Phylogotype-Specific Productivity of Marine Bacterial Populations in Eutrophic Seawater, as Revealed by Bromodeoxyuridine Immunocytochemistry Combined with Fluorescence in situ Hybridization

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Among the fundamental questions in marine microbial ecology are which taxa or phylogenetic groups account for total bacterial productivity and what is the relative contribution of each. We combined bromodeoxyuridine (BrdU) immunocytochemistry and fluorescence in situ hybridization (BIC-FISH) to examine phylogotype-specific contributions to total bacterial productivity in eutrophic seawater. We also examined year-round changes in phylogotype-specific contributions and explored the factors controlling these changes. Monitoring by BIC-FISH throughout the year revealed the importance of the Roseobacter/Rhodobacter group as a constantly proliferating basic population (27% of all BrdU-positive cells), although their contribution was not significantly correlated with water temperature or with chlorophyll a or organic matter concentration. The Bacteroidetes were another important group, as they greatly increased in abundance after the end of phytoplankton blooms. Two other phylotypes tested, the SAR86 and Vibrio groups, changed their contributions to bacterial productivity with changes in water temperature. To our knowledge, this study was the first to estimate the yearly contribution of major subgroups of marine bacteria to total bacterial productivity in a seawater environment.

Key words: marine bacteria, bromodeoxyuridine, fluorescence in situ hybridization

Bacterial biomass and productivity measurements are fundamental to our understanding of the flux of organic materials and energy in aquatic ecosystems (32). During the last three decades, bacterial productivity has been routinely measured by the incorporation of radiolabeled substrates such as tritiated-thymidine and leucine, and its extent and variability in time and space have been described in various environments (7, 10, 19). Also, the application of culture-independent approaches to microbial communities in the ocean has been described in regard to phylogenetically diverse microbial taxa and their spatiotemporal variability. At least 18 major microbial clades have been found in aquatic bacterial assemblages (11). Moreover, a metagenomic analysis of seawater in the Sargasso Sea revealed that 145 new phylotypes inhabited the sampling sites (38). Because of variations in bacterial function and substrate availability, bacterial activity is not homogeneously distributed within assemblages (8, 33). The questions “Which taxa or phylogenetic groups account for total productivity?” and “What is the relative contribution of each?” are important to our understanding of the variability of microbial activity in response to environmental factors in biogeochemical cycles. Because of methodological difficulties, it is a challenge to quantify the phylotype-specific productivity of natural assemblages of marine bacteria; therefore, little work has been done so far in this field.

Microautoradiography in combination with fluorescence in situ hybridization ‘microautoradiography-fluorescence in situ hybridization’ (MAR-FISH) with RNA-targeted oligonucleotide FISH probes (8, 20, 27) can be used to measure the uptake of radiolabeled substrates in single prokaryotic cells, differentiating their phylotypes. In this way, when thymidine (TdR) and leucine are used as substrates, one can estimate the relative contribution of each phylotype to total prokaryotic growth or productivity. Use of the MAR technique has revealed that consumption of organic matter compounds varies at the single-cell level (10). Use of the MAR-FISH technique has revealed that each phylotype of marine bacteria appears to differ in the uptake of high- and low-molecular weight compounds (8). Although the method is useful for addressing the questions above, samples require long processing times (days or weeks to detect single-cell radioactivity). Moreover, there are restrictions limiting the use of radioisotopes in the field.

In this study, we used immunocytochemistry of bromodeoxyuridine (BrdU), a halogenated nucleoside that can serve as a TdR analog, in combination with FISH, ‘BrdU immunocytochemistry-fluorescence in situ hybridization’ (BIC-FISH). This technique has been developed to overcome the above limitations of MAR-FISH in estimating phylotype-specific productivity (28). BrdU incorporation and antibody detection techniques have been used to identify and monitor de novo DNA-synthesizing bacteria (14, 28, 34, 36, 37). The single-cell-based technique has been applied to bacteria in aquatic samples and has detected de novo DNA-synthesizing bacteria. The single-cell BrdU method can immunocytochemically detect cells incorporating BrdU (presumably actively growing cells) by using anti-BrdU antibody conjugated with fluorochrome molecules or enzymatic reporter molecules. The reasonable sample processing time of BIC-
FISH and the fact that radioisotopes do not need to be handled would give BIC-FISH advantages over MAR-FISH.

Our aim was to determine the relative contributions of major bacterial phylotypes to total productivity and to determine which environmental factors controlled the dynamics of these contributions. To our knowledge, this is the first report describing year-round changes in the phylotype-specific abundance of actively growing cells, which we presume indicates the productivity of each phylogenetic population. Our data can provide valuable insights into identification and monitoring of the key bacterial groups that account for the majority of carbon flows through the microbial loop in ecosystems.

Materials and Methods

Sampling site and sample preparation

From August 2005 to July 2006, seawater samples were collected once a month from a depth of 5 m at the Kure sampling station (34°14'N, 132°33'E) in the Inland Sea of Japan. Seawater was collected in 2-L dark bottles. The seawater was incubated in the bottles with 1 μM BrdU for 3 h at the in situ temperature, subsampled in 50-mL bottles, and fixed with 2% (w/v) paraformaldehyde for 1 d at 4°C. An in situ seawater sample prior to incubation with BrdU was used as a negative control for the BrdU analysis. Fixed samples were filtered onto poly-l-lysine (PLL)-coated 0.2-μm-pore-size polycarbonate membrane filters (type GTTP; 25 mm diameter; Millipore, Billerica, MA, USA), washed twice with particle-free distilled water, and then stored at −80°C until further processing. The PLL-coated filter was prepared as described by Maruyama & Sunamura (23).

Environmental factors

Seawater temperature was measured using a mercury thermometer. To determine the chlorophyll a (Chl. a) concentration, duplicate seawater samples were filtered (<27 kPa vacuum) through Whatman GF/F filters (Whatman, Maidstone, UK) and extracted in the dark with N,N-dimethyformamide at 4°C for 24 h (35). The Chl. a concentration was determined fluorometrically (16). Particulate organic matter collected on precombusted (450°C, 5 h) Whatman GF/F filters was examined with a CHN analyzer (Fisons NA-1500, Fisons Instruments, Manchester, UK) to determine the particulate organic carbon (POC) content and particulate organic nitrogen (PON) content.

Bacterial abundance

For the enumeration of total bacterial abundance, 1 to 5 mL of fixed sample was stained with 4',6'-diamidino-2-phenylindole (DAPI) (final concentration, 2 μg mL−1) in the dark, and then filtered through 0.2-μm-pore-size polycarbonate black membrane filters (25-mm type GTBP; Millipore) in a vacuum of ≤27 kPa. The filters were mounted on glass slides in immersion oil. Cells in a whole grid for 10 randomly chosen fields were counted under an Olympus BX-51 epifluorescence microscope (Olympus Optical, Tokyo, Japan).

BrdU immunocytochemistry-fluorescence in situ hybridization (BIC-FISH)

Anti-BrdU monoclonal antibodies, nucleases and incubation buffer were obtained as components of the BrdU Labeling and Detection Kit III (product no. 1444611; Roche Diagnostics, Mannheim, Germany).

Seawater samples (5 to 10 mL) fixed with 2% (w/v) paraformaldehyde were filtered through the PLL-coated membrane filter to collect bacterial cells. The membrane filters were cut into small pieces (up to 12) and then dehydrated by serial treatment with 70%, 90%, and 100% (v/v) ethanol, each for 3 min. To quench the endogenous peroxidase in the samples, the filters were treated with 3% H2O2 in PBS for 10 min at room temperature and then washed with 1 mL of PBS (145 mM NaCl, 1.4 mM NaH2PO4, and 8 mM Na2HPO4 [pH 7.6]) for 10 min. They were treated with 0.1% N HCl for 5 min at room temperature, replaced with pepsin (P6687; Sigma-Aldrich, St. Louis, MO, USA; 0.5 mg mL−1 in 0.01 N HCl) for 2 h at 37°C, washed with 1 mL of PBS for 10 min, treated with lysozyme (L7651; Sigma-Aldrich; 10 mg mL−1 in Tris-EDTA buffer) for 15 min at room temperature, and washed with 1 mL of PBS for 10 min. Intracellular DNA was digested by treating with nucleases (diluted 1:100 v/v in incubation buffer) for 2 h at 37°C and washed with 1 mL of PBS for 10 min.

The filters were placed with the cell-adherent side in contact with a 30 μL drop of hybridization solution (0.9 M NaCl, 20 mM Tris-HCl [pH 7.2], 5 mM EDTA, 0.01% (w/v) sodium dodecyl sulfate, and 30% (v/v) formamide [for the probes Eub338, Non338, Alf968, Bet42a, Gam42a, GRb, GV, and SAR86-1249] or 35% (v/v) formamide [for CF319a]) containing 2.5 ng μL−1 of biotin-labeled probes on a glass slide. The probes used are listed in Table 1. To distinguish single nucleotide differences between the Bet42a and Gam42a probes, the unlabeled oligonucleotide Gam42a or Bet42a was mixed into the hybridization solutions as a competitor probe (21). After hybridization for 180 min at 42°C, the filters were washed with 1 mL of wash buffer for 15 min at 48°C. The FISH signal was amplified by tyramide signal amplification (TSA). The filters were incubated for 30 min at room temperature with HRP-

Table 1. Oligonucleotide probes used in this study

<table>
<thead>
<tr>
<th>Probe name</th>
<th>Specificity</th>
<th>Probe sequence (5'-3')</th>
<th>Target site*</th>
<th>Size of target group</th>
<th>Number of probe hits in target group</th>
<th>Group coverage (%)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eub338</td>
<td>Bacteria</td>
<td>GCTGCTCCCGTGAGGAGT</td>
<td>16S (338–355)</td>
<td>589,064**</td>
<td>535,701**</td>
<td>91</td>
<td>(5)</td>
</tr>
<tr>
<td>Non338</td>
<td>Negative control</td>
<td>ACTCTACGGGACGACG</td>
<td>16S (338–355)</td>
<td>578,217**</td>
<td>58**</td>
<td>&lt;1</td>
<td>(39)</td>
</tr>
<tr>
<td>Alf968</td>
<td>Alphaproteobacteria</td>
<td>GGTAAAGGTTTGGGCGGAT</td>
<td>16S (968–986)</td>
<td>43,366**</td>
<td>31,953**</td>
<td>81</td>
<td>(22)</td>
</tr>
<tr>
<td>Bet42a</td>
<td>Betaproteobacteria</td>
<td>GCTTCCCATCGTGGT</td>
<td>23S (1027–1043)</td>
<td>224**</td>
<td>193**</td>
<td>86***</td>
<td>(21)</td>
</tr>
<tr>
<td>Gam42a</td>
<td>Gammaproteobacteria</td>
<td>GCTTCCCCACATCGTGT</td>
<td>23S (1027–1043)</td>
<td>993**</td>
<td>750**</td>
<td>76***</td>
<td>(21)</td>
</tr>
<tr>
<td>CF319a</td>
<td>Bacteroidetes group</td>
<td>TGGTCTGTTTGTCCAGTAC</td>
<td>16S (319–336)</td>
<td>65,848**</td>
<td>27,718**</td>
<td>42</td>
<td>(22)</td>
</tr>
<tr>
<td>GRb</td>
<td>Roseobacter/Rhodobacter group</td>
<td>GTCATGATGAGCCGAGTGAGG</td>
<td>16S (626–645)</td>
<td>10,531**</td>
<td>7,358**</td>
<td>70</td>
<td>(12)</td>
</tr>
<tr>
<td>GV</td>
<td>Vibrio group</td>
<td>AGGCCACAAACCTCAAGTAC</td>
<td>16S (841–822)</td>
<td>4,484**</td>
<td>2,982**</td>
<td>67</td>
<td>(12)</td>
</tr>
<tr>
<td>SAR86-1249</td>
<td>SAR86 group</td>
<td>GGCTTACGGTCGTCTG</td>
<td>16S (1249–1265)</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>(9)</td>
</tr>
</tbody>
</table>

* Escherichia coli 16S or 23S rRNA numbering.
** The number of sequences from the database site probe Base (http://www.microbial-ecology.net/probebase/).
*** From Amann and Fuchs (4).
ND means no data.
labeled streptavidin diluted 1:50 (v/v) in TNB buffer (100 mM Tris HCl [pH 7.5], 150 mM NaCl, and 0.5% [w/v] blocking reagent from a TSA-Indirect kit [Perkin Elmer Life Sciences; Boston, MA, USA]) and washed with 1 mL of TNT buffer (100 mM Tris HCl [pH 7.5], 150 mM NaCl, and 0.05% [v/v] Tween 20) for 10 min. After the filters had been washed with TNT buffer, they were treated with a biotin-labeled tyramide (Perkin Elmer Life Sciences) for 10 min at room temperature and then a Texas Red-labeled streptavidin (Perkin Elmer Life Sciences) in TNB buffer (1:500, v/v) for 30 min at room temperature and then washed with 1 mL of TNT buffer for 10 min. To bleach peroxidase from the samples, the filters were treated with 0.1 M HCl for 10 min at room temperature, and washed with 1 mL of PBS for 10 min.

After the above step for FISH, anti-BrdU monoclonal antibodies conjugated with peroxidase were diluted in TNB buffer (1:50, v/v) and applied to the samples for 120 min at 37°C; the samples were then washed with 1 mL of PBS. The antibody signal was amplified by incubating the filters for 10 min at room temperature with a biotin-labeled tyramide diluted 1:50 (v/v) in amplification buffer. After the filters had been washed with 1 mL of TNT buffer for 10 min, they were treated with fluorescein isothiocyanate (FITC)-labeled streptavidin (Perkin Elmer Life Sciences) in TNB buffer (1:500, v/v) for 30 min at room temperature and washed again with TNT buffer. The samples were counterstained with DAPI (1 μg mL⁻¹) for 5 min and washed with PBS.

The filters were mounted on glass slides with anti-fading solution (1:4 [v/v] mixture of Vectashield [Vector Labs, Burlingame, CA, USA] and Citifluor [Citifluor, Canterbury, UK]) and cover glasses. The slides were examined under an Olympus BX-51 epifluorescence microscope equipped with a CoolSNAP CCD camera (Roper Scientific, Tucson, AZ, USA).

Image analysis

Epifluorescence microscopic images were stored as TIFF files, and analyzed with the image analysis software Image Pro-Plus 6.0 (Media Cybernetics, Silver Spring, MD, USA). For examining BrdU-positive cells or for FISH, images were thresholded at a gray value at which no detection occurred in the negative controls. Negative control images were obtained from non-labeled samples in the case of BrdU signals, and using the Non338 probe in the case of FISH signals. Noise particles were removed by using the constriction and dilatation tools. Three edited images were combined the Boolean and Operation, and the objects on the resulting image were counted using the counting tool. More than 1300 DAPI-stained cells in 7 to 10 fields were assessed for each FISH probe in each sample.

Statistical analysis

The significance of monthly changes in the number and relative abundance of BrdU-positive cells was tested by using ANOVA and Tukey’s post hoc test. Pearson’s correlation analysis was used to determine correlations between environmental factors and BrdU-positive cells.

Results

Year-round changes in environmental factors

Water temperature varied from 11 to 25°C (Fig. 1A). Three peaks in the concentrations of Chl. a, POC, and PON (September, February, and June) due to phytoplankton blooms were observed (no Chl. a data in March) (Fig. 1B and C). The Chl. a concentration peaked at 14, 18, and 8 μg L⁻¹. C:N ratios differed among the three peaks (Fig. 1D). Bacterial abundance (mean±SD) ranged from (7.0±1.4)×10^5 to (2.4±0.3)×10^6 cells mL⁻¹, with an average of 1.5×10^6 cells mL⁻¹ during the annual cycle (Fig. 1E); the abundance approximately halved from summer to winter and increased from winter to summer.

Productivity of Marine Bacterial Phylotypes

Bacterial abundance (mean±SD) ranged from (7.0±1.4)×10^5 to (2.4±0.3)×10^6 cells mL⁻¹, with an average of 1.5×10^6 cells mL⁻¹ during the annual cycle (Fig. 1E); the abundance approximately halved from summer to winter and increased from winter to summer. The abundance of BrdU-positive cells ranged from (1.3±0.5 to 6.3±1.7)×10^5 cells mL⁻¹, with an average of 3.5×10^5 cells mL⁻¹ (Fig. 1F). The percentage of BrdU-positive cells among DAPI-stained cells ranged from 15% to 30%, with an annual average of 22%; changes in the percentage did not coincide with changes in bacterial abundance (Fig. 1G).

The numbers of BrdU-positive cells detected with each FISH probe are shown in Table 2. The Eub338-positive fraction (Bacteria) ranged from (1.1±0.1 to 3.2±0.3)×10^9 cells mL⁻¹, with an average of 2.2×10^9 cells mL⁻¹. This temporal change was statistically significant by ANOVA (p<0.05). The abundance of BrdU-positive cells of the Alphaproteobacteria peaked in August, September, and March (ANOVA, p<0.05) and ranged from (3.2±1.3 to 14±5)×10^5 cells mL⁻¹, with an average of 8.1×10^5 cells mL⁻¹. That of the Betaproteobacteria was constantly low, averaging 1.5×10^5 cells mL⁻¹. The temporal change in the abundance of this group was not statistically significant (ANOVA, p>0.05).

Fig. 1. Year-round changes in (A) temperature, (B) chlorophyll a concentration, (C) particulate organic carbon (POC) and particulate organic nitrogen (PON) concentrations, (D) carbon to nitrogen ratio, (E) bacterial abundance, (F) total number of BrdU-positive cells, and (G) percentage of total BrdU-positive cells, at the Kure sampling station in the Inland Sea of Japan from August 2005 to July 2006.
The abundance of the *Gammaproteobacteria* ranged from (0.6±0.3 to 5.1±2.2)×10^4 cells ml⁻¹ and did not change significantly with time (ANOVA, p>0.05). The abundance of the *Bacteroidetes* group was relatively low, changing most drastically (ranging from 1×10^4 to 10×10^4 cells ml⁻¹) and peaking in October (ANOVA, p=0.001). The *Roseobacter/Rhodobacter* group was a constantly abundant subgroup; its abundance among BrdU-positive-cells ranged from (4.9±1.3 to 13±2)×10^4 cells ml⁻¹, but no significant temporal change was observed (ANOVA, p>0.05). The abundance of the SAR86 group was relatively low throughout the year, although significant temporal change was observed, ranging from (0.4±0.4 to 4.7±2.1)×10^4 cells ml⁻¹ (ANOVA, p<0.001). The abundance of the *Vibrio* group changed temporally, ranging from (0.2±0.5 to 4.2±1.7)×10^4 cells ml⁻¹ (ANOVA, p<0.005).

**Year-round changes in percentage of BrdU-positive cells**

Bacteria detected with the Eub338 probe accounted for 44% to 90% of BrdU-positive cells (mean 68%), but no significant temporal change in this percentage was observed (ANOVA, p>0.05) (Table 3). The *Alphaproteobacteria* were the largest subclass population among the BrdU-positive cells, accounting for 15% to 35% (mean 25%), but their percentage did not change significantly temporally (ANOVA, p>0.05). The *Betaproteobacteria* were a relatively low fraction (mean 4%), with no significant temporal change in percentage (ANOVA, p>0.05). The *Gammaproteobacteria* accounted for 2% to 15% of BrdU-positive cells (mean 8%), but the temporal change in their percentage was not significant (ANOVA, p>0.05). The relative contribution of the *Bacteroidetes* group was mostly less than 10% but peaked in October at 35% of BrdU-positive cells; the temporal change in their percentage was significant (ANOVA, p<0.001). The *Roseobacter/Rhodobacter* group, a major cluster of *Alphaproteobacteria* in coastal seawater environments (5), accounted for 16% to 38% of BrdU-positive cells (mean 27%). The SAR86 group accounted for 2% to 19% of BrdU-positive cells and peaked in October, comprising 19% of BrdU-positive cells (mean 6%); the percentage changed significantly with time (ANOVA, p<0.001). The *Vibrio* group accounted for 1% to 14% of BrdU-positive cells (mean 4%), and its percentage also peaked in October, changing significantly with time (ANOVA, p<0.001).

**Correlation analysis**

The results of Pearson’s correlation analysis between environmental factors and both the percentage and the abundance of total BrdU-positive cells showed no significant correlation. The abundance of BrdU-positive *Roseobacter/Rhodobacter* cells was correlated with the abundance of all BrdU-positive cells (r=0.68; p<0.05; n=12) (Table 4). However, the percentage of these cells was negatively correlated with the percentage and abundance of all BrdU-positive cells (r=−0.60, p<0.05, n=12; and r=−0.80, p<0.01, n=12, respectively) (Table 5). The percentages of the SAR86 and *Vibrio* groups among BrdU-positive cells were correlated with water temperature (r=0.59, p<0.05, n=12; and r=0.59, p<0.05, n=12, respectively) (Table 5). Also, the abundance of BrdU-positive cells was correlated with water temperature (r=0.71, p<0.05, n=12; and r=0.66, p<0.05, n=12, respectively) (Table 5).
Productivity of Marine Bacterial Phylotypes

The abundances of BrdU-positive Alpha-, Beta-, and Gammaproteobacteria were correlated with the abundance of all BrdU-positive cells ($r=0.74$, $p<0.01$, $n=12$; $r=0.71$, $p<0.01$, $n=12$; and $r=0.63$, $p<0.05$, $n=12$) (Table 4).

Discussion

Because bacterial production is determined by bacterial abundance and growth rates, the number of BrdU-positive cells (presumably actively growing cells) should be a good indicator of bacterial productivity. In our preliminary study, the number of BrdU-positive cells was positively correlated with bulk BrdU incorporation in the Pacific Ocean (unpublished data). In the present study, we successfully applied the BIC-FISH method to year-round monitoring of the percentage contributions of major bacterial phylogenetic groups to the total number of BrdU-positive cells. To our knowledge, this is the first report of the estimated yearly contributions of major subgroups of marine bacteria to total bacterial productivity in a seawater environment. The results suggest the relative importance of each subgroup to total bacterial productivity in a eutrophic seawater environment. They also clearly show the temporal variability of phylotype-specific productivity.

The Alphaproteobacteria were always the largest component of BrdU-positive cells. In particular, Roseobacter/Rhodobacter-group bacteria were the most abundant, except in October, accounting for 27% of all BrdU-positive cells on average (Table 3), and also their abundance of BrdU-positive cells was positively correlated with the abundance of all BrdU-positive cells (Table 4); these indicate the importance of this group of bacteria as a major part of total bacterial productivity throughout the year. The relative contributions of Roseobacter/Rhodobacter and Bacteroidetes, two major subgroups of marine bacteria, tended to decrease with increasing total bacterial productivity (Fig. 3, Tables 4 and 5). These results suggested that the temporal change in total bacterial productivity was controlled not only by changes in the abundance of the major subgroups of bacteria detected here, but also by changes in the abundance of other bacteria that were usually minor components but grew rapidly under some environmental conditions.

Roseobacter/Rhodobacter-group bacteria are reportedly

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Table 5. $r$ values in the Pearson's correlation analysis of the relationships between environmental factors and the percentage of BrdU-positive cells of major marine bacterial taxa

<table>
<thead>
<tr>
<th></th>
<th>Alf968</th>
<th>Bet42a</th>
<th>Gam42a</th>
<th>CF319a</th>
<th>G Rb</th>
<th>SAR86-1249</th>
<th>G V</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temperature</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.18</td>
<td>0.26</td>
<td>0.17</td>
<td>0.35</td>
<td>−0.10</td>
<td>0.59</td>
<td>0.59*</td>
</tr>
<tr>
<td>Chl. a ($n=11$)</td>
<td>0.56</td>
<td>−0.09</td>
<td>0.21</td>
<td>−0.04</td>
<td>0.39</td>
<td>−0.12</td>
<td>−0.01</td>
</tr>
<tr>
<td>POC ($n=12$)</td>
<td>0.28</td>
<td>−0.12</td>
<td>0.21</td>
<td>−0.04</td>
<td>0.39</td>
<td>−0.12</td>
<td>−0.01</td>
</tr>
<tr>
<td>BrdU-positive cells (cells)</td>
<td>−0.45</td>
<td>0.29</td>
<td>0.01</td>
<td>−0.23</td>
<td>−0.80**</td>
<td>−0.02</td>
<td>0.09</td>
</tr>
<tr>
<td>BrdU-positive cells (%)</td>
<td>−0.47</td>
<td>−0.21</td>
<td>−0.43</td>
<td>−0.39</td>
<td>−0.60*</td>
<td>−0.22</td>
<td>−0.21</td>
</tr>
</tbody>
</table>

Single asterisks (*) show $p<0.05$ and double asterisks, (**) $p<0.01$. (Table 4). The abundances of BrdU-positive Alpha-, Beta-, and Gammaproteobacteria were correlated with the abundance of all BrdU-positive cells ($r=0.74$, $p<0.01$, $n=12$; $r=0.71$, $p<0.01$, $n=12$; and $r=0.63$, $p<0.05$, $n=12$) (Table 4).
abundant in bacterial communities associated with phytoplankton (2, 13, 30, 40). A whole-genomic analysis of *Silicibacter pomeroyi*, a representative strain, has revealed the various functions of this bacterium in using phytoplankton-derived organic substrates, e.g. by dimethylsulfoxonio-propanionate assimilation (24). However, in the present study it was not clear whether the growth of the Roseobacter/Rhodobacter group was controlled by the supply of phytoplankton-derived organic matter. The temporal abundance and percentage of BrdU-positive cells of the Roseobacter/Rhodobacter group were stable, with the lowest coefficients of variation (CV=27% and 26%, respectively) (Tables 2 and 3). The abundance and relative contribution of this group were not correlated with the Chl. a concentration or other environmental factors such as water temperature and POC (Tables 4 and 5). We speculate that the Roseobacter/Rhodobacter-group bacteria are ‘generalist’ species that can always maintain their growth because of their versatility in the use of organic matter, but that they cannot grow as fast as some of the ‘specialist’ species under specific conditions.

Because Roseobacter/Rhodobacter-group bacteria dominated the alphaproteobacterial populations, we did not use the FISH probe targeting the SAR11 group, which belongs to the Alphaproteobacteria and is one of the most abundant groups in marine environments (25). In our previous study using BrdU incorporation in combination with PCR-denaturing gradient gel electrophoresis (DGGE), DGGE bands representing SAR11-group bacteria were not often detected in BrdU-incorporating (actively growing) bacterial communities in the Inland Sea of Japan, an area adjacent to our study site (15). Also, a MAR-FISH study had revealed that the percentage of SAR11 cells taking up glucose was always low, whereas their abundance was high (3). These findings reasonably suggested that the contribution of the SAR11 group to total bacterial productivity was always low throughout the year at our neritic study site.

Correlation analysis suggested that water temperature was a more effective environmental factor than supply of organic matter in controlling the growth of both SAR86- and Vibrio-group bacteria and their contributions to total bacterial productivity. The SAR86 group, belonging to the Gammaproteobacteria, is one of the major subgroups of bacteria, predominantly found in surface seawater but not yet cultured (1, 9, 26, 29). This group has also shown rapid growth during summer at the surface of the North Sea (29). Vibrio species are frequently found as an actively growing phylotype in coastal seawaters (15). We found that they were a relatively minor part of the BrdU-positive fraction (average: 4% of BrdU-positive cells) but were capable of being temporarily active and contributed significantly to total bacterial productivity (up to 14% of BrdU-positive cells).

The abundance of the Bacteroidetes group and their percentage among BrdU-positive cells increased dramatically in October, overwhelming the abundance of Roseobacter/Rhodobacter-group bacteria. We also observed a peak in the abundance of BrdU-positive Bacteroidetes cells in March after the phytoplankton bloom in February, although the peak was not as large as that detected in October, probably because of low water temperatures or a high C:N ratio of organic matter (Fig. 1). The occurrence of the peak 1 month after the phytoplankton bloom could be explained by the preferential growth of these bacteria on high-molecular-weight residual organic matter derived from phytoplankton cellular components. Many of the Bacteroidetes-group bacteria have the capacity to degrade complex carbohydrates such as pectin, cellulose, and chitin (8, 18, 31). One MAR-FISH study has revealed that many cells of the natural Bacteroidetes population have the capacity to use dissolved high-molecular-weight organic matter (8). Members of this group have been detected in degrading mucopolysaccharides from *Phaeocystis* species (17).

In conclusion, monthly monitoring of actively growing fractions of major subgroups of marine bacteria by BIC-FISH revealed the importance of the Roseobacter/Rhodobacter group as a constantly proliferating basic bacterial population in a neritic seawater community. The bacteria of this group did not show any great response to spontaneous increases in organic matter content or changes in water temperature, despite changes in total productivity. Instead, their growth remained stable, possibly because of their adaptive versatility in the seawater environment. Bacteroidetes bacteria were the second most important group, as they temporally increased their contribution after the end of phytoplankton blooms. Growth of the other two major phylotypes, the SAR86 and Vibrio groups, was controlled by water temperature. Although their average contributions to total bacterial productivity in a year were small, they could temporally increase their contributions under warm conditions. We do not yet know whether the growth response of each group of bacteria is typical in eutrophic seawater. Further comparative work in other coastal waters and in oceanic waters is required. Although the BIC-FISH methodology was sensitive enough to be used here and promises to be useful in addressing various ecological questions regarding bacterial growth responses to environmental variables, further development and an increase in sensitivity are required to assess the bacterial populations inhabiting less productive oceanic waters.

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