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Maximum growth rates and possible life strategies of different bacterioplankton groups in relation to phosphorus availability in a freshwater reservoir

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Summary

We investigated net growth rates of distinct bacterioplankton groups and heterotrophic nanoflagellate (HNF) communities in relation to phosphorus availability by analysing eight *in situ* manipulation experiments, conducted between 1997 and 2003, in the canyon-shaped Římov reservoir (Czech Republic). Water samples were size-fractionated and incubated in dialysis bags at the sampling site or transplanted into an area of the reservoir, which differed in phosphorus limitation (range of soluble reactive phosphorus concentrations – SRP, 0.7–96 μg l^{-1}). Using five different rRNA-targeted oligonucleotide probes, net growth rates of the probe-defined bacterial groups and HNF assemblages were estimated and related to SRP using Monod kinetics, yielding growth rate constants specific for each bacterial group. We found highly significant differences among their maximum growth rates while insignificant differences were detected in the saturation constants. However, the latter constants represent only tentative estimates mainly due to insufficient sensitivity of the method used at low *in situ* SRP concentrations. Interestingly, in these same experiments HNF assemblages grew significantly faster than any bacterial group studied except for a small, but abundant cluster of Betaproteobacteria (targeted by the R-BT065 probe). Potential ecological implications of different growth capabilities for possible life strategies of different bacterial phylogenetic lineages are discussed.

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Introduction

Bacterioplankton communities play an important role in the flow of energy and nutrients through plankton food webs as a consequence of their high abundance, efficient nutrient uptake and large growth potential (Azam *et al.*, 1983; Sanders *et al.*, 1992). Heterotrophic bacteria are thought to be superior competitors for phosphorus (P) to phytoplankton in a variety of pelagic environments, mainly in oligotrophic ones (for review see Cotner and Biddanda, 2002). Indeed, some studies conducted in oligotrophic freshwater lakes have indicated that the lack of available P, not carbon, could be the factor limiting bacterial growth (e.g. Toolan *et al.*, 1991; Coveney and Wetzel, 1992). There is now overall growing evidence indicating that growth of heterotrophic bacteria is often limited by inorganic nutrients (especially P) in many different kinds of ecosystems (Chrzanowski *et al.*, 1995; Elser *et al.*, 1995; Cotner and Biddanda, 2002), even in relatively productive ones.

Not surprisingly then, sudden changes in bottom-up controlling factors (i.e. limiting nutrients) yield marked shifts in lake bacterioplankton bulk properties: abundance, production and biomass (e.g. Chrzanowski *et al.*, 1995; Schweitzer and Simon, 1995). However, little is known concerning the details of such changes in bacterioplankton. Which individual bacterial groups shift in relative proportions or absolute abundance in the community as a response to sudden changes in the availability of different nutrients in freshwaters has rarely been studied under *in situ* conditions (e.g. Gasol *et al.*, 2002; Selje and Simon, 2003; Šimek *et al.*, 2003). For such studies, typical canyon-shaped reservoirs are particularly useful. They differ from typical, relatively homogenous lake ecosystems

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quite conspicuously by having relatively short retention times and displaying pronounced longitudinal heterogeneity in nutrient availability and plankton succession from a river inflow to lacustrine reservoir parts (Armengol *et al*., 1999; Gasol *et al*., 2002; Mašín *et al*., 2003). Remarkably different combinations of bottom-up and top-down factors control bacterioplankton dynamics in different parts of the same water body at the same time offering thus ideal sites for studying shifts in bacterioplankton community composition (e.g. Gasol *et al*., 2002; Šimek *et al*., 2003; 2005).

The canyon-shaped Římov reservoir longitudinally spans from eutrophy, in the upper inflow parts (generally richer in nutrients), to mesotrophy in the dam area of the reservoir in terms of total P and soluble reactive phosphorus (SRP) (Mašín *et al*., 2003). The dam area of the reservoir could be temporarily even severely nutrient-limited with concentrations of SRP $< 1\ \mu g\ l^{-1}$ frequently accompanied with microbially mediated enhanced turnover rate of orthophosphate (Nedoma *et al*., 1993). Thus, transplanting bacterioplankton from the nutrient-poor dam area upstream to the P-richer sites markedly stimulated bulk bacterioplankton production as well as changes in the growth and mortality rates of different phylogenetic lineages of bacteria (Šimek *et al*., 2003; 2005; Jezbera *et al*., 2005). As water moves through the system, resources needed for bacterial growth as well as grazing pressure vary.

Though some general features or examples of strategies have been suggested, little is known about ecological strategies that can ensure bacterial survival and competitiveness under conditions of varying nutrient availability and grazing pressure (e.g. Cotner and Biddanda, 2002; Thingstad *et al*., 2005). The lack of the knowledge in this field raises some intriguing questions: How is the growth rate of different bacterial groups related to P-availability in a freshwater environment on one hand, or to the growth rate of their major grazers, bacterivorous heterotrophic nanoflagellate (HNF), on the other? Can we detect fast growing, opportunistic strategists having a capability to respond flexibly to environmental perturbations? Does our knowledge about growth capabilities and vulnerability to predation of different bacterioplankton groups allow us to tentatively detect major features of their survival strategies? One possible way to investigate at least some aspects of this 'hot topic' is to study growth responses of phylogenetically defined bacterial groups to different experimental manipulations *in situ*.

Our objective was to analyse, using a Monod kinetic approach, net growth rate parameters of several bacterioplankton groups detected by means of fluorescence *in situ* hybridization (FISH)-probes related to SRP concentrations, used as a tentative indicator of the amount of bioavailable phosphate (Moutin *et al*., 2002), occurring in different parts of the canyon-shaped Římov reservoir. A series of eight manipulation experiments was conducted, most of them employing an approach allowing the simultaneous assessment of the influence of bottom-up and top-down factors (see e.g. Šimek *et al*., 2003; 2005) by incubating size-fractionated samples in dialysis bags allowing a relatively free exchange of inorganic and organic solutes present in different parts of the reservoir. Bacteria in bacterivore-free ($< 0.8\ \mu m$) and HNF in zooplankton-free ($< 5\ \mu m$) treatments grew for a period of several days at ambient temperature and nutrient/bacterial prey concentrations allowing estimating of *in situ* net growth rates of the target population.

We found that the different groups of bacteria targeted by the probes were characterized by distinct Monod kinetic parameters. While maximum bacterioplankton growth rates were estimated to occur at similar concentrations of phosphorus, the maximum growth rates differed significantly among the groups. We found that the members of phylogenetically narrow R-BT065 cluster (96% minimum 16S rRNA sequence similarity), which is affiliated with the broad '*Rhodoferax*' sp. BAL47 cluster (Zwart *et al*., 2002), showed the highest growth rates followed by the larger groupings of Beta- and Gamma-subclasses of the class Proteobacteria, then the lowest values were estimated for the Cytophag/Flavobacterium/Bacteroidetes (CFB) group and Actinobacteria (ACT) group.

Results

All eight experiments overviewed in Table 1 (assigned as I–VIII) were designed primarily to study the impacts of top-down and bottom-up manipulations on bacterioplankon community composition. The results of these specifically focused studies were either published (the experiments I–VI, Šimek *et al*., 1999; 2001; 2003; 2005), or they have been submitted for publication (experiments VII and VIII, Jezbera *et al*., 2006 and K. Horňák, unpublished, respectively). However, the influence of P-availability on growth capabilities of different bacterial subgroups was not considered in any of these studies. Here we examined growth rates as a function of SRP concentrations among five phylogenetically defined bacterial groups (as well as HNF communities) under a broad range of SRP concentrations (for details see Table 1).

Using a size fractionation approach, bacteria in the $< 0.8\ \mu m$ treatments and HNF in the $< 5\ \mu m$ treatments grew released from predation. Incubation in dialysis bags ensured that bacterial growth was limited by the ambient resource availability inherent in different parts of the reservoir. This experimental set-up consistently resulted in increases of cell concentrations. Representative results of the experiment III and VIII are depicted in Figs 1 and 2 as examples of the fast growth of bacteria and HNF.

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Table 1. Overview and timing of eight *in situ* manipulation experiments (I–VIII) conducted in the Římov reservoir during the period of 1997–2003.

Experiment/timing	Incubation site/ treatment	Incubated in	Temperature range (°C)	SRP ($\mu g\ l^{-1}$)	Bacterioplankton (< 0.8 μm treatments) Interval (h)	Bacterioplankton (< 0.8 μm treatments) Probe-defined bacterial populations detected	Heterotrophic nanoflagellates (< 5 μm treatments) Interval (h)	Heterotrophic nanoflagellates (< 5 μm treatments) Doubling time (h)
I. 9–13 June 1997	DAM	Dialysis bag	18–19	7	0–48	BET, GAM, CFB	0–48	10.8 ± 1.1
II. 12–18 September 1997	DAM	Dialysis bag	17–19	0.7	0–72	BET, GAM, CFB	0–72	22.6 ± 2.1
III. 28 May–1 June 1999	DAM	Dialysis bag	18–20	4.5	0–48	BET, GAM, CFB, R-BT	24–72	15.1 ± 2
IV. 12–16 June 2000	DAM at DAM	Dialysis bag	23–24	2	0–48	BET, GAM, CFB, R-BT	48–72	16 ± 2
	DAM→RIVER	Dialysis bag	18–19	96	0–48	BET, GAM, CFB, R-BT	48–72	8.3 ± 2
V. 21–25 May 2001	RIVER at RIVER	Dialysis bag	12–13	32	0–48	BET, GAM, CFB, R-BT, ACT	48–72	16 ± 0.9
	RIVER→DAM	Dialysis bag	16–17	4	0–48	BET, GAM, CFB, R-BT, ACT	48–72	10.8 ± 0.5
VI. 20–24 May 2002	DAM at DAM	Dialysis bag	18–20	2.1	0–48	BET, GAM, CFB, R-BT, ACT	24–72	8.6 ± 1
	DAM→MIDDLE	Dialysis bag	18–20	23	0–48	BET, GAM, CFB, R-BT, ACT	24–72	5.8 ± 0.4
	DAM→RIVER	Dialysis bag	13–16	59	24–72	BET, GAM, CFB, R-BT, ACT	96–144	10.5 ± 1.6
	DAM at DAM	Bottle	18–20	2.1	0–48	BET, GAM, CFB, R-BT, ACT	24–72	11.8 ± 1.7
VII. 9–13 September 2002	DAM – control	Dialysis bag	17–19	1.9	24–72	BET, GAM, CFB, R-BT, ACT	0–48	15.1 ± 1.5
	DAM + P	Dialysis bag	17–19	62	0–48	BET, GAM, CFB, R-BT, ACT	24–48	7.4 ± 1.4
	DAM + Glu	Dialysis bag	17–19	1.9	24–72	BET, GAM, CFB, R-BT, ACT	0–48	15.5 ± 2.3
	DAM + P +Glu	Dialysis bag	17–19	62	0–48	BET, GAM, CFB, R-BT, ACT	24–48	8.5 ± 0.6
VIII. 19–23 May 2003	DAM	Dialysis bag	16–18	4.8	0–48	BET, GAM, CFB, R-BT	0–48	10.2 ± 2
	DAM	Bottle	16–18	3.2	0–48	BET, GAM, CFB, R-BT	0–48	7.0 ± 0.7
	DAM	Bottle + P	16–18	51	0–48	BET, GAM, CFB, R-BT	0–48	6.6 ± 0.7
	MIDDLE	Dialysis bag	16–18	15	0–48	BET, GAM, CFB, R-BT	24–72	6.5 ± 0.3
	MIDDLE	Bottle	16–18	5.8	0–48	BET, GAM, CFB, R-BT	0–48	6.4 ± 1.2
	MIDDLE	Bottle + P	16–18	65	0–48	BET, GAM, CFB, R-BT	0–48	6.1 ± 1.1

Data are shown for incubation site in the reservoir (DAM, MIDDLE and RIVER), type of incubation (penetrable dialysis bags or non-penetrable bottles), the specific bottom-up manipulation made via transplanting the samples into reservoir areas with different nutrient availability (→, the arrow indicates direction of the transplantation), or nutrient amendments of bottle-incubated samples by phosphate (+P) or glucose additions (+GLU). DAM at DAM (RIVER at RIVER) means that the samples were collected and also incubated at the same site. Further the table shows water temperature, SRP concentrations, intervals of exponential growth of bacterioplankton in the bacterivore-free, <0.8 μm treatments and of exponential growth of HNFs in the < 5 μm treatments that were used to calculate growth rate parameters of the microorganisms (for details see Fig. 3 and Table 2), and of different phylogenetic subgroups of bacteria targeted by specific probes (in parenthesis): BET, Betaproteobacteria (BET42a); GAM, Gammaproteobacteria (GAM42a); CFB, Cytophaga/Flavobacterium/Bacteroidetes group (CF319a); R-BT, a subgroup of Betaproteobacteria (R-BT065); and ACT, the Actinobacteria group (HGC69a). Values of doubling times of flagellates are means of two or three replicate treatments ± range of values.

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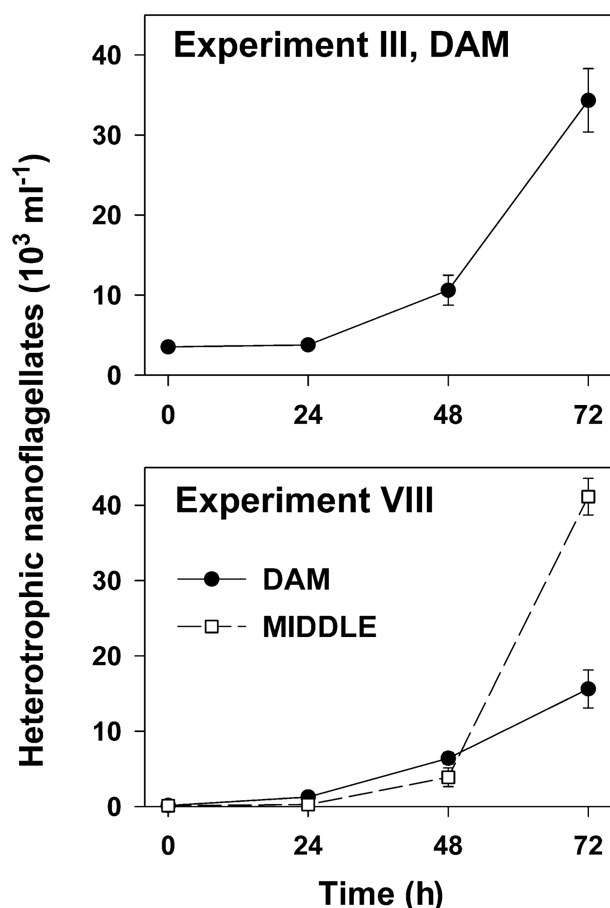

Fig. 1. Representative cell number increase of HNFs in zooplankton-free (< 5 μm) treatments used to calculate growth rate of HNF from 24 to 72 h time intervals of their fastest growth in different treatments as exemplified for the experiment III and VIII (for details see Table 1). The samples were incubated in the DAM (experiment III) or in the DAM and MIDDLE parts of the reservoir (experiment VIII) in dialysis bags. Values are means for three replicate treatments and error bars show standard deviations.

Overall, the magnitude and rate of such a growth response were highly variable among the experiments, both seasonally and spatially, and they did not show any clear relationship to water temperature over its range of more than 10°C recorded within the experiments (for details see Table 1). The maximum net increase rate in the target microbe population was usually observed over a period of ~48 h after sample manipulation (e.g. Figs 1 and 2, for time intervals used to calculate net growth rates consult Table 1), provided a suitable indicator of maximal net growth rate under the given environmental conditions.

The net growth rates, calculated using Eq. 1, were then estimated for time intervals with the fastest growth of target microbial groups (see Table 1). Pooling data from all the experiments, the specific net growth rates were plotted against concentrations of total (TP) and SRP and the data were fitted to a saturation curve using non-linear regression according to Eq. 3. All the bacterial groups showed a significant relationship of growth rate to SRP when fitted with the saturation curve (Fig. 3), with the best fit found for Betaproteobacteria (BET), its small cluster (R-BT) and ACT. No significant correlations were found for relationships to TP (data not shown).

The growth parameters derived from the regression analysis, the maximum growth rate and saturation constant (μ_{max} and K_s, respectively, – see Table 2), were highly significantly different for μ_{max} among the studied bacterial groups (*F*-test, $P < 0.0001$), while these differences were insignificant for the K_s values ($P = 0.803$). The latter parameter fell within the extremely narrow range of values of ~1.7–2.6 μg SRP l^{-1} for all bacterial groups. Overall, the μ_{max} parameter (Table 2) and the data plotted in Fig. 3 indicated that: (i) the bulk bacterioplankton showed significantly lower μ_{max} than BET, R-BT and GAM bacterial groups (*F*-test); and (ii) the R-BT-positive cells were the fastest growing segment of the bacterioplankton though its growth was not significantly different from the BET and GAM groups. Figure 4 shows the distribution of growth rates as generation times for the different groups of bacteria and HNF. Generation times (± SD, in hours) averaged for each group were: HNF = 10.4 ± 4.4, Bacterioplankton = 29.4 ± 15.3, BET = 19.5 ± 8.7, R-BT = 15.7 ± 7.8, GAM = 29.3 ± 26.1, CFB = 45.4 ± 39.7 and ACT = 33.9 ± 29.

Table 2. Growth parameters (μ_{max} and K_s ± standard error) of bacterioplankton (BACT) and different bacterial subgroups targeted with five oligonucleotide probes.

Bacterioplankton and different bacterial subgroups						
Parameter	BACT	R-BT	BET	GAM	CFB	ACT
K_s ± SE (SRP, μg l^{-1})	1.83 ± 0.68	1.80 ± 0.75	1.70 ± 0.68	2.59 ± 1.36	1.99 ± 1.35	2.45 ± 1.32
μ_{max} ± SE (day^{-1})	0.89 ± 0.08	1.61 ± 0.13	1.33 ± 0.11	1.35 ± 0.17	0.92 ± 0.14	1.13 ± 0.18
Significance of differences in μ_{max} among the microbial groups studied (*P*-value)						
	BACT μ_{max} differs from	**0.0001**	**0.004**	**0.018**	0.851	0.153
	R-BT μ_{max} differs from	–	0.108	0.206	**0.001**	**0.048**

Parameters were determined using non-linear regression (Monod kinetics) using pooled data from eight *in situ* experiments conducted in the reservoir under different concentrations of SRP (for more details and explanations see Table 1). The lower part of the table shows in bold face the significant differences (*F*-test) in μ_{max} of the whole bacterioplankton and the subgroup of BET targeted by the probe R-BT065 (R-BT) compared with other subgroups of bacterioplankton.

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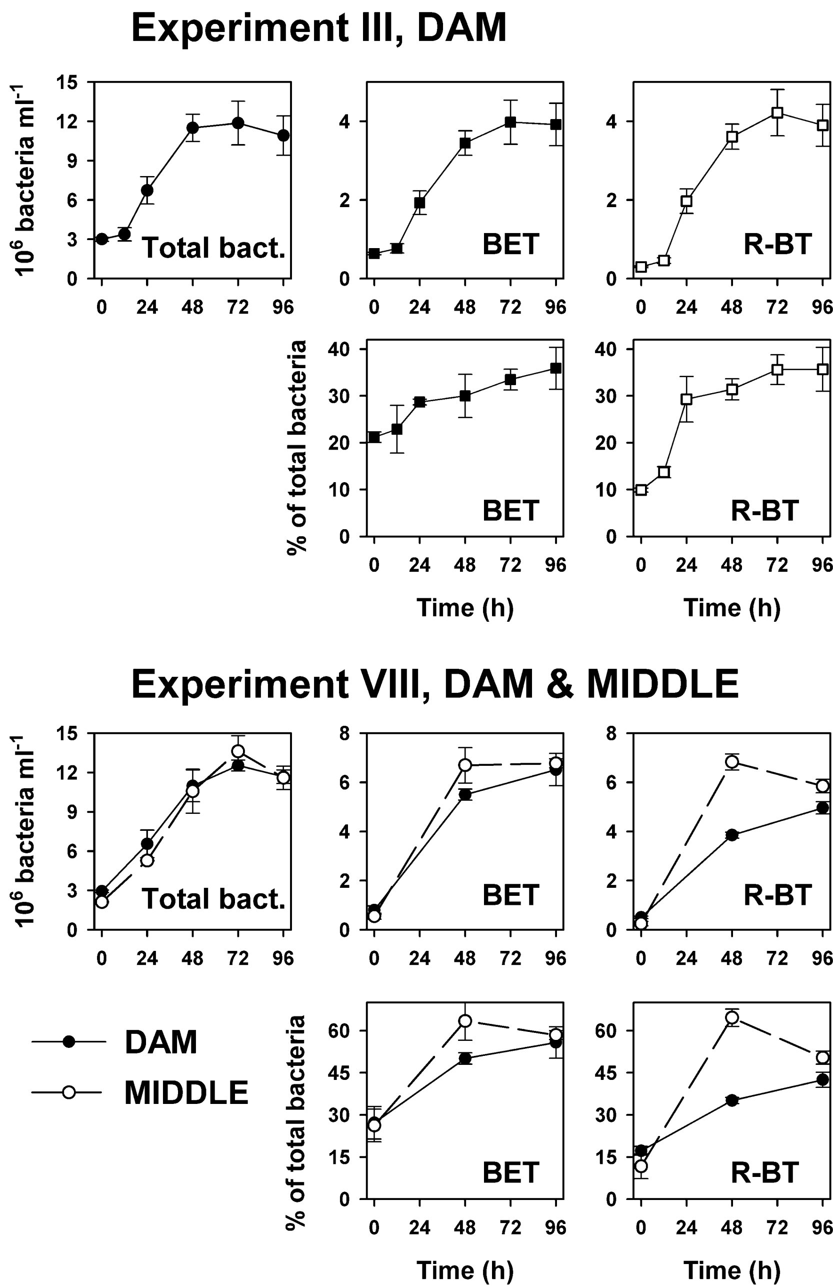

Fig. 2. Representative cell number increase of bacterioplankton and of its phylogenetic subgroups (examples of BET – Betaproteobacteria and of its R-BT subcluster) derived from the changes in their relative proportion in the community (corresponding bottom panels in the data referring to the experiments III and VIII) in bacterivore-free (< 0.8 μm) treatments incubated in the DAM (experiment III) and in the DAM and MIDDLE parts of the reservoir in dialysis bags (experiment VIII, for details see Table 1). Growth rates of different bacterioplankton groups were calculated for time intervals of t_0 to t_{48} h when three to four data points showed clear exponential increase in total bacterial numbers (the very left panels). Values are means for three replicate treatments and error bars show standard deviations.

Discussion

In our experiments 'bacterioplankton' was subdivided into several broader and one narrow phylogenetic subgroup by using FISH with group-specific probes. The net growth rates of distinct groups were determined under natural environmental conditions in the absence of grazing. However, our experimental design did not eliminate virus-induced bacterial mortality as viruses were always present in the treatments (cf. Šimek *et al*., 2003; Weinbauer *et al*., 2003). As virus-induced mortality appears to increase with growth rate (e.g. Weinbauer *et al*., 2003), proportionately greater impacts of viruses on the growth rate estimates in the treatments incubated under elevated SRP concentration may have occurred in our experiments. Unfortunately we have no data permitting a direct examination of this possibility.

Keeping in mind the possible differential impacts of virus-induced bacterial mortality in differently nutrient-limited parts of the reservoir, and the rather low taxonomic resolution of the FISH-probes used (except for the R-BT065 probe, cf. Šimek *et al*., 2001), the findings presented in Fig. 3 are compelling. Our data are compatible with the biological assumption that growth of different

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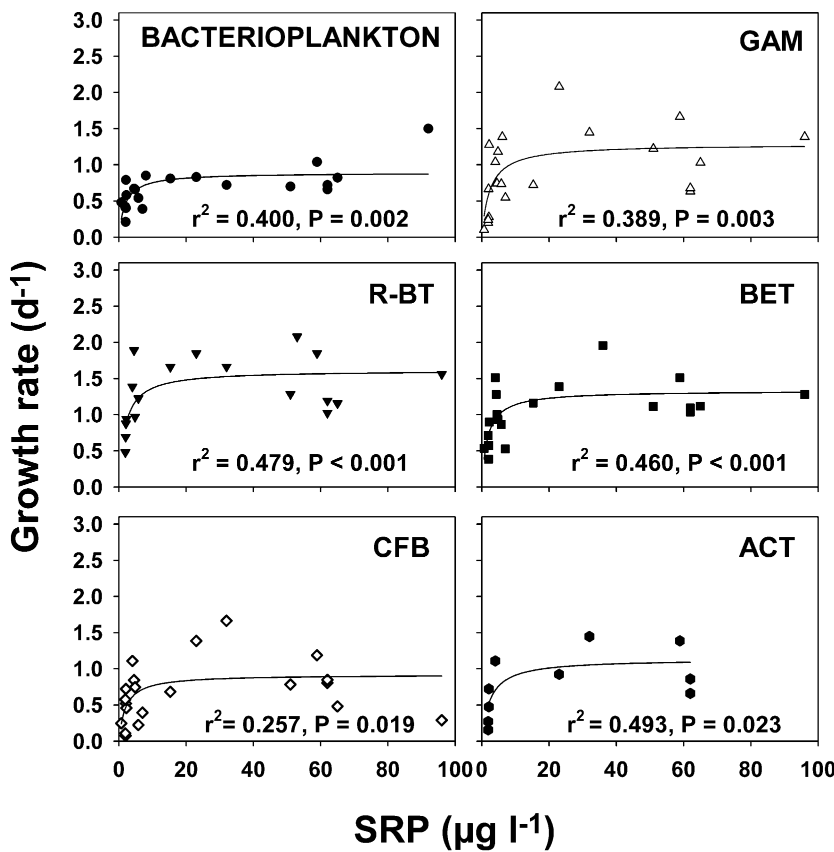

Fig. 3. Summary of growth rate estimates of the total bacterioplankton, of its different phylogenetic subgroups detected with the probes BET42a (BET), R-BT065 (R-BT), GAM42a (GAM), CF319a (CFB), HGC69a (ACT – the Actinobacteria group) measured under different SRP concentrations in the reservoir (for more details see Table 1). Pooled data from the experiments I–VIII were fitted by a hyperbolic Monod function (unbroken line) according to Eq. 3. r^2, coefficient of determination; *P*, probability.

taxonomic groups of bacteria is related to P-availability in freshwaters and indicate significant differences in growth rates as related to SRP concentrations among groups of bacteria. To our knowledge, this is the first application of Monod kinetics as related to P-availability for probe-defined bacterioplankton groups in natural assemblages. A direct influence of P-availability on bacterial growth seems to be likely, because indirect effects are unlikely to produce such clear Monod kinetic relationships between the two parameters. Furthermore, it is unlikely that the bacterial groups identified would rely in similar ways on an indirectly related parameter such as primary produc-

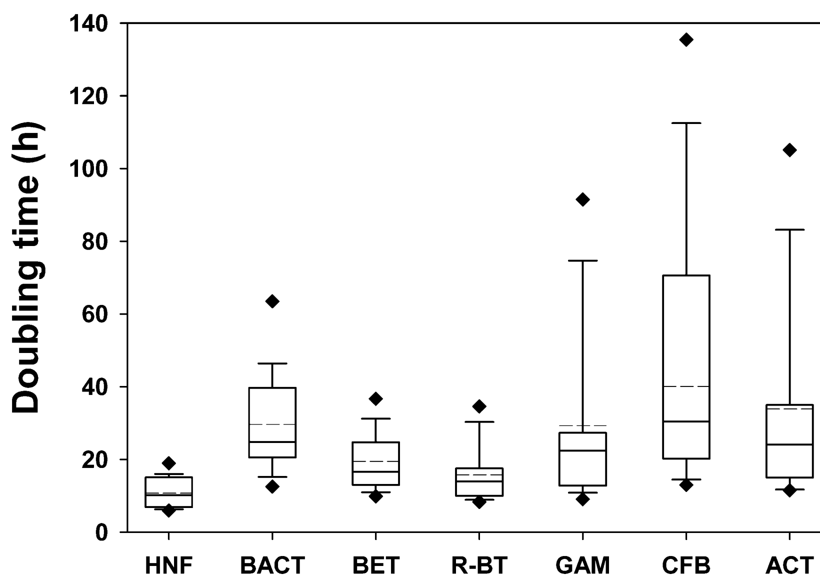

Fig. 4. Whisker box plots showing distributions of doubling times of HNF, total bacterioplankton (BACT) and of different bacterioplankton groups (for explanation of the abbreviations used see the text to Fig. 3 and Table 1). The median of the data is the full line in the box, average is the dash line while the 75th and 25th percentiles and 95th and 5th percentiles of the data are represented by the upper and lower error bars and upper and lower diamond symbols respectively.

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tion (or exudation by phytoplankton). In addition, the fact that P-availability, rather than organic carbon, plays a determining role in regulating bacterial growth rate in the reservoir (Fig. 3), is indicated by our finding that none of the phylogenetic bacterial groups studied showed a significant correlation of growth rate and chlorophyll-*a* concentrations (data not shown).

Our calculated K_s values were quite similar among the groups of bacteria, falling into a narrow range (1.7–2.6 μg SRP l^{-1}, Table 2), suggesting that none was adapted to eutrophic conditions. However, we are aware that our data on the K_s estimates should not be overstated, they represent only crude estimates of the real values as they are likely prone to a large statistical error from the following reasons: (i) we have a generally low number of data points measured close to origin, i.e. those measured under low and limiting SRP concentrations (generally below 2–3 μg SRP l^{-1}, cf. Table 1). These values are not frequently met in the meso-eutrophic reservoir where generally higher SRP concentrations are one of the inherent characteristics of the system. (ii) The detection limit for our SRP measurements is ~1 μg SRP l^{-1}, which implies that all our values below 1 μg SRP l^{-1} are likely inaccurate estimates of their low *in situ* concentrations, which limits the accuracy of the K_s estimate. (iii) The SRP concentration cannot be used as a direct estimate of orthophosphate available to bacteria, as independently measured concentrations of the bioavailable P are generally one to two orders of magnitude lower than the SRP concentrations (Rigler, 1966; Nedoma *et al*., 1993; Hudson *et al*., 2000). On the other hand, there is a fairly good correlation between SRP concentrations and the amount of bioavailable phosphate reported for marine waters (Moutin *et al*., 2002). Thus overall, while we could not accurately estimate the small differences of the K_s parameter from the reasons outlined above, the data scatter of measurements below the concentration 3.2 μg SRP l^{-1} (Fig. 3, i.e. seven of 21 measurements, cf. Table 1) undoubtedly indicates that below ~2–3 μg SRP l^{-1} the phosphate became the factor limiting the growth rates of even phylogenetically quite distinct bacterioplankton groups in the reservoir. This seems to be ecologically valuable new information.

In contrast to the K_s estimates, the μ_{max} estimates are robust enough, based on a wide range of SRP concentrations and they indicate large ecological differences between the groups. Simply in terms of maximum growth rates, the BET, R-BT and GAM groups grew significantly faster than the bulk bacterioplankton. The CFB group appeared to be the slowest growing group. The R-BT cluster, a subgroup of the BET bacteria showed the fastest growth rate and with an overall low variability (Fig. 4). The large growth capacity of the members of the phylogenetically narrow R-BT065 cluster can explain some general trends in the experiments. For instance, based on transplanting the size fractions from P-limited DAM area into the P-rich RIVER site (the experiment IV, a shift in SRP from 2 to 96 μg l^{-1}), quite significant changes in growth rate, production and bacterial community composition were detected (see Šimek *et al*., 2003). This latter phenomenon, the strong community shift in the resource-rich environment, could be also interpreted as the selection of bacterial species with high μ_{max}. Not surprising, the members of the R-BT065 cluster were the driving force of the observed community shifts and they always rapidly overgrew the other bacterial groups under a broad range of SRP concentrations whenever bacterivores were removed from the treatments.

In theory, osmotrophs can adopt strategies ranging from 'uptake specialist' to 'predation defence specialist' (Thingstad *et al*., 2005). The R-BT-positive cells showed fastest growth of all bacterial groups under the SRP concentrations tested (Fig. 3). Tight correlations of the relative proportions of this lineage in the community with the proportions of bacteria with high nucleic acid content, considered generally as the most active members of bacterial communities (Gasol *et al*., 1999), have been reported for the experiment VI (Šimek *et al*., 2005) and confirmed also in the experiment VIII (K. Horňák, unpublished). However, the members of this cluster likely suffer large grazing-induced mortality. Morphologically, the R-BT-positive cells are medium-to-larger-size rods (~0.09–0.23 μm^3), and are never found as filaments. In the experiments III and VI (Šimek *et al*., 2001, 2005) net growth rates were low in 'grazing-enhanced' (< 5 μm) treatments. Direct experimental evidence of high grazing-related mortality can also be found in FISH analysis of HNF food vacuole content (Jezbera *et al*., 2005). Thus, we suggest that the members of the R-BT065 cluster represent a specific case of 'uptake specialists', an example of a high growth potential (cf. Figs 3 and 4) but one counterbalanced by a marked vulnerability to protist grazing. Mainly the latter characteristic differentiates these phylotypes clearly from an alternative, third type of survival strategy called a 'Winnie-the-Pooh-strategist' (cf. Thingstad *et al*., 2005), i.e. characterizing microbes that maximize uptake and predator defence simultaneously. The finding that the net doubling time of only the R-BT-positive cells was comparable to and insignificantly different from that detected for HNF (*F*-test, data not shown), while all other bacterial subgroups showed significantly slower growth than HNF, supports our overall conclusion concerning this group.

On the other hand, not all the characteristics of R-BT-positive bacteria fit the theoretical view of a typical 'uptake specialists' proposed by Thingstad and colleagues (2005), i.e. fast growing, smaller cells with a high surface : volume ratio. Strictly following the view, the relatively large mean cell volume (MCV) of the members of this lineage (see above) should make them less competitive in a nutrient-

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limited environment compared with other bacterial lineage displaying generally very small MCV, such as small-sized ACT observed in the reservoir (cf. Jezbera *et al*., 2005). However, there are obviously some alternative strategies of nutrient acquirements (Cotner and Biddanda, 2002) that might explain this apparent discrepancy: (i) to reduce cell requirements for a given nutrient; or (ii) to acquire the nutrient more efficiently under the same ambient concentration than other similarly sized osmotrophic competitors.

The bacteria of the ACT group represent autochthonous and phylogenetically highly diverse component of freshwater bacterioplankton (Warnecke *et al*., 2004). The growth responses of the ACT group in the reservoir seem to qualify them rather as specific representatives of 'predation defence specialists'. The cells from this lineage were generally very small (e.g. in the experiment VI, MCV of 0.04–0.08 μm^3), less vulnerable to predation by HNF (cf. also Pernthaler *et al*., 2001; Hahn *et al*., 2003). In two of our experiments, VI and VII, HNF consistently discriminated against actinobacterial phylotypes based on food vacuole content analysis (Jezbera *et al*., 2005; 2006). Probably not only the small cell size itself make these phylotypes more grazing-resistant, but also partly other size-independent traits and growth capabilities can play an important role in their survival in a plankton environment (Hahn *et al*., 2003). While apparently one of the smallest members of the bacterioplankton and therefore less efficiently grazed upon by HNF, they showed growth rates not significantly different from that of the bulk bacterioplankton (Figs 3 and 4, Table 2). The typical growth rate of the ACT (Fig. 3 and Table 2, see e.g. a moderate μ_{max}) suggests a competitive advantage only under high grazing pressure when other apparently faster growing phylogenetic groups (e.g. BET, R-BT) of bacteria suffer much higher grazing-induced mortality (Jezbera *et al*., 2005; 2006; Šimek *et al*., 2005).

As the GAM42a and CF319a probes have generally very low taxonomical resolution and the growth data showed relatively large variability (Fig. 4), rather limited conclusions can be drawn regarding the ecology of these phylogenetic groups in the reservoir. Perhaps, the significantly higher μ_{max} value for GAM (Table 2) compared with the bulk bacterioplankton data and frequent appearance of large, grazing-resistant GAM-positive 'vibrio-like' cells [the experiment V, for details see Fig. 6C and F in the study by Horňák *et al*. (2005)] should be noted. The GAM phylotypes, which represent a rather small group in fresh- compared with marine waters, were consistently found to be generally large and fast growing cells under nutrient-rich conditions in the upper inflow part of the reservoir (Horňák *et al*., 2005).

In the grazing-enhanced treatments of the experiment III, large filaments targeted by the R-FL615 probe (the *Flectobacillus* lineage affiliated with the CFB group) were detected and the increase in their total filament numbers yielded a doubling time of 12.7 h (Šimek *et al*., 2001). However, even single cells within a *Flectobacillus* filament could be easily distinguished [see Fig. 5 in the study by Šimek *et al*. (2001)]. When re-evaluated, taking into account the increase of single *Flectobacillus* cells in the growing filaments in the experiment III, we estimated a fairly short net doubling time of only 6.2 h. This indicates that with the high taxonomic resolution of the R-FL615 probe one can detect grazing-resistant, fast growing 'predation defence specialist' in plankton [cf. the contrasting survival strategies of microbes detailed in the study by Thingstad *et al*. (2005)]. They apparently profit from the combination of large growth potential and almost complete grazing-resistance against small phagotrophic protists (Hahn *et al*., 1999; Šimek *et al*., 2001). Thus, in the absence of large zooplankton in $< 5\ \mu m$ treatments and the high HNF grazing pressure towards the end of the experiment III, the members of *Flectobacillus* lineage accounted for more than 30% of total bacterial biomass. This situation was paralleled with a marked decrease of relative proportions of R-BT-positive cells (considered as the potential 'uptake specialists' in the system) suffering large mortality due to HNF grazing (Šimek *et al*., 2001; Jezbera *et al*., 2006).

Remarkably little is known about how the growth rate of HNF communities *in situ* compares with the growth rates of different phylogenetic groups of bacteria preyed upon by HNF. Surprisingly, in all the experiments, HNF assemblages grew faster than any bacterial group studied, moreover, with little variability in growth rate (for details see Table 1, Fig. 4). These data, falling into the upper range of published HNF growth rates estimated *in situ* (e.g. Berninger *et al*., 1991; Weisse, 1991; Gasol *et al*., 1995), clearly indicate that HNF assemblages in the reservoir are probably efficiently top-down controlled by zooplankton. Strengthening this argument is the fact that resource, or bottom-up, control in form of available bacterial prey is rather unlikely. Typically abundances of $2–7 \times 10^6$ bacteria per millilitre are present both in the reservoir and in $<5\ \mu m$ treatments and these abundances exceed those that limit growth of small bacterivorous HNF (cf. Jürgens, 1992; Arndt *et al*., 2000).

In summary, we have shown significant relationships between phosphorus availability and growth responses of even relatively broad taxonomic groups of bacterioplankton that can be described by Monod kinetics. The groups of bacteria found in the reservoir all displayed adaptation to mesotrophic, in terms of P, waters with statistically indistinguishable differences in their K_s values. However, highly significant differences were detected among their μ_{max} values and consequently different growth capabilities in P-limited waters. We found the phylogenetically narrow R-BT065 cluster (BET) to be the fastest growing segment

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of bacterioplankton under the broad range of SRP concentrations. Thus, these phylotypes likely well exemplify the opportunistic strategists (Šimek *et al.*, 2005), or eventually 'uptake specialists' (Thingstad *et al.*, 2005) highly vulnerable to protistan predation. As bacterivorous HNF showed even larger growth potential than the R-BT-positive cells the efficient top-down control of HNF by zooplankton may indirectly contribute to sustaining a certain 'equilibrium' between fast growing bacterial 'uptake specialists', suffering large grazing-induced mortality, and other bacterial groups displaying different life and survival strategies.

Experimental procedures

Study site and common features of experimental design

From 1997 to 2003 we conducted eight manipulation experiments (assigned as experiment I–VIII, see Table 1) in the canyon-shaped Římov reservoir (South Bohemia – CZ; 470 m a.s.l.; area, 2.06 km^2; volume, 34.5×10^6 m^3; length, 13.5 km; maximum depth, 43 m; mean depth, 16.5 m; mean retention time, 100 days; dimictic; meso-eutrophic). In the experiments, bacterioplankton were subjected to shifts in either top-down or bottom-up controlling factors or to a combination of both types of manipulation in parallel. Thus, changes in growth rate and community composition of bacterioplankton and growth rate of HNFs were studied as a response to variable experimental scenarios (see below and Table 1).

Water samples were size-fractionated, yielding thus different levels of grazing-induced mortality of microbes. The size fractions represented: (i) a 'bacterivore-free', < 0.8 μm treatment, via filtration through 0.8-μm-pore-size filters (OSMONIC INC., Livermore, CA, USA), which were assumed to remove all bacterivores and thus allowed determining net growth rate of bacterioplankton and of its different phylogenetic subgroups; and (ii) a 'zooplankon-free', <5 μm treatment, via filtration through 5.0-μm-pore-size filters, which removed the HNF predators allowing determinations of HNF net growth rate. For more details concerning filtration procedures see the study by Šimek and colleagues (2001; 2003). In all experiments, these size fractions were placed into ~2.5 l pretreated (deonized water rinsed and boiled) dialysis bags (diameter 75 mm, molecular weight cutoff 12–16 kDa, Poly Labo, Switzerland) incubated at a depth of 0.5 m *in situ* assuring relatively free exchange of low-molecular-weight compounds present in surrounding water. Thus, both top-down uncontrolled bacterioplankton in < 0.8 μm treatments and HNF in <5 μm treatments grew under *in situ* limiting nutrient/resource concentrations inherent in different parts of the reservoir. Besides, in the experiment VI and VIII (for overview see Table 1) the same size fractions were also incubated in bottles (no nutrient penetration). Subsamples (~300–450 ml) were taken from each dialysis bag and bottle at times 0 and then at interval from 24 to 48 h.

To alter resources, or bottom-up controlling factors, the fractionated plankton subsamples were incubated in dialysis bags in different parts of the reservoir (i.e. DAM, MIDDLE and RIVER – the inflow part) with distinct levels of nutrient limitation (see e.g. the SRP concentrations in Table 1). Alternatively, bottom-up controlling factors were manipulated by additions of phosphate or glucose into large containers in which dialysis bags were incubated (experiment VII, for details see below) or to experimental bottles (experiment VIII).

Specific features of manipulation experiments

An overview of the experimental designs applied in the experiments I–VIII, water temperature, SRP concentrations, time intervals used to calculate growth parameters of HNF, bacterioplankton and of phylogenetic bacterial subgroups detected with group-specific genetic probes are shown in Table 1. All experiments were run in duplicates except for the triplicate experiments III and VIII. Briefly, the most important specific characteristics of the experiments are as follows.

In the experiment I (9–13 June 1997), II (12–18 September 1997) and III (28 May–1 June 1999) only top-down manipulations of samples via size fractionation were performed. The experiments were run in either late clear-water phase (experiments I and III) or late summer phytoplankton bloom (experiment II) with 5, 7 and 17 μg l^{-1} of chlorophyll-*a* (Chl-*a*) respectively. The samples were incubated in dialysis bags only in the DAM area and subsamples for growth rate determinations were analysed in 1- to 2-day intervals (experiment II). For more details concerning experimental arrangement and background data see the study by Šimek and colleagues (1999; 2001).

The experiment IV (12–16 June 2000) was begun during the onset of a summer phytoplankton bloom with ~11 μg Chl-*a* l^{-1}. Water samples were subjected to both top-down and bottom manipulations. The dialysis bags with <0.8 μm and <5 μm treatments of the samples collected at the DAM area of the reservoir were incubated at the sampling site and in parallel samples were transplanted into a RIVER inflow site, an area with almost two orders of magnitude higher concentrations of SRP [Table 1, for more details see the study by Šimek *et al.* (2003)]. The opposite direction of transplanting the size fractions was used in experiment V (21–25 May 2001): The samples originating from the RIVER inflow area were incubated in dialysis bags in this site and transplanted downstream into the DAM area with an almost one order of magnitude lower SRP concentrations [Table 1, for more details see the study by Horňák *et al.* (2005)].

A more complicated design, combining top-down and bottom-up manipulations with plankton samples, was applied in the experiment VI (20–24 May 2002) conducted at three experimental sites along the longitudinal profile of the reservoir (DAM, MIDDLE and RIVER) with a remarkable longitudinal gradient in SRP concentrations from 2.1 to 59 μg l^{-1} [Table 1 in this paper, for more details see the study by Šimek *et al.* (2005)]. The < 5 μm and < 0.8 μm treatments of water samples collected at the DAM site (4.5 μg Chl-*a* l^{-1}) were incubated in this site in dialysis bags as well as in glass bottles. Besides, < 5 μm and < 0.8 μm treatments from the DAM site were transplanted and incubated in dialysis bags in the P-richer MIDDLE and RIVER sites.

The experiment VII was conducted during a late stage of summer phytoplankton bloom period (9–13 September 2002, 10 μg Chl-*a* l^{-1}) and it was run in transparent 50 l polyethylene containers (acid soaked and washed several times with deonized water) that were filled with water collected from a

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depth of 0.5 m at the reservoir DAM area. The water appeared to be P-limited as indicated by the 1.9 μg SRP l^{-1} (Table 1). By manipulating P and organic carbon availability inside the experimental containers we intended to differentially stimulate bacterial and in turn also HNF growth. Immediately after filling the containers with unfiltered reservoir water, selected duplicate containers were amended by additions of either P ('+P treatment'; total initial concentration of 62 μg SRP l^{-1}) or glucose ('+GLU treatment'; corresponding to 2.5 mg C l^{-1}), or by a combination of both ['+P +GLU treatment', for details see the study by Šimek *et al*. (2004)]. Then, the containers were thoroughly mixed, the <5 μm and <0.8 μm treatments in dialysis bags were added into the containers and incubated for 4 days at the sampling site in the reservoir.

The experiment VIII, conducted during the clear water phase (19–23 May 2003), explored a different design: <5 μm and <0.8 μm treatments produced from water samples collected at the DAM and MIDDLE areas (4.4 and 2.7 μg Chl-*a* l^{-1} respectively) were deployed at the site of their origin. However, to assess the influence of resource availability, aliquots of the size fractions were incubated in parallel in dialysis bags, bottles and bottles amended by phosphate (see Table 1). The DAM site was approximately three times less in SRP concentrations than the MIDDLE site. Abundances of total bacteria and HNF were determined daily, while bacterial community composition was analysed bi-daily using FISH probes (see examples in Figs 1 and 2).

Total abundance of bacteria and HNFs

Subsamples for bacterial counting were fixed with formaldehyde (2% final concentration, v/v), stained with DAPI (final concentration 0.1 μg ml^{-1}) and enumerated by epifluorescence microscopy (AX 70 Provis). Subsamples for HNF enumeration were fixed with the Lugol-formaldehyde-thiosulfate decolourization technique (Sherr and Sherr, 1993). Subsamples of 5–10 ml volume were stained with DAPI, filtered through 1 μm black Poretics filters and inspected via epifluorescence microscopy as previously described elsewhere (Šimek *et al*., 2001).

Fluorescence in situ *hybridization with rRNA-targeted oligonucleotide probes*

In experiments I–VII, analysis of planktonic bacterial community composition was carried out by *in situ* hybridization with group-specific Cy3-labelled oligonucleotide probes on membrane filters (Alfreider *et al*., 1996). To assess proportions of the ACT group (HGC69a probe) in the experiments V–VII (cf. Table 1) and the whole community in the experiment VIII, a CARD-FISH protocol was applied (see Pernthaler *et al*., 2002; and Sekar *et al*., 2003 for details). Five different group-specific oligonucleotide probes (ThermoHybaid, Germany) were targeted to the Beta- and Gamma-subclasses of the class Proteobacteria (the BET42a and GAM42a probes respectively), to a narrower subcluster of the BET – (R-BT065), to the CFB group (CF319a), and to the ACT group (HGC69a). Throughout the whole text and figures the codes of these probes are abbreviated and refereed to as BET, GAM, R-BT, CFB and ACT respectively. After the whole procedure, the filter sections were stained with DAPI and the proportions of hybridized bacterial cells were enumerated using an epifluorescence microscope (Olympus AX70 Provis). The proportions of the probe-defined bacterial groups and total bacterial numbers were used to calculate absolute numbers of the bacterial subgroups at different times of an experiment that allowed to calculate group-specific net growth rate (see e.g. Fig. 2).

Net growth rate of HNF, bacterioplankton, and of different phylogenetic groups of bacteria

Top-down manipulations with plankton samples resulted consistently in increases in concentrations of HNF populations in <5 μm treatment and of total bacterioplankton in bacterivore-free, <0.8 μm treatments. The increase in total bacterial cell number and changes in relative proportions of probe-defined bacterial populations (for the list of the probes applied in the respective experiments see Table 1) were used to calculate their group-specific net growth rate. Examples of the data gained in experiment III and VIII are shown in Figs 1 and 2. To calculate the net growth rates we used the data obtained in time interval with the fastest growth, the most frequently from 0 to 48 h of an experiment, usually based on three to four data points characterizing the exponential growth of the total bacterioplankton in the <0.8 μm treatments. For more examples characterizing exponential growth of total bacterioplankton and the corresponding time-course changes in relative proportions of the FISH-detected bacterial groups compare Figs 1 and 4 in the study by Šimek and colleagues (2001) for the experiment III, and Figs 1 and 4 in the study by Šimek and colleagues (2005) for the experiment VI. Alternatively, data from 24 to 72 h intervals were used to calculate net growth rates when certain lag in growth response to the manipulation appeared (see Table 1 for details). Only in one case was a 96–144 h time interval of an experiment used for an HNF growth estimate (the RIVER site in experiment VI) when a markedly prolonged lag occurred as a response to the transfer of the sample from the DAM site into the RIVER site with much lower water temperature. Specific growth rates were calculated from the change in bacterial abundances of the probe-defined bacterial groups over time intervals detailed in Table 1 using linearized equation for exponential growth:

$$\ln N_t = \ln N_0 + \mu \times t \quad (1)$$

where N_t is bacterial abundance (cells ml^{-1}) at time t (day), μ is specific growth rate (day^{-1}), and $\ln N_0$ is intersect of the best fit line with the y-axis (cells ml^{-1}), ln is natural logarithm. The parameters μ and N_0 were determined by linear regression.

Doubling time of the respective microbial group was calculated according to the equation:

$$T = \ln 2/\mu \quad (2)$$

where T is doubling time, μ is specific growth rate, ln2 is the natural logarithm of 2.

Total and soluble reactive phosphorus

Soluble reactive phosphorus and total phosphorus concentrations were determined by the molybdate method (Murphy

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and Riley, 1962) and by the perchloric acid digestion and molybdate method according to Kopáček and Hejzlar (1993) respectively.

Statistical analysis

Using data on temporal increases in cell concentrations in different treatments, net doubling time and growth rate of different microbial subgroups were calculated for intervals with exponential or quasi exponential growth (for details see Table 1) using Eq. 1. Then all growth rates for a given microbial group were plotted against the corresponding concentration of SRP (see Table 1) and fitted with saturation curve (Monod kinetics) using non-linear regression on non-transformed raw data shown in Fig. 3 according to the equation:

$$\mu = \mu_{max} \times S/(K_s + S) \quad (3)$$

where μ_{max} is the maximum growth rate, K_s is the saturation constant and S is the concentration of SRP. Then we tested (F-test) for significant differences in μ_{max} of total bacterioplankton and the bacteria targeted by the R-BT065 probe compared with other phylogenetic groups of bacteria (Table 2). All statistics was performed using GraphPad Prism (GraphPad Sotfware, San Diego, CA, USA).

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