

A small population of planktonic *Flavobacteria* with disproportionately high growth during the spring phytoplankton bloom in a prealpine lake

Michael Zeder, Simone Peter, Tatiana Shabarova and Jakob Pernthaler*

Limnological Station Kilchberg, University of Zurich, Zurich, Switzerland.

Summary

Bacterioplankton growth in temperate Lake Zurich (Switzerland) was studied during the spring phytoplankton bloom by *in situ* techniques and short-term dilution bioassays. A peak of chlorophyll *a* (Chl *a*) concentrations was followed by a rise of bacterial cell numbers and leucine assimilation rates, of the proportions of cells incorporating 5-bromo-2-deoxyuridine (BrdU), and of community net growth rates in dilution cultures. Incorporation of BrdU was low in *Betaproteobacteria* ($2 \pm 1\%$), indicating that these bacteria did not incorporate the tracer. Pronounced growth of *Betaproteobacteria* in the enrichments was only observed after the decline of the phytoplankton bloom. An initial peak in the proportions of BrdU-positive *Actinobacteria* (30%) preceded a distinct rise of their cell numbers during the period of the Chl *a* maximum. *Cytophaga-Flavobacteria* (CF) changed little in numbers, but featured high proportions of BrdU-positive cells ($28 \pm 12\%$). Moreover, CF represented > 90% of all newly formed cells in dilution cultures before and during the phytoplankton bloom. One phylogenetic lineage of cultivable *Flavobacteria* (FLAV2) represented a small (0.5–1%) but highly active population in lake plankton. The growth rates of FLAV2 in dilution cultures doubled during the period of the Chl *a* maximum, indicating stimulation by phytoplankton exudates. Thus, CF, and specifically *Flavobacteria*, appeared to be substantially more important for carbon transfer in Lake Zurich spring bacterioplankton than was suggested by their standing stocks. The high *in situ* growth potential of these

bacteria might have been counterbalanced by top-down control.

Introduction

Phytoplankton communities in temperate lakes undergo predictable seasonal transitions (Sommer *et al.*, 1986). The first bloom of algal biomass during spring time is typically triggered by the interplay of increasing solar irradiation levels and the onset of thermal stratification (Bleiker and Schanz, 1997), and it is eventually terminated by the grazing pressure of herbivorous zooplankton. The primary algal colonizers of the spring water column (small diatoms and flagellates) share common ecophysiological features, i.e. they compensate for increasing predation by achieving high growth rates despite low ambient temperatures (Sommer, 1981).

The abundances and biomasses of heterotrophic bacterioplankton in lakes usually show less annual fluctuations than the phytoplankton. This is thought to be a consequence of the tight coupling between bacteria and their protistan consumers, or between bacteria and viral lysis respectively (Weinbauer, 2004; Pernthaler, 2005). Even so, bacterioplankton numbers increase during the spring period at least by a factor of two, typically with a small temporal delay to the primary producers (Simon *et al.*, 1998). Unlike the phytoplankton, the pelagic bacterial assemblages are typically not suppressed to their pre-bloom abundances during early summer, likely due to the complex cascading trophic interactions within aquatic food webs (Zöllner *et al.*, 2003).

Little is known about the ecological attributes of bacterial taxa that occur during the period of the spring phytoplankton maximum in temperate lakes. Cultivation-independent approaches allow for the quantitative analysis of the dynamics of freshwater bacterioplankton populations (Pernthaler *et al.*, 1998; Eiler and Bertilsson, 2007). However, a determination of changes in cell (or rRNA gene copy) numbers may not be sufficient to unambiguously identify those bacterial taxa that are most strongly favoured by ambient growth conditions. Both protistan grazing and viral lysis tend to disproportionately affect the most rapidly growing microbial populations

Received 15 November, 2008; accepted 4 June, 2009. *For correspondence. E-mail pernthaler@limnol.uzh.ch; Tel. (+41) 44 716 1210; Fax (+41) 44 716 1225.

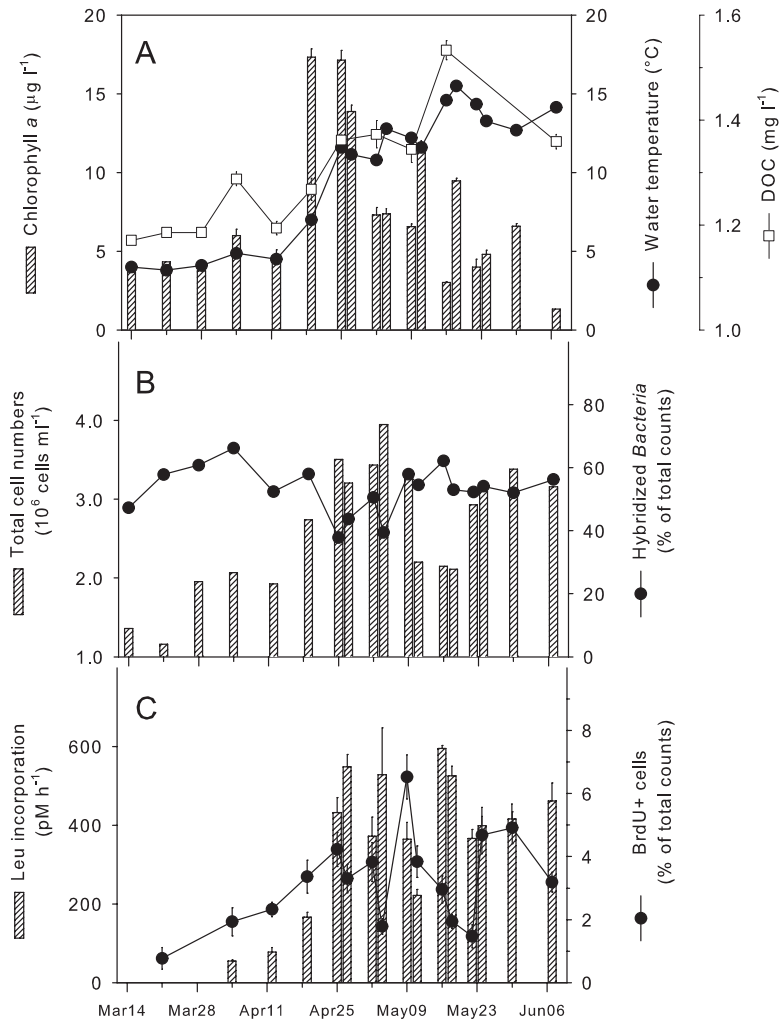


Fig. 1. A. Temperature, concentrations of chlorophyll *a* and dissolved organic carbon (DOC).

B. Total microbial cell number (DAPI staining) and fraction of hybridized *Bacteria* (probe EUB I-III).

C. Incorporation rates of [³H]-leucine and proportions of BrdU-positive DAPI-stained microbial cells in Lake Zurich during the investigation period.

Error bars in this and all subsequent graphs represent one standard error of the mean values of triplicate determinations.

(Sherr *et al.*, 1992; Weinbauer, 2004). Thus, it is possible that small and/or apparently stable populations of highly active bacterial taxa might nevertheless be responsible for a considerable fraction of carbon transfer to higher trophic levels (Simek *et al.*, 2005).

Several strategies allow linking the changes in the standing stocks of bacterioplankton populations with their growth activity. For one, growing cells in lake water may be distinguished from inactive ones by pulse labelling techniques (Warnecke *et al.*, 2005; Salcher *et al.*, 2008). The typically short incubation times required to probe for particular activities allow evaluating physiological properties of populations at close to *in situ* conditions. Second, the *in situ* growth potential of microbes can also be assessed by short-term dilution cultures (Elser *et al.*, 1995). The reduction of predation mortality in these assays typically induces a profound shift of community composition: those genotypes are favoured that might be most proficient in utilizing the available resources but that are at the same time disproportionately affected by grazing (Beardsley *et al.*,

2003). Dilution enrichments can therefore provide valuable additional information about the growth strategies of different microbial taxa (Burkert *et al.*, 2003).

We investigated the dynamics and growth state of bacterioplankton taxa during the spring phytoplankton bloom in the mesotrophic Lake Zurich. Samples were collected at high temporal resolution to adequately map the development of physicochemical and biological parameters during this dynamic period of plankton succession. Microbial growth was assayed by the two complementary approaches outlined above. In addition, the *in situ* population dynamics and growth potential of cultivable bacteria from a readily enriched phylogenetic group were assessed.

Results

Physicochemical changes during phytoplankton bloom

Water temperatures at the sampling depth (2.5 m) rose from 3.3 to 15.5°C during the study period (Fig. 1A). A

distinct spring phytoplankton bloom was observed in the epilimnion of Lake Zurich during the second half of April 2006 (Fig. 1A). Chlorophyll *a* (Chl *a*) concentrations in 2.5 m depth ranged between 3.7 and 17.3 $\mu\text{g l}^{-1}$, with maximal values between 19 and 27 April. The algal community during that period was dominated by phytoflagellates (*Rhodomonas* sp., *Cryptomonas* sp., *Erkenia* sp.) and diatoms (*Cyclotella* sp., *Asterionella* sp.), and a pronounced maximum of the mixotrophic flagellate *Dinobryon* sp. ($> 3000 \text{ ml}^{-1}$) was observed at the beginning of May. In parallel, the concentrations of dissolved phosphorus dropped from 10 to 4 $\mu\text{g l}^{-1}$ during April (source: water monitoring programme of Water Supply Zurich). The concentrations of dissolved organic carbon (DOC) increased during and after the bloom, from 1.2 mg l^{-1} in the pre-bloom period to 1.5 mg l^{-1} thereafter (Fig. 1A). Dissolved organic carbon on average represented 72% ($\pm 9\%$) of total organic carbon (TOC), and the lowest DOC : TOC ratios (0.53–0.59) were observed during peak values of Chl *a*. Oxygen saturation followed the development of Chl *a*, ranging from 90% to 150% (data not shown).

Bacterial abundance and bulk activity

Total microbial cell numbers ranged between 1.5 and $2.0 \times 10^6 \text{ cells ml}^{-1}$ prior to the peak of Chl *a* concentrations, and reached approximately twice these values thereafter (Fig. 1B). An intermediate drop of total cell counts was observed for a 1-week period in mid-May. The incorporation rates of radiolabelled leucine increased by approximately one order of magnitude over the study period (Fig. 1C). The temporal pattern of leucine incorporation rates closely reflected the changes in cell abundances during the actual phytoplankton bloom period (4 April–11 May, linear regression, $r^2 = 0.80$, $n = 9$), whereas the two parameters were clearly uncoupled during the subsequent minimum of cell numbers.

The proportions of 5-bromo-2-deoxyuridine (BrdU)-incorporating [BrdU-positive (BrdU+)] cells (Fig. 1C) were determined from three parallel incubations per sampling time point and from five separate preparations per incubation. Only a small fraction of total (DAPI-stained) cells visibly incorporated BrdU into their DNA, corresponding to a range of $0.1\text{--}2.3 \times 10^5 \text{ BrdU+ cells ml}^{-1}$ (mean, $1.0 \times 10^5 \text{ cells ml}^{-1}$). The fractions and total numbers of BrdU+ cells steadily increased until the peak of Chl *a* concentrations but became distinctly more variable thereafter. While this general pattern was also observed for total abundances and leucine incorporation rates, no significant correlation was observed between either parameter and the proportions of BrdU+ cells over the course of the investigation.

Contribution of phylogenetic groups to total and BrdU+ cells

On average, only 53% of DAPI-stained cells could be detected by fluorescence *in situ* hybridization (FISH) in combination with catalysed reporter deposition (CARD) and using a probe targeted to *Bacteria* (Fig. 1B). The sum of the three studied groups represented 68% ($\pm 8\%$) of all hybridized *Bacteria* at four consecutive sampling time points during the decline of the phytoplankton bloom (25 April–4 May), but only 33% ($\pm 14\%$) of *Bacteria* before and after this period. *Betaproteobacteria* accounted for 3% (7 June) to 11% (16 May) of cell counts (i.e. 6–22% of hybridized *Bacteria*). Slightly higher relative proportions and abundances of *Betaproteobacteria* were observed during and after the phytoplankton bloom (Fig. 2). Microbes affiliated with the *Cytophaga-Flavobacteria* lineage (CF) of *Bacteroidetes* showed a trend similar to

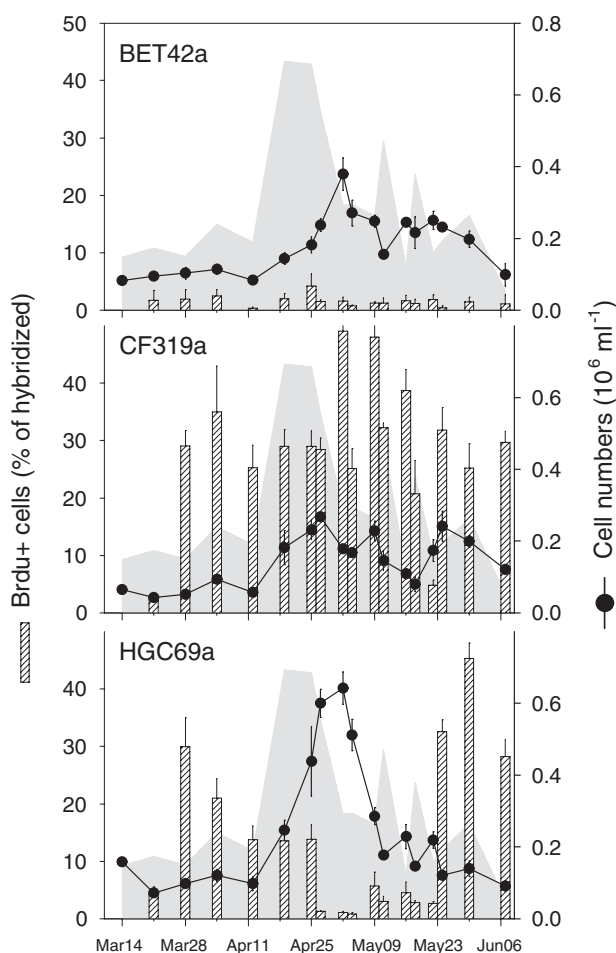


Fig. 2. Cell numbers and proportions of BrdU-positive *Betaproteobacteria* (probe BET42a), *Cytophaga-Flavobacteria* (probe CF319a) and *Actinobacteria* (probe HGC69a) during the spring phytoplankton bloom period 2006. Chlorophyll *a* concentrations (from Fig. 1A, shaded area) are depicted for reference.

Betaproteobacteria in terms of both relative proportions and absolute counts (Fig. 2). In contrast, a distinct peak of actinobacterial abundances was observed that followed the period of the Chl *a* maximum with a delay of 1 week (Fig. 2). During that period *Actinobacteria* represented > 40% of all hybridized *Bacteria* (c. 20% of total cell counts), whereas they reached approximately half this value at the other sampling time points.

Bacteria hybridized by the three probes represented 62% ($\pm 18\%$) of all BrdU+ cells, and the combined contribution of the three groups to active cells was significantly higher than to all hybridized *Bacteria* (paired Student's *t*-test, $P < 0.05$). This was mainly due to microbes affiliated with CF and *Actinobacteria* (Fig. 2), whereas only low fractions of *Betaproteobacteria* were found to incorporate the halogenated compound into their DNA (Fig. 2). Remarkable temporal dynamics of the fractions of BrdU+ actinobacterial cells were observed: two maxima of 30% and > 40% of active cells at the beginning and ending of the investigation period could be distinguished. In contrast, only a minor fraction of *Actinobacteria* visibly incorporated BrdU for a period of 4 weeks following the phytoplankton bloom period. CF hybridized by probe CF319a on average featured significantly higher proportions of BrdU+ cells than the other two studied groups over the study period (one-way ANOVA, $P < 0.05$). The highest fractions of active cells within this lineage (approximately 50%) were found in the immediate aftermath of the phytoplankton bloom period (Fig. 2).

Dilution enrichments

Bacteria targeted by the three FISH probes on average represented 63% (range, 32–74%) of all DAPI-stained cells after 48–120 h of enrichment, or > 90% of hybridized *Bacteria*. The average enrichment factors of CF, *Betaproteobacteria* and *Actinobacteria* over the whole investigation period (i.e. the ratio between their abundances at the beginning and ending of the dilution enrichments) were 26 (range, 5–81), 6 (2–11) and 4 (2–11) respectively. During the first month of the investigation CF represented approximately half of the microbial assemblage at the end-points of the dilution cultures or 90% of all newly formed cells (Fig. 3A). Their relative abundances in the enriched assemblages remained high until the onset of the phytoplankton bloom and continuously decreased thereafter; to about twice the proportions of CF in environmental samples on the last sampling date. *Betaproteobacteria* were not conspicuously enriched before and during the period of the Chl *a* maximum or at the end of the investigation period. However, these bacteria represented 50–80% of the newly formed cells (30–40% of final abundances) in two subsequent dilution bioassays in May.

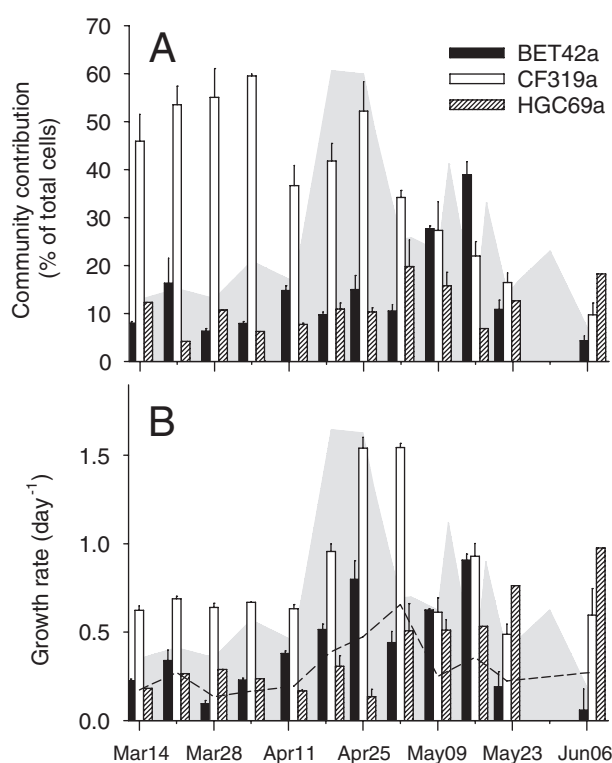


Fig. 3. A. Proportions of *Betaproteobacteria* (probe BET42a), *Cytophaga-Flavobacteria* (probe CF319a) and *Actinobacteria* (probe HGC69a) in weekly dilution bioassay after approximately one doubling of total cell numbers. B. Estimated growth rates of the three groups in the enrichments, as compared with the overall community growth rate (broken line). Chlorophyll *a* concentrations (shaded area) are depicted for reference.

Actinobacteria never formed more than 20% of cells in the enrichments.

The lowest overall growth rates of the experimental assemblage in the dilution cultures were observed prior to the phytoplankton bloom at water temperatures < 5°C (Figs 1 and 3B). However, the community growth rates were not linearly related to the continuous increase in temperature, but rather peaked during and after the Chl *a* maximum. This was paralleled by a distinct maximum of the growth rates of CF between 25 April and 3 May (Fig. 3B). The growth of CF was significantly more rapid than the community average also at all other sampling time points. *Betaproteobacteria* exhibited the highest growth rates between mid-April and mid-May, whereas *Actinobacteria* significantly exceeded the average community growth in the dilution cultures only at the end of the investigation period.

No differences in the proportions of bacteria hybridized with probe CF319a were observed after 48 h of incubation of unmanipulated lake water performed in spring 2007 (initial and final concentrations: 7–8% of DAPI-stained cells). In contrast, CF319a-positive cells almost doubled

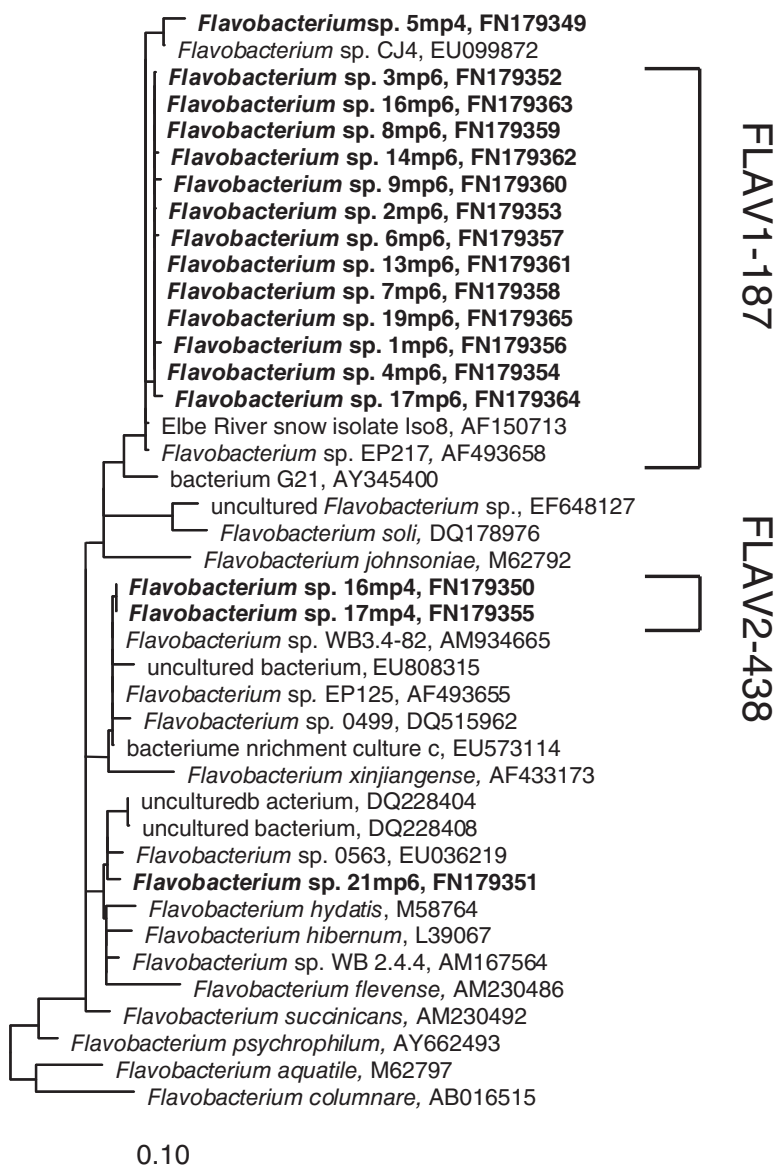


Fig. 4. Phylogenetic affiliation of flavobacterial isolates obtained from dilution enrichments in spring 2007 (in bold face). The depicted tree is based on Maximum Likelihood (ML) analysis of 650 flavobacterial sequences, and multifurcations indicating discrepancies between ML and other analyses (Maximum Parsimony, Neighbour Joining). Brackets indicate sequence types targeted by probes FLAV1-187 and FLAV2-438 respectively.

(to 14%) in 1:10 dilutions with 0.1 μm pre-filtered water. The total bacterial cell numbers in the unmanipulated samples increased by < 20% over the incubation period, whereas they changed by more than twofold in the dilution treatment.

Bacterial isolates, design of new FISH probes

Twenty of 86 obtained isolates were affiliated with the *Flavobacteria*. These strains could be clustered into three separate phylogenetic lineages (Fig. 4). The first lineage harboured a set of almost identical sequence types and one strain (5mp4) with only 98% sequence identity with the other isolates. The second group was formed by two isolates (strains 16mp4, 17mp4) which only differed by one base position and that formed a

monophyletic lineage together with a psychrophilic strain and with isolates from riverine biofilms (99% sequence identity). One isolate (21mp6) could not clearly be grouped with a specific sequence type. It displayed around 97% of sequence similarity to the other strains. Specific FISH probes were designed for two phylogenetic clades of *Flavobacteria* that harboured isolates and tested on pure cultures from these groups. Probe FLAV1-187 (5'-AAT AAT CTA CTC ATG CGA AT-3') detects the set of highly similar sequences from the FLAV1 clade as well as two isolates from riverine aggregates or biofilms. Probe FLAV2-438 (5'-GAA CTG TTT CTT CCT GTA CAA-3') is targeted to the two isolates 16mp4 and 17mp4. Stringent hybridization conditions for CARD-FISH according to the protocol by Pernthaler and colleagues (2004) were obtained at 50% and 45% of

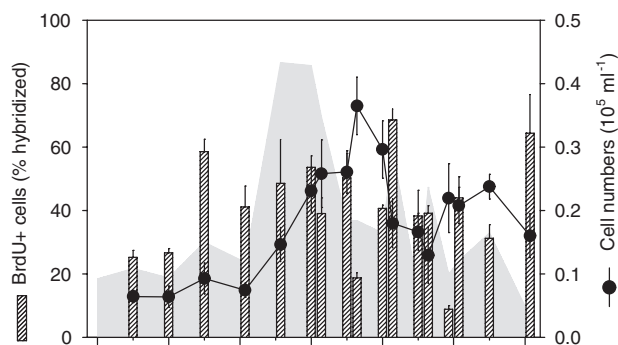


Fig. 5. Cell numbers and proportions of BrdU-positive cells of *Flavobacteria* targeted by probe FLAV2-438 in Lake Zurich during the spring phytoplankton bloom period 2006.

formamide in the hybridization buffer for probes FLAV1-187 and FLAV2-438 respectively.

FLAV1 and FLAV2 bacteria in environmental samples and enrichments

The concentrations of bacteria targeted by probe FLAV1 were below the detection limit by our FISH quantification technique at all sampling time points both in the environmental samples and in the dilution cultures ($< 0.25\%$ of total counts, data not shown). Cells detected by probe FLAV2-438 represented a small but quantifiable population in Lake Zurich ($0.7 \pm 0.1\%$ of total counts). The cell numbers of FLAV2 bacteria varied by a factor of approximately 6 over the study period, and a distinct maximum of this population was observed subsequent to the phytoplankton bloom (Fig. 5). Typically, high proportions of FLAV2 bacteria were found to incorporate BrdU (mean 41%, range 9–69%), at instances even exceeding the fractions of BrdU+ cells within the CF lineage.

Bacteria hybridized by probe FLAV2-438 produced substantial populations in the dilution cultures, in particular at the first two sampling time points and in the period of the Chl *a* maximum (19 and 25 April) (Fig. 6). During the phytoplankton bloom FLAV2 bacteria enriched by a factor of > 100 within 48 h and represented $> 40\%$ of all newly formed cells ($> 50\%$ of newly formed CF). The growth rates of FLAV2 bacteria in the dilution cultures always exceeded the community average by a factor of 4–6. Moreover, these bacteria approximately doubled their growth rates during and immediately after the phytoplankton bloom in comparison with the rest of the study period (Fig. 6).

Discussion

Community level activity

The proportions of hybridized *Bacteria* in all DAPI stained cells were rather low (Fig. 1B), as has also been reported

from other lakes (Allgaier and Grossart, 2006). This suggests either that a large fraction of DAPI-stained objects from the pelagic zone of Lake Zurich were not viable or of non-bacterial origin (Zweifel and Hagström, 1995) or that some microbes were not targeted by the FISH probe EUB I-III. The presence of non-viable cells or cell fragments would imply that the low fractions of BrdU+ cells of all DAPI-stainable objects (Fig. 1C) might in fact have underestimated the real proportion of growing cells in the microbial assemblages. In any case, the method probably only detects microbial growth above a certain threshold (Pernthaler and Pernthaler, 2005), as is the case for similar approaches (Sherr *et al.*, 1999). In addition, some bacteria might not be able to incorporate BrdU at all (Urbach *et al.*, 1999). Thus, while cells without BrdU incorporation generally should not be regarded as dead or dormant, many of them likely grow substantially more slowly than BrdU+ bacteria. A small subset of highly active cells within a generally slowly growing microbial assemblage would also agree with the low overall community growth rate estimates observed in the dilution bioassays (on average 0.3 day^{-1} , Fig. 3).

Dilution culture bioassays are a traditional means of assessing the growth rates of planktonic algae (Landry and Hassett, 1982). High dilution reduces microconsumer grazing and permits a closer determination of bacterial gross growth rates (Elser *et al.*, 1995). Moreover, dilution cultures provide a means of growing microbes under the substrate and nutrient concentrations of the original samples for several generations (Sterner, 1994) without introducing unintended bottom-up limitations (Posch *et al.*, 2007). Ambient substrate concentrations might in fact also be artificially augmented in dilution treatments, e.g. by the destruction of phytoplankton cells during filtration and/or preparation of the diluent (Ferguson *et al.*, 1984). However, particle removal by tangential flow filtration does not measurably increase the ambient

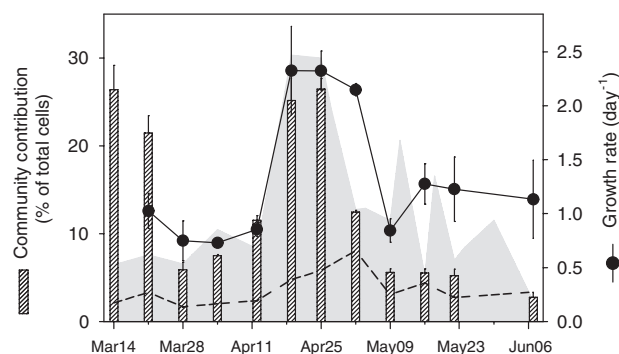


Fig. 6. Proportions of FLAV2 bacteria and estimated growth rates in dilution bioassays. Chlorophyll *a* concentrations (shaded area) and the average community growth rate (broken line) are depicted for reference.

concentrations of dissolved assimilable organic carbon in lake water filtrates (Jüttner *et al.*, 1997).

Growth behaviour of different phylogenetic groups

The low fractions of BrdU+ cells in *Betaproteobacteria* appear to suggest that these bacteria reproduced more slowly than the two other studied groups (Fig. 2). In view of their increasing abundances in lake water (Fig. 2) and their high growth rates during the later phase of the experimental incubations (Fig. 3), it is more likely that some freshwater *Betaproteobacteria* might not be able to assimilate BrdU. In contrast, the high fractions of BrdU incorporating cells within *Actinobacteria* and CF (Fig. 2) constitute plausible evidence for the autochthonous growth of these bacterial groups in lake water (Warnecke *et al.*, 2005).

Some members of the CF lineage in freshwater plankton appear to readily respond to changes in growth conditions (Simek *et al.*, 2005; Salcher *et al.*, 2007; Bertoni *et al.*, 2008). Thus, the observation of high CF growth rates in our dilution cultures by itself does not represent unambiguous evidence that these bacteria were also active in the environment. However, at the same time high fractions of BrdU+ CF cells were also present *in situ* (Figs 2 and 3). In view of these complementary lines of evidence we may conclude that some genotypes affiliated with CF were a highly active component of the spring bacterioplankton assemblage. While CF have been associated with phytoplankton blooms in marine habitats before (Simon *et al.*, 1999; Riemann *et al.*, 2000), no evidence has so far been produced for a similar ecological function in freshwaters.

Actinobacteria differed from CF both in their temporal dynamics and growth behaviour (Figs 2 and 3). Maximal abundances of *Actinobacteria* during spring or early summer have also been reported from other lakes with different limnological characteristics (Allgaier and Grosart, 2006). Freshwater representatives of this lineage are substantially less sensitive to predation by bacterivorous protists than other bacteria (Hahn *et al.*, 2003; Jezbera *et al.*, 2006). This might explain why *Actinobacteria* featured such a pronounced increase in population size whereas the equally active CF did not. The bimodal patterns of BrdU incorporation (Fig. 2) and the inverse relationship between the fraction of BrdU+ cells and total actinobacterial abundances suggest a succession of different genotypes from the bloom to the post-bloom period. This hypothesis should be followed up in future investigations at higher phylogenetic resolution. Moreover, there was a striking contrast between the high proportions of BrdU+ *Actinobacteria* in lake water prior to the Chl *a* maximum and their apparent failure to grow in dilution bioassays. Thus, different activity assays may need to be

combined in order to reach reliable ecophysiological conclusions, and the absence of evidence for growth by one particular approach may not automatically be evidence for non-growth. So far, enrichment of *Actinobacteria* after experimental manipulation of lake water has only been achieved at high protistan predation (Hornak *et al.*, 2005; Simek *et al.*, 2005).

Cultivable Flavobacteria in spring bacterioplankton

Flavobacteria are a rather poorly studied component of limnic bacterioplankton. Sequence types affiliated with *Flavobacteria* have been obtained from a variety of lakes with contrasting limnological characteristics during various seasons, including phytoplankton bloom events (Eiler and Bertilsson, 2007). High rRNA gene copy numbers of bacteria from this phylogenetic lineage were found in the spring and summer plankton of several eutrophic lakes during periods of high bacterial production (Eiler and Bertilsson, 2007), suggesting that *Flavobacteria* may be particularly successful if resources are plenty. In addition, many freshwater representatives of this lineage appear to be readily cultivable, e.g. more than 40 putative novel phylospecies of *Flavobacteria* have recently been isolated from a hardwater creek (Cousin *et al.*, 2008).

In our study, the phylogenetic lineage that harboured most isolated strains of *Flavobacteria* (FLAV1, Fig. 4) could only be detected in extremely low numbers in environmental samples, and no enrichment of these bacteria was observed in dilution cultures. It is possible that members of FLAV1 isolated in 2007 were not present in the spring bacterioplankton in 2006. Alternatively, these bacteria may not be predominantly planktonic but rather originate from the particle-associated aquatic microflora. Strains that are phylogenetically closely related to our FLAV1 isolates (Fig. 4) have been obtained from riverine organic aggregates (Böckelmann *et al.*, 2000) and river biofilms. So far, we have not investigated if bacteria from the FLAV1 lineage were also abundant on lake snow particles in Lake Zurich. The 16 isolates within the FLAV1 clade displayed high sequence identity between each other of approximately 99%. Interestingly, several strains featured base ambiguities in regions corresponding to bases 95, 455–478, 590–593 and 646–651 in *Escherichia coli* 16S rRNA numbering (Brosius *et al.*, 1981). This is probably due to sequence variability between different rRNA operons that might be erroneously interpreted as microdiversity by cultivation-independent approaches.

In contrast, the presence of bacteria from the FLAV2 lineage with high proportions of BrdU+ cells (Fig. 5) suggests that these bacteria were an actively growing component of the spring bacterioplankton assemblage in Lake Zurich. Preliminary FISH analysis of samples from the two subsequent years moreover indicates that these bacteria

might in fact form annually recurrent population (J. Pernthaler, unpubl. data). The rapid enrichment of FLAV2 in dilution cultures (Fig. 6) illustrates that they were well adapted to grow in lake water at ambient substrate concentrations, i.e. that they could successfully pursue an entirely planktonic lifestyle.

The growth rates of FLAV2 bacteria in the dilution cultures were distinctly higher for the period of the phytoplankton maximum than during the rest of the study period (Fig. 6). This stimulation might be due to the short-term availability of specific algal exudates (Hama and Yanagi, 2001) that were particularly favourable for some members of FLAV2. The composition of labile organic substrates in lake water can be temporally highly variable, e.g. carbohydrates may be released in pulses that last for a few hours only (Meon and Juttner, 1999). The high growth rates of FLAV2 in the dilution cultures during and after the maximum of Chl *a* concentrations (Fig. 6) moreover appeared to mirror their population increase in the environment during that period (Fig. 5). However, the net increase in FLAV2 cell numbers during the period of the Chl *a* maximum (11 April to 3 May) was much smaller than suggested by their rapid growth in the bioassays. Following the logic of the dilution assay approach (Landry and Hassett, 1982) this discrepancy could be an indication for strong top-down control (Beardsley *et al.*, 2003) of FLAV2 (e.g. by the abundant mixotrophic chrysophyte *Dinobryon*). Such an interpretation is also supported by the lack of growth of *Flavobacteriaceae* in incubations of unmanipulated lake water in spring 2007. Mixo- and heterotrophic flagellates are known to strongly affect bacterioplankton assemblages in temperate lakes during the spring period (Pernthaler *et al.*, 1996; Comte *et al.*, 2006). Size-selective grazing on FLAV2 (and CF in general) due to size (Simek and Chrzanowski, 1992) is indicated by their larger mean cell volumes (0.07 ± 0.014 and $0.071 \pm 0.027 \mu\text{m}^3$ respectively), which significantly exceeded those of *Actinobacteria* ($0.041 \pm 0.011 \mu\text{m}^3$) or of the total community ($0.044 \pm 0.007 \mu\text{m}^3$) (one-way ANOVA, $P < 0.01$). In fact, a selective elimination of freshwater CF by flagellate predation has been previously demonstrated via direct analysis of protistan food vacuole content (Jezbera *et al.*, 2006). Thus, the high growth rates of FLAV2 in dilution cultures during periods of stable *in situ* cell numbers (i.e. before and after the maximum of Chl *a*, Fig. 6) suggest that this bacterial population was turned over at least once per day by predation.

Experimental procedures

Sampling, physicochemical parameters, Chl *a*

Surface water samples were collected weekly from Lake Zurich (47°18'14"N, 8°34'14"E) from the middle of March to the beginning of June 2006 and twice a week during 4 weeks

that represented the actual phytoplankton bloom period (25 April–24 May). Water was collected with a 5 l Friedinger water sampler at 2.5 m depth (the typical zone of the maximal algal densities during spring in Lake Zurich) and filled in 1 l glass bottles pre-washed with hydrochloric acid. Water temperature, conductivity and oxygen concentrations were monitored along a depth profile of the photic zone (0–20 m) with a multiparameter probe at the time of sampling (model 6600 *Yellow Springs Instruments*). Chlorophyll *a* concentrations were determined fluorometrically (model F-2000, Hitachi) after acetone extraction (Schanz, 1982). Total organic carbon was determined in triplicates from unfiltered water samples on a TOC 5000 analyser (Shimadzu). Dissolved organic carbon was measured following hollow fibre tangential flow filtration with a particle exclusion size of 0.1 (Hydac International). Subsamples (20 ml) for the analysis of total bacterial abundances and community composition were fixed for 1–24 h with buffered paraformaldehyde (PFA, final concentration, 2%).

Bulk incorporation of leucine

The uptake of tritiated leucine was determined starting on April 4 until the end of the study period. Five millilitres of samples were incubated in triplicates (plus one pre-fixed control) with [³H]-leucine (Amersham, final concentration, 20 nM) for 2 h at *in situ* temperature in the dark. Incubations were stopped by addition of formaldehyde solution (2% final concentration). After 1 h of fixation the water was filtered on cellulose-ester filters (Sartorius, diameter, 25 mm, pore size, 0.22 μm). Filters were dissolved and macromolecules were extracted as described (Kirchman *et al.*, 1985). After addition of scintillation cocktail (UltimaGold, Perkin-Elmer) the amount of assimilated radiolabelled substrate was estimated by scintillation counting (Tri-Carb 3170 TR, Perkin-Elmer). Measurements were corrected for quench (external standard method), and by subtraction of counts from the prefixed controls.

Total microbial abundances and community composition

Total bacterial abundances in lake water samples and in experimental enrichments were determined by epifluorescence microscopy after filtration of subsamples (2 ml) on black membrane filters and staining with 4',6-diamidino-2-phenylindole (DAPI) (Porter and Feig, 1980). The proportions of different microbial taxa in lake water and dilution enrichments were determined by FISH with horseradish peroxidase-labelled oligonucleotide probes (purchased from Biomers Inc, www.biomers.net) on filter sections as described before (Pernthaler *et al.*, 2004). Signal amplification was performed with tyramides custom labelled with the fluorochrome Alexa488 (Invitrogen). FISH probes were used that target bacteria affiliated with *Betaproteobacteria* (BET42a), *Actinobacteria* (HGC69a), and with the *Cytophaga-Flavobacteria* sublineage of *Bacteroidetes* (CF319a) (Amann *et al.*, 1995). In addition, two new specific probes were designed and tested by hybridization on isolates at increasing levels of stringency (i.e. increasing formamide concentrations in the hybridization buffer). These probes are targeted to two phylogenetic lineages of isolates that were

obtained from additional dilution enrichments conducted in 2007 (see below).

Proportions of cells with DNA *de novo* synthesis

The proportions of cells with visible DNA *de novo* synthesis were determined from 21 March until the end of the study period. Triplicate lake water samples (200 ml) were incubated in 250 ml glass bottles with BrdU (20 µM) and thymidine (33 nM) at *in situ* temperature in the dark (Pernthaler and Pernthaler, 2005). After 2 h of incubation subsamples (20 ml) were fixed with PFA for 1 h at room temperature. One additional treatment pre-fixed with PFA (20 ml) served as negative control. Portions of 2–7 ml from all replicates were filtered onto polycarbonate membrane filters (type GTTP, Millipore, diameter, 47 mm, pore size, 0.2 µm). Preparations were stored at –20°C until further processing.

The incorporation of BrdU into *de novo* synthesized DNA in whole cells was visualized cytochemically using a BrdU-specific horseradish peroxidase-labelled antibody FAB fragment (Roche) followed by CARD signal amplification (Pernthaler and Pernthaler, 2005). BrdU detection was subsequently combined with cell identification by CARD-FISH (Pernthaler and Pernthaler, 2005). In order to avoid a spectral overlap of fluorochromes between the FISH and BrdU stainings, CARD detection of BrdU-conferred HRP was performed with tyramides labelled with Alexa633 (Invitrogen).

Epifluorescence microscopy

The evaluations of CARD-FISH staining and BrdU incorporation experiments were performed on an integrated automated high-throughput screening platform based on motorized epifluorescence microscopy and image analysis (M. Zeder, unpublished). The core of the system consisted of a conventional epifluorescence microscope (AxioImager.Z1, Carl Zeiss) equipped with a motorized stage for eight microscopic slides. The epifluorescence illumination device (Colibri, Carl Zeiss) included LED modules for 365, 470 and 590 nm excitation. A triple filter set (62 HE) was used in combination with the Colibri system to image three different fluorescent dyes (DAPI, Alexa488, Alexa633), thus eliminating the need of changing filter blocks between different excitation wavelengths. Fluorescence images were recorded with a CCD Camera (AxioCam MRm, Carl Zeiss) using a 63× objective (Plan-Apochromat). The microscope was controlled by a computer (CPU: Intel Core2Duo 3 GHz; RAM: 3 GB) running Windows XP Professional (Microsoft) and the software AxioVision 6.3 (Carl Zeiss). Automation of the image acquisition and evaluation workflow was realized by object-oriented programming either in the Visual Basic for Application module in AxioVision or in Visual Basic.NET (Microsoft). Between 1000 and 4000 DAPI-stained objects were counted per preparation.

The sizes of cells hybridized with different probes were estimated at three time points and in four replicate preparations per time point. Images for cell sizing were acquired at higher magnification than for cell counting, using a 100× Plan Neofluar objective (Carl Zeiss). Cell volumes were inferred from the projected cell areas and perimeters of their DAPI-stained images according to a previously described geometric model (Posch *et al.*, 1997).

Dilution experiments

The growth response of lake bacteria to a relief of top-down control was assessed weekly in dilution cultures. Bacteria-free lake water was prepared by hollow fibre filtration (pore size, 0.1 µm) (Hydac International). Fifty millilitres of lake water samples were added to 450 ml of filtrate and incubated at *in situ* temperature (range, 3.3–14.1°C) in the dark in 1 l glass bottles. Incubations were conducted in triplicates for 120 h before the phytoplankton bloom period (14 March–9 April) and for periods of 48–72 h thereafter. The variable incubation times were chosen to obtain final cell numbers that were approximately twice as high as at the beginning of the experiments (as assessed by daily counting). The enrichments were stopped by fixation of subsamples (20–50 ml) with 1% PFA.

The apparent growth rates of the total microbial assemblage and of different FISH-defined populations in the dilution cultures were estimated from the differences between their cell numbers at the start and end points of the enrichments, assuming exponential cell increase and neglecting a potential growth delay after transfer (lag phase). The presented estimates thus represent conservative approximations of actual growth rates.

In order to test for intrinsic changes of *Flavobacteriaceae* during bottle incubations, additional lake water was collected on 27 March 2007. A 1:10 dilution culture was prepared as described above and compared with a control treatment of unmanipulated lake water. The samples (volume, 1000 ml in 2 l glass bottles) were incubated in the dark for a period of 48 h at ambient temperature (7°C) for a period of 48 h. Subsamples were fixed and prepared as described above, and the total cell numbers and proportions of *Flavobacteriaceae* in either treatment were determined by DAPI staining and CARD-FISH with probe CF319a.

Isolation of enriched bacteria

In view of the results from the dilution cultures, additional enrichments experiments were performed as described above during the spring phytoplankton bloom period 2007 with the purpose of isolating bacterial strains from the phylogenetic groups which grew most rapidly in the 2006 dilution experiments. After 72–120 h of enrichment subsamples (0.1–0.001 ml) were plated on complex media [YST: yeast extract, starch, peptone from casein, each 0.25 g l⁻¹; Plate-Count Agar (PCA, 20 g l⁻¹), PCA/10; Merck]. The plates were incubated for 1–3 days at 17°C and then at 8°C for several weeks to prevent overgrowth of more slowly growing visible colonies by more rapidly spreading ones. Morphologically distinguishable colonies were selected and repeatedly streaked and subcultured in order to obtain pure clonal cultures. The obtained set of isolates was screened by PCR with a group-specific primer set (Alonso *et al.*, 2007). All PCR-positive strains were subsequently identified by determination of their almost complete 16S rRNA gene sequences (> 1300 base pairs) following previously described procedures (Eilers *et al.*, 2000). Sequences were deposited in EMBL under the Accession No. FN179349–FN179365.

A phylogenetic analysis of almost full-length rRNA gene sequences of isolates affiliated to *Flavobacteriaceae* was

performed using the software ARB (Ludwig *et al.*, 2004). Phylogenies by Maximum Parsimony (MP) and Neighbour Joining (NJ) analysis were inferred using > 2500 almost complete sequences of *Flavobacteria* and related genera. A Maximum Likelihood (ML) tree was calculated on a subset of 650 most closely related sequences using the RaxML algorithm (Stamatakis *et al.*, 2005) as implemented in ARB (Hasegawa–Kishino–Yano model of nucleotide substitution). Several trees of each type were calculated using different filters custom made for *Flavobacteriaceae* (nucleotide conservation profiles, positional variability). A consensus tree based on the ML tree was constructed from the various analyses in which only bifurcations were maintained that were conserved in at least two of the three types of reconstruction approaches.

Acknowledgements

We thank Markus Steinkellner and Angela Mechsner for help with sample analysis and two anonymous reviewers for suggestions that improved the manuscript. The Zurich Water Supply Company is acknowledged for data on phytoplankton. This work was supported by a grant from the Foundation for Scientific Research of the University of Zurich, and by the Swiss National Fund (SNF No. 3100A0-117765).

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