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ORIGINAL ARTICLE

Community shifts of actively growing lake bacteria after *N*-acetyl-glucosamine addition: improving the BrdU-FACS method

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In aquatic environments, community dynamics of bacteria, especially actively growing bacteria (AGB), are tightly linked with dissolved organic matter (DOM) quantity and quality. We analyzed the community dynamics of DNA-synthesizing and accordingly AGB by linking an improved bromodeoxyuridine immunocytochemistry approach with fluorescence-activated cell sorting (BrdU-FACS). FACS-sorted cells of even oligotrophic ecosystems in winter were characterized by 16S rRNA gene analysis. In incubation experiments, we examined community shifts of AGB in response to the addition of *N*-acetyl-glucosamine (NAG), one of the most abundant aminosugars in aquatic systems. Our improved BrdU-FACS analysis revealed that AGB winter communities of oligotrophic Lake Stechlin (northeastern Germany) substantially differ from those of total bacteria and consist of *Alpha*-, *Beta*-, *Gamma*-, *Deltaproteobacteria*, *Actinobacteria*, *Candidatus* OP10 and *Chloroflexi*. AGB populations with different BrdU-fluorescence intensities and cell sizes represented different phylotypes suggesting that single-cell growth potential varies at the taxon level. NAG incubation experiments demonstrated that a variety of widespread taxa related to *Alpha*-, *Beta*-, *Gammaproteobacteria*, *Bacteroidetes*, *Actinobacteria*, *Firmicutes*, *Planctomycetes*, *Spirochaetes*, *Verrucomicrobia* and *Chloroflexi* actively grow in the presence of NAG. The BrdU-FACS approach enables detailed phylogenetic studies of AGB and, thus, to identify those phylotypes which are potential key players in aquatic DOM cycling.
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Introduction

In aquatic environments, dissolved organic matter characteristics greatly affect bacterial diversity and metabolic activity (Azam, 1998). During the past two decades, 16S rRNA gene-based techniques have been intensively used to characterize bacterioplankton communities and their spatio-temporal fluctuations in aquatic ecosystems. However, communities of actively growing bacteria (AGB), which are mainly responsible for dissolved organic matter turnover and cycling, remain largely unknown.

To determine community composition and to assess the phylotype-specific substrate uptake at the single-cell level, the microautoradiography-fluorescence *in situ* hybridization method is a powerful tool in aquatic microbial ecology (Lee *et al.*, 1999; Cottrell and Kirchman, 2000; Alonso and Pernthaler, 2005). A major advantage of this method is the flexible usage of a variety of organic substrates as tracers (Fuhrman and Azam, 1982; Kirchman *et al.*, 1985; Cottrell and Kirchman, 2000; Alonso-Sáez and Gasol, 2007). However, fluorescence *in situ* hybridization (FISH)-based methods may encompass a broad range of the 'true' phylogenetic diversity due to the limited number of available FISH probes. Depending on the phylogenetic coverage of the oligonucleotide probes, various phylotypes and even ecotypes with greatly differing growth rates may be detected. Thus, it is difficult to evaluate the AGB community composition at the taxon level by just using FISH-based methods. In addition to microautoradiography-fluorescence *in situ* hybridization, the relative 16S rRNA abundance and the ratio of 16S rRNA to total rRNA genes

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have been used as indices for activity and for the potential growth rates of specific taxa in complex, marine bacterial communities (Schäfer *et al.*, 2001; Gentile *et al.*, 2006; Campbell *et al.* 2009, 2011; Lami *et al.*, 2009). Growth and metabolic activity of individual cells may be also represented by the number of ribosomes per cell (Kemp *et al.*, 1993; Fegatella *et al.*, 1998; Kerkhof and Kemp, 1999). Thus, this method allows to determine the potential growth of bacterial assemblages without using tracers and hence incubation biases. However, the relationship between rRNA copy number and growth is of low reliability in complex bacterial assemblages of natural environments, which often consist of a vast number of bacterial taxa with different rRNA-growth relationships and growth stages.

In this study, we used a novel single-cell-based method that combines bromodeoxyuridine immunocytochemistry and fluorescence-activated cell sorting (BrdU-FACS). Bromodeoxyuridine (BrdU), a halogenated nucleoside, serves as a thymidine analog and has been used as a tracer of bacterial *de novo* DNA synthesis, presumably of AGB (Taniguchi and Hamasaki, 2008). BrdU incorporation and fluorescent-labeled antibody detection techniques have been frequently used for identifying the AGB in aquatic environments (Steward and Azam, 1999; Urbach *et al.*, 1999; Pernthaler *et al.*, 2002; Hamasaki *et al.*, 2004; Warnecke *et al.*, 2005; Tada *et al.*, 2010, 2011). In addition, the method can be combined with FACS for 16S rRNA gene analysis, and, thus, provides a powerful tool for phylogenetic characterization of BrdU-fluorescence-labeled cells (Mow *et al.*, 2007). Thereby, BrdU-FACS enables to evaluate the AGB community composition at the single-taxon level.

The BrdU-FACS has been successfully applied to bacterioplankton in eutrophic coastal seawater (Mou *et al.*, 2007). As it can be assumed that bacterial growth in oligotrophic regions should be much lower than in the eutrophic coastal ocean, an improved method is required for analyzing the AGB community composition in oligotrophic environments. Therefore, we improved the BrdU-detection sensitivity by using the tyramide signal amplification technique. The tyramide signal amplification system enhances the fluorescence intensity of the BrdU-detection and consequently increases the sensitivity of FACS of bacterial cells in oligotrophic environments during winter.

Aminosugars such as *N*-acetyl-glucosamine (NAG) ($C_8H_{15}NO_6$) represent a major fraction of the natural dissolved organic matter pool and serve as important bacterial carbon and nitrogen sources in a variety of aquatic systems (Nedoma *et al.* 1994). In previous studies, high-NAG concentrations have been found in lakes, possibly due to algal excretion (Giroldo *et al.*, 2003), fecal pellets and exoskeleton of zooplankton (Lee and Fisher, 1992; Tang *et al.*, 2009), viral lysis or protozoan grazing of bacterial cells (Jørgensen *et al.*, 2003; Cloud-Hansen *et al.*, 2006) and other sources (Wurzbacher and Grossart, 2012). Recent microautoradiography-fluorescence *in situ* hybridization studies show that several bacterial phylotypes can utilize NAG as a carbon and nutrient source and that NAG has the potential for niche separation of closely related bacterial taxa (Beier and Bertilsson, 2011; Eckert *et al.*, 2012). However, information on bacterial taxa actively growing in response to NAG supply is still scarce.

The purpose of this study was to examine the AGB community composition of an oligotrophic lake in winter and their growth response to NAG addition by using an improved BrdU-FACS method.

Materials and methods

Study site and sample collection

Surface water (0 m) from oligotrophic Lake Stechlin, northeastern Germany (53°10′N, 13°02′E) was collected in triplicates on 28 February 2012 by using 2 l pre-combusted glass bottles (rinsed three times with surface water). After sampling, incubation experiments were immediately set up by adding 35 ml water to 50 ml of sterilized centrifugation tubes (three times rinses with surface water). Then, BrdU (1 μM final concentration; Sigma-Aldrich, St Louis, MO, USA) or BrdU + NAG (1 μM and 10 μM final concentration, respectively) were added to the respective tubes. Control tubes were also established. All water samples were incubated at 4 °C (*in situ* temperature ± 2 °C) for 48 h. At the end of the incubation, 10 ml samples were filtered onto 0.1-μm pore-size polycarbonate membrane filters (25-mm Nuclepore Track-Etch polycarbonate membrane, 110605, Whatman) and fixed with 50% ethanol for 1 h. Filters were stored at −30 °C until further analysis. All incubations were carried out in the dark using triplicates. For determining bacterial abundance, bacteria on the 0.1-μm membrane filters were stained with 4′, 6-diamidino-2-phenilindole (DAPI, 1 μg ml^{-1}, for 5 min) and counted using epifluorescence microscopy.

Immunodetection of BrdU-incorporating cells

Before BrdU-FACS analysis, we improved the BrdU-immunodetection procedure in terms of BrdU-detection buffer and anti-BrdU antibody concentration (see Supplementary Material). For the BrdU assay, all treatments were directly carried out in the glass vacuum filter holders (16315, Sartorius, Goettingen, Germany). Bacterial cells on the membrane filters were dehydrated with serial treatments in 80% and 100% ethanol each for 1 min. Filters were then treated with 0.01 mol l^{-1} HCl for 5 min at room temperature and with a pepsin solution (0.5 mg ml^{-1} in 0.01 N HCl) for 2 h at 37 °C. Thereafter, cells were washed three times with 15 ml phosphate-buffered saline (PBS) for 10 min and then treated with lysozyme (10 mg ml^{-1} in Tris-EDTA

buffer; 10 mmol l^{-1} Tris-HCl, 1 mmol l^{-1} EDTA; pH 8.0) for 15 min at room temperature. After the permeabilization steps, intracellular DNA was denatured by a nuclease treatment (1:100 in incubation buffer with the BrdU Labeling and Detection Kit III, 1444611, Roche, Mannheim, Germany) for double-stranded DNA for 2 h at 37 °C and washed three times with 15 ml PBS for 10 min. Thereafter, anti-BrdU monoclonal antibodies conjugated with peroxidase were diluted 1:200 (final) in freshly prepared antibody reaction buffer (0.1% Tween-20, and 0.5% acetylated bovine serum albumin in PBS buffer). Samples were incubated with the antibody solution for 120 min at 37 °C, which then was washed away (three times) with 10 ml phosphate-buffered saline with Tween-20 (0.05% Triton X-100 in PBS). The antibody signal was amplified by incubating the filters with a Alexa488-labeled tyramide diluted 1:500 in amplification buffer (10% [w/v] dextran sulfate, 2 M NaCl, 0.1% [v/v] blocking reagent and 0.0015% [v/v] H_2O_2 in PBS) for 45 min at 46 °C. Filter pieces were then washed three times with 15 ml phosphate-buffered saline with Tween-20 buffer for 10 min. Bacterial cells were counterstained with DAPI (1 μg ml^{-1}) for determining total bacterial numbers. The cells on the membrane were resuspended by shaking filters with vortex (maximum speed) twice in 1.5 ml phosphate-buffered saline with Tween-20 for 15 min at room temperature.

Flow cytometry and cell sorting

Sorting of BrdU-positive cells was performed with a FACSAria II flow cytometer (Sorb, Becton Dickinson, Heidelberg, Germany). The sheath solution consisted of 0.2-μm filtered and sterile PBS. BrdU-positive cells were detected by their green fluorescence emitted from Alexa488 (488 nm excitation and 515–545 nm emission), and fluorescence intensities were used as a proxy of growth rate. Bacterial cells (total) were detected by their blue fluorescence after ultraviolet excitation (405 nm excitation and 430–470 nm emission). Gate notation was based on the extent of BrdU-fluorescence intensity (green fluorescence intensity) and cell size (side scatter) (Figure 1a: P1, low; P2, medium; and P3, high). Bacterial cells were sorted by FACS into sterile 96-well plates containing 50 μl MQ water. Sorting was terminated when the number of sorted cells was $>100\,000$ counts for each fluorescence intensity fraction. The DAPI-stained cells of the control amendment were sorted as a control for denaturing gradient gel electrophoresis (DGGE) and clone library analyses (Figure 1b).

PCR amplification of the partial 16S rRNA gene for DGGE and clone library analyses

Sorted cells from each fraction were transferred from 96-well plates to sterile 0.2 ml tubes, and 3–5 freeze-thaw cycles were applied for cell lysis. The resulting lysates served as templates for the PCR amplification. For checking the replications of incubation experiments, FACS, and PCR amplification, we used the PCR-DGGE method with partial 16S rRNA gene amplification. Thereafter, the sequences of sorted cells were determined by the PCR-cloning method. For PCR-DGGE and PCR-cloning analyses, partial 16S rRNA gene amplification was carried out with the eubacterial primer set (Schäfer and Muyzer, 2001). Protocols for PCR-DGGE and PCR-cloning are given in the Supplementary material. Sequences were aligned to known sequences in the DNA Data Bank of Japan (DDBJ) using BLAST (http://blast.ddbj.nig.ac.jp/blast/blastn).

Phylogenetic relationships were inferred from multiple alignments by using the ATGC 6.04 software (Genetyx Co., Tokyo, Japan) and phylogenetic trees were calculated using the neighbor-joining method and the MEGA 5.05 software (http://www.megasoftware.net/; Tamura *et al.*, 2011). All sequences were checked by the DECIPHER program (Wright *et al.*, 2012) and the Mallard program (http://www.bioinformatics-toolkit.org/Mallard, Cardiff, UK). All nucleotide sequences have been deposited in the DDBJ nucleotide sequence database under accession numbers AB781370-781443 and AB831234-831258.

Results

Methodological improvements

Before combining BrdU-immunodetection and FACS, we optimized the BrdU-detection buffer and anti-BrdU antibody concentrations (Supplementary material). The BrdU-immunodetection efficiency with the new solutions was two to three fold higher than those previously published (Supplementary Figure S1). In addition, the modification of the antibody concentration resulted in the elimination of all 'false' BrdU-positive cells and yielded a proportion of BrdU-positive cells of $36 \pm 1\%$ of total bacteria (Supplementary Figure S2). BrdU-fluorescence signals from cells treated with the peroxidase-labeled antibody (the tyramide signal amplification system) were enhanced as compared with those with the fluorescein isothiocyanate-labeled antibody (Supplementary Figure S3). After all BrdU immunocytochemical reactions in the glass vacuum filter holder, the residual cells on the filter still accounted for $76 \pm 6\%$ and $84 \pm 13\%$ of the initial cells of the control and BrdU amendments, respectively (Supplementary Figure S4).

FACS sorting of BrdU-incorporating cells

Bacterial abundance in the Lake Stechlin winter sample was $7.7 \pm 1.7 \times 10^5$ cells ml^{-1}. Total cell numbers and the proportion of BrdU-positive cells of DAPI-stained cells after 48 h incubation are given in Table 1. On the basis of the levels of

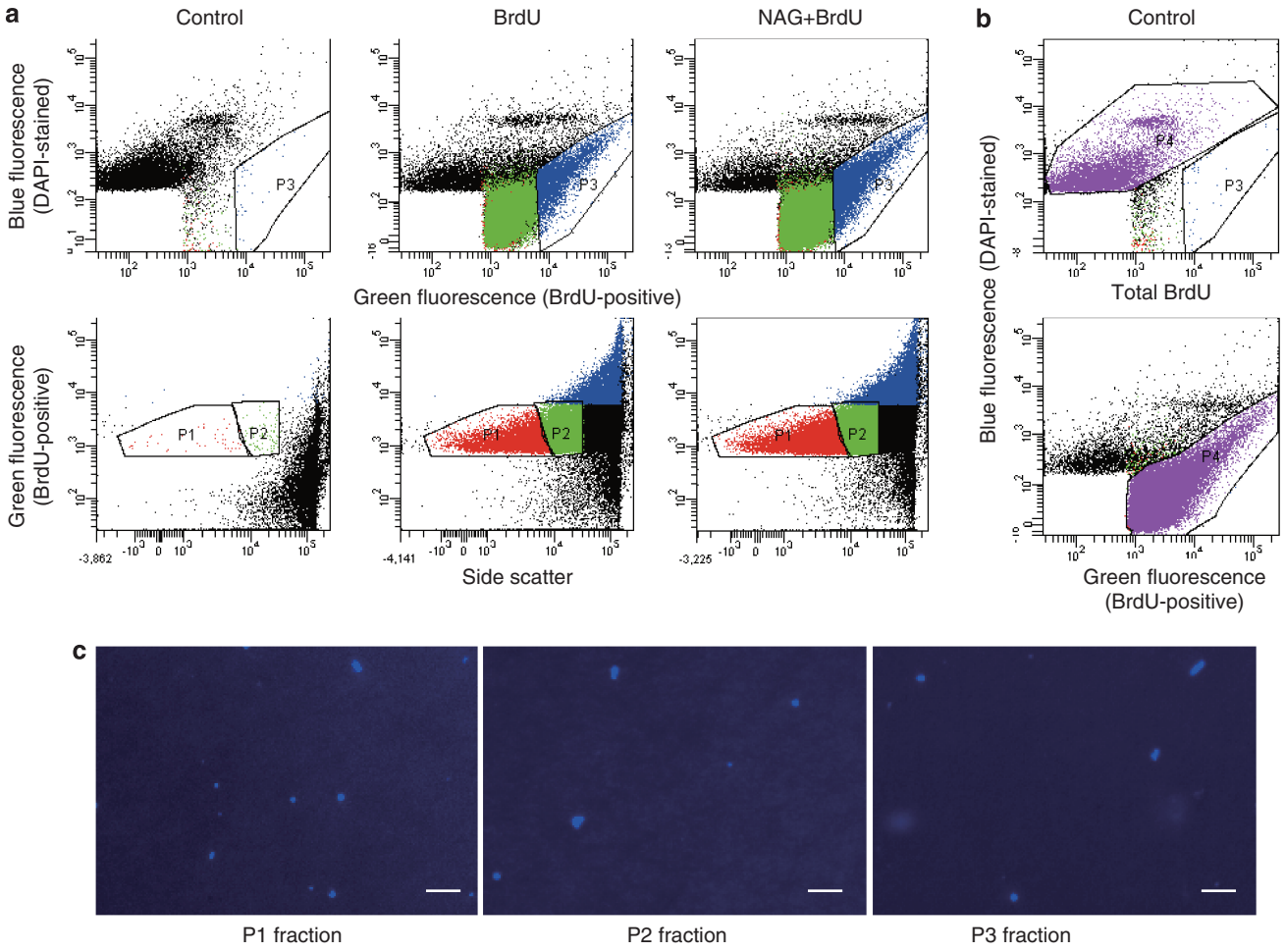

Figure 1 Flow-cytometric analysis of bacterial communities in the control, BrdU and NAG + BrdU amendments after incubation. (**a**) Gate notation (P1, low; P2, medium; and P3, high) based on the extent of BrdU-fluorescence intensity (Green fluorescence intensity) and cell size (Side scatter). (**b**) Gates for sorting the control fraction and counting total BrdU-positive cells. (**c**) Photomicrograph of the sorted cells. Bar = 5 μm.

Table 1 Total cell numbers and proportion of BrdU-positive cells of sorted fractions

Treatments	*Total cells ($\times 10^5$ cells ml^{-1})*		*Resuspension rate (%)*	*Proportion of BrdU-positive cells of DAPI-stained cells*			
	Direct count	*FCM count*		*P1: low (%)*	*P2: medium (%)*	*P3: high (%)*	*Total (%)*
Control	12.2 ± 1.4	2.6 ± 0.5	21	0.5 ± 0.2	1.0 ± 0.4	0.4 ± 0.1	2.0 ± 0.6
BrdU	19.5 ± 2.7	13.3 ± 2.2	68	19.7 ± 3.2	28.1 ± 6.0	9.5 ± 1.4	63.9 ± 7.0
NAG + BrdU	23.6 ± 4.5	16.9 ± 5.6	72	22.4 ± 2.9	27.7 ± 1.8	9.9 ± 0.6	69.2 ± 3.6

Abbreviations: BrdU, bromodeoxyuridine; DAPI, 4′, 6-diamidino-2-phenilindole; FCM, flow cytometry; NAG, *N*-acetyl-glucosamine.

BrdU-fluorescence intensity which is positively related to growth potential (Tada *et al.*, 2010) and cell sizes, cells in the BrdU and NAG + BrdU amendments were categorized into three fractions, low (P1), medium (P2) and high (P3) BrdU-fluorescence intensity and cell size populations (Figure 1a). BrdU-positive cells of each fraction in the controls representing 'false' positives accounted for <2% of total cells. Cell abundances in the BrdU and NAG + BrdU amendments were higher than in the controls, indicating active bacterial growth after tracer addition. BrdU-positive cells in the BrdU and NAG + BrdU amendments accounted for 64% and 69% of total cells, respectively. BrdU-positive cells for total bacteria were a bit higher than the sum of the P1, P2, and P3 fractions, as not all cells were gated for the FACS analysis.

DGGE analysis of the sorted populations

DGGE-banding patterns of 16S rRNA genes of the sorted bacterial populations revealed that the community composition of control and AGB substantially differed (Figure 2). Moreover, the addition

of NAG resulted in a clear community shift, in particular of AGB.

Phylogenetic analysis of AGB community

To analyze AGB community compositions, eight clone libraries were constructed (Table 2). These included the initial, control and samples of all three fractions (P1, P2 and P3) of each BrdU and NAG + BrdU amendment. The 19–41 sequences obtained for each library (average length of 580 bp) were classified into 4–25 operational taxonomic units at an evolutionary distance of 0.03 (Table 2). Operational taxonomic units are distributed among 13 major taxa which are commonly found in freshwater lakes (Figures 3–6, and, Table 3). In the clone library of initial amendment, sequences related to *Actinobacteria* (including acI and acIV lineages) and *Bacteroidetes* (including bacI lineage) frequently appeared and accounted for 32% and 29% of total sequences, respectively (Figure 3 and Table 3). Bacterial taxa which appeared in the BrdU amendment differed from those in the control (Table 3). Whereas *Alphaproteobacteria* represented the frequent sequences (37% of total sequences) in the clone library of the control (Figure 3), *Betaproteobacteria* accounted for the largest fraction (53% of total sequences) of total sequences in that of the BrdU amendment. Furthermore, the AGB community composition of the NAG + BrdU amendment greatly differed from those of the control and BrdU amendments (Figure 3 and Table 3). The most prevailing taxon in the NAG + BrdU clone library consisted of *Gammaproteobacteria* (31% of total sequences). Betaproteobacterial sequences were the second most frequent ones (28% of total sequences) in the NAG + BrdU clone library. Sequences related to *Alphaproteobacteria*, *Bacteroidetes*, *Actinobacteria*, *Firmicutes*, *Planctomycetes*, *Spirochaetes*, *Verrucomicrobia* and *Chloroflexi* also appeared in the NAG + BrdU clone library, but only accounted for 9%, 6%, 9%, 6%, 5%, 2%, and 2% of the total sequences, respectively. *Nitrospira* sequences appeared only in the clone library of the initial amendment and accounted for 5% of total sequences.

Apparent differences between BrdU and NAG + BrdU amendments were related to *Alpha-*, *Beta-*, *Gammaproteobacteria*, *Bacteroidetes*, *Planctomycetes*, *Spirochaetes*, *Verrucomicrobia* and *Firmicutes* taxa (Table 3). Alphaproteobacterial sequences related to *Sphingomonas* appeared in clone libraries of both amendments, whereas those of the *Candidatus Pelagibacter* sp. (alfV lineage) and *Pseudaminobacter* sp. only occurred in the NAG + BrdU amendment (Table 3 and Figure 5). *Betaproteobacteria* related to the betI lineage (including *Acidovorax* sp., *Limnohabitans* sp., *Variovorax* sp. and *Hydrogenophaga* sp.) and the betVII lineage (including *Janthinobacterium* sp. and *Herbaspirillum* sp.) were observed in both amendments. However, members of the betIV

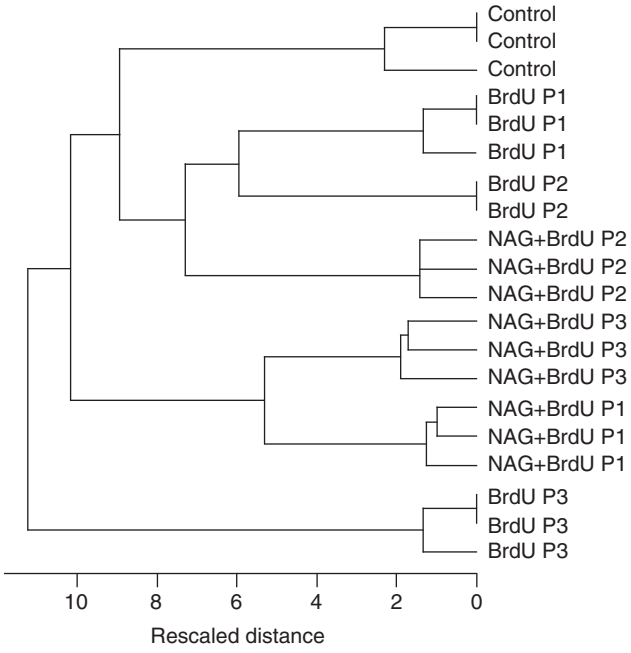

Figure 2 Cluster analysis of DGGE-banding patterns of the different BrdU-fluorescence intensity fractions (P1, P2 and P3) of the control, BrdU and NAG + BrdU amendments. The BrdU-P2 sorted samples represent duplicates, all other samples represent triplicates. The DGGE-banding patterns of sorted cells indicate very good within-treatment reproducibility (incubated, sorted and amplified independently).

Table 2 Distributions of sequences and OTUs from clone libraries

	Initial	*Control*	*BrdU*			*NAG + BrdU*		
			P1	*P2*	*P3*	*P1*	*P2*	*P3*
No. of sequences	41	23	21	24	23	21	24	19
No. of OTUs[a]	25	14	4	7	11	13	12	13

Abbreviations: BrdU, bromodeoxyuridine; NAG, *N*-acetyl-glucosamine; OTUs, Operational taxonomic units.
OTUs was defined with CD-HIT (Li and Godzik, 2006) assuming a 97% sequence similarity level.

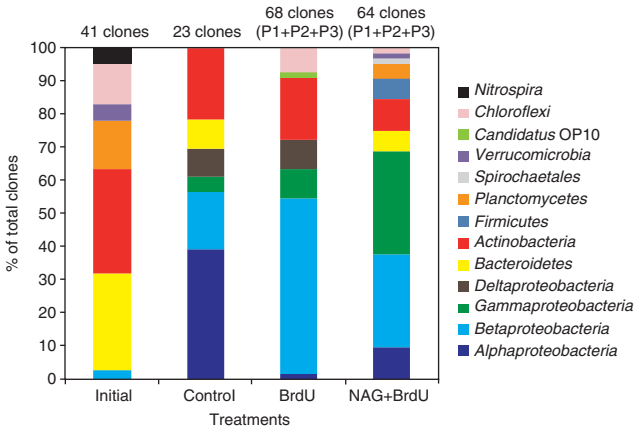

Figure 3 Percentages of clones represented by the major phylogenetic groups of bacteria in libraries of 16S rRNA genes in the initial, control, BrdU and NAG + BrdU amendments.

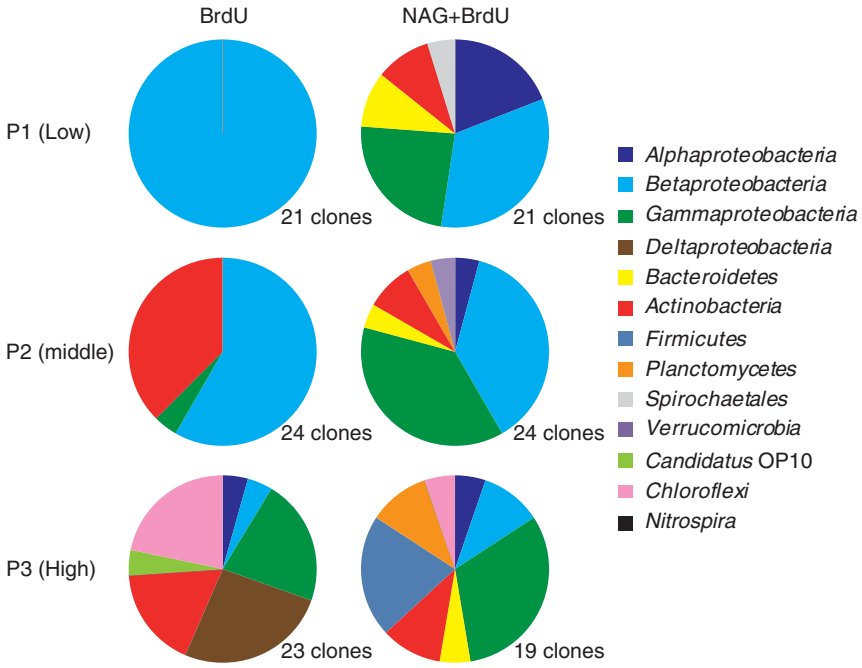

Figure 4 Bacterial community composition (clone libraries) in different BrdU-fluorescence intensity fractions (P1, P2 and P3) of both BrdU and NAG + BrdU amendments.

lineage including *Methylophilus* sp. exclusively appeared in the NAG + BrdU amendment. Whereas *Gammaproteobacteria* affiliated to *Acinetobacter* sp. were found in both BrdU and NAG + BrdU amendments, those affiliated to *Pseudomonas* sp. were solely present in the NAG + BrdU amendment. *Actinobacteria* related to acI, acII, and acIV as well as members of the *Firmicutes*, *Bacteroidetes*, *Spirochaetes*, *Verrucomicrobia* and *Planctomycetes* appeared in the NAG + BrdU amendment (Table 3 and Figure 6). In contrast, members of the *Deltaproteobacteria* and *Candidatus* OP10 did not appear in the NAG + BrdU, but in the BrdU amendment.

Phylogenetic analysis of sorted AGB based on BrdU-fluorescence intensities and cell sizes

AGB community composition of the three sorted fractions with different BrdU-fluorescence intensities and cell sizes (P1, P2 and P3) were determined both in BrdU and NAG + BrdU amendments (Figure 4). In the BrdU amendment, *Betaproteobacteria* formed the largest portion of total sequences in the P1 and P2 fractions (100% and 58%, respectively). In particular, sequences related to *Rodoferax* sp. (betI lineage) and *Janthinobacterium* sp. (betVII lineage) were found in the P1 fraction (Figure 5 and Table 3). The proportion of *Actinobacteria* increased in the P2 fraction (38%), and especially members of the *Nocardioides* and *Propionibacterium* appeared in the P2 and P3 fractions. The number of operational taxonomic units in the P3 fraction was higher than in all other fractions, in particular the proportion of *Deltaproteobacteria* in the BrdU amendment increased in this fraction (26%). Moreover, members of the *Alphaproteobacteria*, *Candidatus* OP10, and *Chloroflexi* were exclusively observed in the P3 fraction and accounted for 4%, 4%, and 22% of total sequences, respectively.

The AGB community in the NAG + BrdU amendment also consisted of major members of common bacterial freshwater clusters, namely *Alpha-*, *Beta-*, *Gammaproteobacteria*, *Bacteroidetes*, *Actinobacteria*, *Firmicutes*, *Planctomycetes*, *Spirochaetes*, *Verrucomicrobia* and *Chloroflexi* (Figures 5, 6, and Table 3). *Betaproteobacteria* represented the most prevailing taxon in the P1 fraction (33% of total sequences). Sequences related to *Rhodoferax* sp., *Methylophilus* sp. and *Janthinobacterium* sp. belonging to the betI, betIV and betVII lineages, respectively were observed in the NAG + BrdU clone library of the P1 fraction (Figure 5). Furthermore, sequences of *Limnohabitans* sp. and *Hydrogenophaga* sp. both belonging to the betI lineage appeared in the P3 fraction. The proportion of *Actinobacteria* in the P1, P2 and P3 fractions of the NAG + BrdU amendment accounted for 10%, 8%, and 11% of all sequences, respectively, whereby acI and acIV *Actinobacteria* mainly appeared in the P2 and P3 fractions, respectively (Figure 6). Sequences of the acII lineage were observed in both P1 and P2 fractions. The proportion of *Gammaproteobacteria* in the P1, P2 and P3 fractions accounted for 24%, 38%, and 32% of total sequences, respectively. Members of the *Bacteroidetes* were observed in all fractions and contributed between 4 and 10% of total sequences. In contrast, a single member of the *Firmicutes*, solely observed in the P3 fraction, accounted for 21% of the total sequences. *Spirochaetes*, *Verrucomicrobia* and *Chloroflexi* were solely observed in the P1, P2

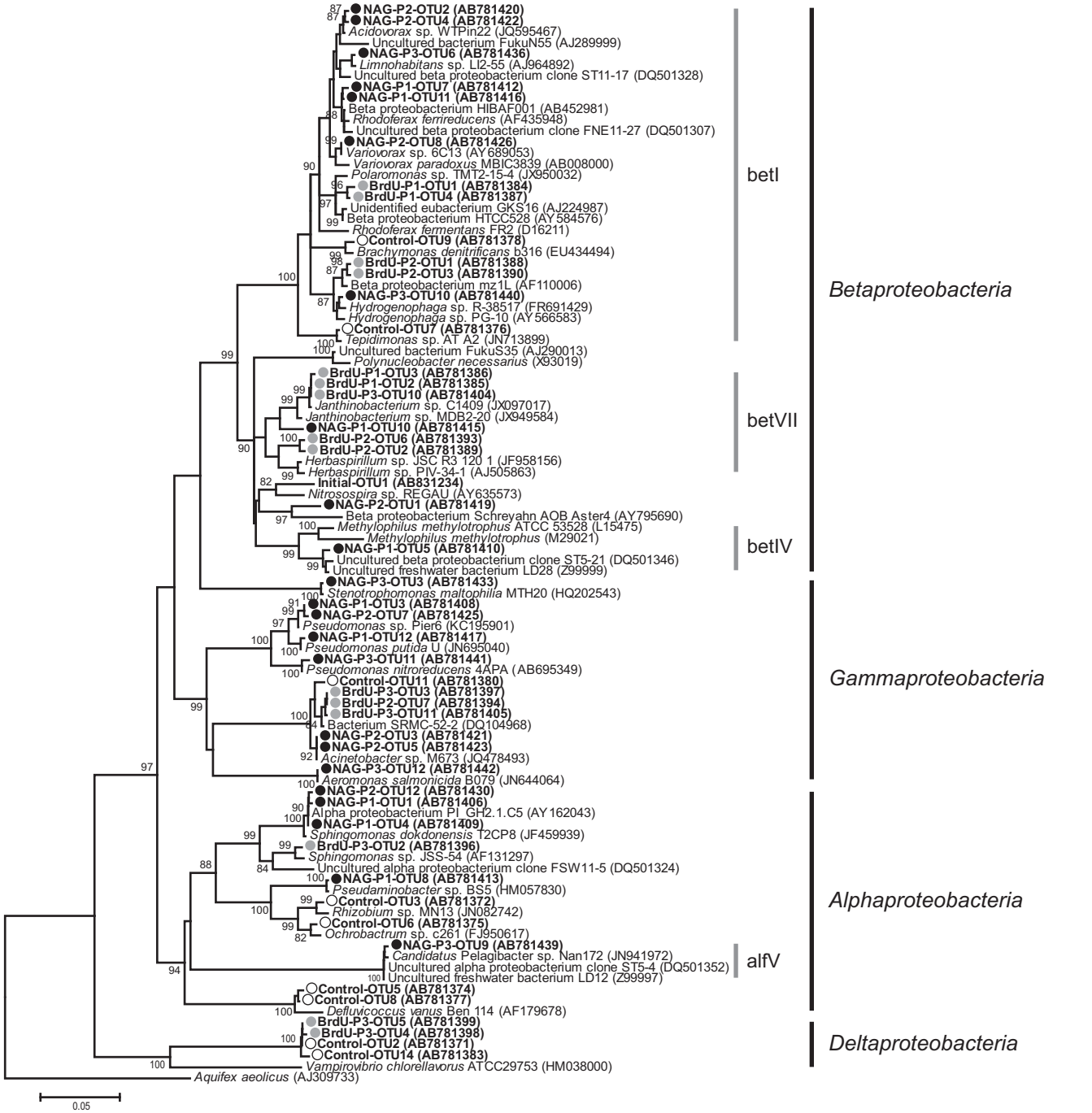

Figure 5 Phylotype distribution in the initial sample, control and fractions of different BrdU-fluorescence intensity (P1, P2 and P3) of BrdU and NAG + BrdU amendments: phylogenetic tree of all obtained 16S rRNA gene sequences (clone libraries; 0.03 distance OUTs) related to known *Proteobacteria*. Bootstrap values >80% are indicated above the branches. The scale bar represents the estimated 5% sequence divergence. Open circles indicate operational taxonomic units (OTUs) from control amendment, gray circles indicate OTUs from the BrdU amendment and solid circles indicate OTUs from the NAG + BrdU amendment.

and P3 fraction of the NAG + BrdU amendment, respectively.

Discussion

Methodological improvements

For the BrdU immunocytochemical detection, loss of cells is one of the most serious problems. In the present study, >75% of all cells could be kept on the filter (Supplementary Figure S4) after performing all BrdU immunocytochemical steps directly in the glass vacuum filter holders to minimize cell loss. In previous BrdU studies, adhesive materials such as agarose (Pernthaler *et al*., 2002) and poly-l-lysin (Tada *et al*., 2010) were used to keep the cells on the filter. For BrdU-FACS application, however, cell

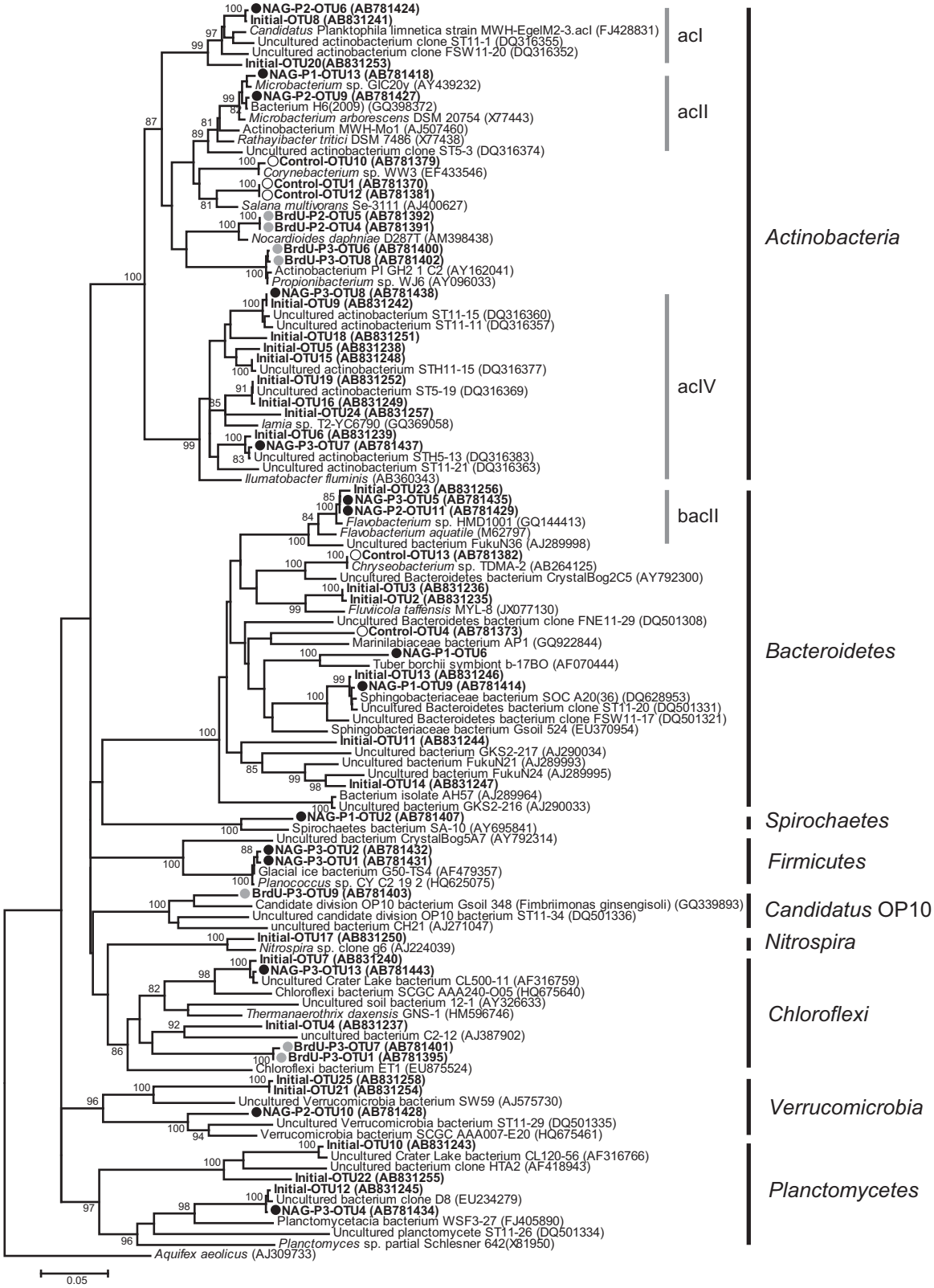

Figure 6 Phylotype distribution in the initial sample, control and fractions of different BrdU-fluorescence intensity (P1, P2 and P3) of BrdU and NAG + BrdU amendments: phylogenetic tree of all obtained 16S rRNA gene sequences (clone libraries; 0.03 distance OUTs) related to known *Actinobacteria*, *Bacteroidetes*, *Candidatus* OP10, *Spirochaetes*, *Firmicutes*, *Nitrospira*, *Chloroflexi*, *Planctomycetes* and *Verrcomicrobia*. Bootstrap values >80% are indicated above the branches. The scale bar represents the estimated 5% sequence divergence. Open circles indicate OTUs from control amendment, gray circles indicate OTUs from the BrdU amendment and solid circles indicate OTUs from the NAG + BrdU amendment.

Table 3 The presence and absence of phylogenetic group of 16S rRNA gene sequences at each sample

Phylum	*Sequence names and OTUs*	*Closest sequences*	*Treatments*			
			Initial	*Control*	*BrdU*	*NAG+BrdU*
Alphaproteobacteria	Control-OTU3	*Rhizobium* sp. MN13		+		
	Control-OTU5, OTU8	*Defluvicoccus vanus* Ben 114		+		
	Control-OTU6	*Ochrobactrum* sp. c261		+		
	BrdU-P3-OTU2	*Sphingomonas* sp. JSS-54			+	
	NAG-P1-OTU1, OTU4/NAG-P2-OTU12	*Sphingomonas dokdonensis*				+
	NAG-P1-OTU8	*Pseudaminobacter* sp. BS5				+
	NAG-P3-OTU9	*Candidatus* Pelagibacter sp. Nan172				+
Betaproteobacteria	Initial-OTU1	*Nitrosospira* sp. REGAU	+			
	Control-OTU7	*Tepidimonas* sp. AT A2		+		
	Control-OTU9	*Brachymonas denitrificans* b316		+		
	BrdU-P1-OTU1, OTU4	*Polaromonas* sp. TMT2-15-4			+	
	BrdU-P1-OTU2, OTU3/ BrdU-P3-OTU10/NAG-P1-OTU10	*Janthinobacterium* sp. C1409			+	+
	BrdU-P2-OTU1, OTU3	Beta proteobacterium mz1L			+	
	BrdU-P2-OTU2, OTU6	*Herbaspirillum* sp. JSC R3 120 1			+	
	NAG-P1-OTU5	*Methylophilus methylotrophus* ATCC 53528				+
	NAG-P1-OTU7, OTU11	*Rhodoferax ferrireducens*				+
	NAG-P2-OTU1	Beta proteobacterium Schreyahn AOB Aster4				+
	NAG-P2-OTU2, OTU4	*Acidovorax* sp. WTPin22				+
	NAG-P2-OTU8	*Variovorax* sp. 6C13				+
	NAG-P3-OTU6	*Limnohabitans* sp. LI2–55				+
	NAG-P3-OTU10	*Hydrogenophaga* sp. R-38517				+
Gammaproteobacteria	Control-OTU11/NAG-P2-OTU3, OTU5	*Acinetobacter* sp. M673		+		+
	BrdU-P2-OTU7, BrdU-P3-OTU3, OTU11	Bacterium SRMC-52–2			+	
	NAG-P1-OTU3/NAG-P2-OTU7	*Pseudomonas* sp. Pier6				+
	NAG-P1-OTU12	*Pseudomonas putida* U				+
	NAG-P3-OTU3	*Stenotrophomonas maltophilia* MTH20				+
	NAG-P3-OTU11	*Pseudomonas nitroreducens* 4APA				+
	NAG-P3-OTU12	*Aeromonas salmonicida* B079				+
Deltaproteobacteria	Control-OTU2, OTU14/BrdU-P3-OTU4, OTU5	*Vampirovibrio chlorellavorus* ATCC:29753		+	+	
Bacteroidetes	Initial-OTU2, OTU3	*Fluviicola taffensis* MY-8	+			
	Control-OTU4	Marinilabiaceae bacterium AP1		+		
	Control-OTU13	*Chryseobacterium* sp. TDMA-2		+		
	NAG-P1-OTU6	Tuber borchii symbiont b-17BO				+
	Initial-OTU13/NAG-P1-OTU9	Sphingobacteriaceae bacterium SOC A20(36)	+			+
	Initial-OTU23/NAG-P2-OTU11/ NAG-P3-OTU5	*Flavobacterium* sp. HMD1001	+			+
	Initial-OTU11	Uncultured bacterium GKS2–217	+			
	Initial-OTU14	Uncultured bacterium FukuN24	+			
Actinobacteria	Initial-OTU5, OTU15	Uncultured actinobacterium STH11–15	+			
	Initial-OTU16, OTU19	Uncultured actinobacterium ST5–19	+			
	Initial-OTU24	*Iamia* sp. T2-YC6790	+			
	Control-OTU1, OTU12	*Salana multivorans* Se-3111		+		
	Control-OTU10	*Corynebacterium* sp. WW3		+		
	BrdU-P2-OTU4, OTU5	*Nocardioides daphniae* D287T			+	
	BrdU-P3-OTU6, OTU8	*Propionibacterium* sp. WJ6			+	
	NAG-P1-OTU13	*Microbacterium* sp. GIC20y				+
	Initial-OTU6, OTU20/NAG-P2-OTU6	*Candidatus* Planktophila limnetica MWH-EgelM2-3.acI	+			+
	NAG-P2-OTU9	Bacterium H6 (2009)				+
	Initial-OTU6/NAG-P3-OTU7	Uncultured actinobacterium STH5–13	+			+
	Initial-OTU9, OTU18/NAG-P3-OTU8	Uncultured actinobacterium ST11-11	+			+
Firmicutes	NAG-P3-OTU1, OTU2	*Planococcus* sp. CY C2 19 2				+
Planctomycetes	Initial-OTU10	Uncultured Crater Lake bacterium XL120-56	+			
	Initial-OTU22	Uncultured bacterium clone HTA2	+			
	Initial-OTU12/NAG-P3-OTU4	Uncultured bacterium clone D8	+			+
Spirochaetes	NAG-P1-OTU2	Spirochaetes bacterium SA-10				+
Verrucomicrobia	Initial-OTU21, OTU25	Uncultured Verrucomicrobia bacterium SW59	+			
	NAG-P2-OTU10	Verrucomicrobia bacterium SCGC AAA007-E20				+

Table 3 (Continued)

Phylum	*Sequence names and OTUs*	*Closest sequences*	*Treatments*			
			Initial	*Control*	*BrdU*	*NAG + BrdU*
Candidatus OP10	BrdU-P3-OTU9	Candidate division OP10 bacterium Gsoil 348			+	
Chloroflexi	Initial-OTU4	Uncultured bacterium C2-12	+			
	BrdU-P3-OTU1, OTU7	*Thermanaerothrix daxensis* GNS-1			+	
	Initial-OTU7/NAG-P3-OTU13	Uncultured Crater Lake bacterium CL500-11	+			+
Nitrospira	Initial-OTU17	*Nitrospira* sp. clone g6	+			

Abbreviations: BrdU, bromodeoxyuridine; NAG, *N*-acetyl-glucosamine; OTUs, Operational taxonomic units.

adhesive materials exert negative effects for cell sorting owing to related changes of the cell's surface properties. Our method circumvents this critical issue.

The cell resuspension rate of the control was lower than those of BrdU and NAG + BrdU amendments both with higher percentages of BrdU-positive cells (Table 1). This indicates that AGB might better detach from the filter than the inactive or dormant cells. Furthermore, bacterial community composition differed between the treatments (Table 3), whereby surface characteristics of cells (for example, cell wall and extracellular matrix) may differ between phylotypes, suggesting that the resuspension rate varies with the changing bacterial community composition.

We optimized antibody concentration and antibody reaction buffer for the improved BrdU-immunodetection (Supplementary Figures S1 and S2). These improvements greatly enhanced the BrdU-detection sensitivity as compared with previous studies (Supplementary Figure S3). These methodological changes thus allowed us to apply the BrdU-FACS method for phylogenetic analyses of AGB in oligotrophic waters during winter even at the taxon level.

In the BrdU amendment, about 63.9% of the total cells (DAPI-stained) were able to incorporate the BrdU (Table 1), which was relatively similar to the proportion of AGB in the NAG + BrdU amendment (69.2%). We added the BrdU at a lower concentration (final 1 μM) than for other BrdU studies. Although the added BrdU did not substantially contribute to the total dissolved organic carbon pool in oligotrophic Lake Stechlin, we cannot rule out that the added BrdU has served as an additional bacterial substrate. Thus, AGB should include bacteria with the potential for active growth.

Biases during incubation experiments

The bacterial community composition of the control amendment differed from that of the initial sample (Table 3), indicating that there were some biases during our incubation experiments. Possible biases should be a confinement bias and long incubation time. However, DGGE analysis revealed a good replication of incubation experiments (Figure 2), indicating that these biases exist in a consistent manner in all bottles.

AGB community composition

Clone library analyses revealed that AGB community composition substantially differed from that of total bacteria in the control, suggesting that community composition of total bacteria is the result of an interplay of many factors including bacterial dormancy (Jones and Lennon, 2010), protozoan grazing (Šimek *et al.*, 2001; Pernthaler *et al.*, 2001; Eiler and Bertilsson, 2007; Jones *et al.*, 2009; Salcher *et al.*, 2010; Eckert *et al.*, 2012) and viral lysis (Weinbauer and Höfle, 1998; Thingstad, 2000). AGB with different BrdU-fluorescence intensities composed of different bacterial taxa (Figures 4, 5, and 6) indicate that single-cell growth potential varies at the taxon level.

In the BrdU amendment, *Polaromonas* sp. as well as *Hydrogenophaga* sp. (both belonging to the betI lineage) and *Janthinobacterium* sp. (betVII lineage) frequently appeared, particularly in the P1 and P2 fractions. This notion indicates that these phylotypes should grow in spite of lower temperature. In previous FISH studies, however, their population size was tightly correlated with water temperature (Hahn *et al.*, 2005; Wu and Hahn, 2006a, b). In contrast, our incubation experiments were performed at a low temperature (ca. 4 °C), suggesting that low nutrient availability was the critical factor in the control, whereas the low water temperature did not limit the bacterial growth in both BrdU and NAG + BrdU amendments.

Actinobacteria represent one of the most prominent lineages in surface waters of temperate, stratified lakes (Glöckner *et al.*, 2000; Hahn *et al.*, 2003; Warnecke *et al.*, 2005; Allgaier *et al.*, 2007; Salcher *et al.*, 2010; Rösel and Grossart, 2012; Rösel *et al.*, 2012). In our study, the actinobacterial sequences were frequently observed in clone libraries of the P2 and P3 fractions (Figures 3 and 4). Especially, *Propionibacterium* sp. observed in the P3 fraction expressed a high growth potential and thus are of potential importance for organic matter cycling in oligotrophic Lake Stechlin in winter.

In addition, our results revealed that sequences of *Candidatus* OP10 and *Chloroflexi,* which are known to contribute to only a minor fraction of lake bacteria (Newton *et al.* 2011) occur in the P3 fraction, suggesting that these quantitatively minor groups may be highly active and hence contribute to the organic matter cycling in Lake Stechlin in winter.

BrdU-immunofluorescence intensity should be influenced by genome size and GC content of individual cells. Genome sizes of marine and freshwater bacteria greatly vary at the species level, and range from 1 to 10 Mbp (for example, Giovannoni *et al.*, 2005; Zeng *et al.*, 2012; Garcia *et al.*, 2013). For instance, the genome size of acI *Actinobacteria* is small (<2 Mbp) and their GC content high (ca. 40%) (Garcia *et al.*, 2013), suggesting that their real growth potential might be underestimated by BrdU incorporation. Such differences should affect the variation of BrdU-fluorescence intensities of specific phylogenetic taxa (potentially within the same taxon level) and hence should reflect their actual activity level.

Community shift of AGB in response to NAG addition

Several FISH-based studies have shown that NAG supply in lakes lead to bacterial community shifts at the phylum or genus level (Beier and Bertilsson, 2011; Eckert *et al.*, 2012). However, closely related bacterial phylotypes exhibit variable preferences for organic substrates (Hunt *et al.*, 2008; Alonso *et al.*, 2009; Buck *et al.*, 2009). Thus, the analysis of community shifts at a higher phylogenetic resolution is a prerequisite to elucidate the effects of specific organic substrates, for example, NAG, in freshwater lakes. The present study revealed a pronounced growth response of various limnetic bacterial phyla and classes to NAG supply (Figures 5, 6, and Table 3). This notion is consistent with previous findings indicating that widespread taxa of marine and freshwater bacteria have the ability to take up NAG (Nedoma *et al.*, 1994; Riemann and Azam, 2002; Cottrell and Kirchman, 2000; Beier and Bertilsson, 2011).

Particularly, *Betaproteobacteria* related to *Acidovorax*, *Rhodoferax*, *Limnohabitans*, *Variovorax*, *Hydrogenophaga* (betI lineage), *Methylophilus* (betIV lineage) and *Janthinobacterium* (betVII lineage) formed substantial portions of the P1 and P2 fractions of the NAG + BrdU amendment (Figures 4 and 5). Sequences related to the widespread *Limnohabitans* and *Hydrogenophaga* (betI lineage) also occurred in the P3 fraction, indicating that these bacteria grow well on NAG or at least on NAG degradation products. This is in accordance to microautoradiography-fluorescence *in situ* hybridization results in mesotrophic Lake Zürich (Eckert *et al.*, 2012). However, genomic and physiological studies on type strains of *Limnohabitans* and *Variovorax* do not support active NAG utilization (Yoon *et al.*, 2006; Miwa *et al.*, 2008; Hahn *et al.*, 2010a, b; Kasalický *et al.*, 2010). A recent study has revealed that members of *Limonohabitans* perform well on a variety of alga-derived substrates; and regarding their genome size they have a more substrate-responsive ability 'high metabolic IQ' (Kasalický *et al.*, 2013) rather than abundant acI and *Polynucleobacter* groups. Thus, it seems likely that this lineage solely should take up the NAG and alga-derived hydrolysis products and, therefore, depends on the chitinolytic activity of other phylogenetic groups.

NAG utilization by *Polynucleobacter* (betII lineage), especially PnecC, has been demonstrated (Alonso *et al.*, 2009). However, sequences related to *Polynucleobacter* were absent in all clone libraries. A possible reason for this notion could be a limited resolution of our phylogenetic analyses (Table 2). Another reason could be the relatively low water temperature, which has been indicated as a critical factor regulating *Polynucleobacter* populations (Hahn *et al.*, 2005; Wu and Hahn, 2006b). Our findings thus suggest that the contribution of the betII lineage to NAG cycling is diminished during winter.

Gammaproteobacteria frequently appeared in the P2 and P3 fractions of the NAG + BrdU amendment, indicating that they have a key role in the NAG degradation during winter. A large part of *Gammaproteobacteria* utilizing NAG was closely related to potentially chitinolytic soil bacteria (Figure 5). Their active growth suggests a tight terrestrial–aquatic coupling and point to a possibly important biogeochemical role, for example, in NAG degradation, of otherwise rare soil bacteria in this lake.

Members of the *Bacteroidetes* were not detected in the BrdU but in the NAG + BrdU amendment (Figure 6 and Table 3). This suggests that NAG may stimulate specific members of limnic *Bacteroidetes*. Previous studies also revealed that *Bacteroidetes* (including *Cytophaga-Flavobacteria*) take up NAG (Beier and Bertilsson, 2011; Eckert *et al.*, 2012). In contrast to Lake Zürich (where NAG-utilizing *Bacteroidetes* predominantly belonged to the bacVI lineage) (Eckert *et al.*, 2012), in our study, they mainly belonged to the bacII lineage. The phylum *Bacteroidetes*, however, expresses a huge diversity in freshwater ecosystems (Newton *et al.*, 2011; Eckert *et al.*, 2012). On the basis of our results it seems likely that key lineages of NAG-utilizing *Bacteroidetes* differ with environmental variables such as season and physiological state of the phyto- and zooplankton.

Members of *Actinobacteria* are known as important consumers of NAG in freshwater lakes (Beier and Bertilsson, 2011; Eckert *et al.*, 2012). Our results revealed that *Microbacterium* sp., uncultured lineages of acI and acIV formed the majority of *Actinobacteria* in the NAG + BrdU treatment. Especially, the acIV sequences frequently appeared in the P3 fraction, implying that this lineage represents highly active *Actinobacteria* involved in NAG degradation. AcI and acIV *Actinobacteria* are

highly abundant and widespread (Glöckner *et al.*, 2000; Burkert *et al.*, 2003; Warnecke *et al.*, 2005; Allgaier and Grossart, 2006; Newton *et al.*, 2007; Buck *et al.*, 2009). However, little is known about their ecological role, especially as no pure cultures of these lineages are available (Newton *et al.*, 2011). In this study, acI and acIV lineages appeared in the P2 or P3 fraction. Furthermore, single-cell genome analysis has revealed that acI *Actinobacteria* have a chitinase-like gene (Garcia *et al.*, 2013). This suggests that acI *Actinobacteria* are actively involved in chitin degradation. Our results indicate that NAG may be an important energy source for members of acI and acIV *Actinobacteria* and implies participation in NAG cycling in Lake Stechlin.

In general, members of *Verrucomicrobia* and *Planctomycetes* are known as common but minor phyla in freshwater lakes and little is known about their diversity and ecological role (Newton *et al.*, 2011). Our results suggest that these groups could also be involved in NAG degradation in Lake Stechlin. However, their overall contribution to freshwater NAG cycling still remains to be determined.

Conclusions

Combining an improved BrdU-FACS method with clone library analysis revealed an AGB winter community in an oligotrophic lake. AGB in fractions with different BrdU-fluorescence intensities and cell sizes revealed that single-cell growth potential varies at the taxon level, which might be used to identify bacterial groups with different growth rates using flow cytometry. Moreover, NAG incubation experiments revealed that widespread limnetic phyla and classes actively grow in response to NAG addition even at low temperature. These results imply that NAG cycling can be important for organic matter cycling and bacterial community composition in freshwater ecosystems in winter.

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