

# Spatio-temporal niche separation of planktonic *Betaproteobacteria* in an oligo-mesotrophic lake

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## Summary

We investigated the diversity of planktonic *Betaproteobacteria* and the seasonal population changes of betaproteobacterial taxa in an oligo-mesotrophic lake (Piburger See, Austria). Focus was put on the vertical distribution of the investigated populations and on differences between their respective cell fractions with apparent amino acid incorporation. On average, 66% of betaproteobacterial cells and 73% of their diversity could be attributed to four clades within three lineages that were further analysed by fluorescence *in situ* hybridization. The numbers of bacteria from the R-BT subclade of the beta I lineage and from the PnecB subgroup of the beta II lineage were rather constant throughout the water column. In contrast, members of another subgroup of beta II (PnecC) and bacteria related to *Methylophilus* (beta IV) were particularly numerous in the oxygen-depleted zone. In general, only moderate seasonal changes in abundance were observed in the upper water layers, whereas there was a clear relationship between decreasing oxygen levels and the rise of bacteria from the PnecC and beta IV clades in deeper strata. On average, almost 80% of beta I bacteria, but < 15% of cells from the beta IV clade, showed amino acid incorporation. Our results suggest that the studied populations occupy distinct vertical and ecophysiological niches in Piburger See.

## Introduction

Seasonality of freshwater bacterioplankton at the community level has been the topic of numerous studies.

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It has been shown that bacterial abundances, biomasses and bulk production may follow seasonal patterns of phytoplankton development and/or physicochemical changes (e.g. Coveney and Wetzel, 1995; Simon *et al.*, 1998). However, the seasonal successions of lake bacterioplankton are much less understood than that of phyto- or zooplankton (Sommer *et al.*, 1986). This is mainly due to the limited information on temporal changes of the taxonomic composition of freshwater microbial assemblages.

There is, however, first evidence that different freshwater bacteria exhibit specific seasonal succession patterns. For example, a species-like clade within *Betaproteobacteria* showed pronounced summer maxima of population sizes that were tightly correlated with water temperature (Hahn *et al.*, 2005; Wu and Hahn, 2006a,b). *Actinobacteria* from a typical freshwater lineage may form spring and autumn maxima in temperate lakes (Burkert *et al.*, 2003; Allgaier and Grossart, 2006), and a rise of filamentous bacteria affiliated with *Saprospiraceae* was observed in parallel with the decay of phytoplankton spring bloom (Schauer *et al.*, 2006). Short-lived peaks of filamentous morphotypes related to *Sphingobacteria* and *Alphaproteobacteria* were also found during periods of high densities of bacterivorous nanoflagellates (Pernthaler *et al.*, 2004; Nishimura and Nagata, 2007).

One drawback of many seasonal studies is the exclusive focus on the surface water layers (e.g. Burkert *et al.*, 2003; Wu and Hahn, 2006a). Thermally stratified lakes typically exhibit pronounced depth gradients of physicochemical parameters, and higher abundances and biomasses of microorganisms can be found in deeper water layers (Cole *et al.*, 1993). The oxygen-depleted hypolimnion may represent a niche for specialized microorganisms, such as fermenting bacteria, denitrifiers, methanotrophs, methylotrophs or autotrophic sulfur bacteria (Casamayor *et al.*, 2000; Lehours *et al.*, 2007). Moreover, some heterotrophic bacteria from the oxic layers may be capable of a facultative anaerobic metabolism (Alonso and Pernthaler, 2005). Such ecophysiological questions can be addressed by combining phylogenetic identification with the assessment of single-cell activity (Lee *et al.*, 1999).

*Betaproteobacteria* are a prominent group of freshwater bacterioplankton that may form high abundances in

lakes of different trophic status (Glöckner *et al.*, 1999; Wu *et al.*, 2006). Several authors have defined four to six phylogenetic clusters of freshwater *Betaproteobacteria* (Glöckner *et al.*, 2000; Zwart *et al.*, 2002). So far, research has mainly been focused on the ecology of bacteria from the beta I (also called *Rhodofera* and GKS16) and beta II (also called *Polynucleobacter necessarius*) clades (Šimek *et al.*, 2001; 2006; Burkert *et al.*, 2003; Wu and Hahn, 2006a,b; Pérez and Sommaruga, 2006; 2007; Wu *et al.*, 2006). In contrast, less is known about the cosmopolitan betaproteobacterial clusters beta III and beta IV (Glöckner *et al.*, 2000).

We investigated the diversity of *Betaproteobacteria* and the seasonal abundance fluctuations of different betaproteobacterial lineages in a prealpine soft water lake in the context of changes in physicochemical parameters and chlorophyll *a* concentrations. As this lake is characterized by stable summer stratification and an anaerobic hypolimnetic layer, focus was also put on vertical distribution patterns. In addition, potential differences between the studied populations in their preferences for amino acids were assessed.

## Results

### *Seasonal changes of water chemistry and chlorophyll a*

Piburger See was ice-covered from the beginning of our study in February 2005 until March 2005 and again from December 2005 until the end of the investigation period in February 2006. Spring mixis occurred in April 2005, but the oxygen profiles suggested only partial hypolimnetic oxygen replenishment. The lake was stratified between May and November 2005, and the metalimnion extended from 3 m depth in May to 12 m depth in November (Fig. 1). Oxygen was strongly depleted in the hypolimnion during stratification, and the anoxic water body extended up to a depth of 18 m in November 2005 (Fig. 1). Autumnal mixis occurred in early December and the aerobic zone expanded to at least 21 m depth in January 2006. Dissolved phosphorus concentrations ranged from 1.7 µg l<sup>-1</sup> in 3 m in August to a maximum of 40.9 µg l<sup>-1</sup> in 24 m depth in March. Nitrate was presumably consumed by algae in the upper layers of the lake during the growing season, whereas its concentrations in the hypolimnion strongly decreased with oxygen depletion (Fig. 1), concomitant with an accumulation of ammonium during late summer and autumn (data not shown).

Two pronounced peaks of chlorophyll *a* concentrations were observed in June–July and September–November respectively. The first maximum was mainly formed by *Chrysophyceae*. A second, late autumn phytoplankton bloom almost exclusively consisted of the diatom *Asterionella* sp.

### *Diversity and phylogenetic affiliation of freshwater Betaproteobacteria*

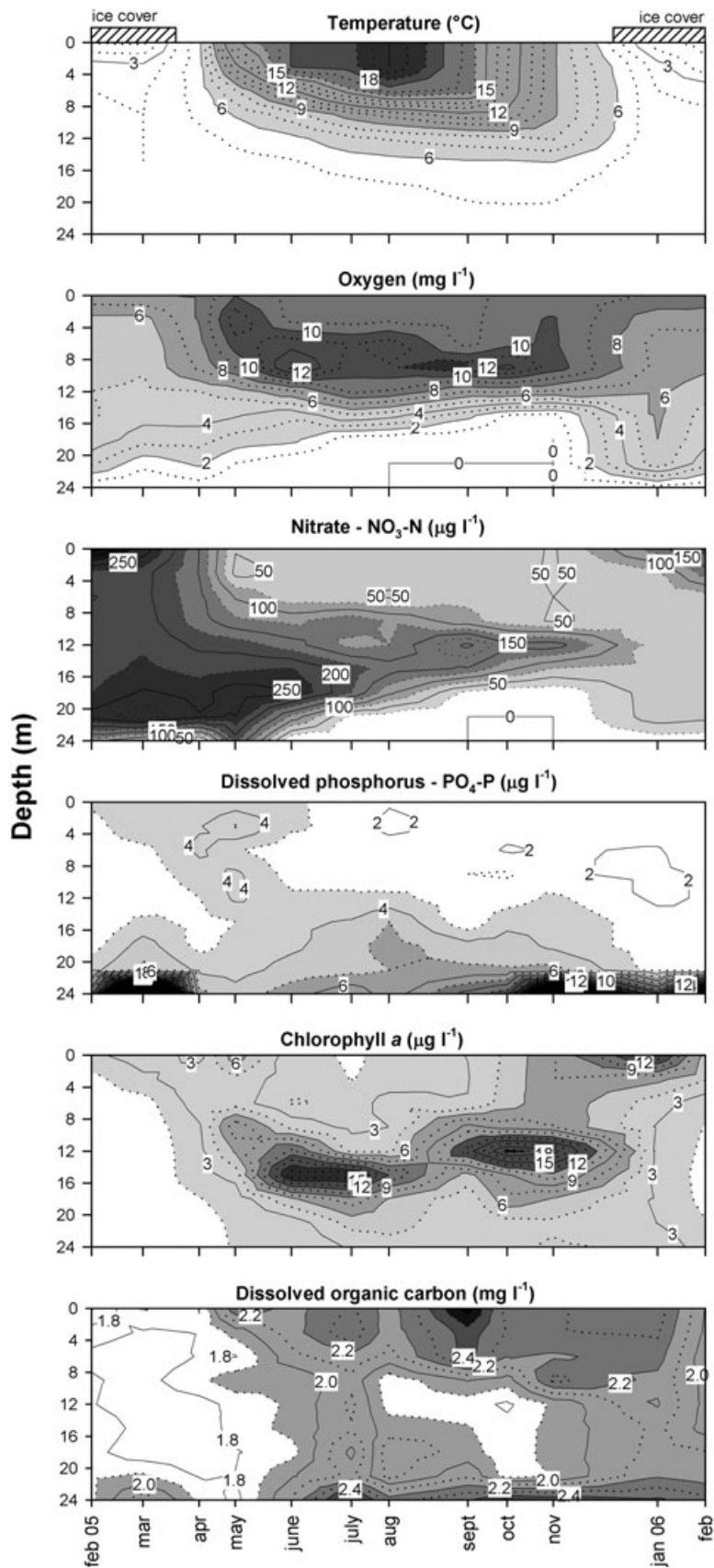
Altogether 39 nearly full-length and eight partial 16S rRNA gene sequences affiliated with *Betaproteobacteria* were obtained in four clone libraries from different sampling depths and dates by screening 229 clones in total. Three almost complete sequences were subsequently identified as chimeras and excluded from further analysis. Our sequencing effort was sufficient to sample a high proportion of the total diversity of *Betaproteobacteria* in the libraries at 98% of sequence similarity. This was indicated by: (i) a high value of Good's coverage (> 0.9) and (ii) a stable estimate of total predicted phylotypes (Chao1 estimator: 13 predicted versus 11 observed) after the analysis of at least 30 sequence types.

The majority of sequences (55%) were related to two well-separated branches within a phylogenetic lineage that is targeted by fluorescence *in situ* hybridization (FISH) probe MET1217 (Fig. 2). One of these subgroups (beta IVa) exclusively consisted of sequence types from the pelagic zone of freshwaters, whereas the other one (beta IVb) contained sequence types from a variety of habitats (wastewater treatment pools, groundwater, sediments, soil and the like), but none from the water column. Nine per cent of our sequences fell into the branch of beta I bacteria detectable by probe R-BT065 and 9% into the beta II clade (targeted by probe Bet2-870). All clones affiliated with the beta II clade were related to the species-like subcluster PnecC and targeted by probe PnecC-445. Of the remaining 12 sequences, two were affiliated with the beta III clade (GKS98 cluster), and the rest with the beta I lineage, although not detectable by probe R-BT065: two sequences were related to the *Rhodofera* sp. BAL47 clade (Zwart *et al.*, 2002), two to *Coccomonas*, four to *Rhodofera fermentans* and one to the GKS16 clade (Zwart *et al.*, 2002) and to *Leptothrix* respectively.

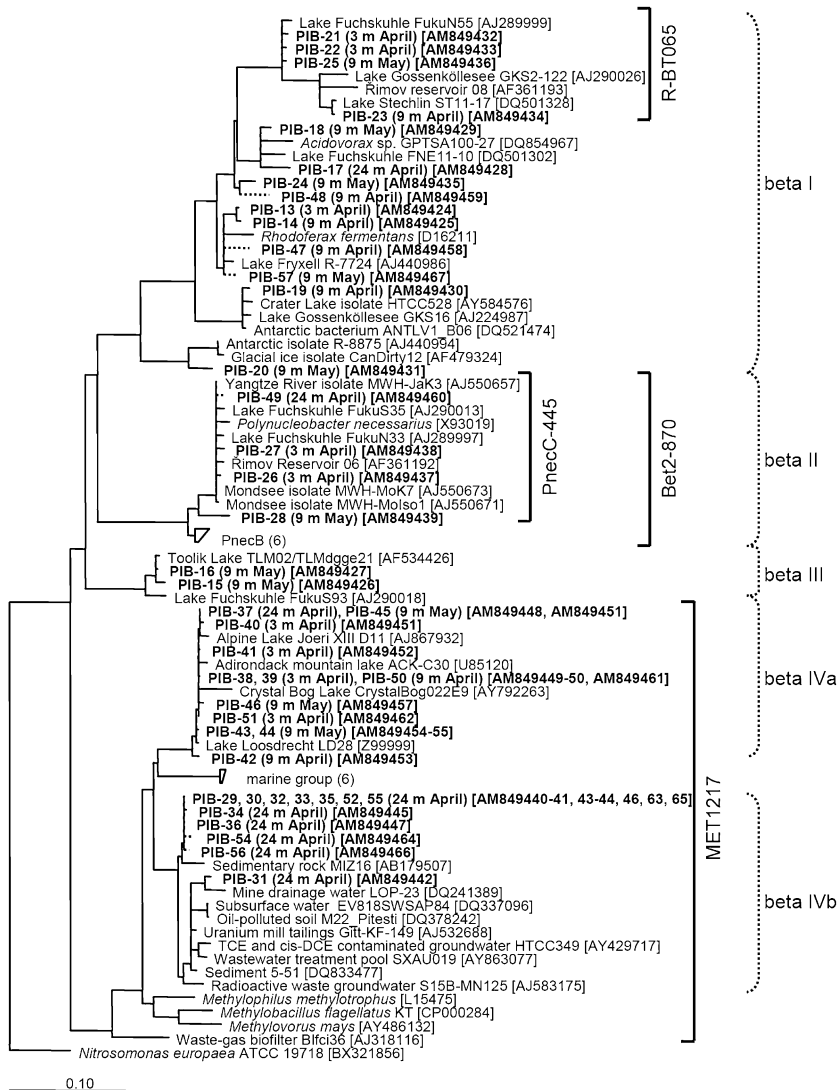
### *Seasonal and vertical distribution patterns of different lineages of Betaproteobacteria*

Between 34% and 100% of all *Betaproteobacteria* could be assigned to three phylogenetic groups by FISH with specific oligonucleotide probes (annual mean: 66%, Table 1). Microbes of the R-BT branch of the beta I lineage formed one-third of all *Betaproteobacteria* in 3 and 9 m depth, but their contribution was significantly smaller in the hypolimnetic layers (Table 1). By contrast, the fractions of *Betaproteobacteria* affiliated with the beta II clade significantly increased with depth. This was even more pronounced for beta IV bacteria, which formed only a small proportion of *Betaproteobacteria* in the epi- and metalimnion, but over 40% in the anoxic layer.

Members of the R-BT sublineage of the beta I clade were present throughout the whole water column



**Fig. 1.** Seasonal and vertical fluctuations of different environmental parameters in Piburger See during the study period (February 2005–February 2006).



**Fig. 2.** Phylogenetic affiliation of betaproteobacterial sequence types from four 16S rRNA gene clone libraries obtained at different time points and depths (in bold). Partial sequences are shown with dotted lines, and identical sequences (similarity 99.9% or higher) are presented in one line. The scale bar represents 10% estimated sequence divergence.

(Table 2, Fig. 3), where they constituted between 1.4% and 7.6% of total (4,6-diamidino-2-phenylindole, DAPI) cell counts. These bacteria showed seasonal fluctuations in the epilimnion with two maxima between April and June (3 m), and between October and November (3 and 9 m). Distinctly higher abundances of R-BT bacteria were observed in the anoxic hypolimnetic water body (24 m) during summer stratification (June and July).

Bacteria from the beta II and beta IV clades (Probes Bet2-870 and MET1217) were both most abundant in hypolimnetic, oxygen-depleted waters (Figs 4 and 5). Members of the beta II clade occurred in rather low densities in the oxygenated water column (Fig. 4). In 18 m depth, their numbers increased approximately threefold in parallel to the progressing oxygen depletion between June and July (Fig. 1). Here, they maintained high abundances until November and subsequently decreased to

**Table 1.** Annual means of the relative contributions of the studied populations to all *Betaproteobacteria* (i.e. cells hybridized with probe Bet42a) and to *Betaproteobacteria* with visible amino acid incorporation (MAR+ cells) of the studied sampling depths.

Depth	R-BT065	Bet2-870	MET1217	Sum
% of total <i>Betaproteobacteria</i>				
3 m	33.0 (A)	19.3 (A)	5.0 (A)	57.4
9 m	34.7 (A)	22.8 (AB)	6.3 (A)	63.8
18 m	17.6 (B)	22.7 (AB)	17.9 (B)	58.3
24 m	15.2 (B)	28.1 (B)	41.2 (C)	84.4
% of MAR+ <i>Betaproteobacteria</i>				
3 m	54.4 (A)	15.5 (A)	ND	69.9
9 m	58.0 (A)	13.2 (A)	ND	71.2
18 m	27.9 (B)	30.2 (AB)	9 (A)	67.1
24 m	28.9 (B)	27.6 (B)	13.7 (A)	70.2

Different letters in brackets indicate significant differences between the different depths (one way ANOVA,  $P < 0.01$ ). ND, no MAR data were determined for bacteria hybridized with probe MET1217 in these layers.

**Table 2.** Means and annual ranges (in brackets) of relative abundances (in percentage of DAPI) and of cells with visible amino acid incorporation (MAR+ cells) (in percentage of MAR+ DAPI) of the studied betaproteobacterial clades.

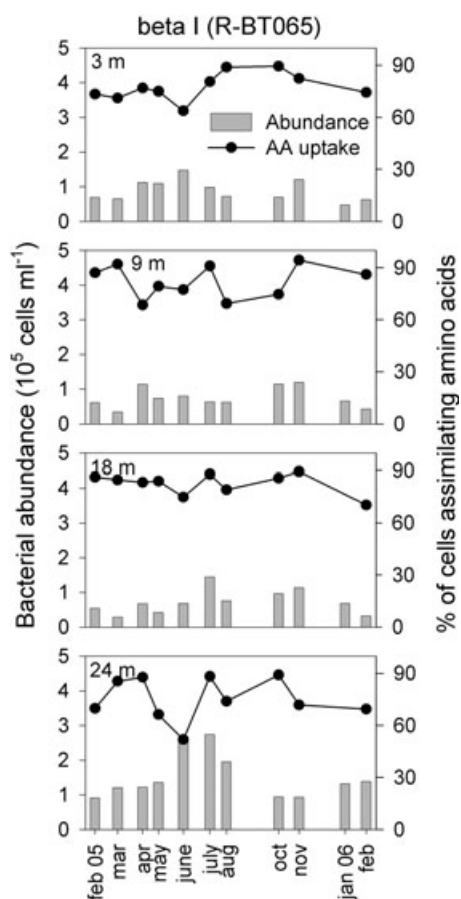
Depth	BET42a	R-BT065	Bet2-870	MET1217	PnecB	PnecC
Relative abundance (% of DAPI)						
3 m	10.9 (8–15)	3.5 (2.3–7.6)	2.1 (0.6–3.6)	0.6 (0–1.2)	0.8 (0.3–1.5)	0.8 (0.4–1.4)
9 m	9.2 (5–14)	3.0 (1.4–6.2)	2.0 (1.4–2.9)	0.6 (0.1–1.3)	0.6 (0.2–1.3)	0.6 (0.2–1.2)
18 m	14.4 (9–21)	2.4 (1.4–3.5)	3.4 (1.2–6.6)	2.7 (0.4–6)	0.6 (0.3–0.9)	2.9 (0.8–7.3)
24 m	21.5 (13–31)	3.4 (1.5–5.2)	5.7 (2.7–9.1)	8.7 (4.4–12.2)	0.5 (0.2–0.9)	5.6 (3.4–7.1)
Relative abundance of MAR+ (% of MAR+ DAPI)						
3 m	36 (12–63)	19.6 (7.9–32.8)	5.2 (2.9–12.9)	ND	ND	ND
9 m	25.9 (5–39)	14.3 (6.3–22.6)	3.4 (1.1–6.1)	ND	ND	ND
18 m	32.8 (12–63)	9.6 (7.7–12.6)	8.9 (1.2–22.2)	2.9 (0.7–5.2)	ND	ND
24 m	57.9 (22–94)	18.7 (8.3–31.2)	17.7 (5.5–28.2)	7.4 (3.7–10.7)	ND	ND

ND, no MAR data were determined.

initial numbers ( $0.6 \times 10^5$  cells  $\text{ml}^{-1}$ ). In 24 m, beta II bacteria increased in numbers between May and June and remained stable thereafter. Virtually all bacteria hybridized with probe Bet2-870 could be assigned to the sum of two specific probes for two subclades within beta II (PnecB, PnecC, Table 2). Moreover, clear differences in the vertical distribution of the two sublineages were

observed. While bacteria affiliated to PnecB and PnecC occurred in roughly equal abundances in the oxygenated water column, the bloom of beta II bacteria in the sub- and anoxic layers of Piburger See could be almost entirely attributed to microbes related to PnecC (Fig. 4).

A similar trend as observed for PnecC but even more pronounced was found for microbes affiliated with the beta IV lineage (Fig. 5). In the epi- and metalimnion (3–9 m), these bacteria were close to the lower limit of precise quantification by our FISH approach (i.e. below 0.8% of total counts) throughout the investigation period [mean  $\pm$  standard deviation,  $(0.15 \pm 0.1) \times 10^5$  cells  $\text{ml}^{-1}$ ]. Beta IV bacteria increased in numbers by > 20 times in 18 m depth between May and June, maintained high densities until November and sharply declined thereafter. By contrast, bacteria from this lineage were always abundant in 24 m depth (between  $2.6 \times 10^5$  and  $5 \times 10^5$  cells  $\text{ml}^{-1}$ ), forming distinct maxima in early summer and late winter (June and February respectively).

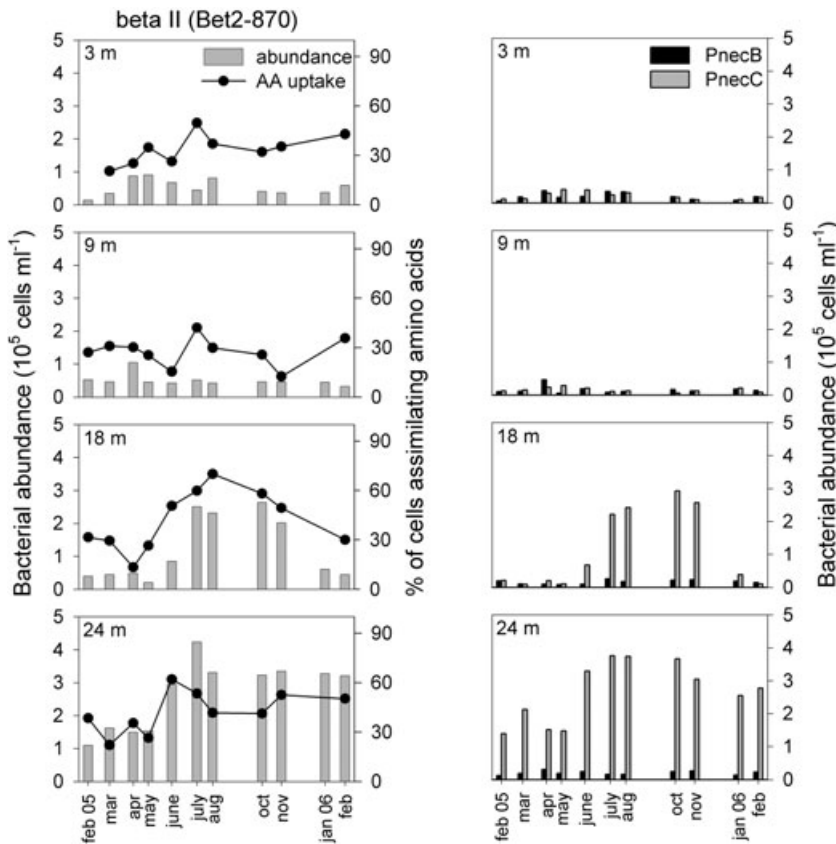


**Fig. 3.** Seasonal and vertical patterns of abundance ( $10^5$  cells  $\text{ml}^{-1}$ ) and amino acid uptake activity (AA uptake, in per cent of all probe-positive cells) of bacteria from the beta I clade (as detected by probe R-BT065).

#### *Incorporation of amino acids by different groups of Betaproteobacteria*

On average, 76% of all *Betaproteobacteria* with visible incorporation of amino acids were members of the studied subgroups. While beta I and beta IV bacteria showed only slight changes in amino acid uptake activity, microbes of the beta II lineage exhibited pronounced temporal and vertical fluctuations (Table 2, Figs 3–5).

Bacteria from the beta I clade had significantly higher fractions of active cells than the other two groups (ANOVA,  $P < 0.001$ ), and on average 79% (minimum: 52%, maximum: 94%) of cells hybridized by probe R-BT065 incorporated amino acids (Fig. 3). These bacteria represented almost 60% of all active *Betaproteobacteria* in the upper layers (3 and 9 m depth), but less than one-third in the hypolimnion (18 and 24 m depth, Table 1). Bacteria from the beta II clade showed the opposite trend, with higher contributions to active *Betaproteobacteria* in 24 m depth than in the epilimnion (Table 1). On average,



**Fig. 4.** Seasonal and vertical patterns of abundance ( $10^5$  cells  $\text{ml}^{-1}$ ) and amino acid uptake activity (AA uptake, in per cent of all probe-positive cells) of bacteria from the beta II clade (as detected by probe Bet2-870, left panels) and abundances of bacteria from the PnecB and PnecC subclades of beta II ( $10^5$  cells  $\text{ml}^{-1}$ , right panels).

approximately one-third of these bacteria incorporated amino acids (mean: 36%, minimum: 13%, maximum: 70%) (Fig. 4). The highest fractions of active beta II cells were found between June and November in 18 m, and between June and February in 24 m depth respectively. Because of the low abundances of beta IV bacteria in the upper water body, it was not possible to determine microautoradiography (MAR)-positive fractions of these bacteria in 3 and 9 m depth. Members of this lineage showed significantly (ANOVA,  $P < 0.001$ ) lower amino acid uptake activity (mean 13%, minimum: 7%, maximum: 20%) than the other studied groups in 18 and 24 m. Consequently, their contribution to total MAR-positive *Betaproteobacteria* was low (Table 1).

#### *Relationship between environmental parameters and distribution patterns of different Betaproteobacteria*

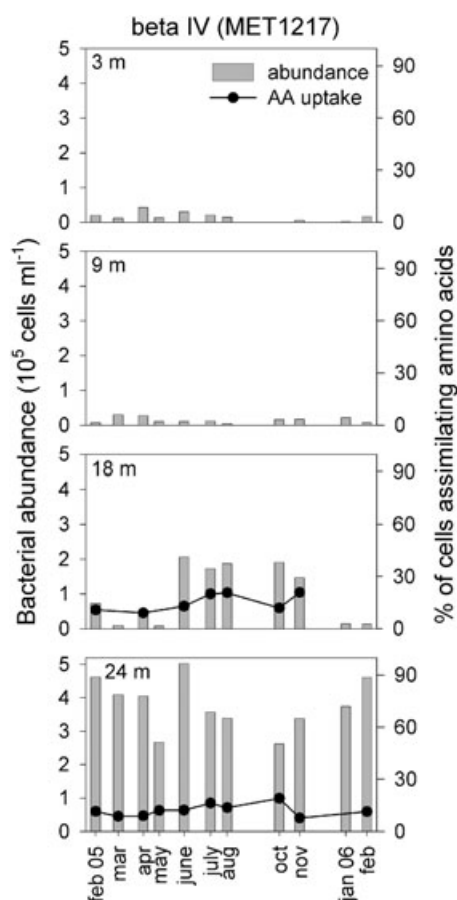
The first two axes of the redundancy analysis (RDA) model explained 99.1% of the variability in abundance and amino acid uptake of the different betaproteobacterial groups (Fig. 6). Axis 1 explained 93% of the variability and was found to correlate most highly with oxygen concentrations ( $r = -0.85$ ) and dissolved phosphorus ( $r = 0.68$ ). The second axis explained only 6.1% of the variability. Dissolved organic carbon and chlorophyll *a* concentra-

tions were identified as the most important second axis variables ( $r = -0.40$  each). Population sizes of beta IV and PnecC bacteria were clearly separated from R-BT and PnecB bacteria on the first axis of RDA, and from each other on the second one. By contrast, the abundances of R-BT bacteria were mainly related to the second axis, where they aligned with PnecC bacteria. RDA did not significantly explain changes in the fractions of active cells in either R-BT or beta II bacteria.

## Discussion

### *Occurrence of Betaproteobacteria as detected by FISH and clone libraries*

While over 80% of all *Betaproteobacteria* could be hybridized by the applied set of probes in the anoxic water body of Piburger See, FISH coverage was substantially lower in the epi- and metalimnetic samples (Table 1). Thus, other *Betaproteobacteria* might also form large populations in the lake at least seasonally. This is also suggested by sequence analysis: approximately one quarter of the sequence types in our 16S rRNA gene clone libraries were not detected by the specific FISH probes (Fig. 2). Most of these sequences were affiliated to other branches of the highly diversified beta I lineage (10) plus two



**Fig. 5.** Seasonal and vertical patterns of abundance ( $10^5$  cells  $\text{ml}^{-1}$ ) and amino acid uptake activity (AA uptake, in per cent of all probe-positive cells) of bacteria from the beta IV clade (as detected by probe MET1217). No MAR data were produced for fractions of probe-positive cells below 1.5% of total (DAPI) counts.

sequence types from 9 m depth related to the beta III (GKS98) clade. Interestingly, bacteria from the species-like PnecB lineage of beta II could be detected by FISH throughout the investigation (Fig. 4). However, no corresponding PnecB genotypes were present in our sequence collection, although different indices suggested that most betaproteobacterial diversity in the libraries at the species level had been covered.

#### Substrate incorporation patterns of different Betaproteobacteria

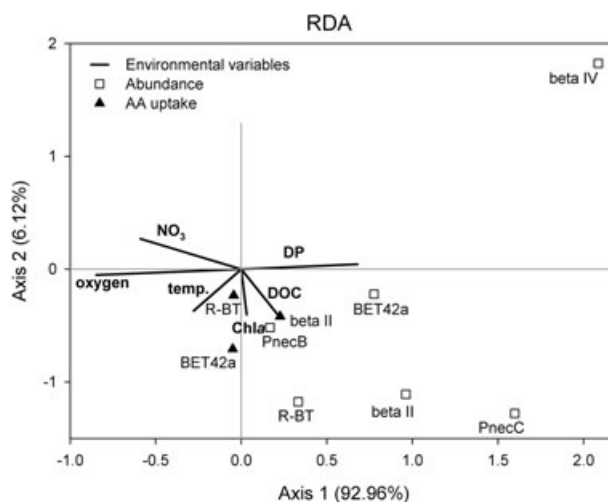
The three studied betaproteobacterial populations clearly differed in their respective incorporation of radiolabelled amino acids (Tables 1 and 2, Figs 3–5). In the epi- and metalimnion, the fractions of active bacteria from the R-BT clade were always larger than from the beta II clade, and there were always fewer MAR-positive cells in the beta IV lineage than in the beta II lineage in anoxic waters. This suggests that there might be consistent, growth rate-

independent differences in the preference of the three populations for amino acids characteristic of their respective ecophysiological niches.

Unfortunately, it was not possible to incubate MAR samples at strictly ambient oxygen conditions in the suboxic and anoxic layers of Piburger See (18–24 m). Therefore, the high fractions of MAR-positive cells from the beta I and II lineages (Figs 3 and 4) might not accurately reflect their *in situ* levels of activity, but rather indicate their potential for a facultatively anaerobic lifestyle. As amino acid uptake is dependent on ATP-driven transporter systems (Hosie and Poole, 2001), oxygen-independent energy sources might be used by these bacteria for the incorporation of the offered substrates. A facultative anaerobic metabolism of beta I and beta IV bacteria in 18 m depth was also suggested by the rather constant percentages of active cells in this layer throughout the season irrespective of the drastic changes in ambient oxygen concentrations (Figs 1, 3 and 5).

#### Members of the R-BT subclade of Betaproteobacteria

All sequences affiliated with the R-BT subcluster (and detected by probe R-BT065) originated from clone libraries from 3 or 9 m depth, suggesting that these bacteria preferably inhabited the oxygenated layers of Piburger See. However, R-BT bacteria were also present and highly active in hypolimnetic layers of the lake (Fig. 3). This hints at high ecological plasticity within this lineage, as has been observed for other freshwater *Betaproteobacteria* (Hahn 2006).



**Fig. 6.** Redundancy analysis biplot showing the different betaproteobacterial clades in cell numbers ( $\square$ ) and visible amino acid incorporation ( $\blacktriangle$ ) in relation to the strongest environmental variables. The eigenvalues of the two axes are given in brackets. Oxygen, oxygen concentrations; DP, dissolved phosphorus concentration; temp, temperature; Chla: chlorophyll a concentrations;  $\text{NO}_3$ : nitrate concentration.

The population sizes of R-BT bacteria in Piburger See showed some seasonal variability both in the epi- and hypolimnion (Fig. 3). This was also reflected in their alignment with the second axis of redundancy analysis (Fig. 6), which is most strongly related to seasonal parameters, such as chlorophyll *a* and dissolved organic carbon (DOC) concentrations (Fig. 1). Bacteria detectable with probe R-BT065 are preferably ingested by heterotrophic nanoflagellates (Jezbera *et al.*, 2006), and they are typically enriched if protistan predators are eliminated via size fractionation (Šimek *et al.*, 2001, 2005) or dilution (Pérez and Sommaruga, 2006). In fact, these microbes may compensate predation mortality by their unusually high growth rates (Šimek *et al.*, 2001, 2005; Horňák *et al.*, 2006). The R-BT bacteria were observed to rapidly multiply after addition of phosphorus (Šimek *et al.*, 2006; Salcher *et al.*, 2007), algal-derived DOC (Pérez and Sommaruga, 2007) or if transplanted from nutrient-poor to nutrient-rich environments (Šimek *et al.*, 2006). Altogether, these findings indicate that microbes from this phylogenetic lineage follow an 'opportunistic' life strategy, i.e. they respond to environmental changes by rapid increase in growth rates, but are also tightly controlled by predation.

#### *Beta II subgroup of Betaproteobacteria*

Bacteria related to the beta II lineage have been found in various freshwater habitats in different climatic zones (Hahn, 2003; Wu *et al.*, 2006). Water chemistry, in particular pH and salinity, has been identified as an important factor for the ecological differentiation of two subclusters within this lineage, PnecB and PnecC (Wu and Hahn, 2006a,b). Numerous strains of free-living, aerobic, heterotrophic ultramicrobacteria from this lineage (predominantly from the PnecC and PnecD subclades) have been isolated from various freshwaters (Hahn, 2003; Hahn *et al.*, 2005; Wu and Hahn, 2006a; Vannini *et al.*, 2007). The generally small cell sizes of beta II bacteria have also been confirmed *in situ* (Wu and Hahn, 2006a), and some strains seem to range at the lower size-dependent uptake limit of heterotrophic nanoflagellates (Boenigk *et al.*, 2004).

In the upper layers of Piburger See, the subgroups PnecB and PnecC each approximately represented half of all beta II bacteria respectively (Fig. 4). Microbes related to PnecB were present in low numbers throughout the year in all depth layers, and no pronounced seasonality could be observed. In contrast, distinct seasonality of PnecC bacteria was found in surface waters of Lakes Mondsee and Taihu, with maxima in summer and winter respectively (Wu and Hahn, 2006a). The species-like PnecC subcluster of the beta II clade harbours ecologically distinct free-living strains as well as

phylogenetically closely related obligate endosymbionts of the freshwater ciliate *Euplotes* sp. (*P. necessarius*) (Hahn, 2003; Vannini *et al.*, 2007). PnecC bacteria may persistently occur in humic lakes (Newton *et al.*, 2006), where they can form pronounced seasonal fluctuations (Burkert *et al.*, 2003) and transient blooms of up to 60% of total cells (Hahn *et al.*, 2005). However, members of PnecC are widespread across various freshwaters and have also been reported to form seasonal blooms in a shallow eutrophic subtropical lake (Wu and Hahn, 2006a).

So far, there is no information about the vertical distribution of PnecC bacteria in freshwaters. Our findings thus expand the current knowledge about the ecology of these bacteria by illustrating their potential preference for oxygen-depleted and anoxic waters: members of the PnecC lineage formed a distinct summer maximum in 18 m depth in Piburger See, and they were significantly more numerous in 24 m depth than in the two epi- and metalimnetic layers (Fig. 4). Moreover, > 50% of all cells affiliated to beta II from anoxic waters (and thus presumably to PnecC) readily incorporated amino acids, indicating *de novo* biomass production by these bacteria. It is therefore likely that members of the PnecC clade can use other electron acceptors than oxygen, or that they are capable of fermentation.

#### *Bacteria affiliated with the beta IV lineage*

All 16S rRNA gene sequences from the beta IV lineage obtained from Piburger See fell into two well-separated branches (Fig. 2). One clade (beta IVa) exclusively contains freshwater sequences of the beta IV (Glöckner *et al.*, 2000) or LD28 cluster (Zwart *et al.*, 2002). The majority of betaproteobacterial sequences in our 24 m clone library were affiliated with a second lineage (beta IVb) that harbours sequences from habitats such as wastewater treatment plants, contaminated groundwater, mine drainage water, sedimentary rocks or oil-polluted soils (e.g. Connon *et al.*, 2005; Battaglia-Brunet *et al.*, 2006). The closest taxonomically described relative of beta IV bacteria is *Methylophilus methylotrophus*. It is an obligate type I methylotrophic bacterium isolated from activated sludge (Jenkins *et al.*, 1987) which can grow only on one-carbon organic compounds, such as methanol or formaldehyde, but does not use methane as substrate (Anthony, 1982). Members of the family *Methylophilaceae* are important denitrifiers in wastewater treatment plants and some of these bacteria use methanol as a substrate *in situ* (Ginige *et al.*, 2004). Stable isotope probing in lake sediment microcosms with the <sup>13</sup>C-labelled C1 substrates methanol, methylamine and formaldehyde selectively yielded sequence types related to *Methylophilaceae* (Nercessian *et al.*, 2005).

The FISH probe MET1217 was independently designed by Friedrich and colleagues (2003) and Ginige and colleagues (2004) for studying bacteria in an industrial bio-filter and in an anoxic methanol-fed sequencing batch reactor respectively. The catalysed reported deposition-FISH (CARD-FISH) revealed a distinct vertical pattern of these bacteria in Piburger See (Fig. 5). While cells hybridized with probe MET1217 were almost absent in the epilimnion and metalimnion, they formed the dominant fraction of *Betaproteobacteria* in the hypolimnion (Table 2, Fig. 5). Because of the rather broad specificity of the probe (Fig. 2), it remains unclear if bacteria from both branches of beta IV shared the same vertical distribution. Recently, eight strains of the beta IVa cluster have been isolated from three German lakes on a mix of organic substrates under aerobic conditions (Gich *et al.*, 2005), indicating that these bacteria might be more important in epilimnetic waters. All our 16S rRNA gene sequences affiliated to the beta IVb lineage were exclusively derived from 24 m depth and other sequences from this subclade were mainly obtained from anoxic habitats (Fig. 2). Therefore, we hypothesize that predominantly methylotrophic bacteria affiliated with the beta IVb lineage were abundant in the suboxic and anoxic layers of Piburger See. This is also supported by the low fractions of cells hybridized with probe MET1217 that could incorporate amino acids (Fig. 5).

## Conclusions

The numerical importance of beta IV and PnecC bacteria in anoxic waters of Piburger See would have been missed by sampling of the upper layers only. Thus, a vertical profiling of freshwaters might substantially expand our knowledge of which bacteria are typical components of lake bacterioplankton. Moreover, we recommend studying bacterial communities at a high-resolution level, as even bacteria of such closely related clades as PnecB and PnecC or beta IVa and beta IVb might substantially differ in their niche occupation.

## Experimental procedures

### Study site and sampling

Piburger See is an oligo-mesotrophic dimictic lake situated in the Tyrolean Alps, Austria, at 913 m above sea level. Further information on the lake can be found elsewhere (Tolotti and Thies, 2002). Water samples were taken monthly over a period of 1 year (from February 2005 to February 2006) at the deepest part of the lake (24.6 m) from 3, 9, 18 and 24 m depths with a 5 l Schindler-Patalas sampler. Furthermore, samples for chemical and phytoplankton analyses were taken in a 3 m interval (nine samples from 0 to 24 m depths). Water temperature was directly determined during sampling.

No samples could be collected in December 2005 because of the unstable ice coverage of the lake.

Portions of 40 ml from each sample were fixed either with formaldehyde (2% final concentration) for determination of bacterial abundance, or with freshly prepared buffered paraformaldehyde (pH 7.4, 2% final concentration) for analysis by CARD-FISH. All formaldehyde-fixed samples were stored at 4°C until further processing. In addition, unfixed water samples were collected for MAR-FISH (MAR combined with CARD-FISH) analyses (40 ml), for the construction of 16S rRNA gene clone libraries (1 l), and for chemical analyses (2 l). These samples were delivered to the lab at *in situ* temperature within 2 h. The following chemical parameters were evaluated: oxygen concentrations ( $\text{mg l}^{-1}$ ; Winkler's method, detection limit:  $0.1 \text{ mg l}^{-1}$ ), concentrations of chlorophyll *a* ( $\mu\text{g l}^{-1}$ ; spectrophotometric measurement), nitrate ( $\text{NO}_3\text{-N}$ ,  $\mu\text{g l}^{-1}$ ; spectrophotometrical determination after reduction with sodium salicylate Seignette salt), dissolved phosphorus ( $\text{PO}_4\text{-P}$ ,  $\mu\text{g l}^{-1}$ ; molybdate method after digestion with  $\text{H}_2\text{SO}_4$  and  $\text{H}_2\text{O}_2$ ) and DOC ( $\text{mg l}^{-1}$ ; high-temperature catalytic oxidation with a Shimadzu TOC analyser). Samples for phytoplankton analysis (100 ml) were fixed with Lugol's solution and counted with an inverted microscope with phase contrast.

### Bacterial abundances

A total of 2–2.5 ml of formaldehyde-fixed samples were stained with DAPI ( $6.7 \mu\text{g ml}^{-1}$  final concentration, Porter and Feig, 1980), filtered onto black polycarbonate filters (Osmonics,  $0.22 \mu\text{m}$  pore size, 25 mm diameter) and evaluated with an epifluorescence microscope (Zeiss Axioplan, Carl Zeiss, Germany). At least 1000 bacteria (Zeiss filter set 01) were counted per sample at a total magnification of 1600 $\times$ .

### 16S rRNA gene clone libraries and phylogenetic sequence analysis

Unfixed water samples were pre-filtered through  $3 \mu\text{m}$  filters to remove larger organisms. Subsequently, 300–600 ml were filtered onto white polycarbonate filters (Millipore, Type GTTP,  $0.2 \mu\text{m}$  pore size, 47 mm diameter). Filters were air-dried and stored at  $-80^\circ\text{C}$  until further processing. Samples taken in spring 2005 from different depths (i.e. April: 3, 9 and 24 m depths, and May: 24 m depth) were processed to produce four clone libraries. Small pieces of the filters were used as template for polymerase chain reaction (PCR) as previously described (Kirchman *et al.*, 2001) using the primers GM3F and GM4R (*Escherichia coli* positions 8–24 and 1492–1507; Muyzer *et al.*, 1995). The PCR products were purified with the QIAquick PCR purification kit (QIAGEN), inserted into TOPO vectors (TOPO TA cloning kit for sequencing; Invitrogen) and cloned into competent cells of *E. coli* according to the manufacturer's instructions. After screening of the clones for right-sized inserts, plasmid preparations were done with the QIAprep Spin Miniprep Kit (QIAGEN) or with the Montage Plasmid Miniprep96 Kit (Millipore). The sequencing reactions were accomplished with the M13F vector primer and the ABI BigDye chemistry on an ABI 3130x Genetic Analyzer (Applied Biosystems). Partial sequences were first analysed with the

BLAST queuing system (<http://www.ncbi.nlm.nih.gov/blast/>) for their phylogenetic affiliations. Nearly full-length sequences of all unique sequence types affiliated with *Betaproteobacteria* were obtained by additional sequencing with vector primer M13R (Messing, 1983) and primer GM1 F (Muyzer *et al.*, 1993).

Partial sequences were assembled with the Sequencher software (Gene Codes) and checked for chimeric origin using the software Pintail (Ashelford *et al.*, 2005). Phylogenetic analyses were performed with the ARB software package (Ludwig *et al.*, 2004). The ARB database was complemented with betaproteobacterial sequences deposited in GenBank that were closely related to our sequences. All sequences were first automatically aligned using the ARB tool FAST\_ALIGNER and alignments were subsequently manually optimized. For the reconstruction of phylogenetic trees, only nearly full-length (i.e. longer than 1400 nucleotides) sequences were considered. A 50% base frequency filter and a filter designed with betaproteobacterial sequences were used to exclude highly variable positions. Maximum parsimony, neighbour-joining and maximum likelihood analyses were performed with the respective ARB tools. The resulting trees were compared manually to obtain a consensus tree. Partial sequences were added to this consensus tree in accordance with maximum parsimony criteria, without allowing changes in the tree topology.

#### Abundances of different Betaproteobacteria

A total of 10–15 ml of paraformaldehyde-fixed samples were filtered onto white polycarbonate filters (Millipore, Type GTTP, 0.2 µm pore size, 47 mm diameter), rinsed with distilled water, air-dried and stored at –20°C until further processing. CARD-FISH was carried out as previously described (Sekar *et al.*, 2003) with horseradish peroxidase-labelled oligonucleotide probes specific for *Betaproteobacteria* (BET42a, used in combination with the unlabelled competitor GAM42a, Amann *et al.*, 1995) and three subclades within this group: R-BT065 (closely related to *Rhodoferrax* sp. BAL47, belonging to the beta I cluster, Šimek *et al.*, 2001), Bet2-870 (*P. necessarius* or beta II cluster, Burkert *et al.*, 2003) and MET1217 (order *Methylophilales*, Friedrich *et al.*, 2003) which includes freshwater bacteria from the beta IV cluster (Glöckner *et al.*, 2000). In addition, the probes PnecB-23S-116 and PnecC-445 (Wu and Hahn, 2006a) were used for the discrimination of two subclusters within the beta II clade. Signal amplification was performed using tyramides labelled with Alexa488 (Invitrogen). Filter sections were counterstained with DAPI (1 µg ml<sup>-1</sup>) and inspected with an epifluorescence microscope (Axiophot, Carl Zeiss) at a magnification of 1250× and the filter sets 01 (DAPI) and 10 (Alexa 488). For manual evaluation of the fractions of hybridized cells, at least 500–1000 DAPI-stained cells were counted.

The majority of FISH and MAR-FISH preparations were evaluated by automated cell counting using a set of manual counts for system calibration (Pernthaler *et al.*, 2003). Evaluations were performed with a fully automated system consisting of an epifluorescence microscope (AxioImager.Z1, Carl Zeiss) with a motorized stage for eight microscopic slides (Zeiss WSB Piezodrive 05) and a CCD Camera (AxioCam

MRm, Carl Zeiss), linked to a personal computer with the image analysis software AxioVision 4.6 (Carl Zeiss). A 63× 'PlanApo' objective was used for image acquisition. Automation was achieved using the Visual Basic for Application module of AxioVision (M. Zeder, unpublished). Briefly, the microscope was loaded with up to eight slides, overview images were automatically acquired using a 1× EC 'Plan-Neofluar' objective and filter pieces were detected. A x,y-position list was generated containing a user-defined number of locations equally distributed on each filter piece. Two images (DAPI and Alexa 488 fluorescence) were acquired at each microscopic field. After image acquisition and an automated quality control routine to discard images of low quality, a counting routine automatically processed all images and detected the fractions of hybridized bacteria within all DAPI-stained cells.

In order to better assess counting precision, five duplicate hybridizations were evaluated for each probe. Only insignificant differences between duplicate determinations were observed (paired *t*-test, *P* > 0.2).

#### Microautoradiography combined with CARD-FISH

Five millilitres of samples were incubated with [<sup>3</sup>H]-amino acids (5 nM final concentration, 48 Ci mmol<sup>-1</sup> specific activity, Amersham) for 1 h at *in situ* temperatures. After fixation with buffered paraformaldehyde (pH 7.4, 2% final concentration) duplicate subsamples of 2 ml were filtered onto white polycarbonate filters (Millipore, type GTTP, 0.2 µm pore size, 25 mm diameter), rinsed twice with sterile water and stored at –20°C until further processing. After CARD-FISH staining of filter sections (see above), MAR was performed as previously described (Alonso and Pernthaler, 2005). Briefly, filter sections were glued onto glass slides with 2% agarose (Seakem), dipped into autoradiography emulsion (NTB emulsion, Kodak) in the darkroom, placed on ice for 10 min and exposed in the dark at 4°C for 2–4 days. Thereafter, slides were developed with Dektol developer (Kodak) and Kodak fixer (Kodak) following the manufacturer's instructions. After drying, filters were embedded in a mounting medium containing DAPI (1 µg ml<sup>-1</sup>). The evaluation of MAR-FISH filters was carried out by fully automated image analysis as described above, but an additional stack of nine bright field images in 0.5 µm steps above the z-position of the DAPI image was acquired to detect the silver grains (M. Zeder, unpublished). No samples for MAR-FISH were collected in January 2006, and no MAR-FISH samples were evaluated in which the fractions of probe-positive cells were below 1.5% of total (DAPI) counts.

#### Statistical analyses

Prior to statistical analyses, all data of relative abundances (percentages) were arcsine-transformed to obtain normal distribution, whereas cell numbers and chemical variables were log(x + 1)-transformed. We used ANOVA to test for significant differences between the different depth layers and for differences in the amino acid uptake between betaproteobacterial clades. Furthermore, paired *t*-tests were carried out. The programme R (<http://www.r-project.org>) was used for these statistical analyses.

The coverage of the diversity of *Betaproteobacteria* in our clone libraries (Good's C index, Good, 1953) and the estimated total diversity ( $S_{\text{Chao1}}$  estimator, Chao, 1984) were calculated using 98% of sequence similarity as cut-off for the classification of different sequence types. Analyses were performed with the online input form created by Kemp and Aller (2004).

Redundancy analysis was used to determine the effects of environmental variables on betaproteobacterial abundances and the fraction of cells with visible amino acid incorporation (Stewart and Love, 1968). Only those environmental variables exhibiting a significant correlation ( $P < 0.001$ ) to betaproteobacterial abundances or amino acid uptake were included in the analysis. Activity patterns of beta IV bacteria were excluded from analysis because of the high number of missing data points. The significance of added variables was tested by a Monte Carlo permutation test (499 permutations,  $P < 0.001$ ). Analyses were performed with the Microsoft EXCEL add-in program XLSTAT-ADA (<http://www.xlstat.com>).

#### Nucleotide sequence accession numbers

All 16S rRNA gene sequences have been deposited to EMBL under the Accession Numbers AM849424 to AM849467.

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