' balanced salt solution (HBSS) without phenol red (Sigma) was added to 1 ml seawater sample (final concentration 100 μg ml^{-1}) and incubated for 15 min at 37˚C. Thereafter, 200 μl of trypsin solution (in HBSS without phenol red, Sigma) was added (final concentration 5 mg ml^{-1}) to the sample and incubated for an additional 30 min at 37˚C (Darzynkiewicz et al., 1994). At the end of the incubation, 200 μl of trypsin inhibitor solution (in HBSS without phenol red, Sigma) was added in the proportion of one unit of trypsin inhibitor

per unit of trypsin in the sample. In this study, different incubation temperatures (i.e. 20 and 37ºC) were tested in order to examine their effect on the cell digestion assay in September 2003. As a result, the differences in cell viabilities at different temperatures were found to be insignificant (p>0.05, two-tailed t-test) except for *Synechococcus* spp. at Stn A17 (70.1 ± 1.7% at 20ºC and 61.2 ± 3.7% at 37 ºC; mean ± first standard deviation, n = 3).

For the cell digestion assay with flow cytometer, triplicate seawater samples were taken following the procedure mentioned above. Control samples were also prepared by adding HBSS without phenol red medium instead of enzymatic cocktails, and incubated in the same manner. After incubations, paraformaldehyde was added to each sample (final concentration 0.2%). Samples were frozen in liquid nitrogen or a deep-freezer (-80˚C) until analysis on land. Details of analytical procedure of flow cytometry are described in Suzuki et al. (2005). Briefly, *Synechococcus* spp. and eukaryotic ultraphytoplankton cells were distinguishable by the orange fluorescence of phycoerythrin from *Synechococcus*, while *Prochlorococcus* spp. cells were distinguished from eukaryotic ultraphytoplankton by their size and dim chlorophyll fluorescence. The fraction of living cells in the samples was calculated by dividing the concentration of living cells after enzymatic treatment by the cell concentration in the

control samples, representing the total (dead plus living) cell concentration.

For the cell digestion assay with HPLC, seawater sample (80-100 l) was concentrated to ca. 150 ml using the Pellicon-2 tangential flow filtration system (Millipore Corp.) fitted with a V screen (Millipore Corp., 0.65 μm in pore size). The cell digestion assay was applied to five replicates of the cell concentrate (1 ml). After procedures with and without the enzymatic treatment described above, samples were filtered onto Whatman GF/F (ca. 0.7 μm in pore size) filters (25 mm in diameter) under gentle vacuum pressure (< 13kPa). The filter samples were stored in liquid nitrogen or a deep-freezer (-80˚C) until analysis on land. The analytical procedure for HPLC pigment analysis is described in Suzuki et al. (2005). Cell viability was calculated in terms of pigment concentration in the same manner as in the cell digestion assay with flow cytometry.

2.3. Specific lysis rates of phytoplankton

Specific lysis rates of phytoplankton at 10 m depth were estimated during September 2003 following the dissolved esterase activity method described by Agustí et al. (1998). Esterase is an intracellular enzyme that is expected to appear in the dissolved fraction of seawater after cell breakage (van Boekel et al., 1992). Total esterase activity (TEA) and dissolved esterase activity (DEA) were determined using unfiltered and filtered (0.2 μm

Nuclepore membrane) samples, respectively. Particulate esterase activity (PEA) was calculated from TEA and DEA where PEA = TEA – DEA (Brussaard et al., 1996; Riegman et al., 2002). All esterase activity measurements were performed under identical standardized conditions: 100 µl EDTA (final concentration 0.2 mM) + 100 µl FDA (fluorescein diacetate: final concentration 0.02 mM) were added at time $t = 0$ to 10 ml of triplicate samples, and mixed with a vortex mixer. After incubating the samples for one h at 20˚C, the fluorescence emission was immediately measured in a Shimadzu RF-5300PC spectrofluorometer at 451 nm and 510 nm excitation and emission (5 nm bandwidth) wavelengths, respectively. Specific lysis rates of phytoplankton (μ_L d^{-1}) were calculated as the decrease in PEA with time (t) due to the production of DEA after cell lysis (Agustí et al., 1998). The production of DEA was estimated using DEA and the decay of esterase, which was measured by adding commercial esterase (porcine liver esterase, Sigma) to seawater samples and incubating at *in situ* temperature (Agustí and Duarte, 2000). The esterase samples for estimating its decay were collected at intervals of eight hours in a day.

2.4. Phytoplankton community structure

Seawater samples collected from 5 or 10 m depths were filtered onto 2 and 10 µm

Nuclepore membrane filters, and Whatman GF/F filters under gentle vacuum pressure (< 13kPa). The filter samples were folded and frozen in liquid nitrogen or a deep-freezer (-80˚C). To estimate the phytoplankton community structure, phytoplankton pigments were analyzed by HPLC on land (Suzuki et al., 2005). In September 2003, divinyl Chl *a* was quantified using HPLC equipped with a C-8 reverse phase column (Barlow et al., 1997), since *Prochlorococcus* cells were detected by flow cytometry.

3. Results

3.1. Hydrographic data

In September 2003, our sampling area included the regions of (1) Oyashio (Stns A4, A7, and A9), (2) Kuroshio-Oyashio transition (Stns A13, and A21), and (3) warm-core ring (Stn A17). In the Oyashio region, temperature in surface waters (10 m) was relatively low (11.4 ± 0.5˚C, n = 4) and nutrient levels were relatively high (6.08 ± 2.64 μM for nitrate, 0.774 ± 0.201 μM for phosphate, and 8.4 ± 3.2 μM for silicate, n = 4) (Table 1). In contrast, in the Kuroshio-Oyashio transition and warm-core ring regions, surface temperature was more than 20˚C, and nutrients were almost depleted (0.20 ± 0.21 μM for nitrate, 0.049 ± 0.013 μM for phosphate, and 1.5 ± 0.4 μM for silicate, n =

4).

Hydrographic conditions in May 2005 were different from those in September 2003. The sampling stations of May 2005 were categorized into the regions of (1) Oyashio (Stn A4), (2) Kuroshio-Oyashio transition (Stns A13, A17 and A21), and (3) warm-core ring (Stn A9). At Stn A4 in the Oyashio region, temperature and nutrients in surface waters were relatively low and abundant, respectively (Table 1). In the Kuroshio-Oyashio transition region, surface temperature was highly variable, ranging from 7.7 to 17.0˚C. The nutrient levels (3.42 ± 2.53 μM for nitrate, 0.370 ± 0.283 μM for phosphate, and 4.8 ± 1.8 μM for silicate, n = 3) in surface waters of the Kuroshio-Oyashio transition region were roughly half those in the Oyashio region during September 2003. At Stn A9 in the warm-core ring region, surface temperature and nutrient levels were similar to those in the Oyashio region during September 2003.

3.2. Phytoplankton community structure

In September 2003, the concentrations of total Chl *a*, which is Chl *a* plus divinyl Chl *a*, varied from 0.16 to 1.00 μg l^{-1} among the stations (Table 2). Ultraphytoplankton (<10 μm in size) contributed more than 90% to the total Chl *a* concentration at all stations (Fig. 2). The concentrations of Chl *a*, Chl *b*, fucoxanthin, and 19'-hexafucoxanthin were

clearly higher in the Oyashio region (Stns A4-A9) than south of it (Stns A13-A21). Ultraphytoplankton were composed of *Synechococcus* spp. and eukaryotic ultraphytoplankton in the Oyashio and warm core-ring regions. The cell abundances of *Synechococcus* spp. (3 to 14 x 10^4 cells ml^{-1}) were 2 to 5 orders of magnitude higher than those of eukaryotic ultraphytoplankton at all stations (Fig. 3). In the Kuroshio-Oyashio transition region, *Prochlorococcus* spp. cells were also detected with *Synechococcus* spp. and eukaryotic ultraphytoplankton, but the cell abundances of *Prochlorococcus* spp. were low (< 1 x 10^3 cells ml^{-1}). Divinyl Chl *a*, which is a marker for *Prochlorococcus* spp. (Goericke and Repeta, 1992), ranged between 0.06-0.17 μg l^{-1}.

In May 2005, Chl *a* concentrations varied from 0.69 to 3.86 μg l^{-1} among the stations (Table 2). Divinyl Chl *a* was not analyzed by HPLC with a C-8 reverse phase column, since *Prochlorococcus* spp. cells were not detected by flow cytometry, nor was zeaxanthin, an indicator of cyanobacteria (*Prochlorococcus* and *Synechococcus*). At Stn A4 in the Oyashio region, the highest Chl *a* concentration was observed (3.86 μg l^{-1}) and the large-sized phytoplankton (>10 μm size fraction) contributed 90% to the total Chl *a* concentration (Fig. 2). The concentrations of Chl *a* and fucoxanthin were relatively high at Stn A4 (Table 2). In contrast, ultraphytoplankton contributed more

than 64% to the total Chl *a* concentrations at the other stations (Fig. 2). Ultraphytoplankton were composed of *Synechococcus* spp. and eukaryotic ultraphytoplankton as estimated by flow cytometry. The cell abundances of eukaryotic ultraphytoplankton and *Synechococcus* spp. varied among the stations from 7 to 35 x 10^3 cells ml^{-1} and from 0 to 8 x 10^3 cells ml^{-1}, respectively (Fig. 3).

3.3. Phytoplankton cell viability

In September 2003, cell viabilities of *Synechococcus* spp. ranged from 60 to 79%, while those of eukaryotic ultraphytoplankton were from 26 to 41% as estimated by the cell digestion assay coupled with flow cytometry (Fig. 4). Those results indicated that cell mortalities of eukaryotic ultraphytoplankton were significantly higher than those of *Synechococcus* spp. ($p<0.05$, two-tailed *t*-test). The differences in the viabilities of *Synechococcus* spp. in each region were insignificant ($p>0.05$, two-tailed *t*-test). Similarly, the differences in the viabilities of eukaryotic ultraphytoplankton in each region were also insignificant ($p>0.05$, two-tailed *t*-test). Cell viabilities of *Prochlorococcus* spp. were not estimated at all stations, since the cell abundances of *Prochlorococcus* spp. enumerated were low as described above. The specific decay rates of DEA ranged from -0.028 to -0.021 h^{-1}, the average specific decay rate of DEA

was -0.024 ± 0.003 h^{-1}, and PEA varied between 1.25 and 11.0 fluorescein l^{-1} h^{-1}. The specific lysis rates of phytoplankton were 0.34 ± 0.12 d^{-1} (n =2), in the Oyashio region (Stns A4 and A9) (Table 3). In the Kuroshio-Oyashio transition region (Stns A13 and A21), large difference in the specific lysis rates was observed. No significant differences (p>0.05, two-tailed t-test) among the specific lysis rates in each water mass were found. In the observations at Stn A4 on September 10 and 12, 2003, pronounced differences in the concentrations of nutrients except silicate, the cell viabilities of *Synechococcus* spp. and eukaryotic ultraphytoplankton, and the specific lysis rates of phytoplankton were observed. These results indicated the heterogeneity of water masses at Stn A4, where Oyashio waters flowed. Similarly, two observations were carried out at Stn A13 of the Kuroshio-Oyashio transition region on September 8 and 14, 2003, but the differences in the cell viabilities of phytoplankton were insignificant (p>0.05, two-tailed t-test).

In May 2005, cell viabilities of eukaryotic ultraphytoplankton were more than 70% at all stations as estimated by flow cytometry (Fig. 4). *Synechococcus* spp. only occurred at Stns A17 and A21 of the Kuroshio-Oyashio transition region, and their viabilities were 80%. At Stn A21 in the Kuroshio-Oyashio transition region, the cell viability of eukaryotic ultraphytoplankton was significantly lower than those of *Synechococcu*s spp.

(p<0.01, two-tailed t-test), while the difference in the viabilities between eukaryotic ultraphytoplankton and *Synechococcus* spp. was insignificant (p>0.05, two-tailed t-test) at Stn A17 of the Kuroshio-Oyashio transition region. Cell viabilities of the total phytoplankton community in terms of Chl *a* were approximately 100% at Stns A9 and A13 and 55% at Stn A4, as estimated by the cell digestion assay coupled with HPLC (Fig. 5). Cell viabilities of fucoxanthin-containing phytoplankton (i.e. mainly diatoms and prymnesiophytes) at Stn A4 in the Oyashio region and at Stn A9 in the warm-core ring region were close to 100%. Microscopic analyses revealed that diatoms and prymnesiophytes appeared in the cruise of May 2005 (A. Kuwata, personal comm.). Cell viabilities of the other phytoplankton groups could not be determined, because the abundance of another phytoplankton pigment was much lower than those of Chl *a* and fucoxanthin.

4. Discussion

Although much attention has recently been focused on the cell death of phytoplankton as it relates to the marine ecosystems and biogeochemical processes (e.g. van Boekel et al., 1992; Agustí, 2004; Bidle and Falkowski, 2004), field observations are still limited

in several regions, and no such data were available from the Pacific Ocean. In this study, we report for the first time the cell death of phytoplankton in the Oyashio and Kuroshio-Oyashio transition regions of the northwest Pacific Ocean in late summer of 2003 and spring of 2005.

The hydrographic conditions clearly differed between September 2003 and May 2005. Nutrient levels (Table 1) and the phytoplankton biomass (Table 2) in surface waters during September 2003 were relatively lower than those in May 2005. These differences were probably caused by seasonal stratification of the water columns. Furthermore, the community structures of phytoplankton also differed between the two periods as inferred from the pigment signatures. In September 2003, ultraphytoplankton (<10 μm in size) were predominant (> 90% in terms of total Chl *a* level) in surface waters at all stations (Fig. 2). This result was consistent with the previous observations in the study area during summer (Liu et al., 2004). From the results of our flow cytometric analyses, *Synechococcus* spp. were predominant in the ultraphytoplankton community, and their cell abundances were high (> 70 x 10^3 cells ml^{-1}) especially in Oyashio waters (Stns A4, A7, and A9), which were categorized as subarctic water masses during September 2003 (Fig. 3). However, these cells were not detected in Oyashio waters in May 2005. Liu et al. (2002) also reported that relatively high abundances of *Synechococcus* occurred in

the northwest subarctic Pacific Ocean at Station KNOT (44ºN, 155ºE) during summer, when sea surface temperature and nitrate were relatively high and low, respectively, compared to other seasons. These results suggest that *Synechococcus* cells are particularly abundant in the northwest subarctic Pacific Ocean during summer. In the Kuroshio-Oyashio transition region during September 2003, *Prochlorococcus* spp. cells were little detected, but significant amounts of divinyl Chl *a* were observed (Table 2). Assuming that cellular divinyl Chl *a* contents of *Prochlorococcus* are 1.06-2.71 fg $cell^{-1}$ (Shimada et al., 1995), its expected cell densities in the Kuroshio-Oyashio transition region were 5.6-16 x 10^4 cells ml^{-1}. The estimates indicate that the measured values were possibly underestimated because the cellular fluorescence of divinyl Chl *a* for *Prochlorococcus* spp. in surface waters was generally so low that their detection was difficult even with flow cytometry (Matsumoto et al., 2004).

In September 2003, cell viabilities of *Synechococcus* spp. were relatively high (from 60 to 79%) in surface waters at all stations (Fig. 4). This result was similar to the previous report of Agustí and Sánchez (2002) who showed that *Synechococcus* sp. dominated the phytoplankton community, and that its cell viability was high (> 80%) in the Mediterranean Sea during summer as estimated by the cell digestion assay. In contrast, in May 2005, the cell abundances of *Synechococcus* spp. were very low (Fig.

3), whereas its cell viabilities (79.8 ± 2.4%, n = 2) were slightly higher than those (65.6 ± 7.5%, n = 7) in September 2003 (Fig. 4). In the North Atlantic Ocean (40ºN, 23ºW) during spring, Veldhuis et al. (2001) also reported that cell viabilities of *Synechococcus* ranged from 75 to 90%, using the SYTOX Green membrane permeability assay. It is known that temperature is the dominant factor controlling the growth and loss of *Synechococcus* in colder waters (Li, 1998). Moreover, Agustí and Sánchez (2002) and Alonso-Laita and Agustí (2006) also demonstrated a positive relationship between the viability of *Synechococcus* sp. and water temperature. However, in the present study, no significant relationships were found between the viability of *Synechococcus* spp. and either temperature or macronutrient (nitrate, phosphate, and silicate) concentrations (p>0.05, two-tailed t-test), indicating that other factor(s) might have been involved in the cell death of *Synechococcus* spp.

In contrast to *Synechococcus* spp., the cell viabilities of eukaryotic ultraphytoplankton (<10 μm in size) were relatively low (from 26 to 41%) at all stations in September 2003 (Fig. 4). Eukaryotic phytoplankton cells were composed of diatoms, cryptophytes, prymnesiophytes, dinoflagellates, chlorophytes and prasinophytes, based on HPLC pigment analyses (Table 2). Similarly, Agustí and Sánchez (2002) also demonstrated that the viabilities of eukaryotic picophytoplankton (<5 μm in size) were less than 60%

from June to October in the Mediterranean Sea. Recently, Alonso-Laita and Agustí (2006) showed that the portion of living cells of nano- and micro-phytoplankton varied with the trophic conditions of waters, showing lowest and highest values in oligotrophic and coastal upwelling areas in the NE Atlantic, respectively. In temperate waters during summer, nutrient depletions in surface waters caused by seasonal stratification often limit the growth of phytoplankton (Mann and Lazier, 1996) and possibly become a key factor in their cell death. However, there were no correlations between the cell viability of eukaryotic ultraphytoplankton and macronutrient (nitrate, phosphate, and silicate) concentrations in our study area ($p>0.05$, two-tailed t-test).

Recently, it was demonstrated that the bioavailability of iron controlled phytoplankton growth in the northwest subarctic Pacific Ocean during summer (Tsuda et al., 2003), and that iron starvation was one of the causes of phytoplankton programmed cell death (Berman-Frank et al., 2004; 2007). Therefore, iron deficiency in seawater could be the cause of the cell death of phytoplankton in our study. In fact, dissolved iron levels decreased from ca. 0.5 nM in spring to <0.1 nM in summer in our study area (J. Nishioka, personal comm.). Further studies must be performed on the relationship between phytoplankton cell death and iron concentrations in seawater.

Cell viabilities of surface ultraphytoplankton in May 2005 were higher than those in

September 2003 (Fig. 4), and cell viabilities of total phytoplankton (Chl *a*) and fucoxanthin-containing algae were also relatively high (Fig. 5). Such high viabilities of the phytoplankton community in May 2005 were possibly due to sufficient nutrient concentrations that were delivered by the winter mixing of the water column and increased solar radiation.

At Stn A4 in the Oyashio region, the large-sized phytoplankton was dominant (Fig. 2), and the concentrations of Chl *a* and fucoxanthin were particularly high in May 2005 (Table 2). These results indicate that our sampling was carried out during a diatom bloom. In fact, microscopic analysis revealed that a diatom bloom occurred during the observation (A. Kuwata, personal comm.). According to Saito et al. (2002), the concentration ratio of silicic acid to nitrate (R_{sw}) in surface water is an indicator of the state of the Oyashio bloom. In the present study, R_{sw} at Stn A4 decreased from 2.03 on May 20, when the cell digestion assay was conducted, to 0.79 on May 24, suggesting that the growth of diatoms had been in exponential phase.

Since large-sized phytoplankton cells (>10 μm in size) are generally dominant in the Oyashio region during spring (Liu et al., 2004), the cell viabilities of the phytoplankton communities were also estimated by cell digestion assay with HPLC pigment analyses. In our laboratory experiment using the diatom *Thalassiosira nordenskioeldii*, which

often forms spring blooms in the Oyashio region (Chiba et al., 2004), the difference in the cell viabilities estimated from Chl *a* and from fucoxanthin was statistically insignificant ($p>0.05$, two-tailed *t*-test) (data not shown). At Stn A4 in the Oyashio region, the cell viability of fucoxanthin-containing phytoplankton (i.e. mainly diatoms and prymnesiophytes) was higher (88.6 ± 7.8%, $n = 3$) than those of the total phytoplankton community in terms of Chl *a* (57.9 ± 1.9%, $n = 3$) (Fig. 5), indicating that phytoplankton cell viabilities, except for the fucoxanthin-containing phytoplankton, would be low.

Specific lysis rates of phytoplankton were estimated in September 2003, following the dissolved esterase activity method of Agustí et al. (1998). This method estimates the specific lysis rate using the intracellular enzyme released by cell lysis, but it contains a few methodological problems (see Riegman et al., 2002). The major problem is that the content of esterase varies among phytoplankton species (van Boekel et al., 1992; Agustí et al., 1998; Riegman et al., 2002). In practice, PEA was often estimated from the Chl *a* concentration in field samples and a PEA/Chl *a* ratio, determined using some phytoplankton cultures, e.g. 331 ± 43 nmol fluorescein (μg Chl *a*)$^{-1}$ h^{-1} (Agustí et al., 1998) and 12.6 nmol fluorescein (μg Chl *a*)$^{-1}$ h^{-1} (van Boekel et al., 1992). Therefore, the specific lysis rate of phytoplankton should vary with the estimation of PEA. In this

study, we assumed that the activity of esterase in seawater was derived from the release of phytoplankton only. This assumption is supported by Brussaard et al. (1995) and Agustí et al. (1998), who reported that the release of esterase by micro-heterotrophic organisms (bacteria, flagellates, and ciliates) was negligible compared to that by phytoplankton. However, our lysis rate could be overestimated due to the use of a non extractive method to quantify the esterase activity in phytoplankton cells. As the other problem of this technique, Riegeman et al. (2002) pointed out that non-enzymatic FDA hydrolysis can occur in seawater. Unfortunately, we could not estimate the effect of the non-enzymatic FDA hydrolysis on the specific lysis rates of the phytoplankton. In this study, the specific lysis rates of phytoplankton ranged from 0.20 to 0.43 d^{-1} in the Oyashio region (Stns A4 and A9) and from 0.12 to 0.67 d^{-1} in the Kuroshio-Oyashio transition region (Stns A13 and A21) (Table 3), which is almost equivalent to the specific grazing rates of microzooplankton in the study area during summer (Shinada et al., 2000).

During September 2003, since ultraphytoplankton contributed more than 90% to the total Chl *a* concentration at all stations (Fig. 2), cell sinking seemed to be a relatively unimportant process as a loss factor. These results suggest that the cell death of phytoplankton is equal to or a more important loss process than microzooplankton

grazing, and can not be negligible in our study area. Moreover, Shinada et al. (2001) pointed out that the microbial food web was more important than the grazing food chain in the Oyashio region during summer. These results cited above imply that the cell lysis of phytoplankton could be an important source of dissolved organic matter that can be utilized by heterotrophic bacteria, and that it stimulates the microbial food web.

In this study, a major difference in the cell viabilities of phytoplankton was found in the Oyashio and Kuroshio-Oyashio transition regions between spring and late summer, implying that the supply of DOC from cell lysis into the microbial food web could be significant, especially in late summer. According to Lee and Rhee (1997), DOC from dead cells consisted of high-molecular-weight compounds (HMW; 2900 Da < MW < 1900 Da), whereas DOC exported from live cells was largely of low-molecular -weight compounds (LMW; 70 Da < MW < 100 Da). HMW-DOC is considered to be an important carbon and energy source for heterotrophic bacteria, though HMW-DOC cannot directly permeate the cell membrane of heterotrophic bacteria which need to hydrolyze HMW-DOC to LMW-DOC (Amon and Benner, 1994; 1996). On the other hand, in May 2005, large-sized phytoplankton cells were sometimes predominant in the phytoplankton communities, and the cell viabilities of the phytoplankton were higher than in September 2003. In the observation period of May 2005, phytoplankton blooms

occurred, and the cell death of phytoplankton was most likely a minor loss process. However, Chiba et al. (2004) recently pointed out that the species composition of bloom-forming diatoms has been gradually altered in the Oyashio region over the years. Agustí et al. (2006) pointed out that losses by cell death were significant for the population dynamics of phytoplankton, and it would be difficult to explain the changes in the algal community without quantifying the differences in the cell death of specific species. To evaluate the relevance between the species change and the cell death of diatoms in the Oyashio region, a species-specific cell viability assay for diatoms would be essential for further studies.

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Legends

Fig. 1. Location of sampling sites (A4-A21 stations) along the monitoring line, *A-line*, in the northwest Pacific Ocean

Fig. 2. Size composition (%) of phytoplankton at each station in (a) September 2003 and (b) May 2005

Fig. 3. Cell abundances of eukaryotic ultraphytoplankton and *Synechococcus* spp. in (a) September 2003 and (b) May 2005. All data are average ± first standard deviation (n = 3). At Stns A4, 9 and 13, since the cell abundances of *Synechococcus* spp. were below the detection limit, the cell viabilities were not estimated.

Fig. 4. Cell viabilities of eukaryotic ultraphytoplankton and *Synechococcus* spp. in (a) September 2003 and (b) May 2005. All data are average ± first standard deviation (n = 3)

Fig. 5. Cell viabilities of total phytoplankton (Chl *a*) and fucoxanthin-containing algae

(mainly diatoms and prymnesiophytes) as estimated by the cell digestion assay coupled with HPLC in May 2005. All data are average ± first standard deviation (n = 5)

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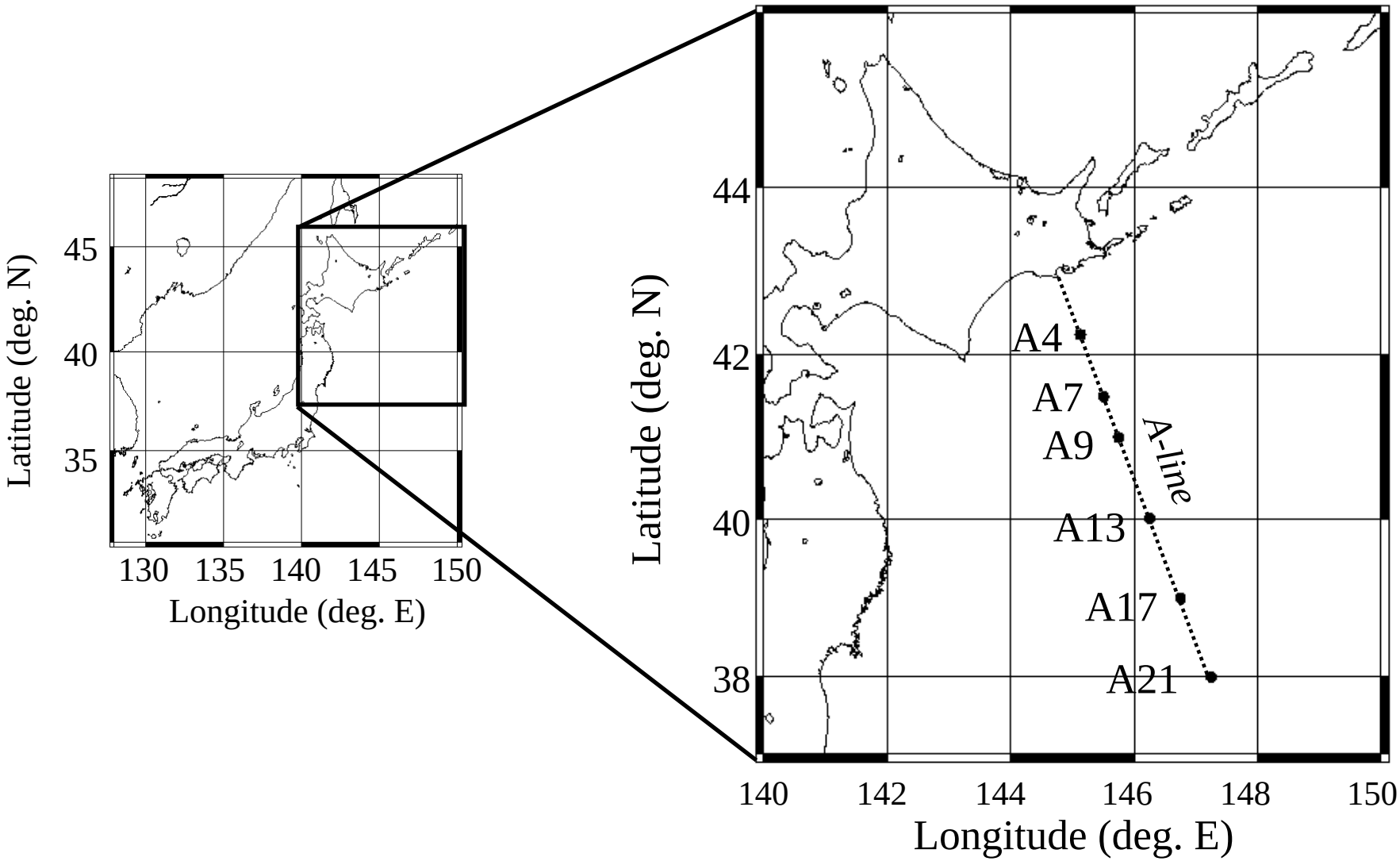
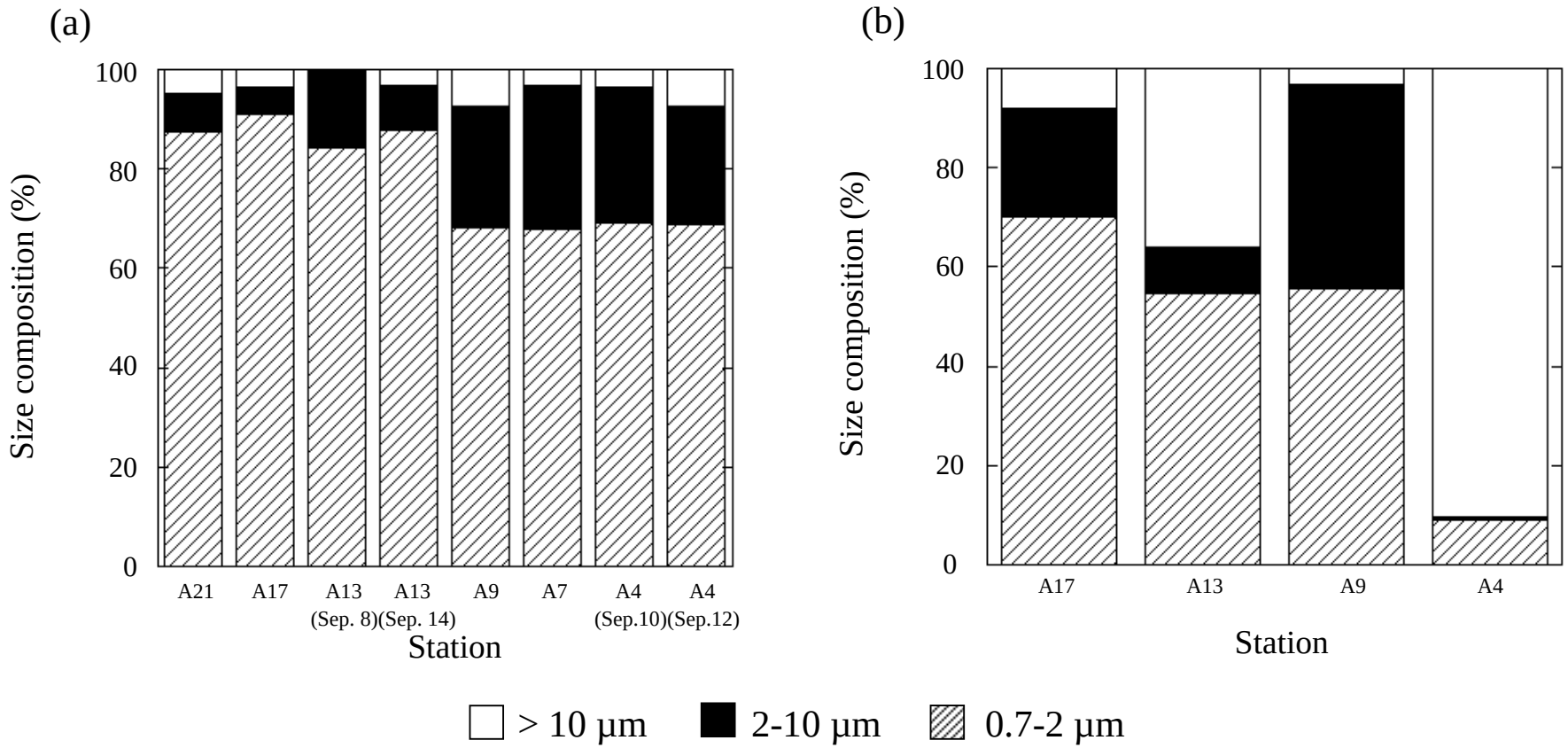


Fig. 1. Hayakawa et al.



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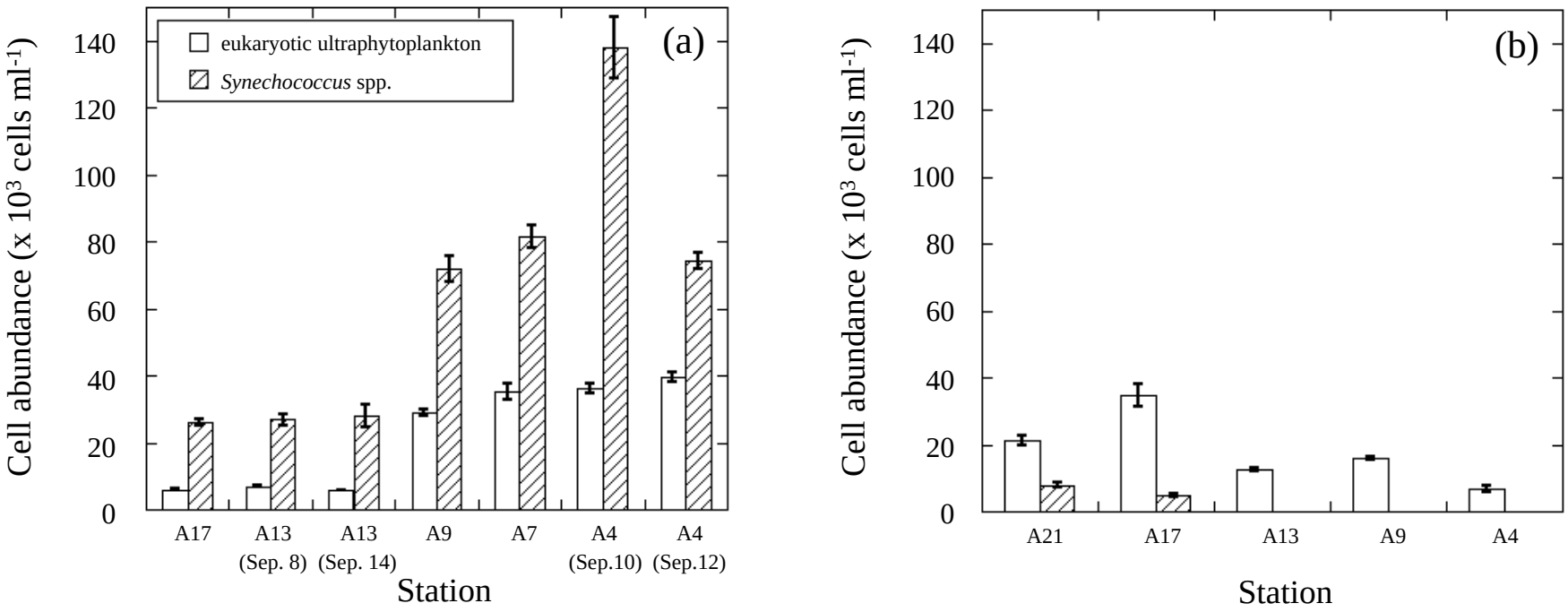

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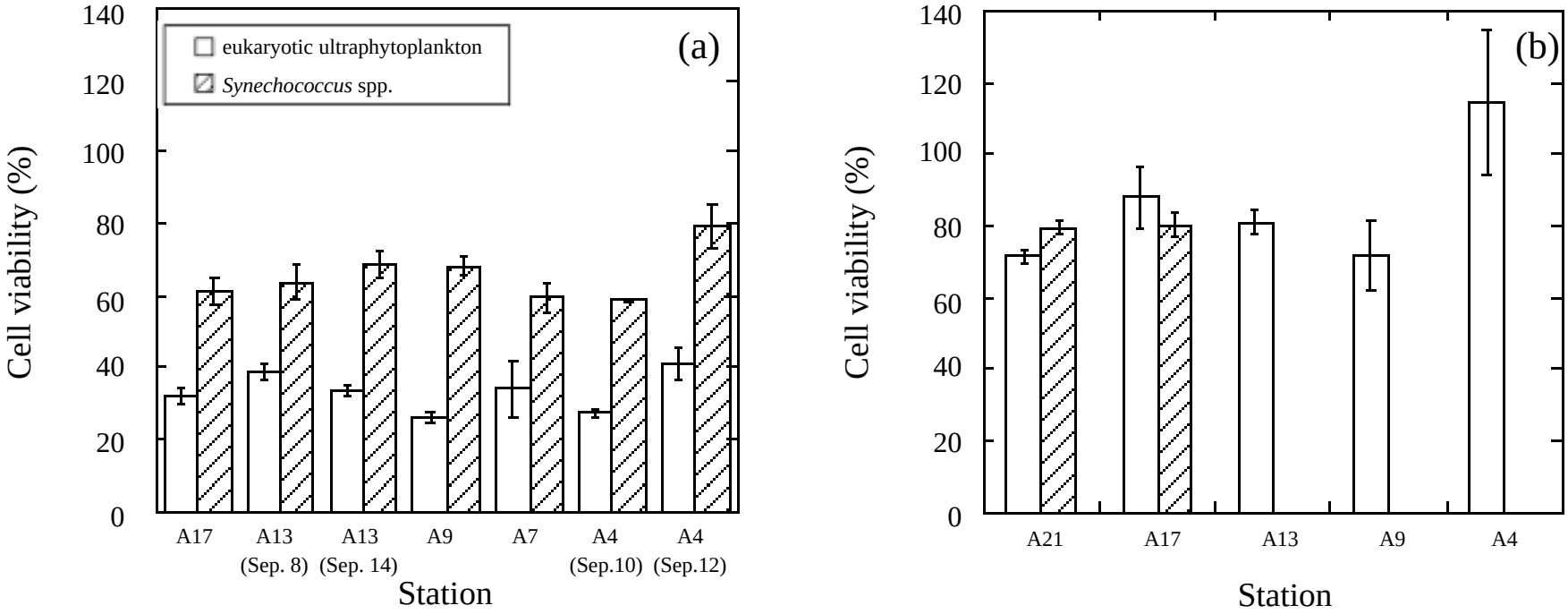

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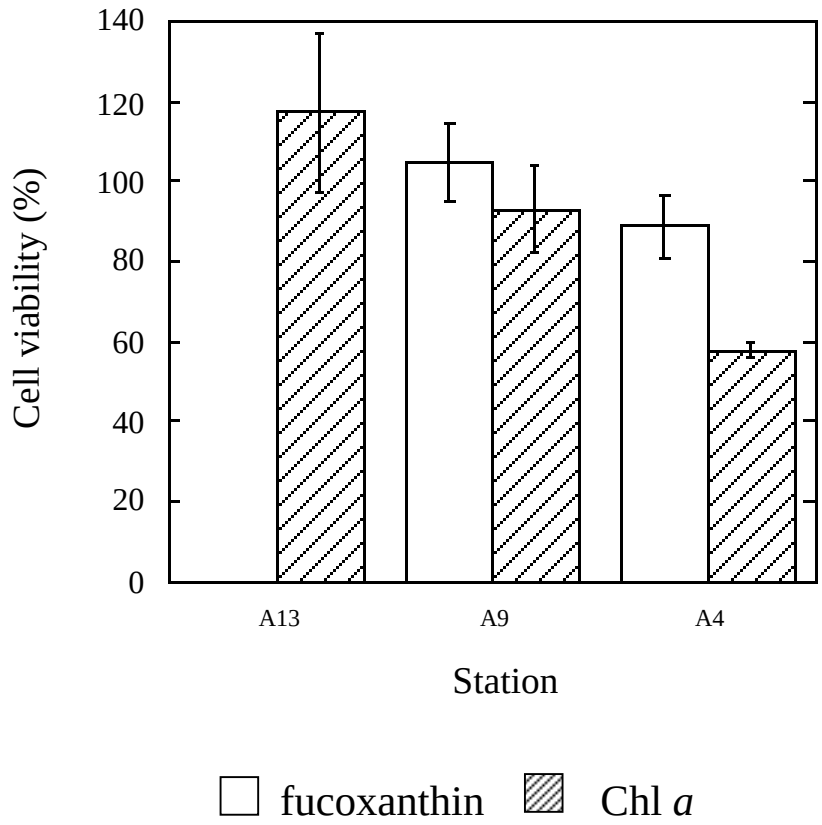

Fig. 5. Hayakawa et al.

Table 1. Temperature and nutrient concentrations in surface waters (5 m in May 2005 or 10 m in Sep. 2003) at each station

	Station	Temperature (°C)	NO_3 (μM)	NO_2 (μM)	PO_4 (μM)	SiO_2 (μM)
Sep. 2003	A4(Sep. 10)	11.7	2.4	0.10	0.49	12
	A4(Sep. 12)	11.7	8.4	0.19	0.96	9.5
	A7	10.9	7.3	0.20	0.82	8.6
	A9	11.8	6.2	0.19	0.83	3.9
	A13(Sep. 8)	20.6	0.040	0.060	0.050	2.1
	A13(Sep. 14)	22.3	0.30	0.10	0.046	1.2
	A17	22.5	0.44	0.020	0.065	1.3
	A21	24.1	0.010	0.070	0.033	1.4
May 2005	A4	1.9	14	0.18	1.4	28
	A9	8.5	6.0	0.12	0.73	6.2
	A13	7.7	6.3	0.27	0.70	6.8
	A17	15.6	1.8	0.12	0.20	3.6
	A21	17.0	2.1	0.34	0.22	3.8

Table 2. Major phytoplankton pigment concentrations (μg l^{-1}) at each station.

	Station	Fucox	19'-But	19'-Hex	Peri	Diadinox	Allox	Violax	Prasionx	Chl *b*	Zeax	Dv Chl *a*	Chl *a*
Sep. 2003	A4(Sep. 10)	0.22	0.02	0.12	0.04	0.06	0.07	0.02	0.02	0.08	0.12	ND	1.00
	A4(Sep. 12)	0.17	0.07	0.30	0.05	0.09	0.02	0.01	ND	0.10	0.06	ND	0.76
	A7	0.13	0.10	0.35	0.03	0.07	0.05	0.01	0.01	0.10	0.08	0.02	0.98
	A9	0.13	0.09	0.37	0.05	0.13	0.03	0.01	0.02	0.08	0.06	ND	0.92
	A13(Sep. 8)	0.02	0.02	0.06	0.01	0.02	ND	0.02	ND	0.02	0.07	0.06	0.26
	A13(Sep. 14)	0.01	0.01	0.02	0.01	0.01	ND	ND	ND	0.02	0.07	0.09	0.14
	A17	0.01	0.02	0.05	0.01	0.01	0.01	0.01	ND	0.03	0.08	0.17	0.21
	A21	0.01	0.01	0.02	ND	0.01	ND	ND	ND	ND	0.06	0.06	0.10
May 2005	A4	2.26	0.01	ND	0.03	0.34	0.01	0.01	0.01	0.07	ND	NA	3.86
	A9	0.09	0.03	0.05	ND	0.05	0.23	0.01	0.01	0.04	ND	NA	0.69
	A13	0.37	0.01	0.04	0.02	0.06	0.06	0.02	0.01	0.10	ND	NA	1.00
	A17	0.13	0.04	0.11	0.03	0.05	0.04	0.05	0.03	0.16	ND	NA	0.78
	A21	0.12	0.02	0.07	0.04	0.02	0.03	0.02	0.02	0.10	ND	NA	0.71

Abbreviations: Fucox, Fucoxanthin; 19'-But, 19'-Butanoyloxyfucoxanthin; 19'-Hex, 19'-Hexanoyloxyfucoxanthin; Peri, Peridinin; Diadinox, Diadinoxanthin; Allox, Alloxanthin; Violax, Violaxanthin; Prasinox, Prasionxanthin; Chl *b*, Chlorophyll *b*; Zeax, Zeaxanthin; Dv Chl *a*, Divinyl Chl *a*; Chl *a*, Chlorophyll *a*; ND, not detected; NA, not analyzed.

Table 3. Specific lysis rates of phytoplankton community (μ_L) and grazing rates of microzooplankton (g)

Year	Date	Station	g(d^{-1})	μ_L(d^{-1})	Reference
1997	Jul. 2	A11	0.09	-	Shinada et al. (2000)
	Jul. 3	A6	0.10	-	Shinada et al. (2000)
	Jul. 5	A3	0.25	-	Shinada et al. (2000)
	Oct. 17	A3	0.31	-	Shinada et al. (2000)
2003	Sep. 5	A21	-	0.12	This study
	Sep. 7	A13	-	0.67	This study
	Sep. 9	A9	-	0.40	This study
	Sep. 10	A4	-	0.43	This study
	Sep. 12	A4	-	0.20	This study