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Bacterial single-cell activities along the nutrient availability gradient in a canyon-shaped reservoir: a seasonal study

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ABSTRACT: We investigated the linkages between the composition and the activities of bacterioplankton assemblages in the meso-eutrophic Římov reservoir (Czech Republic). We examined bacterial fractions with visible leucine and glucose incorporation using microautoradiography, and proportions of bacteria with an intact membrane (live), high nucleic acid (HNA) and low nucleic acid (LNA) content, and an active electron transport system (CTC+ cells; CTC is 5-cyano-2,3-ditolyl-tetrazolium chloride) by flow cytometry. Three stations along the longitudinal axis of the reservoir (designated Dam, Middle, and River) with contrasting phosphorus concentrations and bacterial and extracellular phytoplankton production were sampled at intervals of 3 wk from 29 March to 15 November 2005. At all stations, *Betaproteobacteria* (BET) or *Actinobacteria* (ACT) dominated the bacterial community composition, while *Cytophaga-Flavobacteria* (CF) accounted for smaller proportions. ACT showed high proportions of cells incorporating both leucine and glucose in all samples, whereas only small fractions of CF were scored positive for the incorporation of these substrates. BET incorporated leucine preferentially to glucose in all samples. We identified bacterial phylogenetic groups that correlated with different bacterial populations as determined by flow cytometry: CF and BET significantly contributed to the pool of HNA cells at Stn River, whereas the opposite was found for ACT, forming the pool of LNA cells at all stations. Since ACT efficiently incorporated organic substrates, the LNA fraction represented a highly active component of bacterial assemblages. At Stns Dam and Middle, the dynamics of CF also correlated with the population of CTC+ cells and an enhanced extracellular phytoplankton production.

KEY WORDS: Reservoir · Bacterial activity · Leucine and glucose incorporation · Live cells · HNA cells · CTC+ cells

INTRODUCTION

Canyon-shaped reservoirs are characterized by large longitudinal heterogeneity in water temperature, primary production, nutrient supply, and quality of organic matter related mainly to reservoir loading, morphometry, retention time, and stratification (e.g. Straškraba & Tundisi 1999, Straškraba & Hocking 2002). In eutrophic reservoirs, the main activity processes, driven by microbes, usually occur in the upper part of the reservoir characterized by enhanced nutrient input of allochthonous origin, while the autochthonous organic matter produced by the primary producers dominates the lacustrine reservoir parts (Šimek et al. 2008). Furthermore, phosphorus (P) availability distinctly influences growth of different phylogenetic groups of bacteria (Šimek et al. 2006). Reservoir heterogeneity also has different impacts on the development of different plankton groups (Comerma et al. 2001, Šimek et al. 2001). The canyon-shaped Římov reservoir (Czech Republic) is a typical example of such an ecosystem. The phytoplankton community in the Římov reservoir is typically dominated by consecutive blooms of Cryptophyceae, Bacillariophyceae or

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Cyanobacteria (Znachor et al. 2008). Distinct phytoplankton groups also differ in terms of quality and quantity of extracellular primary production and thus specifically influence bacterial growth and activity (Wehr et al. 1999) or composition (e.g. Pinhassi et al. 2003). All reservoir heterogeneity characteristics thus allow for the assessment of the temporal as well as the spatial variability in composition and metabolic status of the bacterial community.

It has been well documented that only a part of the total bacterial assemblage is metabolically active at a given time and the fraction of active cells is highly variable (e.g. Choi et al. 1999, Smith & del Giorgio 2003). Bacterial activity is related to various metabolic processes such as biomass synthesis, cell division, or respiration that in turn might have significant implications for biogeochemical processes in the particular aquatic system (Cotner & Biddanda 2002). It is therefore essential to specifically identify bacteria with a certain level of metabolism. Apart from other techniques, flow cytometry is commonly used for quantification and detection of microbial activity variables at the single-cell level (e.g. Gasol & del Giorgio 2000). Microautoradiography (MAR) is also applied to track changes in metabolism after incorporation of a radiolabeled substrate (Karner & Fuhrman 1997). In combination with catalyzed reporter deposition-fluorescence *in situ* hybridization (CARD-FISH), substrate-uptake activities of different bacterial groups can be visualized (e.g. Teira et al. 2004).

Using different physiological probes, the bacterial assemblage can be divided into different fractions according to activity status, e.g. membrane integrity (e.g. Barbesti et al. 2000), nucleic acid content (Gasol et al. 1999), or respiratory activity after reduction of a tetrazolium salt (5-cyano-2,3-ditolyl-tetrazolium chloride [CTC], Rodriguez et al. 1992). Bacteria with high nucleic acid content (HNA) were typically considered as more active, being responsible for the vast majority of production processes (Gasol et al. 1999, Lebaron et al. 2001). However, with increasing taxonomic resolution of key bacterioplankton groups, contradictory results highlighting the metabolic status of low nucleic acid content (LNA) cells have appeared recently (Longnecker et al. 2005, Mary et al. 2006, Bouvier et al. 2007). Nevertheless, fractions of HNA cells often show, particularly in eutrophic environments, a tighter coupling with growth and production parameters than total bacterial numbers (del Giorgio & Scarborough 1995) as also documented for the Římov reservoir (Šimek et al. 2005, Horňák et al. 2006).

To reveal the differences in bacterial community composition and activity parameters related to a trophic gradient, we performed a seasonal study on a longitudinal profile of the canyon-shaped Římov reservoir. In the present study, 'active' bacteria were defined as those cells with (1) a seemingly intact membrane, (2) high nucleic acid content, or (3) an active electron transport system within the whole bacterial community, or as cells capable of (4) leucine or glucose incorporation at the group-specific level. We hypothesize that coexisting bacterial groups at 3 differently located stations within the same water body display distinct levels of activity variables. In addition, we focused on the potential linkages between the bacterial community composition and activity parameters determined by flow cytometry and MAR approaches, including selected biological parameters such as bacterial production (BP) and extracellular phytoplankton production (EPP).

MATERIALS AND METHODS

Seasonal sampling at the study site. The sampling was conducted in the meso-eutrophic canyon-shaped Římov reservoir (South Bohemia, altitude 470 m above sea level, volume 34.5×10^6 m^3, maximal depth 43 m, mean depth 16.5 m, mean retention time 100 d, dimictic) displaying a strong longitudinal gradient in major physical, chemical, and biological variables. Water samples were collected from 3 sampling points located in the dam area, middle part, and river inflow of the reservoir, assigned as Stns Dam, Middle, and River, respectively. At each station, samples were taken from the surface level (depth of 0.5 m) at 3 wk intervals from 29 March to 15 November 2005, except for 23 August at Stn River due to a flood event.

P and dissolved organic carbon (DOC). Briefly, concentrations of total P (TP) and dissolved reactive P (DRP) were determined spectrophotometrically (Kopáček & Hejzlar 1993). DOC was analyzed after filtering the samples through glass-fiber filters (pore size 0.4 µm, GF-5, Macherey-Nagel) using a TOC 5000A analyzer (Shimadzu).

Bacterial abundance and nucleic acid content. Samples of 1.5 ml were fixed with a fresh mixture of 0.2 µm-filtered paraformaldehyde and glutaraldehyde (final concentration 1 and 0.05%, respectively) for 30 min, frozen in liquid nitrogen and stored at −80°C until further processing (Gasol et al. 1999). When thawed, samples were stained with Syto13 (10× diluted in DMSO, final concentration 5 µM, Molecular Probes) for 10 min in darkness and analyzed using the FACS Calibur flow cytometer (Becton Dickinson). Fresh 0.2 µm-filtered Milli-Q water was used as a sheath fluid. To avoid the particle coincidence, the rate of particle passage was kept <1000 events s^{-1}. To convert the flow cytometry counts to cell numbers, yellow-green latex microspheres (diameter 1 µm, Poly-

sciences) were added as an internal standard. Total bacteria and bacteria with HNA and LNA were separated from debris using a 90° light scatter versus green fluorescence (530 nm) plot (Gasol & del Giorgio 2000). Alternatively, bacteria were determined in a green versus red fluorescence (630 nm) plot. Percentages of HNA and LNA cells were then subtracted from total bacterial counts.

BP and EPP. BP was measured after incorporation of [^{3}H]-thymidine (MP Biomedicals) according to Straškrabová et al. (1999) using the empirically established conversion factor of 1.6×10^{18} cells mol^{-1} of incorporated thymidine. EPP was measured as incorporation of [^{14}C]-bicarbonate (MP Biomedicals) as described in Straškrabová et al. (1999). The activity passing the 1 µm filter (Poretics) was considered to be equivalent to EPP, since no autotrophic picoplankton was detected in the <1 µm filtrate (P. Znachor unpubl. data). Procedures of the above methods are detailed in Šimek et al. (2008).

Bacterial membrane integrity and respiratory activity. Live samples of 1 ml for cell membrane integrity analyses were incubated with a mixture of SybrGreen I and propidium iodide (both at final concentration 5 µM, 10× diluted in DMSO, Molecular Probes) for 10 min in darkness (Barbesti et al. 2000). To separate bacteria with an intact membrane (considered as live cells), a green versus red fluorescence plot was used. Samples of 1 ml for respiratory activity (also known as electron transport system activity) were stained with fresh 0.2 µm-filtered CTC (10× diluted in Milli-Q water; final concentration 5 mM, Polysciences) for 1 h in the dark (Rodriguez et al. 1992). CTC+ cells were determined in a 90° light scatter versus red fluorescence plot (Gasol & del Giorgio 2000). After staining, samples were immediately processed in the flow cytometer as described above (see 'Bacterial abundance and nucleic acid content').

Incorporation of radiotracers. 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 either with 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 darkness, preserved in formaldehyde (final concentration 2%) and filtered through 0.2 µm polycarbonate filters (Poretics; details in Horňák et al. 2006). Filters for MAR were rinsed with Milli-Q water, air-dried, and kept frozen (–20°C) until further processing.

MAR and CARD-FISH. MAR and CARD-FISH (MAR-FISH; Teira et al. 2004) was used for bacterial identification and active substrate incorporation. Briefly, filters with bacterial cells were embedded in low gelling point agarose and the cells were digested by lysozyme and achromopeptidase (Sekar et al. 2003). Bacteria were hybridized with horseradish peroxidase-labeled probes specific for most *Bacteria* (EUB338 I–III), *Betaproteobacteria* (BET) (BET42a), *Cytophaga-Flavobacteria* (CF) (CF319a), and *Actinobacteria* (ACT) (HGC69a), followed by a tyramide signal amplification as described in Sekar et al. (2003). Details on oligonucleotide probes are available at probeBase (www.microbial-ecology.de/probebase/default.asp) (Loy et al. 2007).

Subsequently, the filters were transferred onto the slides coated with the autoradiography emulsion (NTB, Kodak). After 20 to 40 h of exposure, the cells were stained with DAPI (final concentration 1 µg ml^{-1}). The relative abundances of hybridized cells were enumerated by epifluorescence microscopy (PROVIS AX-70, Olympus). Bacteria incorporating the radiolabeled substrate were surrounded by black silver grains. At least 500 DAPI-stained cells were counted per sample.

Statistical analyses. Prior to analyses, all the percentage data (relative proportions of major bacterial groups, live, HNA, and CTC+ cells) were arcsine-transformed, while data on P concentrations, total bacterial abundance and carbon production, and EPP were log(x+1)-transformed. All the transformed data fitted a normal distribution. Differences in the relative abundances and proportions of Leu+ and Glc+ cells of BET, CF, and ACT at the 3 stations (Dam, Middle, River) were tested by means of ANOVA followed by Tukey's multiple comparison tests. In addition, a correlation matrix was established to explore the relationships among the investigated parameters using Pearson's correlation analysis. All statistics were performed using GraphPad Prism (GraphPad Software).

RESULTS

P and DOC

TP and DRP displayed significantly decreasing concentrations from Stn River to Stn Dam (ANOVA, $p < 0.003$ and $p < 0.0001$, respectively) forming a pronounced longitudinal P gradient (Fig. 1). Stn River also showed the highest variability in both TP and DRP concentrations compared to the lacustrine Stns Dam and Middle. In contrast, no significant differences in concentrations of DOC (Fig. 1) between the stations were found (ANOVA). DOC concentrations ranged mostly from ~4 to ~8 mg l^{-1} at all stations, although temporarily increased concentrations were recorded at Stns Middle and River.

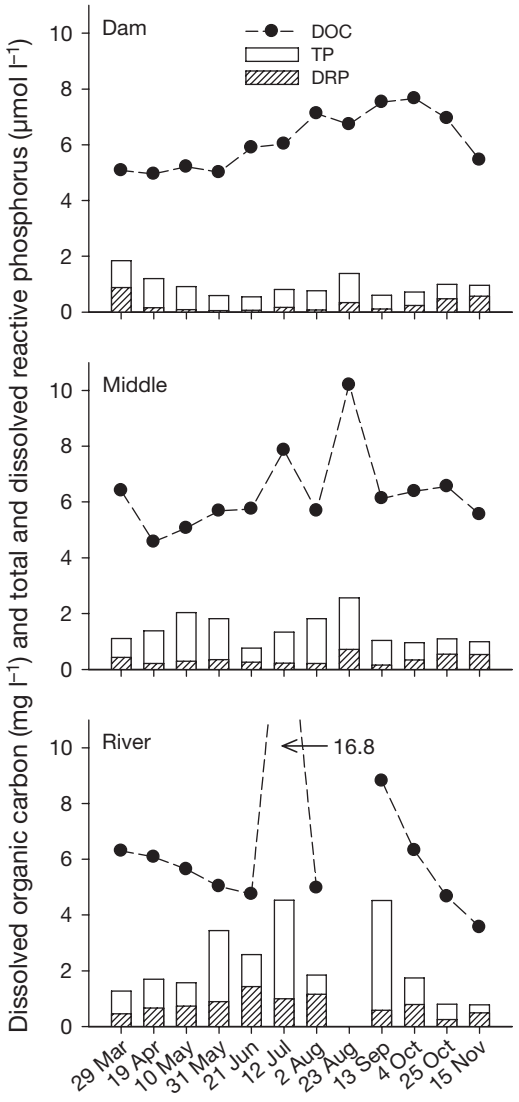

Fig. 1. Time-course changes in concentrations of dissolved organic carbon (DOC), total phosphorus (TP), and dissolved reactive phosphorus (DRP) at 3 stations (upper panel: Stn Dam, middle panel: Stn Middle, lower panel: Stn River)

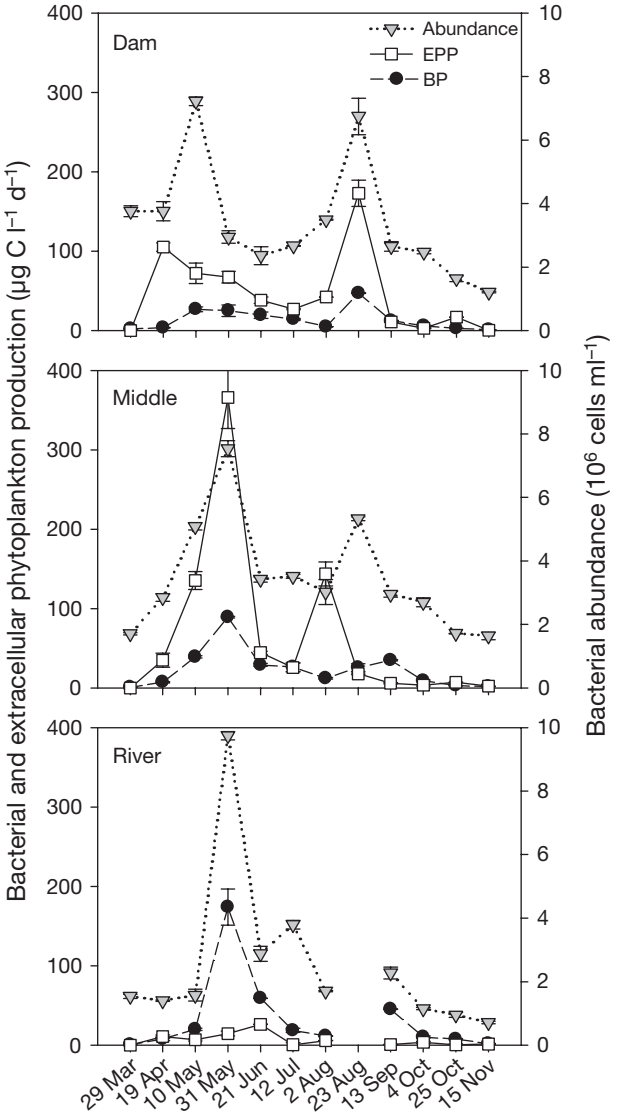

Fig. 2. Seasonal variations in total bacterial numbers, bacterial production (BP), and extracellular phytoplankton production (EPP) at 3 stations (upper panel: Stn Dam, middle panel: Stn Middle, lower panel: Stn River). Values are means of duplicates. Error bars represent range

Total bacterial abundance, BP, and EPP

Total bacterial numbers varied from 0.8×10^6 to 7.5×10^6 cells ml^{-1}. At Stns Dam and Middle, bacterial numbers rapidly increased and peaked on 10 May, followed by a marked decrease until 21 June (Fig. 2). The second maximum occurred on 23 August followed by a continuous decrease lasting until the end of the sampling period. At Stn River, bacterial abundance was slightly lower except for the extremely high numbers on 31 May (Fig. 2) attributed to a flood event. At the lacustrine Stns Dam and Middle, EPP exceeded BP almost over the whole part of the season studied. The opposite pattern was found at Stn River, where EPP was very low while maximal BP was detected on 31 May (Fig. 2).

Bacterial flow cytometry properties

Bacterial cells with an intact membrane (live cells) typically accounted for 60 to 80% of total bacterial counts over the whole sampling period at all stations, thus forming a majority within the bacterial assemblage. Maximal percentage of live cells (close to 90%) was found at Stn Dam on 10 May (Fig. 3). Although the percentages of live cells roughly followed the changes

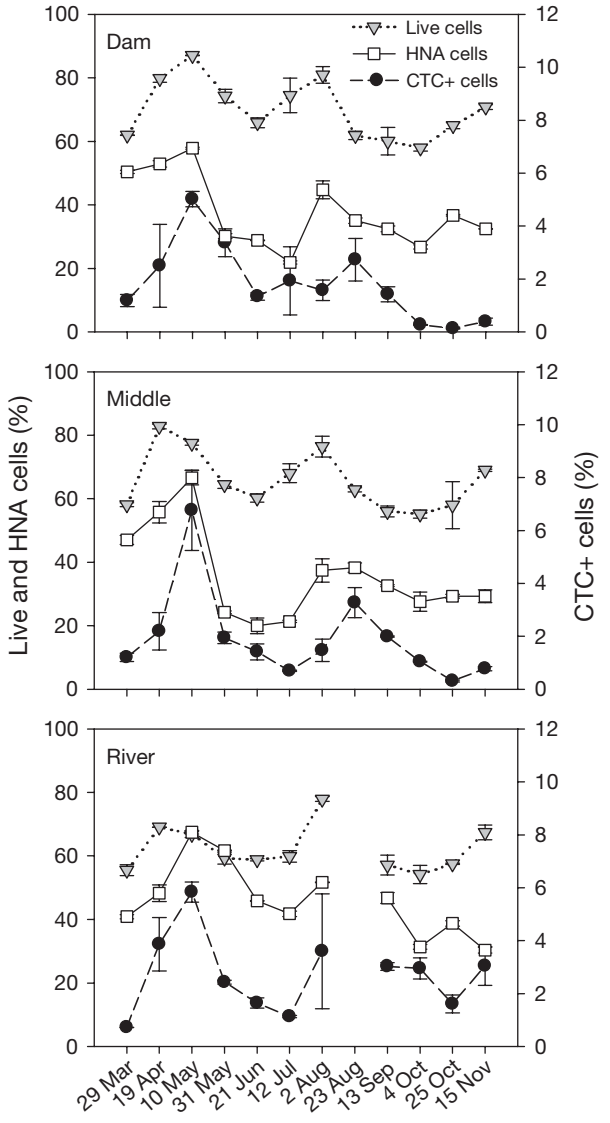

Fig. 3. Seasonal changes in relative proportions (as percent of total bacterial numbers) of live cells, high nucleic acid content (HNA) cells, and cells with an active electron transport system (CTC+ cells) at 3 stations (upper panel: Stn Dam, middle panel: Stn Middle, lower panel: Stn River) determined using flow cytometry. Values are means of duplicates. Error bars represent range

in total bacterial numbers, these parameters were not significantly correlated at any of the stations (Table 1).

Proportions of bacteria with HNA significantly correlated with the changes in total bacterial numbers at Stn Dam (cf. Figs. 2 & 3), whereas such relationships were not found at the other 2 stations (Table 1). Proportions of HNA cells varied from 20 to 67%, with 2 characteristic peaks at all stations (Fig. 3). The initial increase in percentages of HNA cells was typically followed by a marked decrease, particularly at Stns Dam and Middle between 31 May and 12 July.

Table 1. Pearson's correlation coefficients between total bacterial numbers and percentages of cells with an intact membrane (%live), high nucleic acid content (%HNA), and high respiratory activity (%CTC+) at 3 stations. *p < 0.05, ***p < 0.001; significant correlations are in **bold**; n = 24 (except for Stn River: n = 20)

	Total bacterial numbers		
	Stn Dam	Stn Middle	Stn River
%live	0.35	0.19	−0.18
%HNA	**0.57***	0.1	0.53
%CTC	**0.81*****	**0.63***	−0.2

The percentage of CTC+ cells ranged at all stations from 0.1 to 6.8% of total bacterial counts (Fig. 3), with temporal variations significantly correlated to those of total bacterial numbers at Stns Dam and Middle (Table 1), respectively. In addition, proportions of CTC+ cells significantly correlated with changes in percentages of HNA cells at Stn Middle ($r = 0.72$, $p < 0.01$, $n = 24$).

Bacterial community composition

We followed the spatio-temporal changes in the 3 numerically most important phylogenetic groups of bacteria (BET, CF, and ACT) accounting for the majority of bacteria covered by a mixture of EUB338 I–III probes (data not shown). All 3 stations revealed roughly similar bacterial community compositions (Fig. 4), with no significant differences in the relative abundances of the investigated bacterial groups between the stations (ANOVA). BET consistently dominated the bacterial community in most of the samples, ranging from 10 to 40% of total bacterial counts. Percentages of BET were only temporarily outnumbered by members of CF and ACT in spring and autumn periods, respectively (Fig. 4). In general, proportions of CF tended to increase during spring while they often decreased towards the end of the studied period. Maximal percentages of CF (~30%) were found at Stns Middle and River. In contrast, proportions of ACT greatly varied in the range of ~3 to almost 50% at Stns Dam and Middle, while at Stn River, maximal percentages of ACT did not exceed 25% (Fig. 4). ACT were usually more abundant in late summer and autumn periods as opposed to relatively low proportions (~10%) in spring.

Group-specific bacterial activities

Typically, >50% of BET were scored positive for leucine incorporation at all stations during the sam-

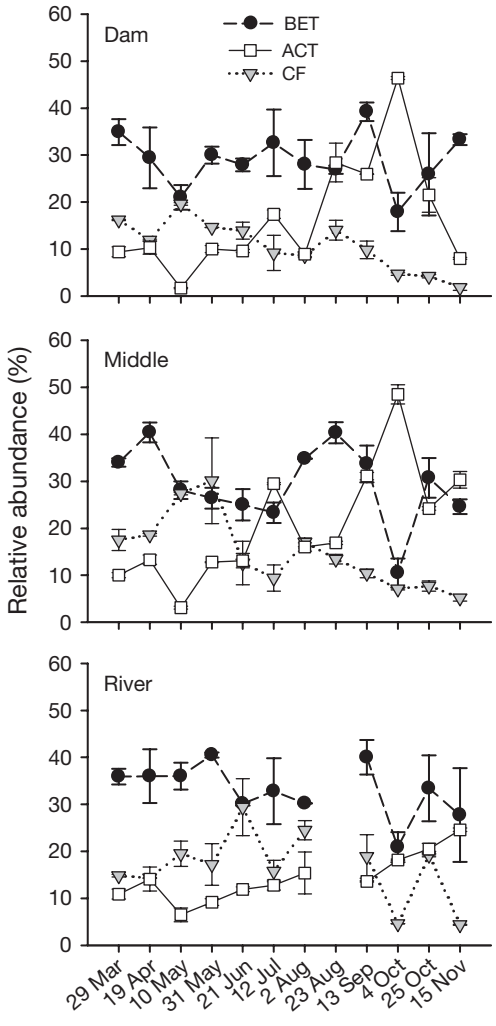

Fig. 4. Relative abundances (as percent of DAPI-stained cells) of *Betaproteobacteria* (BET), *Cytophaga-Flavobacteria* (CF), and *Actinobacteria* (ACT) at 3 stations (upper panel: Stn Dam, middle panel: Stn Middle, lower panel: Stn River) over the whole sampling period. Values are means of duplicates. Error bars represent range

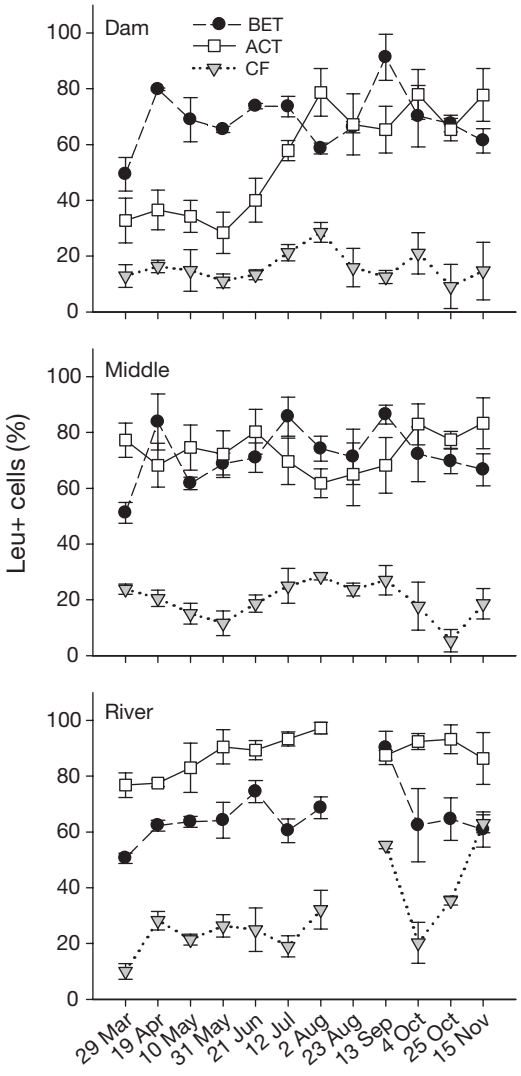

Fig. 5. Percentages of cells incorporating leucine (Leu+ cells as percent of hybridized cells) of *Betaproteobacteria* (BET), *Cytophaga-Flavobacteria* (CF), and *Actinobacteria* (ACT) at 3 stations (upper panel: Stn Dam, middle panel: Stn Middle, lower panel: Stn River) over the whole sampling period. Values are means of duplicates. Error bars represent range

pling period (Fig. 5). In contrast, CF often showed <30% of Leu+ cells. At Stn River, proportions of Leu+ CF temporarily increased up to 60% of Leu+ cells towards the end of the sampling period (Fig. 5). ACT showed similar proportions of Leu+ cells to those of BET at the lacustrine stations. Maximal proportions of ACT Leu+ cells were found at Stn River (Fig. 5). Interestingly, proportions of Leu+ ACT were markedly lower at the beginning of the sampling period at Stn Dam.

In terms of glucose incorporation, ACT showed the highest percentages of Glc+ cells among the groups studied, in the range of 60 to 80% (Fig. 6). Both BET and CF showed comparable proportions of Glc+ cells, but only 10 to ~40% of either BET or CF were scored positive for glucose incorporation (Fig. 6). However, elevated percentages of Glc+ BET and CF were temporarily observed at Stn River.

Comparing the overall pattern in the proportions of Leu+ and Glc+ cells of the 3 major phylogenetic groups of bacteria, the following trends were revealed: First, within each bacterial group, both activity parameters detected at Stn River were significantly different from those observed at the lacustrine stations, which revealed high similarity (Table 2A). Second, CF revealed significantly lower proportions of Leu+ cells compared to BET and ACT at the lacustrine stations (Table 2B). Third, ACT showed significantly higher

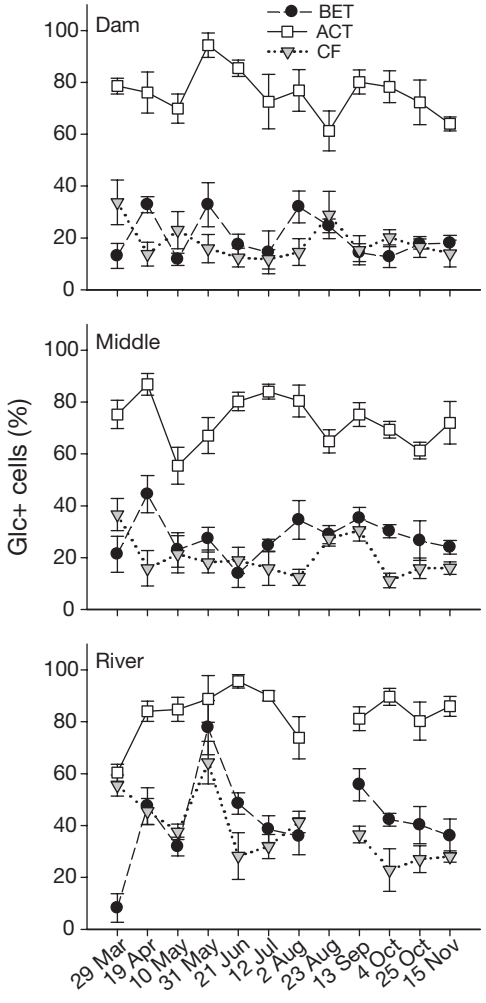

Fig. 6. Percentages of cells incorporating glucose (Glc+ cells as percent of hybridized cells) of *Betaproteobacteria* (BET), *Cytophaga-Flavobacteria* (CF), and *Actinobacteria* (ACT) at 3 stations (upper panel: Stn Dam, middle panel: Stn Middle, lower panel: Stn River) over the whole sampling period. Values are means of duplicates. Error bars represent range

percentages of Glc+ cells than both BET and CF groups at all stations (Table 2B). Finally, BET preferentially incorporated leucine compared to glucose, while CF and ACT revealed generally balanced proportions of both Leu+ and Glc+ cells (Fig. 7).

DISCUSSION

Flow cytometry activity properties

Exploring the seasonal variations in total bacterial numbers (Fig. 2) in relation to the proportions of live, HNA, and CTC+ cells as determined by flow cytometry (Fig. 3), markedly different results (Table 1) were obtained for different stations: none of the flow cytometry parameters tested showed a significant relationship to total bacterial numbers at Stn River, while the dynamics of HNA and CTC+ cells positively correlated to total bacterial numbers at Stn Dam (Table 1). At Stn Middle, only percentages of CTC+ cells showed a significant relation to total bacterial abundance. Apparently, linkages between total bacterial abundance and bacterial flow-cytometric properties were affected by more pronounced oscillations, e.g. in water chemistry (Fig. 1) or by the proportions of cells with visible tracer incorporation (Fig. 7) at Stn River compared to Stn Dam. Stn River also differed from the other 2 stations by about 1 order of magnitude lower EPP (Fig. 2), primary production, and protozoan grazing (see Šimek et al. 2008).

Additionally, proportions of live cells were not associated with total bacterial abundance (Table 1) or BP at any of the stations. Our data thus agree with Berman et al. (2001), although more recent investigations in the Antarctic suggest contradictory results (Pearce et al. 2007). Nevertheless, in the Římov reservoir, the per-

Table 2. Tukey's multiple comparison post test applied after ANOVA to test for differences in percentages of cells incorporating leucine (Leu+ cells) or glucose (Glc+ cells) (both as percent of hybridized cells) of *Betaproteobacteria* (BET), *Cytophaga-Flavobacteria* (CF), and *Actinobacteria* (ACT). Pair-wise comparisons (A) within and (B) between the investigated groups at 3 stations over the whole sampling campaign are shown. Significant differences are in **bold**; n = 24 (except for Stn River: n = 20); ns = not significant

A) Within groups	Leu+ cells			Glc+ cells		
	BET	CF	ACT	BET	CF	ACT
Stn Dam vs. Stn Middle	ns	ns	**<0.001**	ns	ns	ns
Stn Dam vs. Stn River	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.05**
Stn Middle vs. Stn River	**<0.001**	**<0.01**	**<0.001**	**<0.01**	**<0.001**	**<0.01**
B) Between groups	Leu+ cells			Glc+ cells		
	Stn Dam	Stn Middle	Stn River	Stn Dam	Stn Middle	Stn River
BET vs. CF	**<0.001**	**<0.001**	ns	ns	ns	ns
BET vs. ACT	ns	ns	**<0.01**	**<0.001**	**<0.001**	**<0.001**
CF vs. ACT	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**

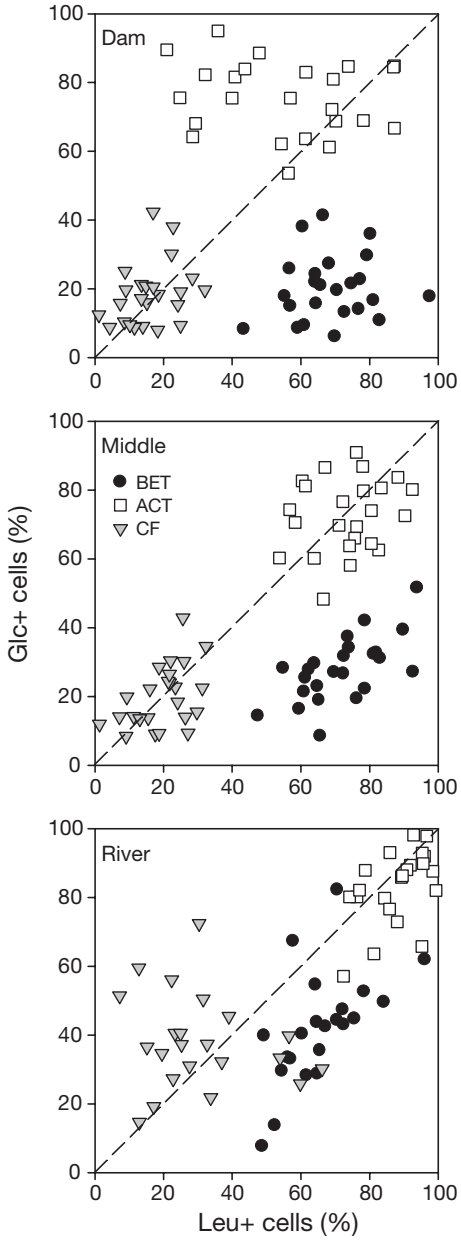

Fig. 7. Percentages of leucine- versus glucose-incorporating cells (both Leu+ and Glc+ cells as percent of hybridized cells) of *Betaproteobacteria* (BET), *Cytophaga-Flavobacteria* (CF), and *Actinobacteria* (ACT) at 3 stations (upper panel: Stn Dam, middle panel: Stn Middle, lower panel: Stn River). Diagonal line is the 1:1 line; n = 24 (except for Stn River: n = 20)

centage of live cells does not seem to be a relevant parameter for explaining bacterial community dynamics. Furthermore, we found a significant correlation between bacterial carbon production (Fig. 2) and percentages of CTC+ cells (Fig. 3) at Stn Dam ($r = 0.87$, $p < 0.001$), suggesting a tight coupling between bacterial biomass production and respiration. The same relationship, although less pronounced, was also found at Stn Middle ($r = 0.57$, $p < 0.05$). Similarly, Morán & Calvo-Diaz (2009) reported a positive relationship between the percentage of CTC+ cells and the total leucine incorporation rates for coastal marine bacteria. At Stn River, however, BP was significantly linked to the fraction of HNA cells ($r = 0.64$, $p < 0.05$), suggesting a dominant role of those cells for overall community production.

Group-specific activities based on MAR-FISH

One limitation of the flow cytometry approach we used is the lack of bacterial identity, even though the samples were analyzed at the single-cell level. To date, MAR-FISH is not commonly combined with flow cytometry unless an efficient sorting followed by molecular analyses of the sorted populations is applied. The advantage of MAR-FISH is its very high sensitivity and the direct identification of active cells. Although we used only probes with rather low specificity targeting the broad phylogenetic groups of bacteria (BET, ACT, and CF), activity of these bacterial groups based on the proportions of Leu+ and Glc+ cells showed significant differences along the longitudinal axis of the reservoir (Table 2). In general, Stns Dam and Middle revealed high similarity. In contrast, the inflow Stn River frequently revealed significant differences in the proportions of MAR+ cells from the lacustrine stations. Moreover, the highest proportions of Leu+ or Glc+ cells were often found in the P-rich Stn River (Figs. 5 & 6).

As documented from previous studies conducted in the Římov reservoir (Horňák et al. 2006, 2008), BET very efficiently incorporated leucine. In the present study, the majority of BET was also scored positive for leucine incorporation at all stations, and thus BET represented a fraction of actively growing cells synthesizing new biomass (e.g. Simon & Azam 1989). Active incorporation of amino acids by BET has been also documented from other freshwater habitats with different trophic status (e.g. Pérez & Sommaruga 2006, Salcher et al. 2008), including humic (Buck et al. 2009) or brackish waters (Alonso et al. 2009). On the other hand, the proportions of Glc+ cells among BET were substantially lower (e.g. Fig. 7) even though increasing percentages at Stn River were observed. Thus, BET can be characterized by a high growth and production potential rather than by the preferential production of storage compounds over the large scale of trophic conditions.

It has been shown previously that members of the CF group do not preferentially incorporate amino acids across a wide range of aquatic environments including marine ones (Cottrell & Kirchman 2000, Elifantz et al.

2007). In the Římov reservoir, CF showed similar characteristics, i.e. very low proportions of Leu+ cells at Stns Dam and Middle (Fig. 5). Although in marine systems, the dynamics of CF were linked to the blooms of distinct diatoms (Pinhassi et al. 2004, Grossart et al. 2005) or dinoflagellates (Garcés et al. 2007), only low proportions of CF were scored positive for leucine and also for glucose incorporation over the whole season in the lacustrine part of the reservoir, where marked changes in EPP (Fig. 2) and phytoplankton composition (Šimek et al. 2008) were observed. However, the percentages of Leu+ and Glc+ cells within CF significantly increased at Stn River (Table 2). We thus suppose that CF bacteria detected at Stn River were mainly of allochthonous origin and their phylogenetic identity differed from those detected at other stations.

Generally, ACT clearly showed the highest fractions of Glc+ cells at all 3 stations (Fig. 6), indicating the active carbon metabolism and the potential production of storage material (e.g. glycogen) since the substrate was incorporated into cell biomass. ACT simultaneously revealed high percentages of Leu+ cells comparable to those of BET. This finding is partially contradictory to a previous study (Buck et al. 2009) conducted in a humic lake, where glucose was the preferentially incorporated substrate rather than leucine or acetate, although different subgroups of the actinobacterial acI lineage (Newton et al. 2007) were involved in the incorporation of the substrates. In the present study, we used only the general probe for ACT, which could partly explain the apparent discrepancy between the present study and the one by Buck et al. (2009). However, our present analyses of bacterial community composition in the surface layer of the Římov reservoir revealed that the relative proportions of the acI lineage tightly matched those of the whole ACT group over the spring phytoplankton maximum (J. Jezbera unpubl. data). Thus, we assume that most of the ACT detected in the present study were members of the acI lineage.

Comparing flow cytometry and MAR-FISH results

At the group-specific level, we have identified bacterial groups possibly associated with distinct bacterial populations as determined by flow cytometry: both BET and CF bacteria contributed significantly to the pool of HNA cells at Stn River (Table 3). We thus hypothesize that under increased P availability, the population of HNA cells seems to be formed mainly by BET and CF bacteria. CF also showed a significant relationship with CTC+ cells (Table 3), which are supposed to be the most active cells in terms of oxidative respiratory metabolism. Moreover, fractions of CF seemed to be also associated with the phytoplankton community via their extracellular products in the lacustrine parts of the reservoir. Flow cytometry analyses thus suggested that CF contained phylotypes with a significant level of metabolism. However, CF simultaneously showed low proportions of MAR+ cells. We can only suppose that other types of substrates (e.g. of algal origin) may be more appropriate for detecting the activity of CF using the MAR-FISH approach. Unfortunately, direct investigations of the phylogenetic composition of distinct bacterial fractions as determined by flow cytometry that could help us to reveal more insights into the functioning of the bacterioplankton assemblage remained beyond the scope of the present study.

While seasonal variations in the relative abundance of BET and CF correlated to the HNA fraction, ACT showed the opposite trends (Table 3). Since both percentages of HNA and LNA cells were subtracted from total bacterial counts, negative correlations of ACT to the HNA fraction (Table 3) also imply a positive and significant relationship to the LNA fraction at all stations. We suggest that some members of ACT (e.g. the acI lineage) may have this group-specific flow-cytometric signature due to their lower nucleic acid content related to smaller genome size (Newton et al. 2007) compared to other bacterial groups studied. Similarly,

Table 3. Pearson's correlation coefficients between relative abundances (as percent of DAPI-stained cells) of *Betaproteobacteria* (%BET), *Cytophaga-Flavobacteria* (%CF), and *Actinobacteria* (%ACT) and percentages of cells with an intact membrane (%live), high nucleic acid content (%HNA), high respiratory activity (%CTC+), bacterial production (BP), and extracellular phytoplankton production (EPP). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$; significant correlations are in **bold**; n = 24 (except for Stn River: n = 20)

	Stn Dam			Stn Middle			Stn River		
	%BET	%CF	%ACT	%BET	%CF	%ACT	%BET	%CF	%ACT
%live	−0.16	0.24	**−0.77****	0.39	0.45	−0.47	−0.02	0.18	0.04
%HNA	−0.11	0.41	**−0.59***	0.48	0.52	**−0.62***	**0.64***	**0.62***	**−0.84*****
%CTC+	0	**0.85*****	−0.52	0.31	**0.68***	**−0.69***	−0.02	−0.09	−0.12
BP	−0.07	**0.75****	−0.27	0.01	0.47	−0.22	**0.56***	**0.57***	**−0.58***
EPP	−0.23	**0.56***	−0.14	0.13	**0.69***	−0.46	−0.02	0.36	−0.38

in marine habitats, the SAR11 cluster prevailed in the LNA fraction (Mary et al. 2006), although other studies pointed to the comparable phylogenetic composition of the HNA and LNA fractions (e.g. Longnecker et al. 2005). Furthermore, the MAR-FISH analysis revealed that a major part of ACT incorporated both substrates at all stations, indicating that the population of LNA cells in the Římov reservoir is a highly active group of bacteria significantly contributing to the overall activity of the whole bacterial assemblage.

CONCLUSIONS

We have shown that spatio-temporal variations in total bacterial abundance were accompanied by changes in bacterial activities as determined by flow cytometry. Considering the group-specific properties of bacteria at 3 stations along the longitudinal axis of the reservoir, no significant differences in bacterial community composition based on the proportions of major bacterial groups occurred, compared to distinct differences in relative proportions of Leu+ and Glc+ cells. Simultaneous application of flow cytometry and MAR-FISH techniques revealed that the fraction of HNA cells correlated with BET and CF, while that of LNA cells correlated with ACT. Moreover, in the lacustrine parts of the reservoir, CF also correlated with CTC+ cells, indicating their high respiratory activity. These major phylogenetic groups of bacteria that comprised the majority of total bacteria in the mesoeutrophic canyon-shaped Římov reservoir also differed in terms of active incorporation of organic substrates, implying differences not only in the composition but also in the functioning of HNA and LNA subpopulations.

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