

Synergistic and antagonistic effects of viral lysis and protistan grazing on bacterial biomass, production and diversity

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Summary

In a mesotrophic reservoir, we examined the effects on the bacterioplankton of distinct consumers of bacteria, viruses and heterotrophic nanoflagellates, both alone and combined in an experiment using natural populations and *in situ* incubations in dialysis bags. Ribosomal RNA-targeted probes were employed as well as 16S RNA gene based PCR denaturing gradient gel electrophoresis (DGGE) to enumerate bacterial groups and assess bacterial community composition. We employed probes for *Actinobacteria* (HGC69a probe), *Cytophaga-Flavobacterium-Bacteroidetes* bacteria (CF319a probe), BET42a probe (*Betaproteobacteria*) and a subgroup-*Betaproteobacteria* (R-BT065 probe). We found consumer-specific effects on bacterial activity and diversity (against a background of CF and BET dominating all treatments) suggesting distinct vulnerabilities to the two sources of mortality. For example, growth rate of *Actinobacteria* was only positive in the presence of flagellates, while towards the end of the experiment ($T_{72-96\text{ h}}$) growth rate of R-BT was only positive in the viruses only treatment. More specific data on how viruses and flagellates influenced *Flectobacillus* are shown in the companion paper. Highest richness (number of DGGE

bands) was found in the virus only treatment and lowest when both consumers were present. In addition, we found suggestions of both antagonistic and synergistic interactions between the two sources of bacterial mortality. Notably, bacterivory by flagellates was associated with reductions in bacterial diversity and increases in viral production.

Introduction

The factors which affect bacterial community composition and activity have been the subject of a large number of studies in recent years. On the one hand both community composition and rates of cell production are ultimately determined by inorganic nutrients and the quality and composition of dissolved organic matter (DOM), seen as 'bottom-up' factors (Kirchman, 2000). On the other hand, bacterial cells are removed through 'top-down' bacterivory, largely by protists, and cell lysis by viruses. These two sources of mortality likely influence bacterial abundance and community composition, but perhaps in different fashions.

Bacterivory by protists appears to control bacterial abundance in a large variety of pelagic systems, especially oligotrophic systems (Gasol *et al.*, 2002). In addition, bacterivorous protists are known to feed selectively on bacterioplankton. Laboratory studies have shown that prey items are distinguished according to size, motility and surface characteristics by both ciliates (e.g. Sanders, 1988; Christaki *et al.*, 1998; Posch *et al.*, 2001) and flagellates (e.g. (Monger and Landry, 1991; Šimek and Chrzanowski, 1992). Selective feeding then potentially influences bacterial community composition. The most obvious effect of protistan bacterivory on bacterial community composition is that it can control morphological characteristics of bacteria. Bacterivory can trigger the development of grazing-resistant morphologies within a species (e.g. (Jürgens and Güde, 1994; Pernthaler *et al.*, 1997; Hahn *et al.*, 1999) or within an assemblage of species (Šimek *et al.*, 1997). Thus it is perhaps not surprising that in recent years protists have been shown to exert a large influence on bacterial community composition, especially in waters in which bacterial resources are limited (e.g. Šimek *et al.*, 1999; Jürgens *et al.*, 2002).

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Viruses are also a significant source of mortality for bacterioplankton (Fuhrman, 1999; Wommack and Colwell, 2000; Weinbauer, 2004; Suttle, 2005). Viral lysis products such as the cell content and cell wall fragments enter the organic matter pool, which are exploited primarily by bacteria. This viral shunt (Wilhelm and Suttle, 1999) stimulates bacterial production ('stimulation of production' hypothesis) and reduces the significance of the bacteria–flagellate link (Fuhrman, 1999). As phages do typically not trespass genus boundaries and as phage infection is density-dependent, phages should limit competitive dominants and thus allow less competitive types to survive (Fuhrman and Suttle, 1993; Thingstad *et al.*, 1993). This concept has been mathematically formalized in the 'killing the winner' hypothesis (Thingstad and Lignell, 1997). Thus, viral mortality can be considered as a mechanism potentially increasing species 'evenness' in the bacterial community.

Because phages and flagellates consume the same prey, an antagonistic interaction may be expected, i.e. an increase in the activity of one type of consumer could result in a decrease in 'resources' for the other consumer of bacteria. Indeed, a mathematical model suggests that grazing of flagellates on infected cells may yield a decrease in bacterial species richness (Maki and Yamamura, 2005). In Římov reservoir, we found that the presence of heterotrophic nanoflagellates (HNF) can stimulate viral production and viral infection of bacterioplankton (Šimek *et al.*, 2001; Weinbauer *et al.*, 2003). The mechanism or generality of the apparent synergism we found is unclear. In fact, very little is known on the interplay of viruses and flagellates on the activity and diversity of bacterioplankton (Winter *et al.*, 2004; Pernthaler, 2005).

Here we compare the two distinct processes of bacterial cell removal, protistan bacterivory and viral lysis, in an experiment using natural populations and *in situ* incubations. We used a 'reconstitution' approach using incubations of bacterioplankton without predators, with viruses, with flagellates and with both sources of bacterial mortality. These populations were incubated inside dialysis bags *in situ* at the sampling site, the lake part of the Římov reservoir. Comparing the effects of viruses and flagellates, we found consumer-specific effects on bacterial activity and diversity suggesting distinct vulnerabilities to the two sources of mortality. In addition, we found suggestions of both antagonistic and synergistic interactions between the two sources of bacterial mortality.

Results

Characteristics of study site

The experiment was performed during late clear-water phase with a water temperature of 15°C. *In situ*, we found

2.3×10^6 bacteria ml⁻¹, 1.1×10^8 viruses ml⁻¹ and 420 flagellates ml⁻¹.

Abundance and biomass

Bacterial abundance increased between two- and three-fold in the different treatments (Fig. 1). While within each treatment, bacterial abundance and biomass showed closely parallel trends, there were differences between treatments. For example, after 48 h bacterial abundance was significantly higher in the viruses-added (V) than in the other treatments of bacteria alone (C), flagellates-added (F) and both viruses and flagellates-added (VF). At the end of the experiment, bacterial abundance was highest in the F treatment and lowest in the VF treatment, and only the difference between these two treatments was significant.

At the start of the experiment viral abundance was slightly higher than *in situ*. The viral abundance dropped in both the C and F treatments from c. $9\text{--}10 \times 10^7$ ml⁻¹ in the C and F treatment and levelled off at c. 4×10^7 ml⁻¹ in the F and c. 2.5×10^7 ml⁻¹ in the C treatment. Recall that the viruses in the C and F treatments had been heat-inactivated at the beginning of the experiment. In the V and VF treatments, in which active viruses were added, viral abundance increased slightly. At time zero, flagellate abundance was reduced in the incubations compared with *in situ* but reached high concentrations ($> 8 \times 10^3$ cells ml⁻¹) by the end of the experiment, with no significant difference between the F and VF treatment (Table 1, Fig. 1). No flagellates were detected in the C and V treatment.

Viral infection and grazing rates

Frequency of infected cells (FIC) at day 3 was 18% in V and 39% in VF (Fig. 2). Viral production was 3.8×10^6 ml⁻¹ day⁻¹ in V and 8.1×10^6 ml⁻¹ day⁻¹ in VF. In the virus only treatment (V), the loss rate of bacterial cells due to viral lysis was about 0.95×10^5 cells day⁻¹ or 5% of cell production. In the sacs containing both viruses and flagellates (VF), 2.0×10^5 bacteria were lysed per day corresponding to about 16% of daily cell production. Using an alternative approach, i.e. FIC equals the amount of production lysed, 18% and 39% were lysed in the V and VF treatment respectively. Grazing rate at the end of the experiment was $5\text{--}6 \times 10^6$ bacteria ml⁻¹ day⁻¹ corresponding to an uptake of 22–26 bacteria per flagellate cell per hour.

Bacterial production; growth rates of bacterioplankton and different probe-defined subgroups

TdR incorporation increased dramatically by c. one order of magnitude after a delay of 1 day (Fig. 1). At the end of

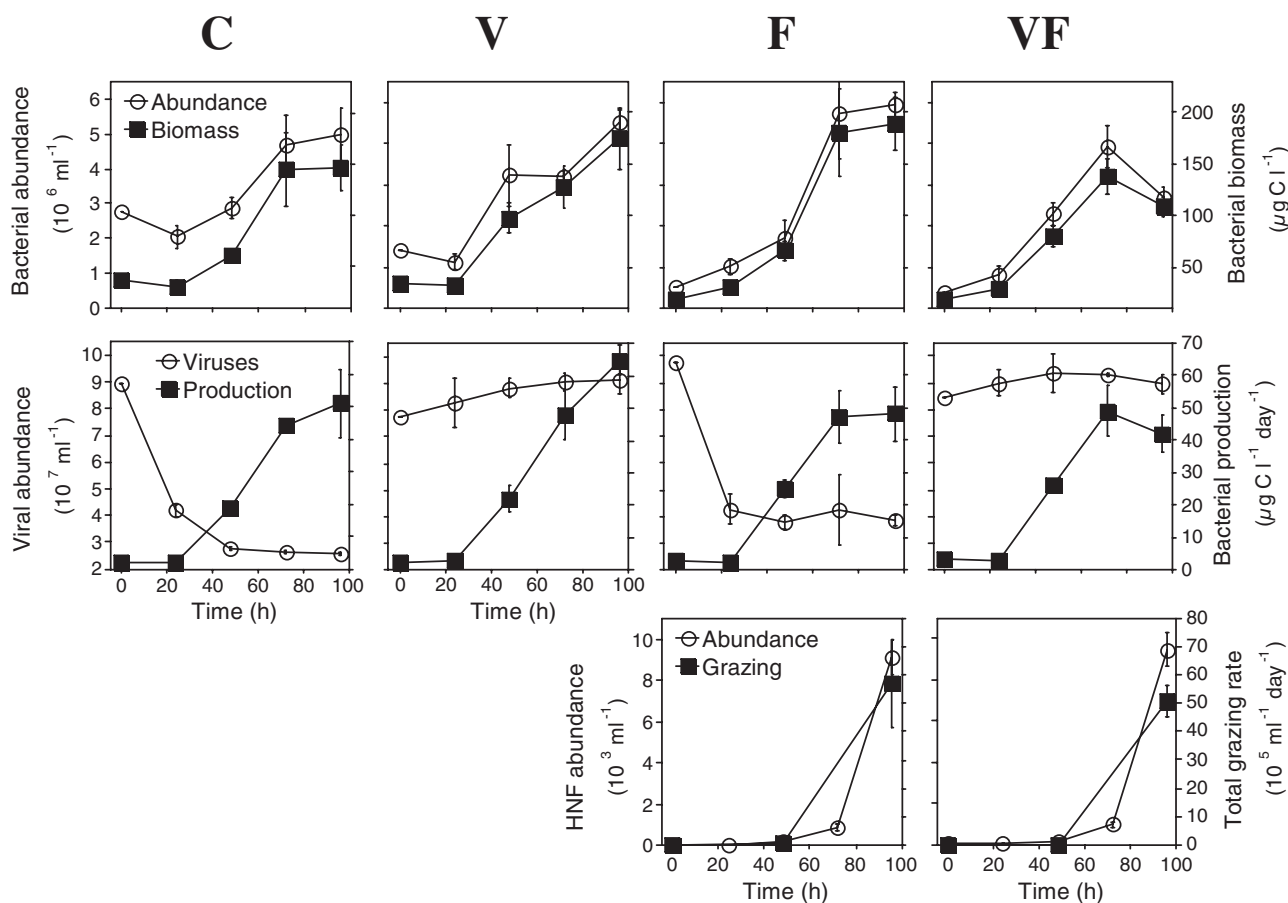


Fig. 1. Time-course of bulk viral, bacterial and flagellate parameters in the experiment. C, control; V, viruses; F, flagellates; VF, viruses + flagellates. Values are presented as means \pm SD of triplicate incubations for each treatment.

the experiment, TdR incorporation was highest in the V and lowest in the VF treatment. Bacterial production in V was significantly different from F and VF. We employed probes for *Actinobacteria* (HGC69a probe), *Cytophaga-Flavobacterium-Bacteroidetes* bacteria (CF319a probe), BET42a probe (*Betaproteobacteria*) and a subgroup-*Betaproteobacteria* (R-BT065 probe). Apparent growth rates of the entire bacterial community were lower than in the probe-defined subpopulations for which growth could be observed between 24 and 72 h (Table 1). BETA, CF and

R-BT, growth rates were significantly higher in the presence of flagellates (F and VF treatment) than in other treatments. BETA, R-BT and CF growth was similar in the F and VF treatment (generation times of 23–28 h), whereas in C and V fastest growth was observed for R-BT (generation time of 29 h). ACT showed a strong decrease in abundance (as indicated by negative growth rates) in all treatments.

Flagellates increased markedly in numbers from 72 to 96 h and thus in potential impact on bacteria. Therefore,

Table 1. For the interval of 24–72 h, net apparent daily growth rates of bacterioplankton and probe-defined bacterial subgroups in the consumer-free treatment (C) and in the treatment exposed to viruses and/or HNF grazing (V, F and VF treatments).

Taxonomic group	Treatment (growth or death rate per day)			
	C	V	F	VF
Bacterioplankton	0.41 \pm 0.062	0.34 \pm 0.018	0.52 \pm 0.027	0.490 \pm 0.037
CF	0.79 \pm 0.128	0.61 \pm 0.093	0.87 \pm 0.115	0.970 \pm 0.160
BET	0.66 \pm 0.064	0.64 \pm 0.108	1.10 \pm 0.066	0.970 \pm 0.092
R-BT	0.83 \pm 0.112	0.83 \pm 0.070	1.04 \pm 0.162	1.035 \pm 0.239
ACT	-0.47 \pm 0.036	-0.48 \pm 0.095	-0.50 \pm 0.047	-0.39 \pm 0.055

The values are mean \pm SD of three triplicate treatments. Rates were calculated for the interval of 24–72 h.

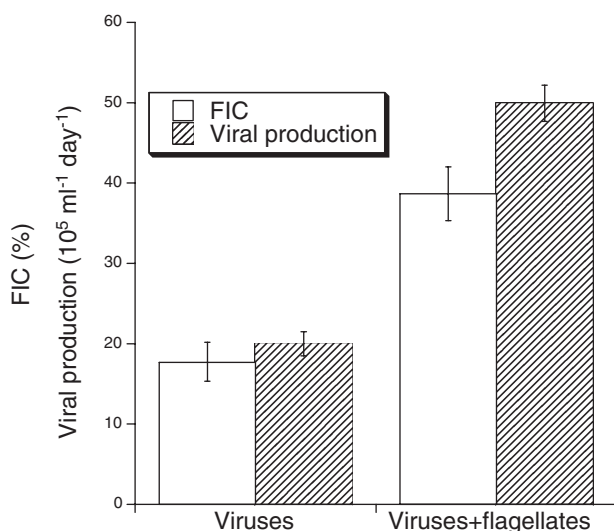


Fig. 2. Frequency of infected cells (FIC) and viral production in the virus (V) and virus + flagellate treatment. Values are presented as means \pm SD of triplicate incubations for each treatment.

growth rates of bacterial populations were also calculated for this time period. For CF and BET, growth rates were positive in the C and V treatment and negative in the F and VF treatment. For R-BT growth rate was positive in the V but negative in the other treatments. Interestingly, this decrease was much higher in the VF than in the F treatment, whereas growth rates were higher in V than in C. Growth rate of ACT increased strongly in the presence of flagellates (generation times 21–35 h) but decreased in C and increased only slightly in V. From 72 to 96 h, growth rates relative to those in the control were positive for CF, BET and R-BT in V, but negative for F and VF and this effect was stronger for VF (Fig. 3). For ACT, growth rates compared with those in the controls were higher in F than in VF (Table 2).

Bacterial community composition and diversity

The EUB probe hybridized to c. 45–50% of total bacterial cells at the start of the experiment and then increased to

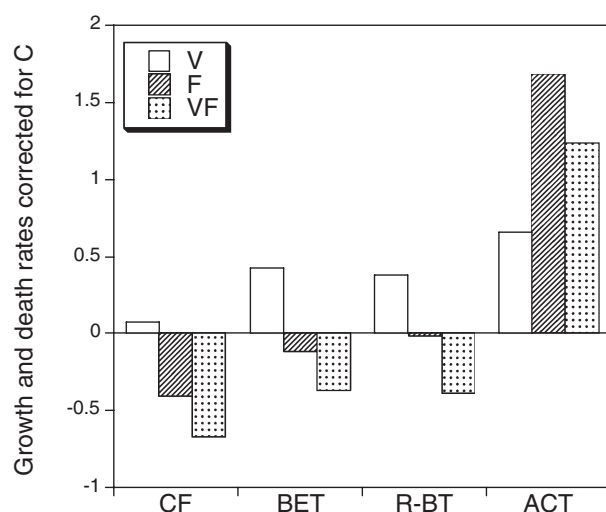


Fig. 3. Growth and death rates of CF, BET, R-BT and ACT corrected for C. The data show that effects of V and F are not always additive compared with VF.

60–70% during the experiment in the different treatments and remained stable except for a drop to about 60% in the VF treatment. BET positive cells ranged from c. 10–20% at T_0 and increased in all treatments (Fig. 4). Similar to EUB, the percentage of BET positive cells increased significantly faster in C and V than in F and VF. About 30% of the cells were ACT positive when the experiment started. In C and V, %ACT decreased until the end of the experiment, whereas in F and VF, %ACT decreased until 72 h and increased again at 96 h to more than 10%. Interestingly, %ACT decreased from day 0 to day 1 in the presence of viruses (V and VF) but remained constant in the other treatments. R-BT increased from c. 10% at the start of the experiment to > 40% towards the end with no significant differences between treatments. However, while %R-BT was not different from %BETA at most time points (except T_0) in C and VF, its contribution was lower in the V and F treatment. CF increased from 5% at the start to c. 30% with slightly, but significantly higher, values in C than in the other treatments. GAM remained well below 2.5% across treatments. The most abundant detectable probe-defined groups at the

Table 2. For the interval of 72–96 h, net exponential rates of population change among probe-defined bacterial subgroups in the predator-free treatment (C), the virus only treatment (V), the treatment consisting of HNF grazing alone (F) or with added viruses (VF).

Taxonomic group	Treatment (growth or death rate per day)			
	C	V	F	VF
CF	0.340 \pm 0.136	0.418 \pm 0.189	-0.069 \pm 0.312	-0.334 \pm 0.288
BET	0.002 \pm 0.217	0.427 \pm 0.169	-0.118 \pm 0.178	-0.368 \pm 0.157
R-BT	-0.051 \pm 0.090	0.331 \pm 0.091	-0.029 \pm 0.118	-0.441 \pm 0.277
ACT	-0.541 \pm 0.404	0.118 \pm 0.265	1.141 \pm 0.145	0.687 \pm 0.184
HNF	-	-	2.02 \pm 0.2	2.10 \pm 0.2

The values are mean \pm SD of three triplicate treatments. Rates were calculated for the last experimental interval, hours 72–96 with dense populations of HNF in the F and VF treatments. Growth rates of flagellates are included for comparison.

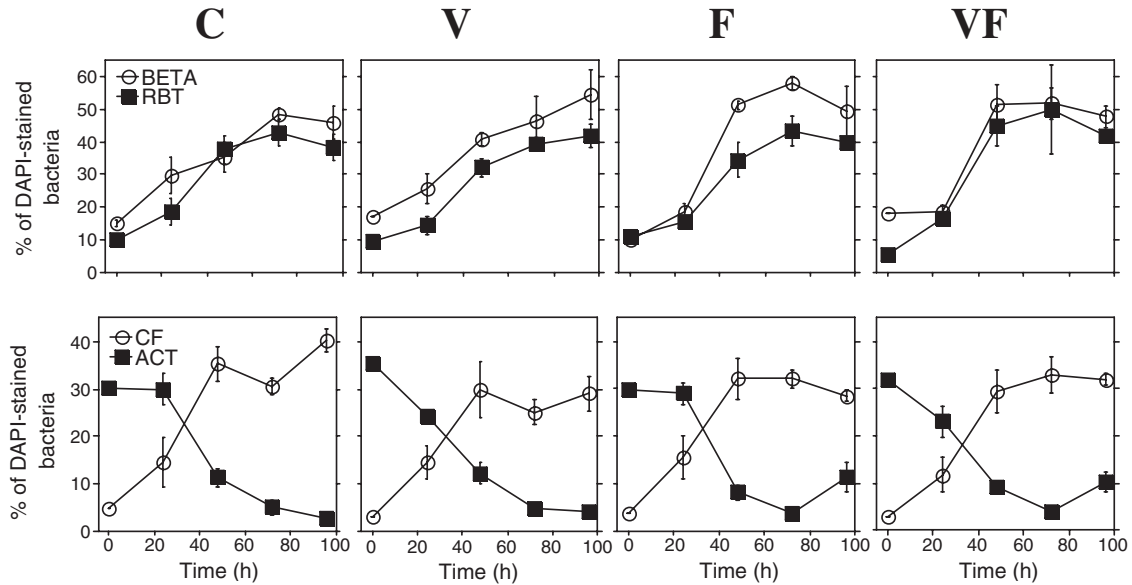


Fig. 4. Time-course of the relative abundance of different bacterial subpopulations as determined by FISH in the experiment. C, control; V, viruses; F, flagellates; VF, viruses + flagellates. Values are presented as means \pm SD of triplicate incubations for each treatment.

start of the experiment were ACT, followed by BET and CF. At the end of the experiment, BETA, dominated by the R-BT subcluster, and CF were the most abundant groups. *Flectobacillus* was only detected in the presence of flagellates at the end of the experiment (for further details see the companion paper by Šimek *et al.*, 2006a).

No *Archaea* could be detected by PCR targeted against the 16S rRNA gene. Using denaturing gradient gel electrophoresis (DGGE), we found an overall containment effect on bacterial diversity in the form of a reduction of the number of bands in all the populations at the end of the experiment compared with time zero (Fig. 5). However, we also found treatment effects. A total number of 13 bands were detected and they were all found in the V treatment. Replicates of single treatments showed the same band pattern; however, relative band intensities varied among replicates. Shannon diversity showed similar results compared with richness as estimated by band number (Fig. 6). Thus, viruses alone stimulated richness, whereas the presence of both predators had the most negative effect on richness and diversity.

Band #4 (98.24% similarity to *Chryseobacterium*) was only found in V. Band #10 (99.43% similarity to *Pseudomonas* sp. and *P. syringae*) vanished only in C. Band #11 and 12 (99.04% and 100% similarity to different betaproteobacterial partial sequences) disappeared in the presence of flagellates (F and VF treatment) and another two bands could not be detected in VF. For band #5 we obtained no sequence, whereas band #13 showed a 100% similarity to a betaproteobacterial partial sequence. The total number of bands was 11 in the C, 10 in the F and

8 in the VF treatment. Of the bands, which occurred in all treatments, four could be sequenced. Two belonged to *Betaproteobacteria* and the other two to different *Flavobacterium* sp. Three other bands, which could be sequenced but were very faint and thus not included in the fingerprint analysis, showed sequence similarities of > 99% to *Flavobacterium* and *Bacteroidetes* sp.

Discussion

Experimental approach and confinement effect

Our experimental protocol yielded some differences compared with natural populations. The experimental protocol resulted in a reduction of HNF abundance at the start of the experiments compared with *in situ* concentrations. This might have decreased the effect of HNF at the start of the experiment and thus we might have underestimated their influence; however, clear treatment effects of HNF were observed at the end of the experiment, when HNF abundance was about twice as high as *in situ*. The marked decrease in viral abundance in C and F is likely due to the decay of heat-inactivated viruses. The finding that viral abundance levelled off at *c.* 25% of initial in the C treatment compared with the start of the experiment suggests that our protocol had significantly reduced the abundance of active viruses. The finding that viral abundance did not recover in the C and F treatment indicates that this reduction remained significant throughout the experiment. Such a lack of recovery has been documented before and indicates that induction of lysogenic

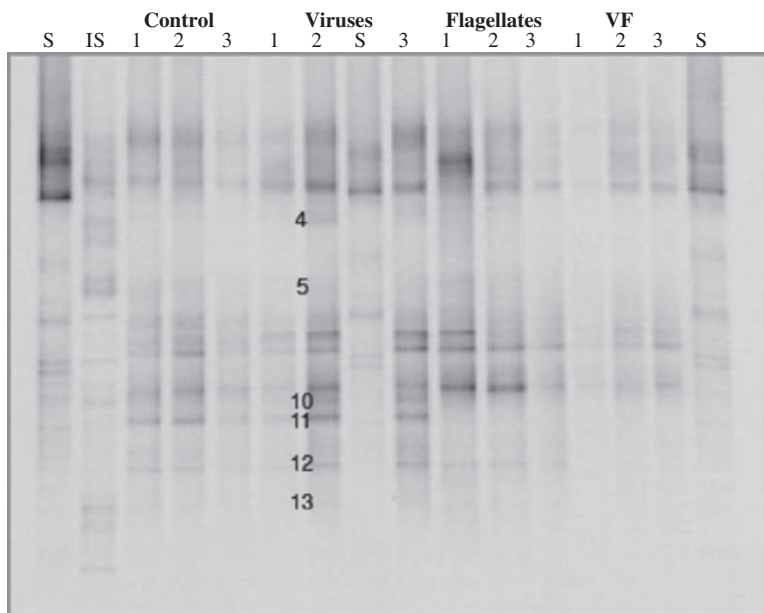


Fig. 5. Genetic fingerprint of bacteria using 16S rRNA gene based PCR-DGGE. S, standard; IS, *in situ* sample; 1–3, replicates. Numbers refer to bands mentioned specifically in the text.

cells was not a major source of viral production in the experiment. However, the finding that viral abundance was stimulated in the F compared with the C treatment suggests that viral production was not completely stopped. These active viruses likely entered the incubations along with bacterial concentrates, since a 0.2 μm cartridge also retains a fraction of the viruses. Thus, the influence of viruses on bacterial activity and production should be considered conservative.

The number of detected bands on the DGGE profiles was lower at the end compared with *in situ*. The manipulation of the samples by tangential flow filtration and or

confinement effects may have influenced bacterial community composition. Confinement effects are common in incubations of natural communities in containers (Massana and Pedrós-Alió, 2001). This was also true for incubation in dialysis bags, where at least a part of the inorganic nutrients and DOM can be exchanged. Despite manipulation or confinement artefacts, treatment effects could be observed with the reconstitution approach we used. It is noteworthy that the study of consumer effects on bacterial production and diversity at the community level can currently not be performed without some strong effects of sample manipulation.

Effects of HNF on viruses

Viral abundance was higher in F than in C. Such a stimulation of viral abundance in the presence of grazers has been reported (Šimek *et al.*, 2001; Weinbauer *et al.*, 2003). Moreover, we estimated, using a dilution approach, that FIC and viral production were higher in VF than in V. However, although viral abundances were highest in the VF and lowest in the V treatment, this difference was not significant. One reason could be that the differences in FIC between V and VF did not produce enough viruses to be detectable against the high viral background of almost 10^8 particles ml^{-1} . Indeed, the difference in the number of viruses produced per day between VF and V was *c.* one order of magnitude less than the standing stock of viruses.

Our data suggest that infection frequencies are higher in the presence of grazers. The hypothesis that the increases in viruses are due to bacteriophages is more likely and supported by findings from the same environment on increased frequencies of visibly infected bacteria

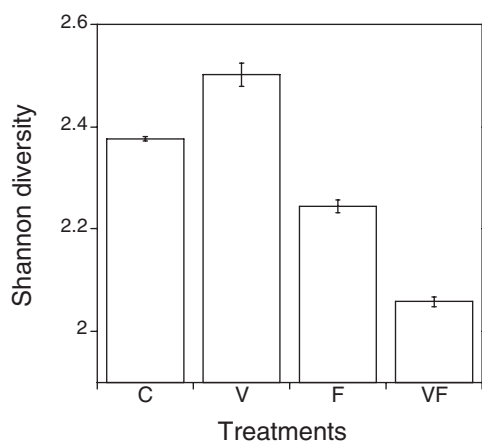


Fig. 6. Shannon diversity in different treatment at the end of the experiments. Data were obtained from an analysis of the gel shown in Fig. 4 using the number of detected bands and their relative intensity. Values are presented as means \pm SD of triplicate incubations for each treatment.

(Šimek *et al.*, 2001; Weinbauer *et al.*, 2003) and FIC (Fig. 2) in the presence of HNF. We hypothesize that increased viral production is due to HNF grazing activity.

Grazing of HNF could influence virus production rates in various ways. Rapidly growing flagellates are relatively inefficient consumers of both phosphorus (e.g. (Dolan, 1997) and carbon (e.g. (Pelegri *et al.*, 1999). A possible effect is then a general stimulation of bacterial growth by increasing the supply of substrates through excretion. Viral production could be boosted through production in previously slow-growing cells. In addition, increased growth rate could induce viral production in lysogens. Another effect, which has not been considered before, could be grazing resistance conferred by infection. Indeed, infected cyanobacteria express a surface protein, which could protect infected cells against grazing (Clokic *et al.*, 2006). Another possible effect is altering viral production as a result of shifts in the community composition of the bacterioplankton. Grazer-induced changes in the community composition of bacterioplankton has been reported previously for the Římov reservoir (Šimek *et al.*, 2001; 2003) and occurred also in our experiment (Fig. 4). Moreover, fluorescence *in situ* hybridization (FISH) analysis of HNF food vacuole content clearly documented different selectivity among bacterial probe-targeted groups studied (Šimek *et al.*, 2006a). If flagellate grazing changes the dominance pattern to one of fewer hosts, increasing then the contact rate of phage to 'correct' host could yield higher infection frequencies. There is some evidence in favour of this scenario in our data. At the end of the experiment, the fewest numbers of DGGE bands, suggesting a lower number of phage hosts, were found in the populations subjected to flagellate grazing.

It should be noted that shifts in flagellate grazing pressure need not always result in detectable changes in abundances or activities of viruses. No clear trends were found by Homák and colleagues (2005) using a size-fractionation in an experiment conducted in 2001. Moreover, there was no evidence of a reciprocal relationship as the presence or absence of viruses had no detectable effect of flagellate abundance and growth rate.

Effect of consumers on bacterial abundance, biomass and activity

With regard to bacterial activity, it has been argued that a virus-induced stimulation occurs, when viral lysis shunts organic matter into the DOM pool (Wilhelm and Suttle, 1999). This is also predicted by a model with and without viruses and explained by 'lysed bacterial production' which is not readily accessible to protists (Fuhrman, 1999). However, bacterial production was not significantly different between F and VF, and thus our data do not support this idea. It has also been shown that viral lysis

products stimulate bacterial production in the absence of prokaryotic grazers (Middelboe *et al.*, 1996; Noble and Fuhrman, 1999; Middelboe and Lyck, 2002). Although bacterial production was highest in V, the values were not significantly different from the control. However, after 48 h, bacterial abundance was significantly higher in V than in C. This might be considered as support of the stimulation of production hypothesis assuming that the nutrient regeneration by viral lysis stimulates bacterial production or biomass accumulation. Thus, our data do not support the viral stimulation of production hypothesis at the food web level (i.e. by reduced transfer of carbon to higher trophic levels by grazing) (Fuhrman, 1999) but suggest that viruses can stimulate bacteria-mediated biomass accumulation compared with the control (Middelboe *et al.*, 1996; Noble and Fuhrman, 1999). Such discrepancies between models and experimental data might also be due to the fact that the models assume steady state conditions, while most experimental approaches (such as in this paper) do not meet this criterion.

It is difficult to envision how biomass accumulates when production does not increase (e.g. found between V and C9). While we cannot exclude the possibility of methodological problems such as the use of a single and constant conversion factor for bacterial production in different treatments or a single relationship between cell volume and biomass, there are also other potential explanations. Bacterial production was measured in 1 h incubations. A part of viral lysis products will not be detected (even if dissolved production is measured), because not all released products will be ³H labelled within the incubation time. This non-labelled DOM can be taken up by bacteria and thus could cause increases in biomass in 1 day incubations. While this might be an explanation for our data, it also shows that there is a need to develop approaches to separate contributions of lysis products (including viral particles), release of extracellular material and true particulate bacterial production.

The following discussion is based on the $T_{72-96\text{ h}}$ part of the incubations, when flagellate abundance and grazing rates started to increase. Typically, in dialysis bags experiments from the Římov reservoir with < 5 µm treatments, flagellate abundances increased earlier in experiments and their effects were more pronounced than in our experiment. This could be due to the lower water temperature (15°C) compared with previous experiments and the degree of sample manipulation using the reconstitution approach. In any event, towards the end of the experiment, consumer abundances were most similar to *in situ* conditions. The VF treatment showed the lowest bacterial abundance and biomass and the F treatment the highest, whereas bacterial production did not differ between these two treatments. This suggests that biomass was only top-down controlled when both predators were present. Our

data also indicate that biomass and production were controlled in different ways. This difference in carbon balance should then be found in the dissolved fraction.

Using a cell number based analysis of the growth rates and responses to treatments of the bacterial subpopulations offers also an explanation for potential discrepancies between biomass and production control in the F and VF treatment (for more details see also companion paper of Šimek *et al.*, 2006a). *Actinobacteria* and *Polynucleobacter* were less abundant in the VF than in the F treatment probably due to indirect or direct viral effects, whereas *Flectobacillus* increased in the VF treatment. Less abundant but generally more active cells of bacteria in VF (*Flectobacillus*) obviously compensated for losses of the more abundant but generally less active cells (*Actinobacteria* and *Polynucleobacter*) in the F treatment. This could have resulted in a comparatively similar bacterial production in both treatments and in lower abundances in VF.

Effect of consumers on bacterial life styles and diversity

The appearance or disappearance of bands in different treatments suggests that bacterial life styles are influenced by consumers. Phylotypes which were only observed in the presence of consumers are likely bad competitors for resources but can display protective mechanisms against grazing and infection, whereas phylotypes only present in C are likely good competitors for nutrients. Phylotypes which vanished in the F and VF treatments are likely vulnerable to flagellate grazers. Phylotypes present in V and F, but not in VF, are probably not able to deal with the combined pressure of viruses and flagellates. More indirect effects are possible as well such as a stimulation of phylotypes by the changing substrate condition due to recycling of nutrients by grazing and lysis. Evidence for some of these life styles is presented in this paper. One example could be the *Polynucleobacter* cluster, which were significantly suppressed in the VF treatment (e.g. fig. 5 in companion paper by Šimek *et al.*, 2006a). *Flectobacillus* is a group that clearly benefited from the presence of both predators but could not be quantitatively detected in C and V (see companion paper by Šimek *et al.*, 2006a). A compilation of *in situ* manipulation experiments, conducted in the Řimov reservoir between 1997 and 2003, indicates that phosphorus availability is one of the major determinants of bacterial life styles (Šimek *et al.*, 2006b). Thus, inorganic and organic phosphorus recycling by viruses and flagellates in addition to their selective mortality might shape the outcome for phosphorus availability of bacterial subpopulations.

Interestingly, we did not detect specific bands for the F or VF treatment, although it is well known that resistance against grazing such as filamentous cells and cell aggre-

gates develop in the system we investigated (Šimek *et al.*, 2001). In the experiment, *Flectobacillus* targeted with the R-FL615 probe (Šimek *et al.*, 2001) could not be detected in V and C by FISH but became a remarkably large proportion of total biomass in the presence of flagellates. However, no sequences belonging to the R-FL615 group could be found. This corroborates the finding that FISH data and sequence data based on PCR-fingerprints can show diverging results. However, BET and CF were the most abundant FISH detectable groups in the experiment and only sequences belonging to these two groups were found.

Some phylotypes could only be detected in the V treatment and richness was highest in this treatment thus, giving support to the 'killing the winner hypothesis'. It has to be noted that other experimental studies contradict this hypothesis (Schwalbach *et al.*, 2004; Winter *et al.*, 2004). Thus, other or additional hypotheses are needed to explain the effect of viruses on microbial diversity. Apparent richness, i.e. the number of detectable bands, was reduced in F compared with C (and V) suggesting that grazing removed specific phylotypes. In the VF treatment, richness was even lower than in F indicating that grazing and lysis acted synergistically in reducing richness and by that possibly accelerating growth of phylotypes such as *Flectobacillus*, which seem to be resistant to grazing and viral infection (Šimek *et al.*, 2006a). It has been suggested that grazing on infected cells 'kills the killer of the winner' and reduces bacterial species richness and the role of the viral loop (Maki and Yamamura, 2005). As richness and production were lower in VF than in V, there seems to be support for the 'kill the killer of the winner' hypothesis. However, viral infection and production were not reduced in the VF treatment compared with V (Fig. 2) and thus the role of the viral loop should be stimulated in the presence of grazers. Our data suggest that grazing influences viral infection although not always in the way simple models predict. In addition, the problem of steady state assumption in models versus non-steady state conditions in experiments also applies here.

Chryseobacterium was specific to the V treatment and is often isolated from diseased aquatic animals (Bernardet *et al.*, 2005). A bacterium usually found on or in animals is likely a bad competitor for nutrients in a non-attached state and has potentially no need for developing strong anti-defence systems. However, its potentially low abundance in the water column would make it less vulnerable against viral infection during the course of our experiment, because infection depends (among other factors) on the encounter rates of viruses with hosts. This would confer an advantage to *Chryseobacterium* and might explain its detectability in the V treatment. Interestingly, the phylotypes vulnerable to the presence of flagellates (F+VF or VF treatment) belonged to uncultured phylotypes of

Betaproteobacteria. This corroborates the finding that percentage BETA increased faster in the C and V than in the F and VF treatment. Surprisingly, *Pseudomonas* sp./*P. syringae* only vanished in the C treatment suggesting that this phylotype was not competitive for nutrients. It has been shown that *P. syringae* strains are versatile plant pathogens. However, one has to keep in mind that *Pseudomonas* is genetically and metabolically diverse and a partial 16S rRNA gene sequence analysis probably does not allow a meaningful comparison. Also, it is possible that some phylotypes are adapted to lysis products.

Interestingly, at the end of the experiment, ACT increased in the presence of HNF suggesting that they were negatively selected. Such data have been shown before (Pernthaler *et al.*, 2001) including the same environment (Šimek *et al.*, 2006a). In addition, HNF grazing rates on ACT, directly estimated using food vacuole contents, have been found to be low compared with other bacterial phylotypes (Jezbera *et al.*, 2005). In virus-free treatments (C and F), % contribution of ACT did not change from day 0 to day 1, suggesting that they could keep pace with other groups for about a day. In the V and VF treatment, however, they decreased rapidly within 1 day, indicating a control by viral lysis. Also, growth rate of ACT was lower in the VF than in the F treatment. This is the first albeit indirect evidence of viral infection of ACT at the community level.

Synergism and antagonism

Our data suggest that the effects of viruses and flagellates are not additive (e.g. Fig. 3). Synergistic effects could, e.g. be observed for the reduction of bacterial biomass and richness, and the growth rates of CF, BET and R-BT. Synergistic and antagonistic effects could be the result of group- or species-specific competition for prey and hosts and the fact that both types of predators produce organic matter and thus potentially fuel growth. Although there were exceptions such as for *Flectobacillus* (see companion paper) and ACT (Fig. 3), the effect of VF on bacterial production, %populations and richness was generally stronger than when only one of the consumers was present. Not enough attention has been paid to such effects, which have to be considered when causal relationships are to be established in natural communities.

Experimental procedures

Study site and experimental approach

The water sample for the experiments were collected about 250 m off the dam of the meso-eutrophic Římov reservoir, a 13.5-km-long, canyon-shaped reservoir in South Bohemia, Czech Republic (for details see Šimek *et al.*, 1999), on 24 May, 2004.

Cells from a 50 l water sample were concentrated by 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.

Each of the four solutions was used to fill three dialysis bags (Spectra/Por, diameter 76 mm, MWC 12–14 kDa) of the final volume of ~2 l as described before Šimek and colleagues (2001) and deployed *in situ* (15°C). Samples for viral and bacterial abundance, bacterial production and FISH were collected once per day. Samples for bacterial richness and viral production were collected at the start and at the end of the experiment.

Bacterial abundance and biomass

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 volumes were measured using the semiautomatic image analysis systems LUCIA D (Lucia 3.52, resolution 750 × 520 pixels, 256 grey-levels, Laboratory Imaging, Prague, Czech Republic) as described in detail in Posch and colleagues (1997). Bacterial carbon biomass was calculated according to Norland and colleagues (1995).

Bacterial production

Bacterial production was measured via thymidine incorporation modified from Riemann and Sondergaard (1986) and described in detail in Šimek and colleagues (2001). An empirical conversion factor (ECF) between thymidine incorporation rate and bacterial cell production rate was determined using data from the triplicate control treatment without grazers. An ECF of 4.05 × 10¹⁸ cells mol⁻¹ of thymidine incorporated as determined for the investigated environment was used for calculations. The cell production rate was calculated from the slope of the increase of *In* bacterial abundance over time.

Protozoan grazing and enumeration

Protozoan grazing on bacteria was estimated using fluorescently labelled bacterioplankton (FLB) (Sherr and Sherr, 1983) concentrated from the reservoir water (see Šimek *et al.*, 2001 for details). Heterotrophic nanoflagellate FLB uptake rates were determined in short-term FLB direct uptake experiments with FLBs equal to 10–15% of natural bacterial concentration. Subsamples for protozoan enumeration and tracer ingestion determinations (for details see Šimek *et al.*, 2001) were fixed with the Lugol's-formaldehyde-decolorization technique (Sherr and Sherr, 1993). For HNF counting 5–20 ml sub-

samples were stained with DAPI, filtered through 1 µm black Poretics filters, and inspected via epifluorescence microscopy. To estimate total protozoan grazing, we multiplied the average uptake rates of HNF by their abundances in F and VF treatments as previously described (Šimek *et al.*, 2001).

Enumeration of viruses and cells; storage of samples

Samples for counting viral and prokaryotic abundance (1 ml each) were fixed in 0.5% glutaraldehyde, stored for 30 min at 4°C in the dark, flash frozen in liquid nitrogen and stored at -80°C until analysis as described in Brussaard (2004). Samples were thawed shortly before analysis and bacteria were stained for a few minutes in the dark with diluted SYBR Green I solution (Molecular Probes) at 2.5 µM final concentration. For viral counts, samples were prepared as previously described (Brussaard, 2004) and stained in a 80°C water bath for 30 min before running in the flow cytometer. We used a FACScalibur (Becton Dickinson) flow cytometer with a laser emitting at 488 nm. Samples were run at low speed and data were acquired in log mode until around 10 000 events had been recorded. In order to maintain the rate of particles passage below 300 events s⁻¹ and to avoid coincidence (Gasol and del Giorgio, 2000), samples for viral counts were 20-fold diluted in autoclaved and 0.2 pre-filtered TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0). Cell and viral concentration was determined from the flow rate, which was calculated by weighing a sample before and after 10–15 min run of the cytometer. Fluorescent 1 µm latex beads (10⁵ beads ml⁻¹) were systematically added to the prokaryotic samples as an internal standard (Polyscience, Europe).

Estimation of viral production and FIC

Viral production and the FIC were assessed using a virus-dilution approach (Weinbauer *et al.*, 2002; Wilhelm *et al.*, 2002). At the end of the experiment, 200 ml of water from each replicate was collected from V and VF. Bacteria were concentrated using a 0.2 µm pore-size cartridge (Vivaflow50, Vivascience) and from the filtrate virus-free water was produced using a 100 kDa cartridge (Vivaflow50, Vivascience). The bacterial concentrate was washed with 100 ml virus-free water to further reduce viral abundance. An equivalent of the bacterial concentrate corresponding to a 50 ml volume was transferred into a 50 ml tube (Greiner) and filled up with virus-free water to 50 ml. Incubations were performed in the dark at *in situ* temperatures. Samples for counting viral and bacterial abundance were removed periodically for 24 h. Viral production was corrected for losses of bacterial abundance due to filtration and used to estimate bacterial cell lysis rates by assuming a burst size of 40 (Weinbauer *et al.*, 2003), thus the production of 40 viruses equalled the lysis of one bacterial cell. This number is similar to the overall average of burst size for eutrophic freshwater systems (Parada *et al.*, 2006).

DNA extraction

Prokaryotic cells from 0.5 l samples were recovered on a 0.2 µm pore-size polycarbonate filter (diameter 0.47 mm;

Whatman) and filters were stored at -80°C until analysis. Nucleic acids were extracted from the filters and purified as described elsewhere (Winter *et al.*, 2004). Briefly, after four freeze-thaw cycles (-196°C to +37°C) an enzyme treatment was performed with lysozyme (1.25 mg ml⁻¹ final concentration; Fluka BioChemika #62970) for 30 min at 37°C followed by a digestion with proteinase K (100 µg ml⁻¹ final concentration; Fluka BioChemika #82456) and 1% sodium dodecyl sulfate for 2 h at 55°C. In contrast to the phenol-chloroform extraction step from the original protocol, nucleic acids were extracted with 4.5 M NaCl and chloroform, followed by 100% isopropanol precipitation. This modified procedure avoids using a toxic chemical and yields genetic fingerprints identical to those obtained by the original method (data not shown). The pellets were resuspended in 60 µl of 0.5× TE buffer [10 mM Tris, 1 mM EDTA (HCl, pH 8.0)].

PCR and DGGE

Conditions of the touchdown PCR and chemicals were as described in Schäfer and Muyzer (2001). One to 4 µl of cleaned nucleic acid extract were used as template in a 50 µl PCR reaction (1.5 mM MgCl₂, 0.25 µM of each primer and 2.5 U *Taq* polymerase; Sigma; #D 5930) together with a positive and a negative control. A fragment of the 16S rRNA gene was amplified using the primer pairs 341F-GC/907R and 344F-GC/917R for *Bacteria* and *Archaea* respectively (Schäfer and Muyzer, 2001). Due to the use of these universal bacterial primers, our community profiles may include sequences not only from heterotrophic bacteria but also from autotrophic cyanobacteria and from chloroplast DNA. However, the abundance of cyanobacteria and autotrophic flagellates was low *in situ* because of the clear-water phase. At the end of the experiment, no autotrophic flagellates could be detected and the number of cyanobacteria was close to the detection limit. In addition, no cyanobacterial sequences could be detected.

Denaturing gradient gel electrophoresis and sequencing

Denaturing gradient gel electrophoresis procedures followed those described by Schäfer and Muyzer (2001). PCR products were separated into bands by electrophoresis for 18 h at 100 V on acrylamide/bis-acrylamide (6%) gels prepared using a gradient of 30% to 70% (urea and formamide) using an INGENYphorU DNA Mutation Detection System (Ingeny International). Denaturing gradient gel electrophoresis gels were photographed with a gel documentation system (GelDoc EQ; Bio-Rad) after 15 min staining with a 10× SYBR Gold solution (Molecular Probes; # S11494). Analysis of band patterns between lanes of the same gel was performed with the Quantity One Software (Bio-Rad) using a variety of exposure times. Visual and software based inspection suggested a reproducible detectability when relative band intensity exceeded 3.5% of total band intensity as relative abundance values.

Bands were excised from gels using a sterile scalpel. DNA was eluted from the gel overnight at 4°C in RNase-free H₂O. Sequencing was performed by MWG-Biotech using the 907R primer. Shannon diversity was calculated using per cent band intensities.

Fluorescence in situ hybridization with rRNA-targeted oligonucleotide probes

Analysis of bacterial community composition was carried out by *in situ* hybridization with group-specific oligonucleotide probes on membrane filters. The catalysed reporter deposition FISH protocol was applied as shown in Pernthaler and colleagues (2002). Seven different group-specific oligonucleotide probes (ThermoHybaid, Germany) were targeted to the domain *Bacteria* (EUB338), to α - and γ -subclasses of the class *Proteobacteria* (the BET42a and GAM42a probes respectively), to a narrower subcluster of the α -*proteobacteria* – (R-BT065), to the *Cytophaga/Flavobacterium* group (CF319a), to the *Actinobacteria* group (HGC69a), and to *Flectobacillus* cluster (R-FL615). After the whole procedure, the filter sections were stained with DAPI and the proportions of hybridized bacterial cells were enumerated using an epifluorescence microscope (Olympus AX70 Provis).

Statistics

An analysis of variance and a Fisher PLSD *post hoc* test were performed to test differences of parameters between treatments. Differences were considered as significant at a probability (P) < 0.05 and tests were performed with data from the end of the experiment unless stated otherwise.

Acknowledgements

This study was largely supported by the Grant Agency of the Czech Republic under research Grant 206/05/0007 (project No. AVOZ60170517) awarded to K. Šimek, by a project No. AVOZ60170517, then also by the project MSM 123100004, the Grant BARRANDE (EGIDE) number 2004-004-2 supported joint experimental activities of K.Š. and J.D. and an ATIPE (CNRS) grant supported MGW. We also thank the breweries of Céské Budějovice in particular and Bohemia in general (Czech Republic) for their stimulating and refreshing products.

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