Environmental Microbiology (2007) **9**(3), 789–800 doi:10.1111/j.1462-2920.2006.01201.x

Grazer and virus-induced mortality of bacterioplankton accelerates development of *Flectobacillus* populations in a freshwater community

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Summary

We present a detailed analysis of the effects of distinct bacterial mortality factors, viral lysis and heterotrophic nanoflagellates (HNF) bacterivory, associated with the development of filamentous *Flectobacillus* populations. Reservoir bacterioplankton communities were subjected to additions of both HNF and viruses together, or HNF alone, and then incubated *in situ* in dialyses bags. For distinct bacterial groups, mortality or growth stimulation was analysed by examining bacterial prey ingested in HNF food vacuoles with fluorescence *in situ* hybridization (FISH) and via FISH with microautoradiography (MAR-FISH). We also developed a semi-quantitative MAR-FISH-based estimation of relative activities of *Flectobacillus* populations (targeted by the R-FL615 probe). Bacterial groups vulnerable to HNF predation (mainly clusters of *Betaproteobacteria*), or discriminated against (*Actinobacteria*), were detected. Bacterial lineages most vulnerable to virus-lysis (mainly the *Betaproteobacteria* not targeted by the R-BT065 probe, of the *Polynucleobacter* cluster) were identified by comparing treatments with HNF alone to HNF and viruses together. Filaments affiliated with the *Flectobacillus* cluster appeared in both treatments, but were about twice as abundant, long and active as in incubations with viruses and HNF as compared with HNF alone. Viruses appeared to selectively suppress several bacterial groups, perhaps enhancing substrate availability thus stimulating growth and activity of filamentous *Flectobacillus*.

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Introduction

The relative importance of two major sources of bacterial mortality, flagellate bacterivory and viral lysis, have been the subject of much discussion (see reviews of Fuhrman, 1999; Weinbauer, 2004; Pernthaler, 2005). Obviously, differential mortality among distinct bacterial groups can influence directly bacterial community composition but indirect effects are also possible. Based on recent laboratory work (Corno and Jürgens, 2006), it has been suggested that, in *Flectobacillus*, substances released by a feeding bactivore stimulate filament formation – a grazing-resistant morphology. In reservoir bacterioplankton communities, our previous experimental manipulation of grazers and viruses resulted in the dominance of filamentous, grazing-resistant, *Flectobacillus* populations (Šimek *et al*., 2001). Moreover, viral infection rates and concentrations increased with grazing. A complicated interaction between the two bacterial mortality sources was suggested by Weinbauer and colleagues (2003) based on the results of an experiment using natural populations from the Římov reservoir. From these studies it has been difficult to disentangle or distinguish the effects of selective feeding on different groups of bacteria from their differential growth or vulnerability to viral attack.

However, new protocols have been developed permitting the identification, using fluorescence *in situ* hybridization (FISH), of distinct groups of grazed bacteria inside grazer food vacuoles (Jezbera *et al*., 2005; 2006). Differential activity of bacterial groups can be assessed using microautoradiography in combination with FISH (e.g. Lee *et al*., 1999) by employing established protocols such as MAR-FISH (Ouverney and Fuhrman, 1999; Cottrell and Kirchman, 2000; Teira *et al*., 2004).

Here, using these new tools, we attempt to elucidate group-specific effects of grazing versus viral mortality by examining samples from the factorial experimental detailed in the companion paper of Weinbauer and colleagues (2006). The design was based on size-fractionation of plankton samples and subsequent reconstitution yielding incubations of bacterioplankton without predators (C – control with heat-inactivated viruses), with active viruses (V), with heterotrophic nanoflagellates (HNF) and heat-inactivated viruses (F) and with both sources of bacterial mortality, flagellates and active viruses (FV).

The major findings of the study of Weinbauer and colleagues (2006) were the common dominance of several phylogenetic groupings in all the treatments, with however, significant differences in their net growth rates and apparent mortality depending on the treatment. Specific effects on bacterial community activity and diversity were found. In addition, preliminary suggestions of both antagonistic and synergistic interactions between the two sources of bacterial mortality were detected.

Here we focus on distinct dynamics of abundant *Flectobacillus* populations in F and FV treatments exploiting group-specific metrics of metabolic activity and grazer-associated mortality. First, group-specific mortality from grazers was estimated by detailed FISH analysis of HNF food vacuole content. Thus, we could identify which groups of bacteria were the most vulnerable to HNF predation, or were avoided by grazers. Second, by comparing the total numbers of the probe-defined bacterial groups in the F and FV treatments we detected the most likely candidates for virus-enhanced bacterial mortality. Third, microautoradiography was used in combination with FISH, MAR-FISH, to provide group-specific activity estimates. These data were gathered and examined with an eye towards identifying the factors promoting the development of filamentous *Flectobacillus* populations.

The major bacterial groups targeted by the probes employed in our study are known to differ in size, morphology, vulnerability to bacterivorous protists and apparent growth potential. We distinguished two large cosmopolitan groups of *Betaproteobacteria* with very different life strategies: (i) the R-BT065 cluster – fast growing 'uptake strategists' (opportunistic, see Šimek *et al*., 2005), highly vulnerable to HNF grazing (Jezbera *et al*., 2006), and (ii) the *Polynucleobacter* cluster, usually small 'ultramicrobacteria', subject to relatively low losses from grazers but with rather slow growth rates (Boenigk *et al*., 2004; Hahn *et al*., 2005). We also enumerated *Actinobacteria*, a diverse group (e.g. Hahn *et al*., 2003; Warnecke *et al*., 2004), generally of Gram positive small to medium size cells; *in situ* they appear to have rather limited vulnerability to HNF grazing (e.g. Jezbera *et al*., 2005; 2006; Pernthaler, 2005) and grow at medium net growth rates (Šimek *et al*., 2006). In marked contrast to the other groups are *Flectobacillus* populations that are reputed for grazing resistance (Hahn *et al*., 1999; Šimek *et al*., 2001; Corno and Jürgens, 2006). They are fast-growing *in situ* (Šimek *et al*., 2006) and they can produce long chains of cells.

Results

General trends, microbial dynamics in F and FV treatments

Time-course changes in total bacterioplankton abundance and relative proportions of specific groups, biomass and production (in terms of μg C l^{-1} day^{-1}) and viral abundance in all treatments as well as viral production are given in detail in the companion paper of Weinbauer and colleagues (2006). Here, only data concerning the incubations with flagellate grazers alone (F) or in combination with viruses (VF) will be reviewed. Briefly, virus abundances were similar in both V and FV treatments, slightly increasing from ~7.8–9 × 10^7 ml^{-1} towards the end of the study. However, at the end of the experiment bacterial production was stimulated higher in V incubations while virus production was higher in the combined FV treatment. This created an interesting scenario for specific comparison of the effects of HNF and viruses in the F and FV treatments.

Heterotrophic nanoflagellate numbers were overwhelmingly dominated by ~3–5 μm large (measured across), *Spumella*-like chrysomonads. Their abundance and aggregate bacterivory showed practically the same trends in both treatments (Fig. 1). After a lag of about 48 h, HNF grew exponentially and exerted strong grazing pressure on bacterial communities in F and FV treatments, far exceeding the bacterial production detected there the last day. The same grazing pressure yielded, however, different trends in bacterial abundance that dropped significantly only in FV compared with F treatment while the differences in bacterial production were insignificant. This may indicate treatment-specific effects of the mortality factors on bacterial community dynamics and composition.

'Losers and survivors' of the combined effect of viruses and flagellates

Micrographs showing examples of R-BT-positive bacterial cells ingested by *Spumella*-like nanoflagellates detected using FISH, and active *Flectobacillus* cells detected with MAR-FISH in the F and FV treatments are shown in Fig. 2.

Figure 3 shows absolute numbers of total bacterioplankton and FISH-defined bacterial groups in the F

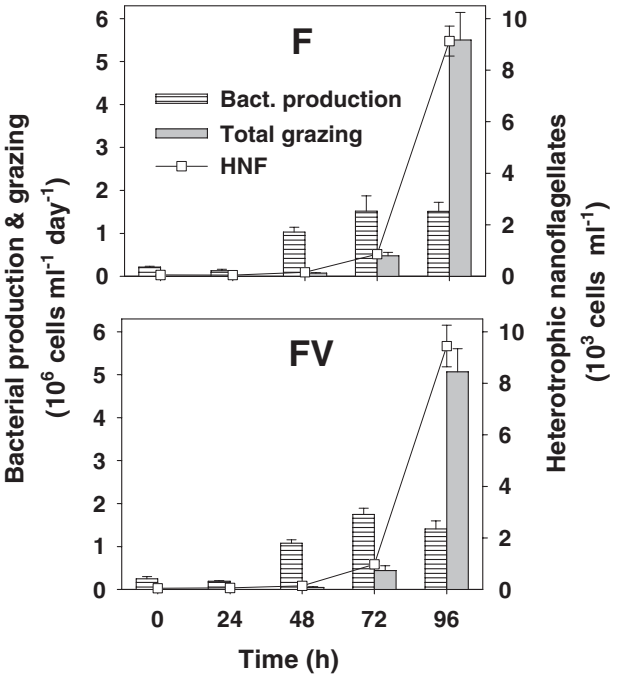

Fig. 1. Time-course changes in microbial parameters in the 'flagellates-added' (F) and 'viruses and flagellates-added' (VF) treatments exposed in dialysis bags in the reservoir: bacterial production, and total heterotrophic flagellate (HNF) abundance and bacterivory. Values are means for three replicate treatments and vertical bars show SDs.

and FV treatments. The most obvious treatment effects were that a part of the whole *Betaproteobacteria* class not targeted by the R-BT065 probe ('NON-R-BT') were almost entirely replaced by R-BT065-positive, fast growing cells in FV treatment, suggesting a marked vulnerability of the NON-R-BT phylotypes to viral lysis. Interestingly, between t_{72} and t_{96} h absolute numbers of *Actinobacteria* (ACT) increased within the heavily grazed bacterial communities, however, less so in the FV treatment (Fig. 3).

By comparing time-course changes in the ratio of total numbers of each bacterial group in the F treatment compared with their concentrations in the FV treatment several trends can be distinguished (Fig. 4). During the second half of the study in the flagellate-only treatment, significantly higher numbers of 'NON-R-BT' phylotypes from *Betaproteobacteria* and to lesser extent, CF and ACT abundances, were found compared with incubations with both flagellates and viruses. In contrast, R-FL615-positive cells (the *Flectobacillus* cluster) were significantly more numerous in the VF treatment (cf. Figs 4 and 5 and Table 1).

These treatment-related community shifts became most obvious the last experimental day as specifically plotted in Fig. 5. The ACT and NON-R-BT cells were significantly less abundant in FV, while this loss was compensated for by the significantly more abundant *Flectobacillus* populations (~2.7-fold) observed in FV. To enhance our taxonomic resolution within the virus-sensitive members of the NON-R-BT group, the last day we used also the probe PnecABCD-445 targeting the whole *Polynucleobacter* cluster of the *Betaproteobacteria*. This revealed that ~80% of NON-R-BT cells belonged to the *Polynucleobacter* cluster. Moreover, we found the significant differences in the total numbers (Fig. 5, top panel) as well as in relative proportion of *Polynucleobacter* in F and FV treatments, 6.9 ± 0.9% and 2.9 ± 1.0% (mean ± SD, data not shown) of total bacteria respectively. Finally, to tentatively estimate the most important biomass re-allocation between 'losers and winners' in the F and FV treatments, we used mean cell volumes measured in the experiment of *Actinobacteria* (0.061 μm³), *Polynucleobacter* (PNEC, 0.058 μm³) and *Flectobacillus* cells (see Table 1). This plot (Fig. 5, lower panel) showed that the sum of biomass decrease of ACT and PNEC in the FV compared with F treatments was roughly compensated for by the proportional increase in the *Flectobacillus* biomass in FV.

We also tested how strongly the observed bacterial community shifts were related to different prey selection

Table 1. Summary of size and activity parameters of the whole filaments and single cells within the filaments affiliated to the *Flectobacillus* cluster targeted with the R-FL615 probe in the 'flagellates-added' (F) and 'viruses and flagellates-added' (FV) treatments on the last experimental day, 28 May, 2004.

Flectobacillus parameters	F	FV	F versus FV
Abundance of the whole filaments (10^3 filaments ml^{-1})	4.42 ± 0.50	8.06 ± 0.62	< 0.0001
Length of the whole filament (μm)	27 ± 15	46 ± 21	< 0.0001
Biovolume of the whole filament (μm³)	5.2 ± 3.9	8.5 ± 3.6	< 0.0001
% in total bacterial biovolume	2.0 ± 1.2	10.3 ± 3.7	< 0.0001
Length of a single cell in a filament (μm)	4.2 ± 0.9	5.4 ± 1.1	< 0.0001
Cell width (μm)	0.49 ± 0.09	0.48 ± 0.08	0.1830
Biovolume of a single cell (μm³)	0.81 ± 0.11	1.0 ± 0.12	< 0.0001
Microautoradiography, relative activity as grain area per filament length (μm² μm⁻¹)	1.2 ± 0.5	2.5 ± 0.6	< 0.0001

Values are means ± SD, for ~200 filaments pooled from the replicate F and FV treatments. Significance (*P*-values) of the differences between the F and FV treatments was tested by *T*-test.

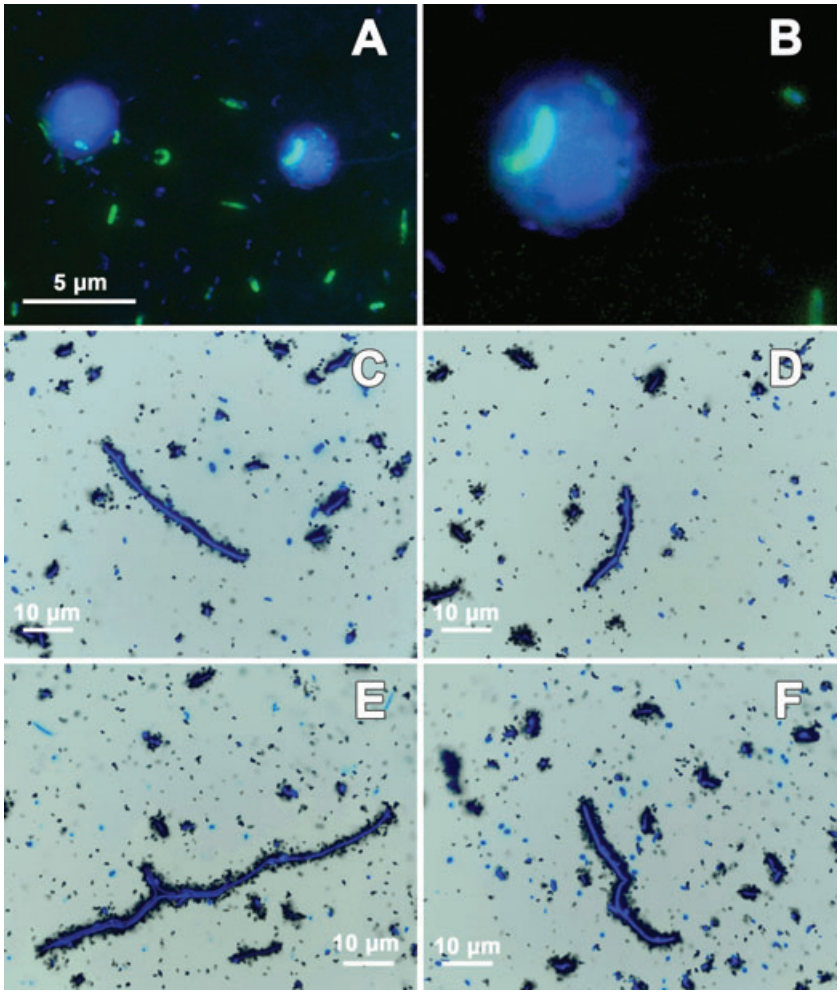

Fig. 2. Microphotographs of bacterial populations and HNF bacterivory in experimental treatments. Overlay images (DAPI- and FITC-stained) of the R-BT-positive bacteria hybridized in food vacuoles of two cells of HNF (A) with the right side HNF cell enlarged in (B). DAPI-stained images (C–F) of the *Flectobacillus* (the R-FL615 probe) filaments taking up ^{3}H-labelled leucine detected with microautoradiography combined with CARD-FISH, showing typical size and activity (the silver grain area around the filaments, cf. Table 1) of the R-FL615-positive filaments in the 'flagellates-added' (C and D) and 'viruses and flagellates-added' (E and F) treatments.

of HNF by analysing their food vacuole contents with the FISH-probes at t_{96} h (Fig. 6). The most frequently ingested prey were BET and its subcluster R-BT (see examples in Fig. 2A and B). However, no significant differences between F and FV treatments were detected in their proportions in food vacuoles and also corresponding selectivity indexes (bottom panel, Fig. 6) indicated efficient, but non-selective random feeding. In contrast, the CF phylotypes were always negatively selected, with significantly less ingested CF cells in the FV treatment. Commonly, the members of *Actinobacteria* group were discriminated against in both treatments. The *Flectobacillus* cells (~4–5.5 µm long, cf. Table 1) were found to be totally grazing-resistant because not a single cell was detected in total of 600 HNF inspected in triplicate F and FV treatments.

Effects of the mortality factors on bacterial growth and activity

Generally, trends in activity parameters, i.e. bacterial production (Fig. 1), proportions of high nucleic acid content bacteria (HNA), and the proportions of BET- and R-BT-positive cells taking up ^{3}H-labelled leucine assessed via microautoradiography (the MAR-FISH approach, see Fig. 7A–C), showed very similar trends. In both treatments, the proportions of HNA cells increased in the same fashion until t_{72} h, from < 50% to > 90% of total bacteria (Fig. 7). Then, between t_{72} and t_{96} h, along with the heavy HNF grazing pressure the HNA proportions decreased (cf. Figs 1 and 7), but more significantly in the presence of both mortality factors (FV).

Using the MAR-FISH approach, different trends in proportions of active cells within *Betaproteobacteria* cluster were also detected (Fig. 7B). On the last day, the strong impact of both mortality factors coincided with a significant difference in the proportions of active BET in the FV treatment compared with the flagellates-only treatment. This decrease in activity can be attributed to the more virus-vulnerable and less abundant 'NON-R-BT' part of BET cluster (the cells not targeted by the R-BT065 probe), because no similar effect was detected for the R-BT-positive and uptake-active cells (cf. Figs 3 and 7C). These

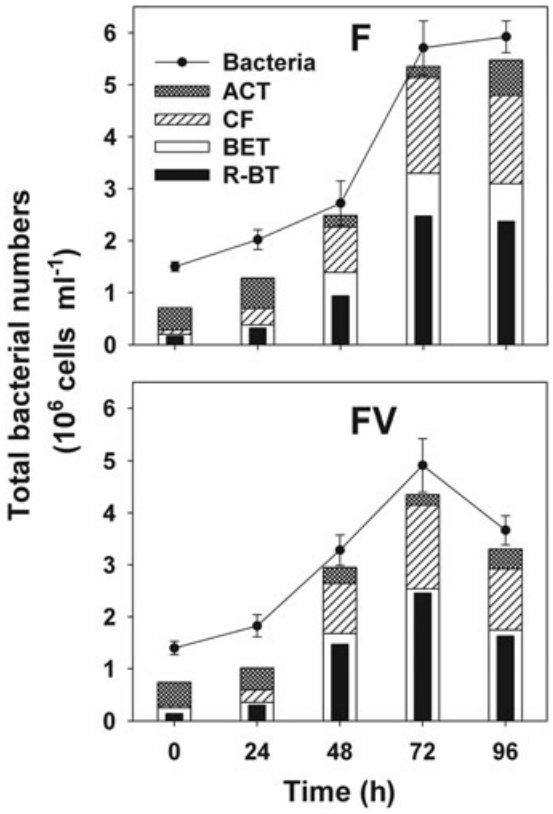

Fig. 3. Time-course changes in total bacterial abundance and total numbers of the probe-defined bacterial subgroups targeted with the probes BET42a (BET), R-BT065 (R-BT), CF319a (CF), and HGC69a (ACT – *Actinobacteria*). Values are means for three replicate treatments and vertical bars show SDs.

phylotypes clearly dominated the *Betaproteobacteria* cluster in both treatments (Fig. 3) and their proportions of active cells were not significantly virus-affected in the FV treatment. Neither significant treatment-specific effects nor remarkable changes in generally fairly low proportions of active CF-positive phylotypes (between 11% and 18% of total CF) were detected by MAR-FISH over the 96 h experiment (Fig. 7D).

Flectobacillus *growth stimulation by the synergistic effect of both mortality factors*

Quantifiable abundances of the grazing-resistant *Flectobacillus* filaments (targeted by the R-FL615 probe) appeared in both F and FV treatments only on the last day of the experiment corresponding to the high grazing pressure exerted on bacterial communities (cf. Fig. 1). However, these phylotypes were roughly two orders of magnitude less abundant in the treatments without flagellates (C and V treatments described in Weinbauer *et al.*, 2006). Compared with flagellates alone (F), in the treatment with both viruses and flagellates (FV), *Flectobacillus* populations displayed significantly faster growth, larger filament length, total volume biomass and higher relative activity based on the semi-quantitative MAR-FISH (see examples in Fig. 2C–F, for details see Table 1). For instance, contribution of the *Flectobacillus* to total biomass in FV was more than fivefold of that measured in the F treatment. Not only the total filament number, but also single cells within a filament were well distinguishable (cf. fig. 5 in Šimek *et al.*, 2001). Using the total filament number and mean cell length (again significantly longer in FV, Table 1), it yielded 2.8×10^4 ml^{-1} and 6.9×10^4 ml^{-1} of individual *Flectobacillus* cells in F and VF respectively (Fig. 5). Finally, taking into account total filament length (total filament number times mean filament length, Table 1) and the more than twofold larger relative

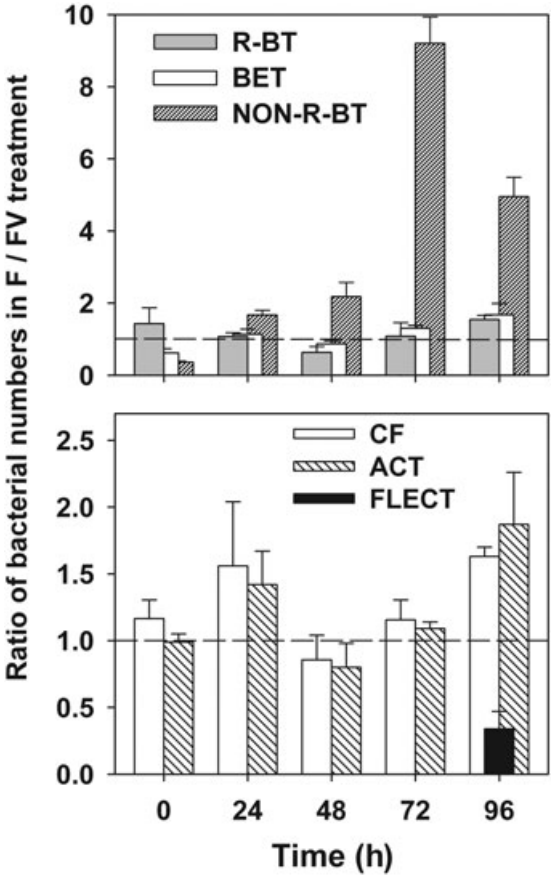

Fig. 4. Time-course changes in the ratio between the numbers of probe-defined bacterial groups detected in the 'flagellates-added' (F) treatment and their numbers in 'viruses and flagellates-added' (VF) treatment exposed in dialysis bags. The number of NON-R-BT cells is total number of the BET-positive cells minus the number of R-BT-positive cells. Abundances of R-FL615-positive cells (FLECT – *Flectobacillus* cluster) were detected only the last experimental day when, for accurate counting, sufficiently high numbers of *Flectobacillus* filaments appeared (cf. Fig. 5, Table 1). For explanation of other abbreviations see the text to Fig. 3. Note the different scales between the top and bottom panels. Values are means for three replicate treatments and vertical bars show SDs.

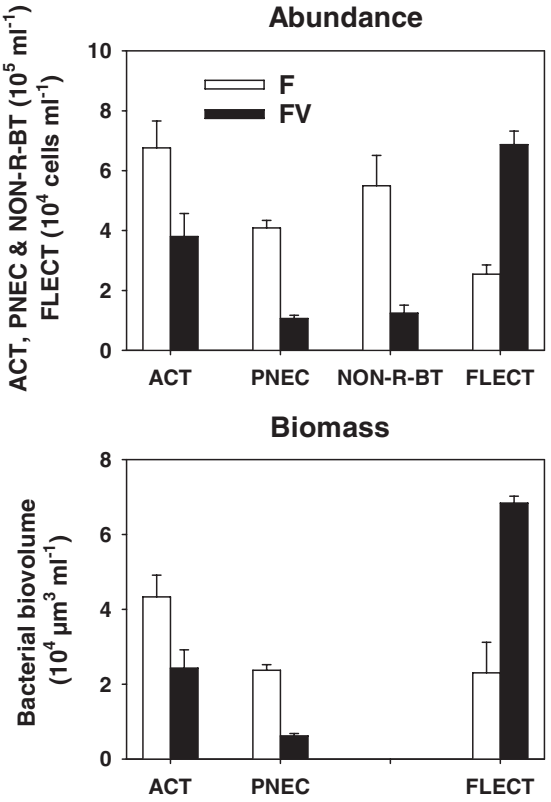

Fig. 5. Abundances (top panel) and biomass (bottom panel) of the selected groups of bacterioplankton showing the most marked differences in F and FV treatments at t_{96} h: the *Actinobacteria* (ACT), the NON-R-BT group (the BET-positive cells not targeted by the R-BT065 probe), the *Polynucleobacter* cluster (PNEC) and *Flectobacillus* (FLECT). Note that only the total number of NON-R-BT cells can be detected (for details see text to Fig. 4), while the mean cell volume and the corresponding biomass of this group cannot be precisely detected because the NON-R-BT part of *Betaproteobacteria* groups is not targeted by any specific probe. Values are means for three replicate treatments and vertical bars show SDs.

activity in FV (see also the silver grain area associated with the filaments shown in Fig. 2C–F), the measure undoubtedly indicates a very large difference in total metabolic activity of the *Flectobacillus* populations between the treatments.

Discussion

Compared with the general trends in the community composition and diversity in all experimental treatments, reported in the companion paper (Weinbauer *et al.*, 2006), in this study we focus on investigating the specific factors shaping the population dynamics of *Flectobacillus* populations. Our data permit the identification of group-specific mortality factors and thereby provide insight into the events promoting the development of filamentous *Flectobacillus* populations.

Based on identification of prey inside bacterivores, losses to grazers were high for the BET and the R-BT subgroup of BET. However, neither appeared to be selectively grazed nor was there a significant difference between numbers of BET or R-BT in the food vacuoles of grazers in the presence or absence of viruses (Fig. 6). In contrast, the CF phylotypes were discriminated against by grazers and more so in the incubations with viruses compared with flagellates alone. A third pattern defined the *Actinobacteria*, which were discriminated against in both treatments equally. This finding is consistent with the previous reports of discrimination against these bacteria by HNF populations in the reservoir (Jezbera *et al.*, 2005; 2006). It is worth noting that

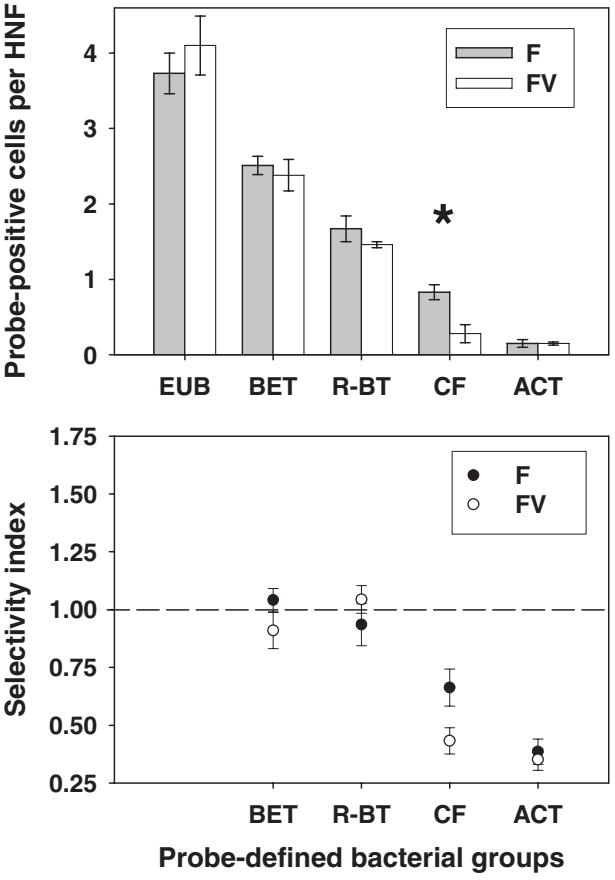

Fig. 6. Analysis of HNF food vacuole contents in the 'flagellates-added' (F) and 'viruses and flagellates-added' (VF) treatments on the last experimental day. (Top) The number of probe-detected bacterial cells targeted with the probes for the EUB, BET, R-BT, CF and ACT groups inside of HNF food vacuoles expressed per HNF cell. (Bottom) Selectivity indexes of the BET, R-BT, CF and ACT groups of bacteria calculated by dividing their proportions detected in HNF food vacuoles by their proportions present in the bacterioplankton, both expressed as the percentage of EUB338-positive cells. Dashed line depicts ratio 1:1 indicating random feeding. Values are means for three replicate treatments and vertical bars show SDs. Asterisk indicates significant difference in selectivity of CF phylotypes in the F and FV treatments.

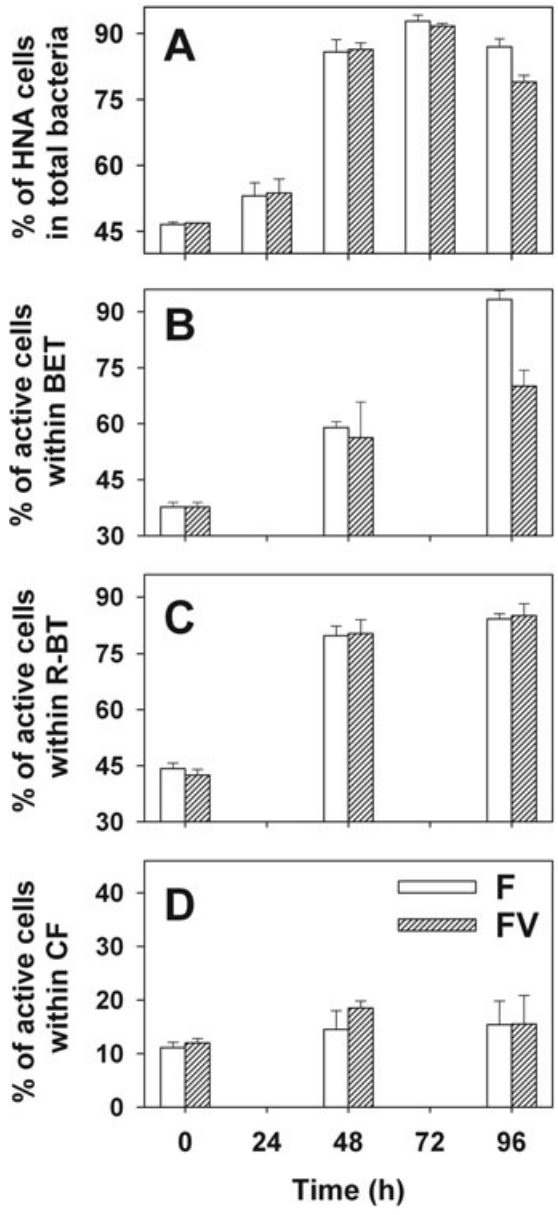

Fig. 7. Time-course changes in microbial parameters in the 'flagellates-added' (F) and 'viruses and flagellates-added' (VF) treatments exposed in dialysis bags in the reservoir. (A) The proportions of bacteria with high nucleic acid content (HNA) related to total DAPI-stained bacteria, (B) the proportions of active cells within BET-positive, (C) R-BT-positive and (D) CF-positive bacteria taking up ^{3}H-labelled leucine as assessed by microautoradiography (for more details see Fig. 3 legend). Note the different scale used for the proportions of the CF group. Values are means for three replicate treatments and vertical bars show SDs.

cells are Gram-positive, usually small cell-size phylotypes (cf. Hahn *et al*., 2003). Finally, *Flectobacillus* cells were not found in any of the 600 HNF examined, thus indicating complete grazing-resistance against bacterivory of small reservoir HNF (cf. Hahn *et al*., 1999; Šimek *et al*., 2001).

The selectivity of bacterivores has been linked to many prey characteristics such as size, surface characteristics, motility, division state, etc. (e.g. Šimek and Chrzanowski, 1992; Christaki *et al*., 1998; Dolan and Šimek, 1999; Hahn and Höfle, 2001). In our experiment, an obvious result of grazer activity was the development of filament-forming *Flectobacillus* populations in both treatments with bacterivores. Additionally, we examined group-specific physiological activity, leucine uptake with MAR-FISH and vulnerability to grazers. We found a positive relationship between the selectivity of the grazers and the activity of the bacterial group as estimated by the percentage of active cells as determined using MAR-FISH (Fig. 8). This crude relationship supports the view that rapidly growing populations are rapidly removed by grazers (cf. Sherr *et al*., 1992; Del Giorgio *et al*., 1996). The obvious exception to this rule was *Flectobacillus*.

In both sets of incubations, flagellates alone, or flagellates with viruses, *Flectobacillus* populations developed. Interestingly, their development was much more pronounced in the incubations with viruses (Table 1, Figs 2 and 5). The grazing pressure exerted by flagellates was nearly identical in the two sets of incubations, as estimated by the uptake of fluorescently labelled bacteria (FLB) (Fig. 1). Therefore the enhanced development of filamentous *Flectobacillus* in the presence of both flagellates and viruses is associated with viral activity. We

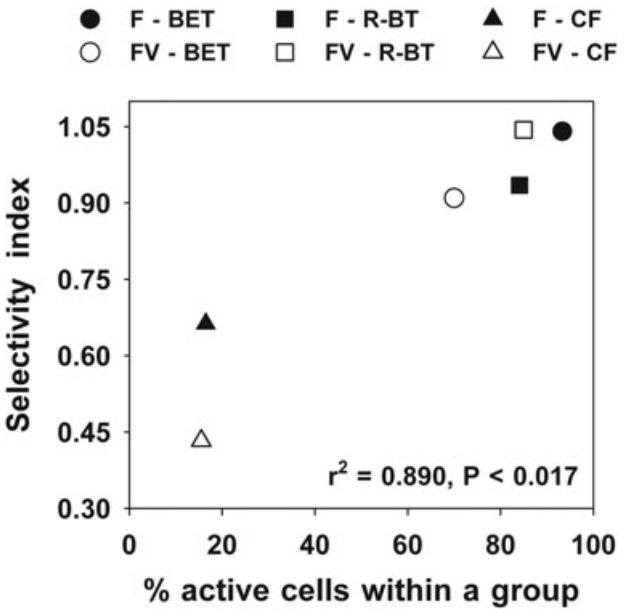

Fig. 8. Spearman rank correlation between the selectivity indexes based on FISH-analysis of HNF food vacuole contents in the 'flagellates-added' (F) and 'viruses and flagellates-added' (VF) treatments (for details see Fig. 6) and the proportions of uptake-active bacterial cells within the probe-defined bacterial groups BET, R-BT and CF (for details see Fig. 7). The r^2 value shows the coefficient of determination of the regression.

hypothesize that the increased bacterial mortality attributable to viral lysis, about 16% of bacterial production (Weinbauer *et al*., 2006), provided additional substrates for *Flectobacillus* compared with the flagellates-only incubations. These results also suggest that *Flectobacillus* is relatively resistant to viral attack and bacterivory of small HNF (round 3–5 μm large, see Fig. 2A and B), or that viruses had insufficient time to respond to the *Flectobacillus* appearance, because its abundance only increased at the last day of the experiment.

Flectobacillus was one of only two bacterial groups, which displayed different rates of activity, as estimated with MAR-FISH when exposed to flagellates alone or with viruses. Bacteria detected using the BET probe showed significantly lower proportions of active cells in incubations with viruses at the end of the experiment (Fig. 7). In contrast, the relative *Flectobacillus* activity, estimated as grain area per micron of filament, was twice as high in the presence of viruses (Table 1). The increase in population size of *Flectobacillus*, which occurred in the treatment with both flagellates and viruses, can be attributed to an increase in growth rate in the presence of viruses. The increase in growth rate could be due to increased substrate availability from the combined effects of the presence of viral lysis products (cf. Middelboe and Jørgensen, 2006) and lowered abundances of competitors such as *Actinobacteria* and the 'NON-R-BT' members of the *Betaproteobacteria* cluster. Indeed, the latter group showed a clear negative virus effect from the t_{24} h of the experiment (Fig. 4).

In a recent laboratory study the development of filamentous *Flectobacillus* sp. strain GC-5 was suggested to be triggered by the products of bacterivory by Corno and Jürgens (2006). They found that exposing cultures of *Flectobacillus* to feeding bactivores, isolated from the *Flectobacillus* in a dialysis bag, provoked the development of filaments. Corno and Jürgens (2006) suggested then that some specific bacterivore excretory product triggered the formation of filaments in *Flectobacillus*. In contrast, Hahn and colleagues (1999) clearly demonstrated, in a continuous cultivation study, that the filament formation of two *Flectobacillus* strains (*F. major* and the strain MWH38) was primarily growth rate controlled and independent of the presence of a predator. In our study, the enhanced development of filamentous *Flectobacillus* in the presence of both viruses and flagellates indicates that viral lysis products may also trigger and accelerate filament formation. *Flectobacillus* may require high substrate concentrations to form filaments (Corno and Jürgens, 2006). Filament formation is generally thought of as a strategy against flagellate grazers (e.g. Jürgens and Güde, 1994; Hahn *et al*., 1999; Šimek *et al*., 2001) but there are some forms capable of feeding on filamentous bacteria (Wu *et al*., 2004). Furthermore filaments are susceptible to grazing by zooplankton (Jürgens and Güde, 1994).

Corno and Jürgens (2006) concluded that a chemical cue provoking filament formation in *Flectobacillus* exists which is smaller than 15 kDa and probably produced in large quantities by the bacterivores and perhaps viral lysis. In their chemostat experiment, the chemical passed out of the dialysis bags containing the bacterivore and its prey into the culture vessels which were running at a dilution rates of 1 day^{-1}. In our experiment filament formation was triggered inside dialysis bags following the marked increase in HNF abundance between $t_{72–96}$ h. In a previous study Šimek and colleagues (2006) estimated that single cells dividing within the *Flectobacillus* filaments could grow with a very short doubling time of only 6 h.

Compared with previous studies (e.g. Šimek *et al*., 2001, 2003; Weinbauer *et al*., 2003; Hornák *et al*., 2005; Jacquet *et al*., 2005; Sime-Ngando and Pradeep Ram, 2005) our experimental design allowed, by comparing dynamics in F and FV treatments (Figs 4 and 5), detection of bacterial phylotypes most vulnerable to viral lysis. Our data suggest large differences mainly between different subclusters of *Betaproteobacteria*. Neither relative proportions (Weinbauer *et al*., 2006), nor the proportions of active cells within the R-BT-positive bacteria, were negatively affected by the presence of viruses (Fig. 7). This relatively narrow, genus-like cluster has been consistently found to be the fastest growing segment of the reservoir bacterioplankton (cf. Šimek *et al*., 2006); however, they are quite vulnerable to HNF grazing (Figs 2 and 6 in this study, cf. Jezbera *et al*., 2006). In contrast, the phylogenetically rather broad group, the 'NON-R-BT' part of *Betaproteobacteria* was obviously largely dominated by phylotypes vulnerable to viral attack. For instance, the dominant proportion of NON-R-BT bacteria, the members of the taxonomically well-defined *Polynucleobacter* cluster (Hahn *et al*., 2005; Wu and Hahn, 2006) clearly suffered significantly enhanced mortality in the FV treatment (Fig. 5). This virus-vulnerable part of *Betaproteobacteria* was almost entirely replaced by the highly active and fast-growing R-BT-positive cells (cf. Šimek *et al*., 2005) in the FV treatment (cf. Figs 3 and 7).

Admittedly, the bacterial communities observed were highly manipulated and followed for the relatively short time of 96 h. Nonetheless, some very interesting trends were apparent. The bacterial community subjected to both viral and flagellate mortality (FV) was less abundant but more active than the community subjected only to flagellate grazing (F), based on bulk production rates. A virus-induced shift in the community composition towards more active bacterial groupings in the presence of both mortality factors can explain why significantly less abundant bacterial populations in FV performed practically the same bulk bacterial production as the more abundant

bacteria in F treatment (cf. Figs 1 and 3). The bacterial community in F treatment contained much higher proportions of slower growing, smaller and to grazing less vulnerable cells such that *Actinobacteria* and *Polynucleobacter* groups (Hahn *et al*., 2005; Jezbera *et al*., 2006). However, these groups were apparently much more virus-vulnerable as they were significantly suppressed in FV treatment. In contrast, the community in FV treatment comprised higher proportions of fast growing and large cells, obviously less vulnerable to virus infection or HNF grazing (*Flectobacillus*), or vulnerable to HNF grazing (the R-BT cluster, Figs 2 and 6), but with the ability to override the effect of heavy HNF grazing by accelerated growth rate (cf. also Šimek *et al*., 2006).

Overall, our study clearly documented the synergistic effects of both mortality factors that significantly accelerated growth of the *Flectobacillus* populations. We detected the most abundant prey of HNF in both treatments (BET and its R-BT subcluster) but also the segment of bacterioplankton most vulnerable for virus lysis, the NON-R-BT part of the BET group with the substantially decreased proportion of the *Polynucleobacter* cluster and *Actinobacteria* group in the FV treatment.

Experimental procedures

Study site, experimental design and sampling

The major features of the experimental design are described elsewhere (Weinbauer *et al*., 2006). Briefly, on May 24, 2004, the water samples for the experiment were collected from the dam area of the meso-eutrophic Římov Reservoir (South Bohemia, Czech Republic, for more details see Šimek *et al*., 1999). Cells from a 50 l water sample were concentrated using a 0.2 μm pore-size polycarbonate cartridge (Durapore, Millipore). Half of the cell concentrate was filtered through a 0.8 μm filter to remove grazers. Viruses in the filtrate were concentrated using a 100 kDa polysulfone cartridge (Prep-Scale, Millipore) and the virus-free permeate was collected. Half of the virus concentrate was exposed to 60°C for 30 min to inactivate viruses. Cells were added to virus-free water at concentrations two times those corresponding to *in situ* volumes and roughly corresponding to *in situ* abundances. From these fractions, following combinations were made: 1. Control (C): bacteria + inactivated viruses; 2. V: bacteria + active viruses; 3. F: bacteria + flagellates + inactivated viruses and 4. VF: bacteria + flagellates + active viruses. Treatments were filled in triplicates in 2 l dialysis bags (Spectra/Por, diameter 76 mm, molecular weight cut-off 12–14 kDa) as described before (Šimek *et al*., 2001; Weinbauer *et al*., 2006) and exposed for 96 h in the reservoir.

The overall evaluation of the whole raw data set and the comparison of the dynamics of the target microbial populations in the four types of different treatments are presented in the companion paper (Weinbauer *et al*., 2006). Here, we compared in detail only the treatments with the presence of HNF (F) or both sources of bacterial mortality (FV).

Samples for bacterial abundance, production, proportions of high nucleic acid content cells in bacterial community, FISH, and HNF numbers and bacterivory were collected daily. Samples for FISH combined with microautoradiography were collected at times 0, 48 and 96 h of the experiment. Only on the last experimental day when HNF were sufficiently abundant ($\sim 9 \times 10^3$ ml^{-1}) did *Flectobacillus* filaments appear numerous enough to be precisely quantified in both F and FV treatments. These specific circumstances allowed use of innovative modifications of microautoradiography and FISH approaches. Thus, the analyses of FISH-positive bacterial cells in HNF food vacuoles, numbers and sizes of the whole *Flectobacillus* filaments and of their single cells, and semi-quantitative microautoradiography-based analysis of relative activity of *Flectobacillus* cells were conducted in the F and FV treatments on the last experimental day.

Bacterial abundance, sizing and production

Samples were fixed with formaldehyde (2% final concentration, v/v), stained with DAPI (final concentration 0.1 μg ml^{-1}, wt/vol), and enumerated by epifluorescence microscopy (Olympus BX 60). Cell sizing was conducted by using the semiautomatic image analysis systems LUCIA D (Lucia 3.52, Laboratory Imaging, Prague, Czech Republic). Both approaches are detailed in Šimek and colleagues (2001) and Posch and colleagues (1997). Bacterial production was measured via thymidine incorporation with the modification described in detail in Šimek and colleagues (2001), using the empirically established conversion factor of 4.05×10^{18} cells mol^{-1} of thymidine incorporated to estimate bacterial cell production rate (cf. Weinbauer *et al*., 2006).

Protozoan grazing and enumeration

Protozoan bacterivory was estimated using FLB (Sherr and Sherr, 1993) concentrated from the reservoir water. HNF abundances and FLB uptake rates were determined in short-term FLB direct uptake experiments described in details in Šimek and colleagues (1999; 2001). To estimate total protozoan grazing, we multiplied the average uptake rates of HNF by their abundances in F and VF treatments.

Catalysed reporter deposition fluorescence in situ *hybridization (CARD-FISH) with rRNA-targeted oligonucleotide probes*

Analysis of bacterioplankton in water samples as well as in HNF food vacuoles was performed using group-specific oligonucleotide probes. We applied CARD-FISH protocol (Pernthaler *et al*., 2002; Sekar *et al*., 2003) employing the following oligonucleotide probes (Interactiva Division, Ulm, Germany) targeted to: the domain *Bacteria* (EUB338), the Beta- and Gamma-subclasses of the class *Proteobacteria* (the BET42a and GAM42a probes respectively), to a narrower subcluster of the *Betaproteobacteria* – (R-BT065, this probe targets a subcluster of the *Rhodoferax* sp. BAL47 cluster of the *Betaproteobacteria*, Zwart *et al*., 2002), to the whole *Polynucleobacter* cluster of the *Betaproteobacteria* (PnecABCD-445,

this probe combined probes PnecABD-445 and PnecC-445, Hahn *et al*., 2005 and Wu and Hahn, 2006), to the *Cytophaga/Flavobacterium/Bacteriodetes* group (CF319a), and to the *Actinobacteria* group (HGC69a). In the following text, the probe-targeted bacterial groups are assigned as EUB, BET, GAM, R-BT, PNEC, CF and ACT respectively. Only on the last experimental day, we detected high numbers of *Flectobacillus* filaments in the heavily grazed bacterial community in the F and FV treatments that hybridized with the R-FL615 probe (for the probe target covering a narrow *Flectobacillus* cluster, see Šimek *et al*., 2001). The last experimental day, the single cells targeted by the probes PnecABCD-445, HGC69a and R-FL615 were also sized using a specific macro built in PC-based image analysis software Lucia G/F 4.80 (Laboratory Imaging, Prague, Czech Republic).

CARD-FISH analysis of HNF food vacuoles

Analysis of bacterial prey inside HNF food vacuoles was conducted on the last experimental day when sufficiently high HNF numbers were available (Fig. 1) allowing comparison of ingested prey with bacterioplankton community composition. At least 100 HNF per each triplicate treatment were inspected for bacteria hybridized directly in the vacuoles (see Jezbera *et al*., 2005; 2006, for details) with the probes EUB338, BET42a, GAM42a, CF319a, R-BT065 and HGC69a. The bacteria hybridized with the GAM42a (*Gammaproteobacteria*) are not reported in Fig. 6 due to their generally very low proportion in the community (< 3% of total bacteria, Weinbauer *et al*., 2006), which severely limited any reliable statistical analysis. Selectivity indexes were calculated by dividing the proportions of probe-targeted subgroups of ingested bacteria by their proportions available in bacterioplankton, both expressed as the percentage of EUB338-positive cells. Values of 1 represent random feeding, values < 1 and > 1 indicate negative and positive selection respectively.

Microautoradiography and fluorescence in situ *hybridization*

A combination of the CARD-FISH protocol and microautoradiography (MAR-FISH) was used to determine the phylogenetic affiliation and bi-daily also the proportions of metabolically active bacteria (Teira *et al*., 2004; Sintes and Herndl, 2006). Samples for microautoradiography were incubated with L-[^{3}H]-Leucine (specific activity, 37 Ci mmol^{-1}; final concentration, 10 nmol l^{-1}; MP Biomedicals) for 1 h in the dark at *in situ* temperature and fixed with formaldehyde (final concentration, 2%) and filtered through 0.2 μm pore-size polycarbonate filters (Osmonics). The bacteria on filters were hybridized with the probes BET42a, R-BT065, CF319a and R-FL615 using the CARD-FISH protocol (for details see Pernthaler *et al*., 2002; Teira *et al*., 2004). Then, in complete darkness, the filters with hybridized bacteria were transferred upside down onto the slides coated with the emulsion (type NTB-2, Kodak). The slides were kept at 4°C in the dark for exposure and then developed following the manufacturer's instructions. The cells were stained with a DAPI (final concentration, 1 μg ml^{-1}) mixed with four parts of Citifluor (Citifluor, UK) and one part of Vectashield (Vector Laboratories, UK). The relative proportions of uptake-active cells within the studied bacterial groups were enumerated in an epifluorescence microscope (PROVIS AX–70, Olympus). The presence of silver grains connected with the cells was inspected via the transmission light. Bacterial cells connected with two or more silver grains were considered as actively taking up the radiolabelled substrate (Teira *et al*., 2004). Between 500 and 1000 DAPI stained cells were counted per sample.

Semi-quantitative evaluation of microautoradiograms of filaments affiliated with the Flectobacillus *cluster*

To quantify microautoradiograms of the cells hybridized with the probe R-FL615 at t_{96} h, we used an image analysis system consisting of a monochromatic digital camera DVC-1300 (DVC, Austin, USA), mounted onto the fluorescence microscope Olympus AX-70, and PC-based image analysis software Lucia G/F 4.80 (Laboratory Imaging, Prague, Czech Republic). First, from each of two replicate F and FV treatment, images with at least 100 randomly selected R-FL615-positive filaments were recorded at 400× magnification. Total filament length, width of the filament and length of the individual *Flectobacillus* cells forming chain in the filaments were measured manually on the DAPI-stained images (see below). For analysis of the filament-associated activity, we took 100 pairs of corresponding images of R-FL615-positive filaments using: (i) UV excitation long-pass emission filter set (excitation/emission: 360–370 nm/ > 420 nm) to record DAPI fluorescence ('DAPI image'), and (ii) incident light to record the developed silver grains in microautoradiograms ('MAR image').

To measure the area of filament-associated grains and their aggregates, first, position of filaments was drawn manually on screen using binary editor in DAPI image/binary image overlay, avoiding parts of *Flectobacillus* filaments tightly neighbouring (< 2 μm distance) to other bacteria. Next, the length of thus drawn binary objects corresponding to the filaments was measured to get the length of the filaments/or the parts of the filaments without close contact with any neighbouring bacterial cell. Subsequently, the objects were dilated by 2 μm to cover the areas of theoretical range of the maximum energy of the electrons emitted from the filament that took-up tritium labelled leucine ('RANGE binary image'). Then, the corresponding MAR grey image was thresholded to mark the area occupied by single or aggregated grains, thus obtaining 'MAR binary image'. Finally, to get the area of the grains occurring within 2 μm range around filaments, area of the intersection of the RANGE binary image with the MAR binary image was measured. The steps of the procedure not requiring operator action were automated using a C++ like macro language. Results were expressed as grain area per filament length (μm^2 μm^{-1}).

Flow cytometric sample analysis

Subsamples were preserved with 1% paraformaldehyde + 0.05% glutaraldehyde (final concentration), frozen in liquid nitrogen and stored at −80°C. The samples were later thawed, stained for a few minutes with Syto13 (Molecular

Probes) at 2.5 μM and run in a desktop FACSCalibur flow cytometer (Becton Dickinson, USA). We used MilliQ water as sheath fluid, samples were run at low speed (approximately 18 μl min^{-1}), yellow–green 0.92 μm Polysciences latex beads were used as internal standards and the fixed samples were diluted 2×–4× with MilliQ water to keep the rate of particle passage below 500 particles per second to avoid coincidence (Gasol and Del Giorgio, 2000). Bacteria were detected by their signature in a plot of side scatter (SSC) versus green fluorescence (FL1) and we used the percentage of high nucleic acid content bacteria (%HNA) as a simple indicator of the proportions of highly active cells in the bacterial community (Gasol and Del Giorgio, 2000).

Statistical evaluation

Means of triplicates were compared for each parameter except for the analysis of morphological and activity parameters of *Flectobacillus*, where ~200 filaments pooled from two replicate treatments of F and FV were analysed. Means were compared using the *T*-test (Table 1).

Acknowledgements

This study was largely supported by the Grant Agency of the Czech Republic under research Grant 206/05/0007 awarded to K. Šimek, by the Academy of Sciences, projects No. AV0Z 60170517 and 1QS600170504, then also by the project MSM 600 766 5801. The grant BARRANDE (EGIDE) number 2004-004-2 supported joint experimental activities of K.Š. and J.R.D. and an ATIPE (CNRS) grant supported M.G.W.

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