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Contrasting trends in distribution of four major planktonic betaproteobacterial groups along a pH gradient of epilimnion of 72 freshwater habitats

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Abstract

The distribution and abundance of *Betaproteobacteria* and three of its genera: *Limnohabitans* genus (R-BT065 lineage), *Polynucleobacter* genus (including two subclusters *P. necessarius* and *P. acidiphobus*/*difficilis*), and *Methylophilus* genus across the epilimnion of 72 limnologically diverse freshwater habitats was investigated using fluorescence *in situ* hybridization. Moreover, seasonal development of *Betaproteobacteria* subgroups along the axis of a longitudinally heterogeneous reservoir was followed. *Betaproteobacteria* comprised on average 29.1%, *Polynucleobacter* 11.6%, *P. necessarius* 10.1%, *P. acidiphobus*/*difficilis* 0.5%, *Limnohabitans* 8.9%, and *Methylophilus* 0.9% of total bacterioplankton cells in the investigated habitats. *P. necessarius* and *Limnohabitans* coexisted in the majority of habitats but showed contrasting abundance patterns along the pH gradient of habitats (pH 3.8–8.5). The observed distribution patterns could be explained by different preferences for substrate sources, i.e. substances of humic origin in acidic waters and algal-derived substances in alkaline waters. However, substrate utilization patterns observed in laboratory experiments indicate no coherent group-specific differences in substrate preferences. Interestingly, similar distribution patterns were revealed for *Limnohabitans* and *P. acidiphobus*/*difficilis*, suggesting similar ecological adaptations of these only distantly related taxa. Our findings further emphasize that at least two major taxa of freshwater *Betaproteobacteria* represent ecologically diversified groups. Investigations at higher phylogenetic resolution are required for obtaining further insights into the ecology of these important taxa.

Keywords

Polynucleobacter; *Limnohabitans*; ecological diversification; competition

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Introduction

Betaproteobacteria represent one of the key components of freshwater bacterioplankton (Hiorns et al., 1997; Glöckner et al., 2000, Barberan et al., 2010) constituting 23% (three lakes, Glöckner et al., 2000) to 70% of freshwater bacterioplankton (one habitat, Hahn et al., 2005). Freshwater bacteria affiliated with the *Betaproteobacteria* have formerly been divided into four (Glöckner et al., 2000), six (Zwart et al., 2002) or seven lineages (Newton et al., 2011). However, recent studies (e.g. Hahn et al., 2005; Lindström et al., 2005; Salcher et al., 2008; Jezberová et al., 2010; Šimek et al., 2010a) indicated that in a vast majority of cases, only two of these lineages, i.e. BetI and BetII (Newton et al., 2011), are responsible for the overall abundance of the *Betaproteobacteria* in freshwater habitats. These two groups are mainly represented by the genus *Limnohabitans* (Hahn et al., 2010a) and especially by its R-BT065 lineage (Šimek et al., 2001; Kasalický et al., 2010) – a monophyletic cluster within this genus – as well as by the genus *Polynucleobacter* (Heckmann and Schmidt, 1987; Hahn et al., 2009). So far, much less is known on the other five lineages of *Betaproteobacteria*, of which only BetIII and BetIV have received some attention (Glöckner et al., 2000, Friedrich et al., 2003, Salcher et al., 2008, Newton et al., 2011).

The genus *Limnohabitans* constitutes, together with the genus *Rhodoferax*, the BetI lineage (Newton et al., 2011). So far, *Limnohabitans* harbors four validly described species (Hahn et al., 2010a; Hahn et al., 2010b; Kasalický et al., 2010).

The genus *Polynucleobacter*, synonymous with PnecABCD (Wu and Hahn, 2006a), and BetII (Newton et al., 2011), is currently represented by five species, which are more or less equivalent to previously designated subclusters (Hahn, 2003).

A wealth of information is now available on *Limnohabitans* and *Polynucleobacter* bacteria, including information on ecophysiology (Hahn et al., 2009; Kasalický et al., 2010), intraspecific ecological differentiation (Jezbera et al., 2011), seasonality (e.g. Crump et al., 2003; Hahn et al., 2005; Wu and Hahn, 2006b), abundance and distribution (e.g. Hahn 2003; Wu et al., 2006; Buck et al., 2009; Jezberová et al., 2010), habitat preferences (Hahn 2003, Hahn et al., 2005, Šimek et al., 2010a), vertical distribution (Wu and Hahn, 2006b; Salcher et al., 2008), niche separation (Šimek et al., 2010b, Jezbera et al., 2011), grazing vulnerability (Šimek et al., 2001, Boenigk et al., 2004, Jezbera et al., 2005), etc. On the other hand, almost all this knowledge has been generated from studies that focused on a single or only a few habitats, or on populations of microorganisms belonging to only one of the lineages, or from results gained from manipulation experiments. What is entirely missing is a comprehensive investigation of the distribution and abundance patterns of the major groups of *Betaproteobacteria* across a large number of freshwater systems representing a broad ecological spectrum of the habitat types that we offer here.

While several studies have focused on *Polynucleobacter* and *Limnohabitans*, considerably less is known on the other groups of freshwater *Betaproteobacteria*. In this comprehensive study we intended to close this gap by investigating the abundance of four major *Betaproteobacteria* groups: the genus *Limnohabitans*, the genus *Polynucleobacter* and two

of its subgroups, and the genus *Methylophilus* across a large set of 72 systems representing a broad spectrum of habitats, covering for example a pH gradient ranging from 3.8 to 8.5. The major aim of the study was to convincingly document the overall numerical importance and the hypothesized contrasting ecological roles of the two key groups of *Betaproteobacteria*, i.e. *Limnohabitans* and *Polynucleobacter*, not only among *Betaproteobacteria* alone, but also among the whole bacterioplankton community. In addition, to cover the distribution patterns of the major *Betaproteobacteria* groups, we decided to use other available genetic probes specific for other betaproteobacterial groups or subgroups of the two mainly studied genera. Last but not least, we intended to explore the fundamental trophic niches of the distinct isolates by following their substrate utilization patterns.

The foremost goals of the presented study thus were (i) to reveal the distribution patterns of the four key subgroups of *Betaproteobacteria*, (ii) to identify environmental drivers that determine their distribution and abundance, (iii) to identify conditions under which the two major genera, *Polynucleobacter* and *Limnohabitans*, co-occur, (iv) and to investigate if they are potentially competing for certain resources. Based on the presented results, we intended to create a generalizing picture on the distribution of *Betaproteobacteria* across ecologically diverse habitat types serving as a tool for directing future studies on *Betaproteobacteria* diversity.

Material and Methods

Sampling of habitats

(i) Distribution of taxa across environmental gradients—In total, 72 lentic freshwater habitats (Supplementary Table S1) representing a pH gradient from 3.8 to 8.5 were sampled between 2006 to 2008 (from June to November, respectively). The primary study area was represented by the Salzkammergut Lake District, a mountainous area with numerous lakes and ponds located on the northern slope of the Alps close to the city of Salzburg (Austria). Apart from this area, several other habitats located in Austria and the Czech Republic were sampled. A detailed list of a set of habitats including the ones presented here can be found elsewhere (Jezberová et al., 2010). The selected 72 habitats represented the largest possible variety of lakes, small natural and artificial ponds and puddles differing in a multitude of parameters. Almost all habitats were sampled at a depth of 0.5 meters.

(ii) Temporal and spatial distribution of taxa in a single habitat—A seasonal distribution study was performed on the canyon-shaped meso-eutrophic Římov reservoir located near the city of České Budějovice (Czech Republic). The reservoir was sampled along its longitudinal axis at three sites (assigned as DAM, MIDDLE and RIVER) in three-week intervals between March and November 2005, as described previously (Šimek et al., 2008).

Determination of physicochemical parameters of the water samples

Temperature, pH, oxygen concentration and conductivity were measured on site. In the habitat survey, dissolved organic carbon (DOC) and concentrations of humic substances

were estimated photospectroscopically, as described previously (Supplementary Table S1 and Jezberová et al., 2010). In the seasonal study of the Římov reservoir, DOC, dissolved reactive phosphorus (DRP), particulate reactive phosphorus (PRP), total phosphorus (TP), particulate phosphorus (PP), total primary production (PP_{tot}), primary production in 1-2 μl filtrate ($PP_{1\text{-}2\mu l}$) and chlorophyll a (ChlA) were measured as described in Šimek *et al.* (2008).

Determination of bacterial abundance

Subsamples were fixed with formaldehyde (2% final concentration), stained with DAPI, (Sigma), filtered onto black 0.2 μm-pore-size Nuclepore filters (Osmonic Inc., Livermore, USA) and enumerated using an epifluorescence microscope under UV excitation at a magnification of 1250×. At least 500 bacterial cells were enumerated.

Absolute and relative abundance of selected bacterial taxa

The abundances of *Betaproteobacteria* and of major *Betaproteobacteria* subgroups – the genus *Limnohabitans*, *the PnecC subcluster* (*P. necessarius*) *and the PnecB subcluster* (*P acidiphobus* and *P. difficilis*) and the genus *Methylophilus* – were determined by Catalyzed Reporter Deposition Fluorescence *in situ* Hybridization (CARD-FISH) following the protocols of Pernthaler *et al.* (2002) and Sekar *et al.* (2003). The CARD-FISH probes deployed and their specificity are listed in Tab. 1.

Incorporation of radioactively labeled substrates assessed by MAR-FISH

To track bacterial cells with active biomass synthesis and the potential production of storage matter, duplicate samples and formaldehyde-fixed blanks of 5 ml were incubated with either L-[^{3}H]-leucine (Leu, final concentration 20 nmol l^{-1}, specific activity 6.4 TBq $mmol^{-1}$, MP Biomedicals) or D-[^{3}H]-glucose (Glc, final concentration 20 nmol l^{-1}, specific activity 2.22 TBq $mmol^{-1}$, MP Biomedicals). Samples were incubated for 2 h at *in situ* temperature in the dark, preserved in formaldehyde (final concentration 2%) and filtered onto 0.2 μm polycarbonate filters, as described previously (Horňák *et al.*, 2006). Filters were rinsed with Milli-Q water, air-dried, and kept frozen (−20°C) until further processing. After the CARD-FISH procedure, the filters were transferred onto slides coated with autoradiography emulsion (NTB, Kodak). After 24 to 48 h of exposure in the dark, the cells were stained with DAPI (final concentration 1 μg ml^{-1}). The relative abundances of hybridized cells were enumerated by epifluorescence microscopy. At least 500 DAPI-stained cells were counted per sample. MAR-FISH experiments were performed with water samples from Loipersbacher Pond 1 and 2 (acidic ponds, Hahn et al., 2005), Lake Mondsee (alkaline, deep lake, Wu and Hahn, 2006a), and the Římov Reservoir (circum-neutral canyon-shaped reservoir, this study).

Substrate assimilation analyses

Previously published as well as unpublished data on substrate utilization by *Limnohabitans* and *Polynucleobacter* strains (Hahn et al., 2009, Kasalický et al., 2010, Hahn et al.,2010a+b +c;Hahn et al.,2011a+b; Hahn et al., in press; Kasalický unpubl. data; Hahn unpubl. data) were compiled and analyzed. All the established data were obtained by following the same

protocol. Briefly, growth of bacterial isolates based on utilizing specific substrates was determined by comparison of the optical density measured at 575 nm (OD_{575}) in liquid one-tenth-strength NSY medium (0.3 g/l, Hahn, 2003) with and without 0.5 g of the test substrate. Differences in OD_{575} of 10, 10–50 and 50% and more compared with growth on the medium lacking the test substance after 10 days of growth were scored as no utilization (−), weak utilization (w) and good utilization (+), respectively.

Statistical analysis

The program CANOCO (TerBraak and Šmilauer, 1998) was used for the multivariable analysis. Redundancy analyses (RDA) were performed, and the results were visualized by CanoDraw for Windows (TerBraak and Šmilauer, 1998).

Results

Occurrence of Betaproteobacteria groups along the pH gradient of investigated habitats

Total bacterial numbers differed strongly between the sampled habitats (Fig. 1, upper panel). *Betaproteobacteria* formed almost one third (29.1%) of all heterotrophic bacteria when averaged across all 72 habitats. Differences in *Betaproteobacteria* numbers were more pronounced in acidic habitats (pH 3.8-7.4), ranging there from 1.5 – 72% of all bacteria. In the alkaline pH range from 7.4 to 8.5, *Betaproteobacteria* numbers were more stable, ranging from 11.3 to 44.8% (Fig. 1). On average, *Betaproteobacteria* contributed less to total bacterial numbers in alkaline habitats than in acidic ones (Fig. 1, lower panel), which can be attributed to the dominant role of PnecC bacteria in the acidic habitats (Fig. 1). In acidic habitats, *Betaproteobacteria* contributed, on average, 34.3% to the total bacteria, in contrast to alkaline habitats where they constituted 23.9% of all bacteria, on average.

Limnohabitans bacteria contributed on average to about 8.9% of bacterioplankton cells and showed a clear trend of increasing abundance with increasing pH. However, they were also present, though in smaller quantities, in habitats of lower or very low pH (close to 3.8). In the alkaline range of the pH gradient, at approximately pH 7.5 and higher, *Limnohabitans* bacteria were clearly the dominant group among *Betaproteobacteria* (Fig. 1).

The entire genus *Polynucleobacter* contributed on average to 11.6% of bacteria in the investigated samples. The vast majority of the detected *Polynucleobacter* cells were affiliated with the species *P. necessarius* (Fig. 1). This species alone accounted for approximately 10% of all bacteria. The B-lineage of *Polynucleobacter* (i.e. PnecB consisting of *P. acidiphobus* and *P. difficilis*) comprised about 0.5% of bacteria detected across all studied habitats. A clear trend in the distribution of these two taxa was observed. The B-lineage was more abundant exclusively in the alkaline part of the sampled habitat range, being most abundant in the large prealpine lakes of the Salzkammergut area (Fig. 1 and 2). *P. necessarius* showed a completely opposite trend, displaying a clear preference for low-pH habitats, where its abundance ranged between 5 to 60% of all detected bacteria. This species was also detectable, however, in alkaline habitats, though in much lower percentages. The three species *P. necessarius*, *P. difficilis* and *P. acidiphobus* constituted together almost 90% of all *Polynucleobacter* bacteria.

In contrast to *Limnohabitans* and *Polynucleobacter*, bacteria belonging to the genus *Methylophilus* were never highly abundant, reaching on average only 0.9% of all bacteria and displaying no clear trend across the investigated habitats (Fig. 1).

Importantly, by using the CARD-FISH probes for *Polynucleobacter*, *Limnohabitans* and *Methylophilus*, we were able to cover on average almost three quarters (72.3%) of all *Betaproteobacteria* across the wide range of sampled habitats.

Comparison of substrate assimilation patterns

No clear pattern in substrate utilization that would clearly separate *Limnohabitans* from *Polynucleobacter* bacteria was observed (Tab. 2). Moreover, large differences in patterns even among strains of the same genus were observed for both genera. Pronounced differences in utilization patterns for strains of the same species (e.g. *P. necessarius*) were also obvious.

Even when using a large array of substances, we were unable to find a sole substrate utilized by all members of one genus and not utilized at all by all members of the other genus (Tab. 2). The only distinct traceable trends were higher preferences of *Limnohabitans* for monosaccharides (namely fructose, glucose, mannose etc.) and certain amino acids (L-alanine) contrasting with no or very weak utilization of these substances by *P. acidiphobus*, *P. difficilis*, and *P. necessarius*. Another important result was a positive and strong preference of *Limnohabitans* and *P. necessarius* for acetate as opposed to no utilization of acetate by *P. acidiphobus* and *P. difficilis* (PnecB lineage).

Activity measurements by MAR-FISH

Uptake of two radioactively labeled substances (leucine and glucose) by *Polynucleobacter* and *Limnohabitans* bacteria (targeted by the R-BT065 probe) were investigated in four habitats representing three limnologically contrasting habitat types. Uptake of both substrates by the two bacterial groups was observed in all four habitats (Supplementary Table S2). In acidic Loiperbacher ponds 1 and 2 (habitats #11 and 12 in Supplementary Table S2), as well as in large, alkaline Lake Mondsee (habitat #51 in Supplementary Table S2), *Polynucleobacter* displayed a markedly lower affinity for leucine than did *Limnohabitans* bacteria. On the other hand, glucose was similarly assimilated by both groups. In the meso-eutrophic, circum-neutral Římov reservoir (Supplementary Table S2), similar trends were observed. On average, 92% of *Limnohabitans* bacteria incorporated leucine as opposed to approximately 56% of *Polynucleobacter* bacteria actively incorporating leucine. A similar pattern was observed for the utilization of glucose, where approximately 81% of *Limnohabitans* and only 45% of *Polynucleobacter* bacteria incorporated this substrate.

Factors influencing the distribution of the major Betaproteobacteria groups across 72 habitats and during one season in the Římov reservoir

In 2005, temporal and spatial development of the B-lineage of *Polynucleobacter* (*P. difficilis* and *P. acidiphobus*), *P. necessarius*, and *Limnohabitans* bacteria were followed at the DAM, MIDDLE and RIVER stations of the Římov reservoir (Fig. 3). The B-lineage and *P.*

necessarius showed clearly contrasting trends. In the DAM area, which is primarily supplied by autochthonous primary production, the B-lineage markedly dominated over *P. necessarius* during most of the season, as opposed to the allochthonously loaded RIVER station, where *P. necessarius* formed the major part of the whole *Polynucleobacter* assemblage. In the MIDDLE station both groups alternated. Note that on the 23rd of August, when sampling was not feasible due to a flood event, the high *P. necessarius* numbers, normally typical of the RIVER station, were projected as far as to the MIDDLE station. Redundancy analyses (RDA) were performed for the set of 72 distinct habitats (Fig. 4A) as well as for the seasonal data from the Římov reservoir (Fig. 4B). Both RDA analyses confirmed contrasting roles of the B-lineage and *P. necessarius*. The B-lineage (*P. difficilis* and *P. acidiphobus*) was positively related to changing ChlA concentrations, primary production, extracellular primary production (EPP) and temperature, and was more abundant in the "lake" part of the Římov reservoir (Fig. 4B). In contrast, *P. necessarius* bacteria showed a positive correlation with dissolved reactive phosphorus and humic substances, being more abundant in the inflow ("river" part) of the reservoir. *Limnohabitans* bacteria did not show any trend within the Římov reservoir, while their distribution patterns were clearly indicated in the highly heterogeneous set of 72 habitats (Fig. 4A). In this analysis, the B-lineage of *Polynucleobacter* (PnecB) and *Limnohabitans* preferred habitats with higher pH, higher conductivity and lower amounts of humic substances, whereas *P. necessarius* was more abundant in habitats at higher altitude. Interestingly, *P. necessarius*, compared to PnecB bacteria and *Limnohabitans*, displayed exactly opposite relationships to the investigated environmental parameters. *Methylophilus* bacteria showed no clear distribution trend across the 72 investigated habitats (data not shown).

Discussion

Polynucleobacter and *Limnohabitans* bacteria as omnipresent components of freshwater systems

In the 72 freshwater habitats investigated, *Polynucleobacter* and *Limnohabitans* bacteria together formed the vast majority of *Betaproteobacteria* (on average more than 70%). However, it is important to note that the FISH probe used for the detection of *Limnohabitans* bacteria does not target the entire genus but only an assumed core lineage within this diverse taxon (Table 1; Kasalický et al., 2010). Other lineages within the genus *Limnohabitans*, not detected by the probe, also represent bacteria frequently inhabiting the pelagic zones of freshwater systems (Zwart et al., 2002; Lindström et al., 2005; Hahn et al., 2010ab). For instance, the type species of *L. curvus* and some closely related strains have been isolated from Lake Mondsee, which was included in the set of habitats investigated here. Due to the lack of complete coverage of the genus by the probe, underestimation of the contributions of the entire genus *Limnohabitans* to total bacterioplankton is quite likely. However, due to the lack of suitable FISH probes, it is currently impossible to estimate how significant this underestimation is.

In this study we used three independent FISH probes to cover the B and C lineages of *Polynucleobacter* as well as the entire genus *Polynucleobacter*. Based on previous studies, where the numerical abundance of A and D lineages was found to be negligible (Wu and

Hahn, 2006a), we decided not to deploy probes targeting these two lineages. This decision is well supported by the fact that probes specific for the B and C lineages detected on average 91.3% of all *Polynucleobacter* bacteria enumerated by using the genus-specific probe.

In contrast to the other two genera studied, the genus *Methylophilus* constituted only a small part of the bacterial communities, as well as of the whole *Betaproteobacteria* assemblages in the investigated habitats. However, it is important to note that we have mainly investigated epilimnetic samples that were more or less oxygen-saturated. We did not sample deeper, oxygen more depleted water layers that were reported to be richer in *Methylophilus* bacteria (Salcher et al., 2008). Potentially, this sampling strategy could have resulted in an underestimation of the importance of this bacterial taxon. Just recently (Hutalle et al., 2010), it has been demonstrated that bacteria such as *Methylophilus* can be enriched on phenol or humic matter additions that might to a certain extent link these microbes to direct degradation of humic substances and probably not so much to the utilization of methane produced in anaerobic zones. The study of Hutalle et al., can not however be generalized, since only few isolates and clones were analyzed. If this assumption would be true one would expect a tight relation of *Methylophilus* spp. numbers to concentration of humic matter which was not so far confirmed.

We have omitted a detailed analysis of the environmental drivers controlling distribution and abundance of *Methylophilus* bacteria because the cell numbers determined by FISH were frequently close to the detection limit, resulting in low accuracy of the determined data. Aside from this, these data indicate also a rather negligible role of these bacteria in the overall carbon flow of the systems studied.

We believe that the observed distribution pattern of major groups of *Betaproteobacteria* can be generalized for the majority of freshwater habitats worldwide.

There are inevitably other factors that may influence the distribution of a certain bacterial taxon, to name just a few: water retention time, dispersal and biotic interactions. Considering the amount of habitats sampled, none of these was unfortunately in the scope of our study. Nonetheless, especially the biotic factors came just recently into attention (Eiler et al., 2011) showing that certain network modeling can predict that biotic factors (including the presence of specific organisms, be it bacteria or higher organisms) can shape network structures. Subsequently it can influence the presence or absence of key species including microorganisms.

(ii) Contrasting distribution patterns of bacteria along the pH gradient—We observed the coexistence of *Polynucleobacter* and *Limnohabitans* bacteria in the majority of investigated lakes, but the two groups showed also rather contrasting abundance patterns regarding pH of the habitats (Fig. 1). *Limnohabitans* occurred, on average, with higher relative and absolute abundances in circum-neutral and alkaline habitats, while the opposite trend was observed for the genus *Polynucleobacter*. However, the two *Polynucleobacter* subgroups considered in our study display both differences in total abundance as well as opposite abundance patterns along the pH gradient (Fig. 1, lower panel). Newton and coworkers (2011) also demonstrated a negative correlation of *P. necessarius* (PnecC

subcluster) occurrence and a positive correlation of the *Polynucleobacter* B-lineage (PnecB subcluster) with lake pH. Interestingly, in this meta-analysis, the R-BT065 lineage of *Limnohabitans* was split in two subclusters (Lhab-A1 and Lhab-A2) for which opposite - i.e. positive and negative – pH correlations were revealed (Newton et al., 2011).

Several previous investigations revealed pH as one of the key drivers, or even as the strongest driver influencing bacterioplankton composition (Lindström et al., 2005; Yanarell and Triplett, 2005), or unveiled significant distribution differences of related taxa based on lake pH (Schauer et al., 2005; Newton et al., 2007). Despite the well-documented role of pH as the factor strongly shaping bacterioplankton composition (Lindström et al., 2005), it is not known if pH acts as a direct factor linked to pH adaptation of the respective bacteria or as an indirect factor influencing other growth conditions. In the case of *Polynucleobacter* and *Limnohabitans* bacteria, the distribution trends observed cannot simply be explained by differences in pH adaptation. The MAR-FISH investigations indicated that populations of both taxa, actively incorporating selected substrates, were present in all four investigated habitats, which represented a pH range of more than three units (pH 4.7 to 8.3, Supplementary Table S2). Other investigations performing similar MAR-FISH experiments on other habitats previously indicated that metabolically active bacteria affiliated with the genus *Polynucleobacter*, and especially with the species *P. necessarius*, are present in habitats strongly differing in pH (Buck et al., 2009; Alonso et al., 2009; Salcher et al., 2010). Additionally, recent findings suggested differences in pH preferences across subgroups of *P. necessarius* (Jezbera et al., 2011).

An alternative explanation for the opposite distribution trends observed could be utilization of different major substrate sources. Previous analyses indicated utilization of different substrate pools for *Limnohabitans* (R-BT065 lineage) and *P. necessarius*. Šimek *et al.* (2008) proposed that *Limnohabitans* bacteria mainly rely on algal-derived substrates, i.e. utilize direct or indirect products of autochthonous primary production. Moreover, it has been documented that *L. planktonicus* and *L. parvus* grow well even in diluted exudates produced by axenic cultures of typical planktonic algae (Šimek et al., 2011). In contrast, bacteria affiliated with the species *P. necessarius* are believed to utilize mainly photooxidation products of humic substances (Watanabe et al., 2009; Jezberová et al., 2010, Hahn et al., submitted) – i.e., they utilize allochthonous organic matter that is, at least partially, of terrestrial origin. In a previous analysis by Jezberová et al., (2010), which investigated a similar set of habitats, it was revealed that pH of habitats and concentrations of humic substances were negatively correlated. On the other hand, the majority of circum-neutral and alkaline habitats studied in the current study represent non-humic lakes with low allochthonous DOC input, which should favor bacteria utilizing autochthonous production.

A previous investigation indicated that not all free-living *Polynucleobacter* rely on humic substances. Wu and Hahn (2006b) proposed that *Polynucleobacter* bacteria of the B-lineage mainly utilize substrates derived from algal primary production and are frequently found in stock cultures of various algae as accompanying bacteria (Šimek et al., 2011). Thus, utilization of similar substrate pools was suggested for *Limnohabitans* (R-BT065 lineage) and *P. acidiphobus*/*P. difficilis*. Interestingly, both PnecB and *Limnohabitans* groups share similar distribution patterns along the investigated pH gradient.

Both PnecB and *Limnohabitans* appear in alkaline and circum-neutral habitats in higher relative abundances and in lower proportions in some of the acidic habitats (Fig. 1). Detection of the *Polynucleobacter* B-lineage in acidic habitats is in disagreement with the previously reported lack of detection in acidic waters (Wu and Hahn, 2006b). These contradicting observations may have resulted from differences in the sensitivity of the FISH methods deployed (CARD-FISH versus FISH).

Previously, there have been numerous studies documenting the effect of protozoan grazing on bacterial community composition. The effect of grazing on *Limnohabitans* and *Polynucleobacter* bacteria specifically can be found for instance in Boenigk et al., 2004, Jezbera et al., 2005 and Šimek et al., 2007. It was documented that bacteria belonging to the *Limnohabitans* genus (partially represented by the R-BT065 cluster) are highly susceptible to grazing and are selectively grazed upon by protists. The manuscript by Hahn et al. (submitted) provides a thorough discussion on the effect of grazing on a specific lineage within the *Polynucleobacter* cluster.

Generally, the above mentioned studies on grazing on *Limnohabitans* and *Polynucleobacter* bacteria seem to suggest that the natural predation mortality of *Limnohabitans* bacteria is higher than that of *Polynucleobacter* bacteria; however, large intra-genus differences in predation vulnerability can be expected for both taxa.

(iii) Environmental gradient analysis versus single habitat analysis—Opposite distribution patterns of *P. necessarius* and *Limnohabitans* (R-BT065 lineage) along broad pH gradients (Fig. 1) were also observed in separate previous investigations (Jezberová et al., 2010; Šimek et al., 2010a). By contrast, our presented seasonal study of the Římov reservoir revealed no significant differences in the relation of the two taxa to parameters related to primary production or phytoplankton biomass. Since *Limnohabitans* growth is expected to depend on a substrate pool derived from algal production (Šimek et al., 2011), the two taxa could be expected to reveal different relationships to these variables. However, the pH and conductivity values in the Římov reservoir remained virtually unchanged during the season, showing only small diurnal fluctuations (data not shown). This hints that other variables may be responsible for the segregation of these two groups, apart from self-correlating ones such as pH and conductivity. Interestingly, no direct significant relationship of *Limnohabitans* bacteria to ChlA was found, strongly contrasting with its positive relationship to certain algal groups, e.g. cryptophytes (Šimek et al., 2008). Jones and co-workers (2009), however, recently suggested a proxy, i.e. the ratio of water color to chlorophyll A (the CtCH ratio), which indicates the dominance of allochthonous versus autochthonous organic carbon sources available for bacteria. This proxy showed significant correlation with the relative proportions of R-BT065 bacteria in total *Betaproteobacteria* (Šimek et al., 2010a), which further corroborates the finding on enhanced proportions of the *Limnohabitans* bacteria in non-humic habitats where autochthonous organic carbon sources dominate.

On the other hand, the B-lineage of *Polynucleobacter* shows a positive correlation with the above-mentioned variables related to primary production and phytoplankton biomass (Fig. 4), which agrees with previous findings (Wu and Hahn, 2006b).

(iv) Lack of taxon-specific trends in substrate utilization patterns—The spectrum of substrates used in substrate utilization tests included several types of substances (e.g. acetate, pyruvate and other short-chain fatty acids) reported as typical products of photooxidation of humic substances (Moran and Zepp, 1997), as well as substances reported as algal exudates (e.g. carbohydrates, Giroldo et al., 2007). If *P. necessarius* and *Limnohabitans* tend to utilize different substrate pools, one could expect different preferences in the assimilation tests. Obviously, the performed tests indicated no taxon-specific trends in substrate preferences, although one has to take into account that these tests were performed at significantly higher substrate concentrations than usually inherent in the water environment. The predictive power of the utilization tests performed should thus not be overemphasized. It may be that the investigated taxa differ in substrate affinity, but not in substrate uptake potential, i.e., the ability to absorb available substrate. Only the latter was tested by the substrate utilization tests.

We are aware of the fact that by using relatively high substrate concentrations for testing of the utilization in the lab, we might have overlooked differences between strains in substrate affinity, which could play an important role in niche partitioning. On the other hand, the revealed small genome sizes of *Polynucleobacter* bacteria (Hahn et al., submitted; Vannini et al., 2007) suggest that these bacteria can only encode a rather small number of substrate utilization pathways.

(v) Potential limitations of ecological analyses caused by ecological diversification of taxa—Several contradictions revealed above indicate that the current pictures of the ecology of *P. necessarius* and *Limnohabitans* (R-BT065) are too simple. Several recent findings suggest intra-taxon ecological diversification resulting in differently adapted ecotypes for both groups (Šimek et al., 2010b; Jezbera et al., 2011). It seems that – at least for future ecological investigations – a taxonomy with a higher phylogenetic resolution as well as molecular tools enabling detection and quantification of these taxa will be required. We demonstrated that the two taxa *P. necessarius* and *Limnohabitans* represent together the majority of *Betaproteobacteria* in a broad range of habitat types. However, it seems that the postulated within-taxon diversity currently limits further insights into the ecological function of these bacterial groups.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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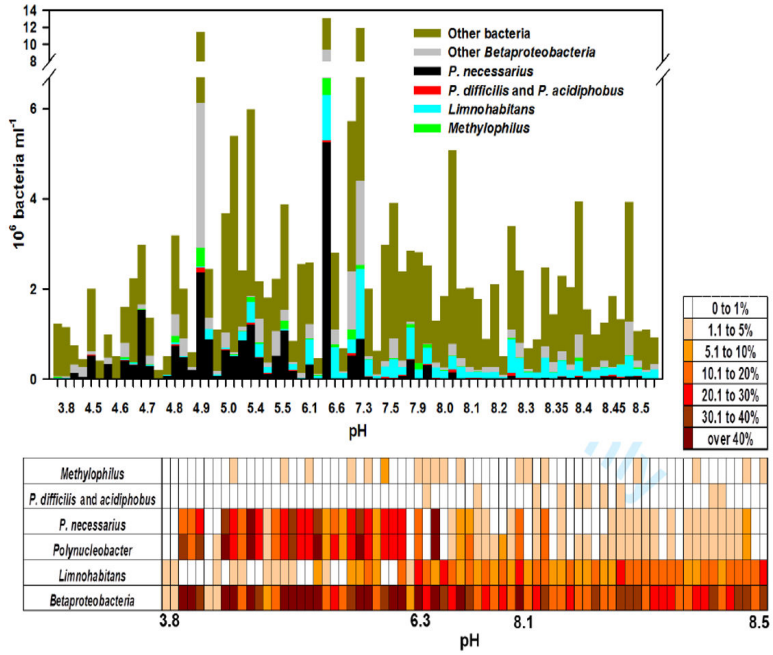

Fig. 1.

Upper panel, distribution of subgroups of *Betaproteobacteria* along the pH gradient (from 3.8 to 8.5) of 72 different habitats in absolute cell numbers. Lower panel, heat map of the relative (%) proportions of distinct *Betaproteobacteria* groups along the gradient of increasing pH (from 3.8 to 8.5) of the investigated 72 habitats. Relative proportion classes used are 0-1%, 1.1-5%, 5.1-10%, 10.1-20%, 20.1-30%, 30.1-40%, and over 40%.

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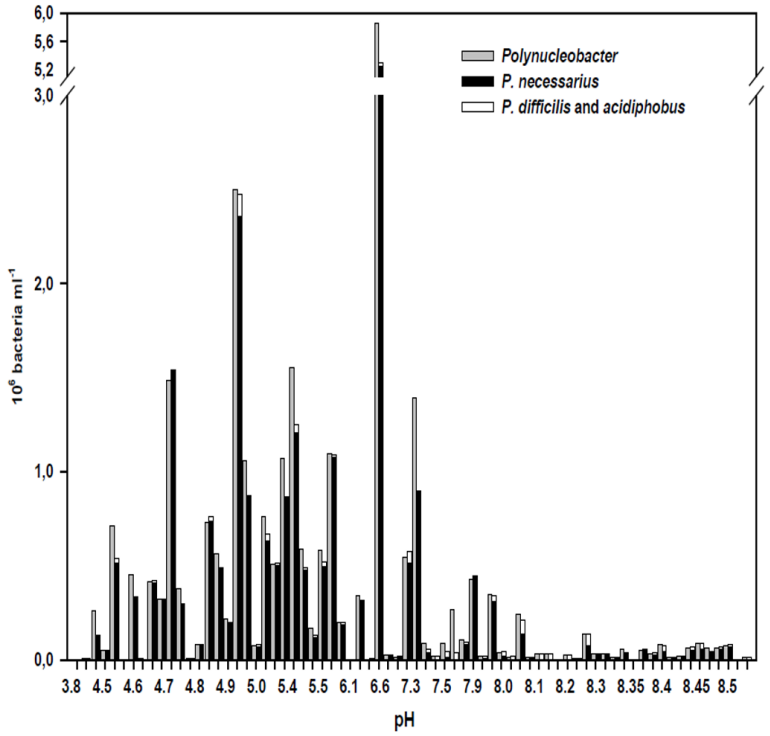

Fig. 2.

Abundance of the B-lineage of the genus *Polynucleobacter* (*P. difficilis* and *P. acidiphobus*), *P. necessarius* and the entire *Polynucleobacter* genus as detected by respective FISH probes along the pH gradient of the 72 investigated habitats.

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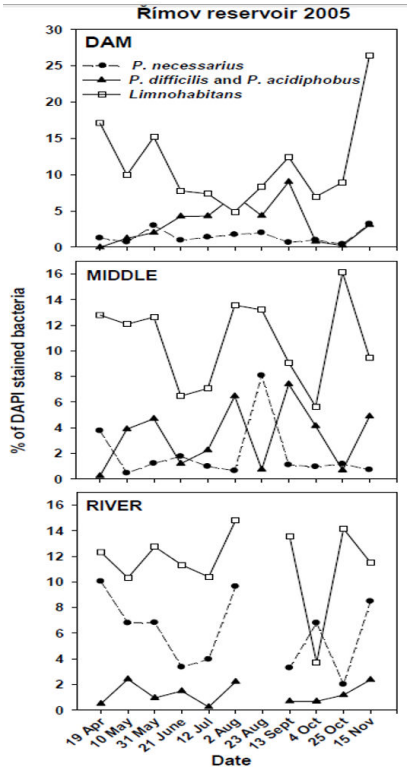

Fig. 3.

Distribution of relative proportions of the major *Betaproteobacteria* subgroups as detected by FISH at the DAM, MIDDLE and RIVER stations located along a longitudinal transect of the canyon-shaped Římov reservoir in the period from mid of March until mid of November 2005.

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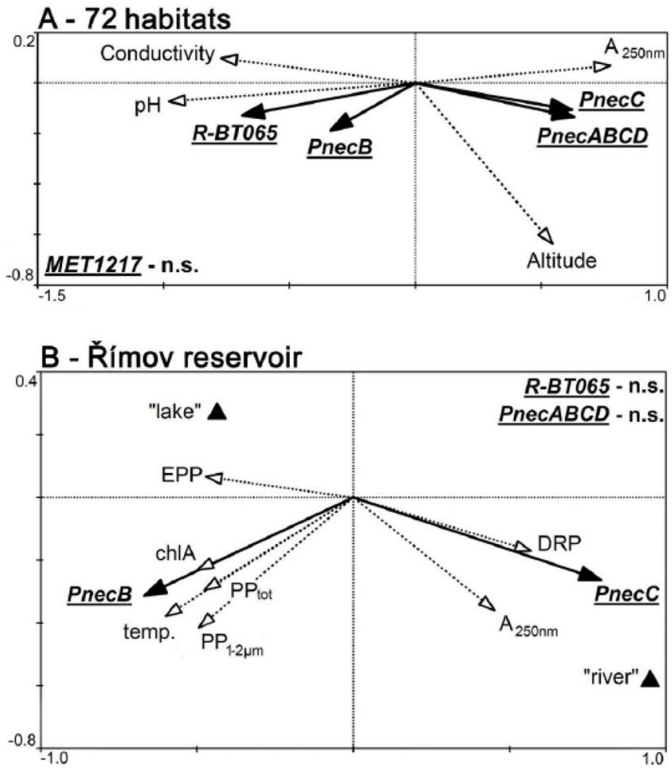

Fig. 4.

Panel **A** – 72 different habitats, Panel **B** – the Římov reservoir, a seasonal study. Redundancy analysis (RDA) showing only the significant parameters responsible for the distribution of the entire *Polynucleobacter* genus (labeled as ***PnecABCD***); *P. necessarius*, (***PnecC***); the B-lineage of *Polynucleobacter*, (***PnecB***, representing *P. difficilis* and *P. acidiphobus*); *Limnohabitans*, (***R-BT065***) bacteria; and *Methylophilus* genus (***MET1217***). **PP_{tot},** total primary production; **A_{250nm},** absorption at 250 nm (DOC proxy); **DRP**, dissolved reactive phosphorus; **PP_{1-2um},** primary production in the 1-2 µm size fraction; **chlA**, chlorophyll a; **temp.**, temperature; **EPP**, extracellular primary production; **"lake"** triangle represents pooled data for the station DAM and MIDDLE; **"river"** triangle represents data for the RIVER stations (see Methods section); **n.s.,** not significant.

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Table 1

Oligonucleotide CARD-FISH probes used in this study, their specificity and reference papers.

Probe	Specificity	Reference
BET42a	*Betaproteobacteria*	Manz et al. (1992)
R-BT065	R-BT lineage within the *Limnohabitans* genus	Šimek et al. (2001)
PnecABCD-445	*Polynucleobacter* genus (= PnecABCD)	Hahn et al. (2005)
PnecC-16S-445	*Polynucleobacter necessarius* (= PnecC)	Hahn et al. (2005)
PnecB-23S-166	*P. acidiphobus* & *P. difficilis* (= PnecB)	Wu & Hahn (2006)
Met1217	*Methylophilus* genus	Friedrich et al. (2003)

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Table 2

Substrate utilization by *Polynucleobacter* and *Limnohabitans* isolates. Adopted from Kasalický et al., 2010, Hahn et al., 2010abc, Hahn et al., 2011ab, Hahn et al., in press, Kasalický unpublished data , Hahn unpublished data. White, no assimilation; grey, weak assimilation; black, 3 good assimilation (for details see Methods section). Note that *L. curvus* and *L. australis* are not targeted by the R-BT065 FISH probe.