

## Diurnal Variation of Cell Proliferation in Three Bacterial Taxa from Coastal North Sea Waters

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**Pulse-labeling with bromodeoxyuridine (BrdU) in combination with fluorescence in situ hybridization was applied to quantify the percentage of proliferating cells in coastal North Sea waters. In order to assess diurnal variability, we sampled eight or nine times, respectively, within 3 consecutive days at two seasons. Bacteria affiliated with the *Roseobacter*, SAR86, and NOR5 lineages constituted on average  $19\% \pm 3\%$ ,  $8\% \pm 2\%$ , and  $6\% \pm 1\%$  of all cells in May 2002 and  $17\% \pm 3\%$ ,  $10\% \pm 2\%$ , and  $11\% \pm 3\%$  in August. The relative abundances of the three populations either remained stable, or they changed very gradually during the observation periods. On average, 38 and 39% of all *Bacteria* exhibited DNA de novo synthesis in May and August, respectively. The fractions of proliferating cells in bacteria of the SAR86 (May, 59%; August, 72%) and the *Roseobacter* (48 and 53%) lineages were significantly above the community average. A substantial cell proliferation of population NOR5 (34%) was only encountered in August, concomitant with a dinoflagellate bloom. Significant short-term fluctuations of DNA-synthesizing cells were observed in *Roseobacter* during May and in NOR5 during August, hinting at a pronounced (temporal or spatial) mesoscale patchiness of growth rates in these populations. Since the BrdU proliferation assay is susceptible to misinterpretation, we also modeled the expected number of labeled cells at increasing BrdU incubation times in a slowly growing bacterial population. We suggest that the absence of visible DNA synthesis in marine bacterioplankton cells after DNA pulse-labeling must not be interpreted as an indication of cell “inactivity.”**

Circadian rhythms of photosynthesis, of algal growth and gene expression, and of the release of dissolved organic carbon (DOC) are well-studied phenomena in marine waters (19, 26, 41, 42). In contrast, investigations of the variability in the proliferation of chemoorganotrophic microbes at time scales of hours are rare. There are, however, indications that bacteria in marine waters are also affected by short-term variability of growth conditions (10, 16, 22). Fluctuation of bacterial growth over the course of a day might be induced either directly or indirectly, e.g., by corresponding changes of primary production (16) or viral mortality (46), but also by UV radiation-induced DNA damage or by changes in DOC composition (21, 25).

Thus far, such diurnal variability of growth in pelagic marine microbes has been investigated only at the community level, i.e., treating the “heterotrophic picoplankton” as a single conceptual unit. It is unknown whether some populations within heterotrophic microbial assemblages exhibit higher diurnal fluctuations of cell proliferation than others. Some bacterial taxa, such as members of the *Roseobacter* clade, might be ecologically more closely related to blooms of particular phytoplankton species and thus to short-term fluctuations of primary production (31, 35). In addition, even a synchronization of cell division by sunlight is imaginable in populations of marine bacteria that possess genes for light-harvesting pigments, e.g., members of the  $\gamma$ -proteobacterial SAR86 lineage (5, 34).

In order to study the mesoscale variability of proliferation in individual microbial populations, methods are required that combine cell identification with a proxy for cell division related processes. This can be achieved by incubation with radiolabeled thymidine (TdR) and subsequent determination of TdR incorporation into different bacteria by microautoradiography and fluorescence in situ hybridization (FISH) (11). A nonradioactive alternative to this approach makes use of the halogenated thymidine analogue 5-bromo-2'-deoxyuridine (BrdU) (29). For the adequate interpretation of field data from TdR or BrdU pulse-labeling experiments, it is, moreover, necessary to understand particular aspects of the bacterial cell cycle. DNA synthesis in slowly growing bacteria is a discontinuous process (20). Therefore, not all growing cells within a population might incorporate tracers.

The short-term variability of cell proliferation was studied in three bacterial populations from coastal North Sea waters at two seasons with contrasting day lengths, water temperatures and phytoplankton assemblages. Specifically, we tested the hypotheses that (i) allegedly proteorhodopsin-containing (34) bacteria from the SAR86 clade would show diurnal synchronicity of DNA synthesis and that (ii) the fractions of proliferating cells within the *Roseobacter* lineage would differ between two seasons with contrasting phytoplankton densities. A third bacterial group, NOR5, was included in the analysis, since a member of this lineage has been isolated at our lab, and these bacteria may seasonally form large populations in the coastal North Sea (15). In order to correctly interpret the numbers of cells with visible BrdU incorporation, we furthermore modeled the expected fraction of labeled cells in a slowly growing *Escherichia coli* population during balanced growth.

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## MATERIALS AND METHODS

**Sampling site, sample fixation, and storage.** Surface water samples (three 1-liter samples) were taken during 2 diel cycles in May and August 2002. Sampling was carried out eight or nine times at ~4-h intervals within a 3-day period at the sampling site Helgoland Roads (54°09'N 7°52'E) near the island of Helgoland, which is situated 23 miles offshore in the German Bight of the North Sea. Water temperatures during the two sampling periods ranged around 9 and 19°C, respectively, and the salinities were 32.8 and 33.3 ppm. In early May algal biomass was approximately one-fifth of the biomass in August. The spring phytoplankton assemblage was composed of similar biomass fractions of diatoms, flagellates, and chlorophyll-bearing ciliates. A short-lived bloom of the dinoflagellate *Gymnodinium chlorophorum* constituted the majority of phytoplankton biomass during the August sampling (MURSYS report 2003 [www.bsh.de]).

Subsamples (200 ml) were poured into acid-pretreated glass bottles, supplemented with BrdU (Roche Diagnostics, Mannheim, Germany) at 20  $\mu$ M and thymidine (Sigma-Aldrich, Seelze, Germany) at 33 nM and incubated in triplicates for 2 h in the dark at the in situ temperature. After the incubations, 50-ml portions were fixed with particle-free formaldehyde solution (final concentration, 1% [vol/vol]) for 10 to 15 h at 4°C. Controls were immediately fixed after sampling and then supplemented with BrdU and thymidine. Fixed samples were filtered onto white polycarbonate membrane filters (type GTTP; pore size, 0.2  $\mu$ m; size, 47 mm; Millipore, Eschborn, Germany), washed with 10 ml of MilliQ water (Millipore), and stored at -20°C until further processing. Bacterial total abundances were determined by epifluorescence microscopy after staining with DAPI (4',6'-diamidino-2-phenylindole) (33).

**FISH and immunocytochemistry.** The combination of FISH and BrdU staining was essentially carried out as described previously (29) but with significant modifications of the epitope retrieval procedure for BrdU detection (see below).

FISH was performed with horseradish peroxidase (HRP)-labeled probes targeted to *Bacteria* (EUB I-III) (13), *Roseobacter* sp. (ROS537) (15), the SAR86 clade (SAR86-1249) (14), and the NOR5 clade (NOR5-730) (15). All probes were synthesized by Biomers.net (Ulm, Germany). Stringent conditions were achieved by 55% of formamide in the hybridization buffer. For details of the hybridization and washing procedure, see references 27 and 28. For the subsequent signal amplification (catalyzed reporter deposition [CARD]), the probe-delivered HRP was detected with tyramides custom labeled with the fluorescent dye Alexa<sub>488</sub> (Molecular Probes, Eugene, OR) (28). Labeled tyramides were diluted 1:400 (final concentration, 2.5  $\mu$ g ml<sup>-1</sup>) in amplification buffer (1× phosphate-buffered saline [PBS; pH 7.6], 0.1% [wt/vol] Blocking Reagent [Roche Diagnostics], 10% [wt/vol] dextran sulfate, 2 M NaCl, and freshly added 0.0015% [vol/vol] H<sub>2</sub>O<sub>2</sub>) by incubation at 46°C for 15 min. *p*-Iodophenylboronic acid was added to the tyramide solution (20 mg per 1 mg of tyramide, dissolved in dimethyl formamide) to further enhance CARD (8).

After CARD-FISH, the probe-delivered HRP was quenched with 0.01 M HCl for 10 min at room temperature. Filters were then washed with 1× PBS and MilliQ. To optimize the unmasking of BrdU incorporated into de novo-synthesized DNA, various conditions were tested (data not shown). In the optimized protocol, cells were digested with Exonuclease III (50 U ml<sup>-1</sup>) and a DNA restriction enzyme (*HAE* III [Promega, Mannheim, Germany], 10 U ml<sup>-1</sup>) in nuclease buffer (5 mM MgCl<sub>2</sub>, 50 mM Tris-HCl [pH 8]) for 30 min at 37°C in order to obtain single-stranded DNA. Samples were subsequently washed twice with MilliQ water. The wet filter sections were then placed onto Parafilm and covered with 500  $\mu$ l of antibody mixture (anti-BrdU-HRP, Fab fragments, clone BMG-6H8 [Roche] at 0.75 U ml<sup>-1</sup>, 1× PBS, 1% [wt/vol] Blocking Reagent). The samples were then covered with Parafilm, placed in a clean plastic petri dish, sealed with Parafilm, and incubated at 37°C for 2 to 2.5 h. They were then washed twice for 15 min each time in 50 ml of PBS at room temperature. HRP-labeled antibodies were detected by CARD with tyramides that were custom labeled with the fluorescent dye Alexa<sub>546</sub> (Molecular Probes; 1:600 diluted in amplification buffer; final concentration, 1.67  $\mu$ g ml<sup>-1</sup>). Signal amplification was performed at 37°C for 15 min. After being washed with PBS and MilliQ water, filters were dehydrated in absolute ethanol, air dried, and stored at -20°C until microscopic processing (within 3 days).

**Microscopic evaluation.** Filter sections were covered in DAPI-amended mountant, and the preparations were evaluated on an Axioplan II epifluorescence microscope (Carl Zeiss, Jena, Germany) equipped with an HBO103 W Hg vapor lamp, appropriate filter sets for Alexa<sub>546</sub> (Cy3 filter set; Chroma, Brattleborough, CT) and Alexa<sub>488</sub> (fluorescein isothiocyanate filter set Zeiss 09; Carl Zeiss), and DAPI fluorescence (Zeiss 01, Carl Zeiss), and a ×100 Plan Apochromat objective. For the quantification of hybridized cells in the May samples, between 800 and 1,000 DAPI-stained objects per preparation were counted. The relative abundances of FISH-stained cells in August were quantified by auto-

mated counting after cross-calibration (30). The fractions of BrdU incorporating cells of all hybridized cells in double-stained preparations were quantified manually from at least 10 individual microscopic fields.

**Statistical evaluations.** Two hypotheses were explored statistically. (i) The fractions of BrdU-positive *Bacteria* (as detected by probe EUB I-III) and BrdU-positive cells from SAR86, *Roseobacter*, and NOR5 populations were different between the two seasons. (ii) The BrdU-positive *Bacteria*, SAR86, *Roseobacter*, and NOR5 fractions were different between individual sampling time points. The first hypothesis was tested by two-way linear analysis of variance (ANOVA; probe versus season) on the set of mean values per sampling time point and post-hoc pairwise multiple comparisons with Bonferroni corrections. The second hypothesis was tested separately for *Bacteria*, *Roseobacter* SAR86, and NOR5 by Kruskal-Wallis one-way ANOVA (time point) on the rank values of the triplicate determinations and post-hoc comparisons of all time points against selected time points (Bonferroni's method). Statistical calculations were performed with SigmaStat (SPSS, Inc., Chicago, Ill.).

**Modeling of BrdU incorporation into *E. coli* during exponential growth.** Model assumptions were that the genome size of *E. coli* is 4.8 Mb, 25% of the nucleotides are thymine (equally distributed over the genome), and every fourth thymine can be replaced by BrdU (7). At least 1,000 BrdU molecules had to be incorporated for a cell to be scored BrdU positive. All cells grow at equal speed at a generation time of 200 min, and individual cell cycles differ by at least 1 min. The first phase of the cell cycle (cell formation to initiation of DNA replication) was set to 85 min, the S-phase (chromosome replication) was set to 85 min, and the D-phase (formation of septum to division) was set to 30 min (20). DNA replication was assumed to be bifurcal, with only one replication fork per cell. The model represents ideal chemostat conditions, i.e., the total cell numbers remain constant.

## RESULTS

**Contributions of the three populations to the microbial assemblages.** Total bacterial abundances ranged from  $0.4 \pm 0.2$  to  $0.5 \pm 0.1 \times 10^6$  cells ml<sup>-1</sup> in May and  $1.7 \pm 0.2$  to  $2.0 \pm 0.3 \times 10^6$  cells ml<sup>-1</sup> in August (mean  $\pm$  one standard error [ $n = 3$ ]). There were no clear trends of cell increase or decrease during the respective sampling periods. The fraction of cells hybridized with a probe for all bacteria (EUB I-III) ranged from 75 to 80% of all cells in May and from 85 to 89% in August (Table 1). No significant changes in the percentages of *Bacteria* detectable with FISH were observed ( $P > 0.05$ ).

On average, 43 and 48% of all cells that were hybridizing with probe EUBI-III could be identified by three specific probes in May and August, respectively. Cells affiliated with the  $\alpha$ -proteobacterial *Roseobacter* clade formed 19% (range, 17 to 21%) of all DAPI-stained cells during the May sampling period and 17% (range, 13 to 20%) in August (Fig. 1). Microbes from the  $\gamma$ -proteobacterial SAR86 lineage were on average 8% (5 to 10%) of total DAPI counts in May and 10% (7 to 12%) in August (Fig. 2). At both time points the relative abundances of these bacteria significantly declined by ca. 50% during the 3-day observation period. *Bacteria* related to the NOR5 cluster of the  $\gamma$ -proteobacterial OM60 clade constituted 6% (6 to 7%) of the microbial community in May and 11% (5 to 13%) in August (Fig. 3). During the second half of the August sampling period, a pronounced and statistically significant decline was observed of the relative abundances of NOR5 cells.

**Bacteria with visible DNA de novo synthesis.** The optimized protocol for detection of BrdU in single hybridized bacterioplankton cells was substantially more sensitive than a previously published one (29; data not shown). The overall coefficient of variation of the fractions of BrdU-positive cells in 68 triplicate determination was 0.19, i.e., the error of the fraction of BrdU-positive cells within a bacterial population, was <20% of the mean value. The error range was not significantly related

TABLE 1. Fractions of total cells visualized after hybridizations with probe EUB I-III at the two sampling dates and fraction of hybridized *Bacteria* showing incorporation of BrdU after 2 h of incubation<sup>a</sup>

May 2002				August 2002			
Date (mo-day)	Time (h)	Hybridized (% total)	BrdU positive (% hybridized)	Date (mo-day)	Time (h)	Hybridized (% total)	BrdU positive (% hybridized)
5-06	16:00	77 ± 3	41 ± 4	8-21	11:00	85 ± 1	32 ± 2
5-06	23:00	80 ± 4	38 ± 1	8-21	16:00	87 ± 1	39 ± 3
5-07	04:40	79 ± 3	45 ± 2	8-21	23:00	85 ± 1	28 ± 2
5-07	10:00	81 ± 1	31 ± 5	8-22	02:00	89 ± 2	33 ± 2
5-08	10:00	77 ± 2	38 ± 4	8-22	11:00	87 ± 2	28 ± 2
5-08	16:00	78 ± 1	35 ± 1	8-22	15:00	88 ± 1	31 ± 2
5-08	22:00	74 ± 2	37 ± 3	8-22	13:00	86 ± 1	49 ± 8*
5-09	04:00	75 ± 3	37 ± 3	8-23	02:00	88 ± 1	46 ± 6*
				8-23	22:30	86 ± 1	61 ± 4*
Mean		77 ± 1	38 ± 1			87 ± 1	39 ± 2

<sup>a</sup> Values are means of triplicate incubations ± one standard error. Asterisks indicate significant differences with respect to other time points (Kruskal-Wallis ANOVA on ranks, *P* < 0.05).

either to population size (the total fraction of cells hybridized by a particular probe) or to the relative abundance of BrdU-positive cells within a population.

During both sampling periods, ca. 40% of all hybridized *Bacteria* were also BrdU positive (May, 31 to 47%; August, 32 to 61%) (Table 1). Thus, on average 29 and 33% of all DAPI-

stained cells showed DNA de novo synthesis for the two sampling periods. The BrdU labeling indices of *Bacteria* remained fairly constant during the May sampling period. In August the fraction of BrdU-positive hybridized cells almost doubled within the 3-day observation period, i.e., from 32 to 61%. The increase of the labeling index at the last three sampling time points—compared to the previous time points—was statistically significant (*P* < 0.01). No corresponding increase of total FISH detection rates was observed (Table 1).

Altogether, BrdU-positive cells affiliated with *Roseobacter*,

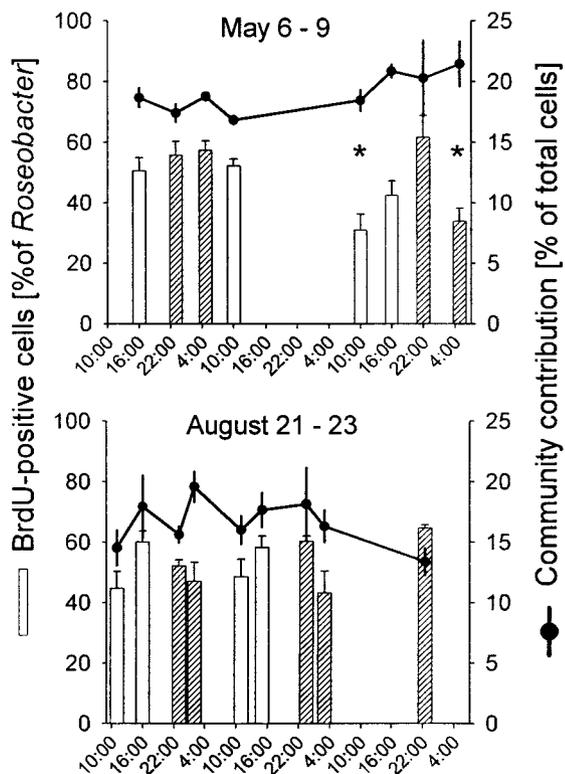


FIG. 1. Relative abundances of bacteria affiliated with the *Roseobacter* clade (symbols) and fractions of *Roseobacter*-related cells with visible DNA de novo synthesis after 2 h of incubation with BrdU (bars) during 3 days of sampling in May and August 2002. Values are means ± one standard error. Bar hatching depicts night hours, and asterisks indicate time points at which the fractions of BrdU-positive cells were significantly different from the other time points (Kruskal-Wallis ANOVA on ranks, *P* < 0.05).

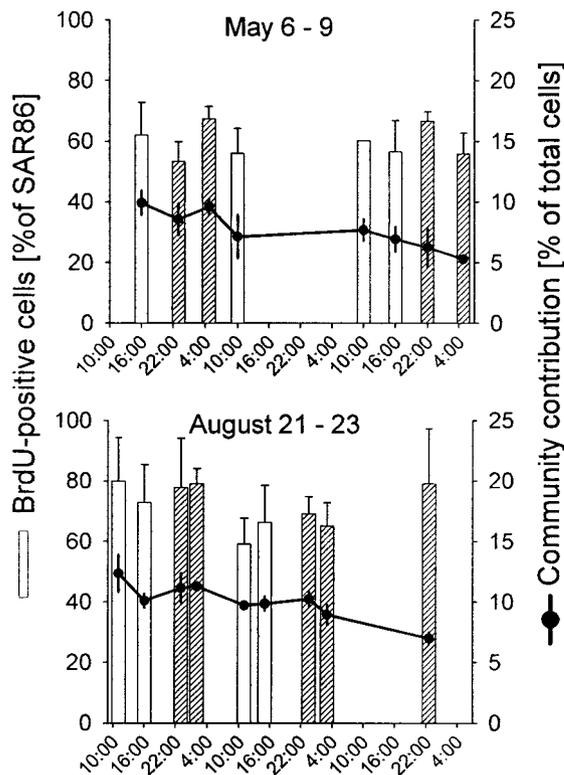


FIG. 2. Relative abundances of bacteria affiliated with the SAR86 clade (symbols) and fraction of SAR86-related cells with visible DNA de novo synthesis after 2 h of incubation with BrdU (bars).

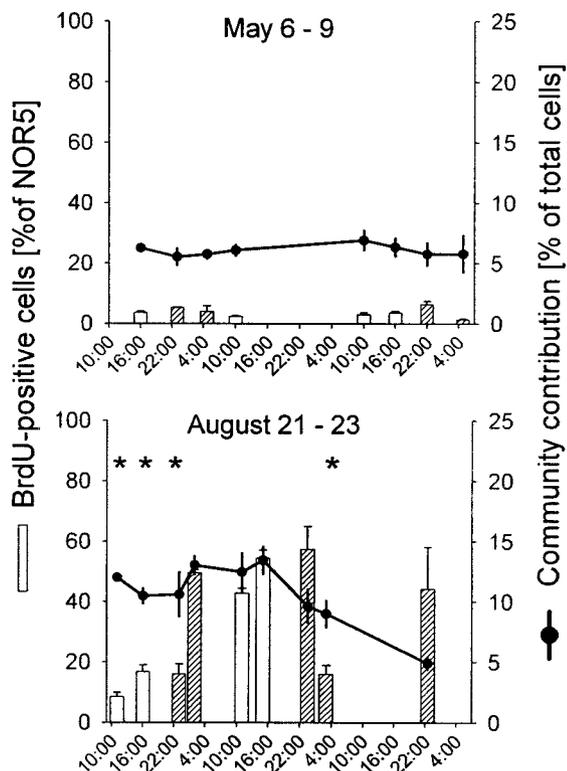


FIG. 3. Relative abundances of bacteria affiliated with the NOR5 lineage of the OM60 clade (symbols) and fraction of NOR5-related cells with visible DNA de novo synthesis after 2 h of incubation with BrdU (bars). Asterisks indicate time points at which the fraction of BrdU-positive cells were significantly different from the other time points.

SAR86, and NOR5 strains accounted for 48 and 67% of all *Bacteria* with visible DNA de novo synthesis in May and August, respectively. In May, on average 30% of all BrdU-positive *Bacteria* were affiliated with *Roseobacter*, 17% with SAR86, and <1% with the NOR5 clade (Fig. 4A). In August, 29% of BrdU-positive *Bacteria* belonged to *Roseobacter* spp., 23% belonged to SAR86, and 14% were members of the NOR5 clade. A distinctly higher variability of this parameter in August than in May was observed for SAR86 and especially for NOR5 (Fig. 4A). The ratio of the fraction of BrdU-positive cells within a specific bacterial group to the average fraction of BrdU-positive *Bacteria* was significantly greater than 1.0 for SAR86 and *Roseobacter* for both sampling periods and less than 1.0 for NOR5 in May (Fig. 4B). It was approximately 1.0 for NOR5 in August.

**Modeled BrdU incorporation into exponentially growing *E. coli*.** The total period of DNA synthesis, and therefore of possible BrdU incorporation, at a generation time of 200 min was 42.5% of the entire cell cycle (Fig. 5A). During this phase, 90.4 BrdU molecules  $\text{min}^{-1}$  would be incorporated, i.e., a cell scored as BrdU positive after 11.1 min of DNA synthesis at the assumed growth rate. The number of BrdU-positive cells matched the actual number of DNA-synthesizing cells after a BrdU incubation period of 21 min (Fig. 5B). After 135 min of BrdU incubation, all growing cells would be labeled. The sudden change of detection above a minimum BrdU incubation

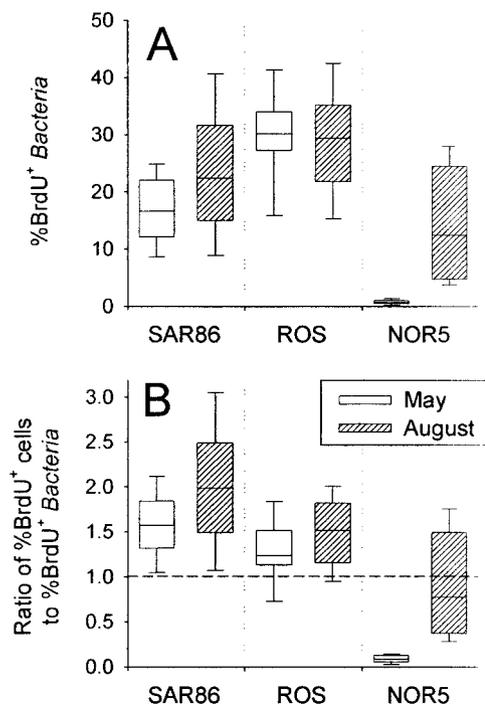


FIG. 4. (A) *Bacteria* with BrdU incorporation from the *Roseobacter* clade (ROS), the SAR86 clade (SAR86), and the NOR5 lineage (NOR5) depicted as fractions of all *Bacteria* with visible DNA de novo synthesis. (B) Ratio of the fraction of BrdU incorporation cells in the three populations to BrdU-incorporating *Bacteria*. Box limits are the 25th and 75th percentiles, whiskers are the 95th percentiles of all datum points from the respective sampling periods.

time reflects the quasi all-or-nothing threshold when CARD signal amplification is used for the detection of BrdU incorporation.

## DISCUSSION

**BrdU incorporation at different seasons.** *Bacteria* from the diverse *Roseobacter* lineage are an important component of bacterioplankton in the temperate oceans (15, 17, 36). Since rRNA gene sequence types and isolates from nonaxenic cultures of marine algae are typically affiliated with this lineage (35, 44), a close association of *Bacteria* from the *Roseobacter* lineage with particular primary producers (e.g., with diatoms [31]) is assumed. In Mediterranean waters and in the Gulf of Maine, these *Bacteria* were found to be superior competitors for the algal osmolyte dimethyl sulfoniopropionate (23, 43), and *Roseobacter* may seasonally represent 25% of all glucose incorporating *Bacteria* in coastal North Sea waters (2).

Microbes related to *Roseobacter* constituted an almost equally large fraction of the bacterial assemblage in spring ( $19\% \pm 1\%$ ) and in summer ( $17\% \pm 2\%$ ) (Fig. 1). *Roseobacter*-related cells formed almost one-third of *Bacteria* with detectable DNA synthesis (Fig. 4A). At both samplings, significantly higher fractions of BrdU-incorporating cells were observed in *Roseobacter* spp. than in all *Bacteria* (as detected by the probe EUB I-III) (Fig. 4B). However, in contrast to our initial assumption, there was no significant difference between the fractions of proliferating cells in the May and August

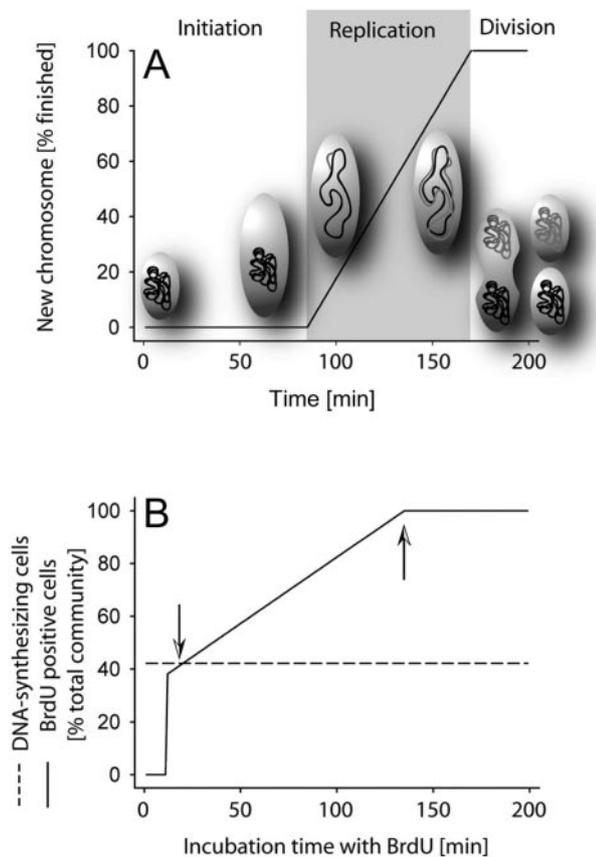


FIG. 5. (A) Schematic depiction of the durations of the different phases of the cell cycle of *E. coli* cells exponentially growing at generation times of 200 min in continuous culture. The gray area represents the fraction of the cell cycle in which DNA is newly synthesized. (B) Modeled changes of the fraction of cells with visible BrdU incorporation at increasing incubation times with BrdU. The arrows indicate the incubation times that would yield the accurate number of DNA-synthesizing cells at the modeled growth rate (42.5%, broken line) and the accurate number of all growing cells (100%).

samples. The *Roseobacter* clade as defined by the probe ROS537 harbors a number of subgroups with rather different physiological properties (1, 38, 40). It thus cannot be excluded that several phylogenetically distinct coexisting *Roseobacter* populations with different levels of cell proliferation were present in our samples.

Little is known about the ecophysiology of bacteria from the SAR86 lineage. It has been hypothesized that some members from this clade might be capable of light-driven energy production (5). Our data suggest that, seasonally, they are among the most rapidly growing bacteria in coastal North Sea surface waters (Fig. 4B). Significantly lower fractions of BrdU-labeled SAR86 cells were observed in May than in August (Fig. 2), indicating that these bacteria were growing more slowly in the spring than in the summer. During the August samplings, bacteria from the SAR86 clade represented roughly one-tenth of all cells (Fig. 2), but almost one quarter of BrdU-positive *Bacteria* (Fig. 4). This illustrates that a small population of rapidly growing cells may form a disproportionately large fraction of total microbial activity. The ecological role of bacteria

from the SAR86 clade might thus be underestimated if only their cell numbers are considered.

**Diurnal variability of proliferating bacteria.** At present, it is not clear whether diel variability of bacterial growth is a periodic phenomenon or if it is rather stochastic. Coffin et al. (10) reported a marked cycle of increasing bacterial growth efficiencies during the day, presumably after inputs of alga-derived DOC. Diel variations in bacterial growth appeared to correspond to diel variations in phytoplankton production (i.e., higher during the day) at an open ocean site in the Mediterranean Sea (16). In oligotrophic waters of the subtropical Atlantic Ocean, a diel cycle of bacterial nutrient limitation was observed, and the addition of N and P stimulated microbial growth at mid-day but not during morning hours (22). In contrast, high mesoscale variability, but no significant day-night differences in bacterial abundance and in radiolabeled leucine incorporation, was found in waters off the Oregon coast (37). Pronounced short-term fluctuations of bacterial per cell biomass productions were also observed in the coastal Mediterranean Sea albeit without any recognizable diurnal pattern (6). Bacteria in coastal waters might thus exhibit bursts of rapid growth induced by diel variations in their growth conditions rather than clear circadian cycles (18).

A statistically significant variability of BrdU-incorporating cells at the scale of hours was observed in bacteria affiliated with the *Roseobacter* and NOR5 clades (Fig. 1 and 3). However, there was no indication for recurring diurnal periodicity in these populations at either season. For example, a significantly lower fraction of cells related to *Roseobacter* exhibited cell proliferation during the morning hours of the second sampling day in May, whereas this trend was not present on the first day (Fig. 1).

Considering the rather unpredictable physicochemical dynamics of the studied system, this lack of clear diurnal periodicity is not surprising. The German Bight is vertically unstratified, and the water column is fully mixing throughout the year. The spring period is generally characterized by complex semi- and quarter-diurnal patterns of chlorophyll concentrations, resuspension, and settling of particulate matter that are related to currents, tidal forces, and wind speed (24, 45). The August 2002 sampling period coincided with a spatially highly patchy bloom of the motile dinoflagellate *Gymnodinium chlorophorum*, which for a period of less than a week represented >70% of algal biomass (K. Wildshire, personal communication). Considering recent reports about the relationship of particular phylogenetic groups of bacterioplankton with different phytoplankton species (31, 35), it is intriguing to speculate that the transient bursts of cell proliferation observed in population NOR5 (Fig. 3) might be related to the dinoflagellate bloom. Alternatively, the observed diurnal fluctuations of DNA de novo synthesis activity in NOR5 and in *Roseobacter* might have been a consequence of small-scale (i.e., mesoscale) horizontal variability, as observed, for example, by Pinhassi et al. (32).

In some cyanobacteria, processes related to DNA synthesis are reported to be under the control of a circadian clock (3). For example, the cell cycle of *Prochlorococcus* is highly synchronized in the field by the daily alternation of night and day. DNA replication occurs in late afternoon, and cell division occurs at night (41). Such a synchronization of cell prolifera-

tion was not found in potentially proteorhodopsin containing (5, 34) members of the SAR86 clade (Fig. 2).

**Limitations of DNA pulse-labeling as a proxy for bacterial “activity.”** The detection of substrate incorporation in single cells has become a powerful tool to investigate various aspects of bacterial physiology in marine waters (2, 11, 12, 23, 43). In a recent review, Smith and del Giorgio concluded that the average fraction of substrate-incorporating “active” bacterioplankton across all habitats was ca. 30% (39). Incidentally, this corresponds well with the average fraction of total (DAPI-stained) cells in our samples that exhibited visible DNA de novo synthesis (i.e., BrdU incorporation).

However, different tracers may address very different aspects of cell physiology (e.g., DNA synthesis or amino acid incorporation [11]), and a simple categorization into “active” and “inactive” cells might result in misinterpretations. If, for example, *Prochlorococcus* cells were investigated for “activity” by BrdU or TdR incorporation, then this species would be regarded as “inactive” during the daytime (but it is not since it is photosynthesizing) and as “active” after sunset (41).

Even without assuming synchronization of DNA synthesis, a cell labeling by tracers that are incorporated into newly synthesized DNA might underestimate the growing fraction of aquatic bacteria. Continuous and multifurcal DNA synthesis is a characteristic of very rapidly growing bacteria only, e.g., of *E. coli* on rich media. In more slowly growing bacterial cells there are four clearly discernible actions during the course of cell duplication: initiation and termination of chromosome replication and initiation and termination of cell division (20). In the model depicted in Fig. 5 (generation time, 200 min), no DNA synthesis occurs during the first part of the cell cycle (85 min). Chromosome replication is initiated once a specific cell mass has been reached, and the actual period of DNA synthesis (S phase) only occupies 42.5% of the total time of the cell cycle (Fig. 5A). During the last part of the cell cycle, the two daughter chromosomes are decatenated, segregated toward the ends of the cell, and a septum is formed at the middle position of the cylindrical cell (20).

In our example, a BrdU incubation time of >65% of the doubling time would be necessary to label 100% of all growing *E. coli* cells (Fig. 5B). Moreover, the duration of the S phase in *E. coli* is much less variable than the initiation phase, which is inversely related to growth rate (20). Thus, the relative period of DNA synthesis per cell cycle could be even shorter in very slowly growing populations. A detection of all growing cells in such populations would require BrdU or TdR incubation times in the range of days. Unfortunately, this is no option for field studies, because it might cause artificial metabolic upshifts of the microbial assemblage (9). Therefore, the percentage of cells with visible in situ uptake of BrdU or TdR at a reasonably short incubation time (2 to 4 h) likely does not allow one to assess how many cells within marine bacterioplankton are indeed “active” or “inactive.”

Using the same line of argument it is, however, legitimate to deduce that a specific bacterial population is more rapidly growing at times when it features higher fractions of cells with visible BrdU or TdR incorporation. DNA pulse-labeling approaches thus appear to be appropriate to detect short-term diurnal fluctuations of cell proliferation, as well as seasonal

differences within specific bacterioplankton populations (Fig. 1 to 3).

**Conclusions.** Our data indicate that there might be significant patchiness of microbial growth rates in coastal North Sea waters above the level of microscale “hot spots” of production (4). This (temporal or spatial) mesoscale variability might not be apparent at the community level (Table 1) but only at the level of individual bacterioplankton populations (Fig. 1 and 3). We furthermore caution against an unreflected categorization into “active” and “inactive” aquatic microbes based on methods that measure DNA synthesis in single cells (Fig. 5).

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