

## Differential effect of algal- and soil-derived dissolved organic matter on alpine lake bacterial community composition and activity

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### Abstract

We studied the effects of algal- and soil-derived dissolved organic matter (DOM) enrichments on the activity and community composition of bacterioplankton in an alpine lake. The effects of the DOM source on bacteria were tested by establishing dilution cultures amended with either an algal lysate or with a soil extract. Cultures were incubated for 6 days under close to in situ conditions, and changes in bacterial community composition and activity were tracked by micro-autoradiography (<sup>3</sup>H]-L-leucine) combined with fluorescent in situ hybridization and signal amplification by catalyzed reporter deposition. Heterotrophic bacterial production increased by 25-fold after the algal lysate addition. After 3 days, up to 90% of the bacteria were active in this treatment, and  $\beta$ -Proteobacteria, particularly the subgroup R-BT, represented 86% and 67% of the total bacterial counts, respectively. From day 3 onward, the percentage of active cells in this treatment decreased dramatically as did the relative abundance of subgroup R-BT. By the end of the experiment,  $\beta$ -Proteobacteria still dominated bacterial abundance (80%), but active cells were only 25% of total bacterial counts. In contrast, the addition of soil-derived DOM led only to a two- to three-fold increase in bacterial production.  $\beta$ -Proteobacteria was still the dominant group (50–60% of total bacterial counts and 50% of the cells positive for leucine incorporation), but Actinobacteria made a substantial contribution (15–23%). This pattern contrasted with that observed in the treatment receiving the algal lysate, where the relative abundance of the latter group rapidly decreased. In the treatment amended with algal-derived DOM, bacterial carbon production matched the observed decrease in dissolved organic carbon concentration. However, bacterial carbon produced on soil-derived DOM accounted only for 30% of the decrease in dissolved organic carbon concentration, suggesting a more inefficient utilization of this material. The expected climate-driven changes in DOM supply to alpine lakes will affect their bacterial community structure and activity.

Dissolved organic matter (DOM) is the largest pool of reduced organic carbon in most aquatic ecosystems (Hedges 1992) and the main source of substrates fueling bacterial metabolism. In aquatic ecosystems, DOM has different origins: allochthonous, such as from soil runoff, or autochthonous, such as from phytoplankton exudates. Allochthonous and autochthonous DOM differs in several optical (McKnight et al. 1994) and chemical characteristics (Benner 2002). Generally, autochthonous DOM is considered as more labile, whereas organic compounds from terrestrial sources are more recalcitrant to bacterial degradation (Del Giorgio and Davis 2003). This difference in their availability to microbial communities can be explained by the nature of the chemical identifiable substances in both DOM sources. For example, plankton-derived DOM is usually enriched in protein and labile polysaccharides, whereas terrestrial DOM contains more humic substances and structural polysaccharides (Benner 2002; Benner 2003). These compositional differences are

thought to influence the structure of natural bacterial assemblages (Crump et al. 2003). The heterotrophic assemblage of planktonic bacteria is often dominated by only a few bacterial groups. In most freshwater systems,  $\beta$ -Proteobacteria is the prevailing group (Glöckner et al. 1999). Actinobacteria have also been found to be numerically important in freshwaters ecosystems of different productivity (Glöckner et al. 2000; Burkert et al. 2003; Warnecke et al. 2005). The third group that seems to be a consistent component of freshwater and marine bacterial communities is the *Cytophaga*-like bacteria (Kirchman 2002). Recent studies (Kirchman et al. 2004) have focused on assessing changes in the structure and activity of the bacterial community in relation to DOM or examined the ability of different bacterial groups to use various compounds of the DOM pool (Cottrell and Kirchman 2000; Elifantz et al. 2005; Malmstrom et al. 2005). From these studies, it seems that bacteria differ in their capacity to use specific dissolved organic compounds, even when considering large taxonomic groups. Therefore, qualitative changes in the DOM source might trigger a shift in bacterial community structure, activity, or both.

Alpine lakes, located by definition above the tree line, have generally small catchment areas with poorly developed soils and sparse terrestrial vegetation. Consequently, dissolved organic carbon (DOC) concentrations are generally low (<1.5 mg L<sup>-1</sup>), and DOM is mainly autochthonous (Sommaruga et al. 1999; Sommaruga and Augustin 2006). It is generally accepted that the location of the

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tree line is principally a function of air temperature and that climate warming promotes its upward migration (Hauer et al. 1997), as well as that of alpine flora (Grabherr et al. 1994). In fact, mean air temperatures in the Alps have increased by 1°C since 1980 (Beniston 2000). If present trends in mean air temperature continue, two expected effects on a time scale of 50–100 years are a strong development of the vegetation cover in the catchment and an increase in the export of allochthonous DOM to these lakes (Hauer et al. 1997; Sommaruga et al. 1999; Sommaruga 2001). On the other hand, it is known that the disappearance of the ice cover improves the exchange of gases and nutrients, wind-driven circulation, and light conditions in lakes (Pechlaner, 1971). Hence, shorter periods of ice cover caused by higher air temperatures may have a large stimulatory effect on phytoplankton growth and production (Pechlaner 1971) and on autochthonous DOM production.

To understand how a shift in the dominant DOM source (autochthonous vs. allochthonous) could influence the bacterial community of alpine lakes, we ran an experiment where the natural bacterioplankton assemblage was supplied with DOM derived either from an algal lysate or from a soil extract. A treatment without DOM addition served as a control of the experiment. In addition, lake samples were collected to obtain background information about natural changes in the bacterial community during the course of the experiment.

## Material and methods

*Study site and experimental design*—The experiment was carried out in Gossenköllesee (GKS), a small alpine lake (area: 0.017 km<sup>2</sup>) situated at 2,417 m above sea level in the Austrian Alps (47°13'N, 11°01'E). GKS is a dimictic and holomictic lake covered by ice for ~7–8 months per year. The catchment area (0.3 km<sup>2</sup>) is composed of crystalline bedrock and covered with a poor soil layer (ca. 10% of its area) and sparse patches of alpine rankers. In the first week of August 2004 (i.e., 3 weeks after ice out), 50 liters of water were collected at 2-m depth and stored in acid-cleaned carboys. The water was gently filtered first through a glass-fiber filter (AP 40, Millipore, 142-mm diameter) precombusted at 450°C for 4 h and subsequently twice through a 0.22- $\mu$ m polycarbonate GSWP membrane (Millipore, 142-mm diameter). Filtered water was distributed among three sets of three replicates of 5-liter glass bottles (Schott). All glassware was soaked in 1 mol L<sup>-1</sup> hydrochloric acid (HCl) and subsequently rinsed several times with Milli-Q water and filtered lake water. The first set of bottles served as control of the experiments and did not receive any DOM addition. The second set of bottles received 300 mL of a soil extract to enrich the lake water with allochthonous DOM (hereafter, SOIL treatment). The soil extract was obtained according to Kablitz et al. (2003) using surface soil (upper 3–4 cm) collected from the catchment area of GKS. Finally, the third set of bottles received 200 mL of a filtrate from an algal lysate to enrich the autochthonous fraction of DOM (hereafter ALGAL treatment). The lysate was obtained from a culture of the

planktonic green algae *Chlorella minutissima* grown in Woods Hole medium (at 17°C with 8:16 light:dark) until the early stationary phase was reached. The culture was then treated with a tip sonicator (2-mm diameter) for 15 min at 30 W to disrupt the cells and then filtrated onto a 0.22- $\mu$ m polycarbonate membrane to eliminate algal rests and bacteria. The concentration of nutrients and DOC were both measured in the control and in the SOIL and ALGAL treatments. The DOC enrichment factor in the SOIL and ALGAL treatments was four-fold as compared to the natural lake concentration.

The lake bacterial assemblage was inoculated in all treatments following a 1:10 dilution obtained by filtering lake water collected from 2-m depth through a 0.8- $\mu$ m polycarbonate membrane (ATTP, Millipore) to exclude bacterivores (microscopic examination at the end of the experiment revealed the absence of grazers). Bottles were incubated at 1-m depth under in situ temperature and light conditions for 6 days. Subsamples for bacterial production and abundance, DOC analysis, and absorbance measurements were collected at day 0, day 2, and thereafter every day. The first sample (i.e., day 0) was removed 2 h after the treatments were settled to allow bacteria to adapt to the experimental conditions. Additional subsamples were removed to perform micro-autoradiography combined with fluorescent in situ hybridization and signal amplification by catalyzed reporter deposition (CARD-FISH). Those samples were incubated with [<sup>3</sup>H]-L-leucine as described in the following section. At every sampling date, a water sample from GKS was collected at 2-m depth and the same analyses and measurements were done.

*Incubation for micro-autoradiography*—Subsamples from every treatment were incubated with [<sup>3</sup>H]-L-leucine (Amersham, specific activity = 2,331 GBq mmol<sup>-1</sup>; 20 nmol L<sup>-1</sup> final concentration) at in situ temperature for 6 h. The optimum incubation time was previously determined by monitoring disintegrations per minute (DPM) increase over time during a time series experiment. Control samples were killed with formaldehyde (2% final concentration) 20 min before adding the substrate and were incubated in parallel with the samples. Incubation ended by adding formaldehyde at a final concentration of 2%. Samples were fixed overnight at 4°C and filtered on the next day through 0.22- $\mu$ m polycarbonate white filters that were subsequently rinsed twice with 5 mL of particle-free Milli-Q water. Afterward, filters were stored frozen (–20°C) until further processing.

*Bacterial production*—Heterotrophic bacterial production was estimated from rates of protein synthesis with [<sup>14</sup>C]-L-leucine (Amersham, specific activity = 11.3 GBq mmol<sup>-1</sup>) (Simon and Azam 1989). Duplicate samples and one formaldehyde-killed control were incubated with 20 nmol L<sup>-1</sup> (final concentration) of [<sup>14</sup>C]-L-leucine. Samples (15–30 mL) were incubated at in situ temperature in the dark for 1 h. Incubations were terminated by adding formaldehyde at 2% final concentration. Subsequently, the samples were filtered through 0.22- $\mu$ m Millipore GSWP filters and rinsed twice with 5 mL 5% trichloroacetic acid

for 5 min. Filters were dissolved in 1-mL ethyl acetate (Riedel de Haen), and after 10 min, 6 mL of scintillation cocktail (Ready-safe, Beckman Coulter) were added, and the radioactivity was assessed after 15 h. The radioactivity of the filter was converted into bacterial carbon production using the formula given in Simon and Azam (1989).

**Bacterial abundance**—Bacterial numbers were assessed by flow cytometry. Subsamples of 450  $\mu\text{L}$  were stained by adding 25  $\mu\text{L}$  of a 50  $\mu\text{mol L}^{-1}$  SYTO 13 solution (Molecular Probes). Fluorescent microspheres (1- $\mu\text{m}$  TransFluoSpheres 488/560, Molecular Probes) were added to a final concentration of  $4.7 \times 10^5 \text{ mL}^{-1}$  as a counting and internal fluorescence standard. The absolute concentration of the stock solution of the microspheres was assessed by flow cytometry combined with gravimetric volume measurement. Counts were made with a MoFlo (DakoCytomation) equipped with a water-cooled argon-ion laser tuned at 488 nm (200 mW). Bacteria were detected by their signatures in a plot of orthogonal side scatter versus green fluorescence.

**Dissolved organic carbon**—Subsamples for DOC analysis were filtered immediately after sampling through a pre-combusted (4 h at 450°C) GF/F filter (Whatman) placed on a stainless steel syringe holder. Filters were rinsed first with Milli-Q water and then with the sample. The filtrate was collected in precombusted glass bottles (Schott), acidified with HCl to pH 2, and stored in the dark at 4°C until further analysis within 48 h. DOC was measured by high-temperature catalytic oxidation with a Shimadzu TOC analyzer Model 5000. The instrument was equipped with a Shimadzu platinumized-quartz catalyst for high sensitivity analysis. Three to five injections were analyzed for every sample and blanks (Milli-Q water).

**DOM absorption**—Samples for DOM absorbance measurements were filtered as described for DOC analysis. Upon arrival at the laboratory, samples were scanned in a spectrophotometer (double-beam Hitachi U-2000) from 250 nm to 750 nm using a 10-cm quartz cuvette (SUPRA-SIL I). The measurements were referenced against Milli-Q water. Apparent absorption coefficients at specific wavelengths ( $\text{abs}_\lambda$ ) were calculated as  $\text{abs}_\lambda = (D_\lambda \times \ln 10)/L$ , where  $D_\lambda$  is the absorbance at the wavelength considered, and  $L$  is the path length (m) of the cuvette. True absorption coefficients ( $a_\lambda$ ) were corrected for the effect of scattering by colloids using a long reference wavelength (740 nm) applying the formula:  $a_\lambda = \text{abs}_\lambda - (\text{abs}_{740} \times 740/\lambda)$ . The ratio  $a_{250}:a_{365}$  was used to provide information about the relative size of DOM molecules (Strome and Miller 1978).

**Hybridization and tyramide signal amplification**—CARD-FISH was carried out on filter sections embedded in low gelling point agarose (0.2%). Sections were permeabilized with lysozyme and achromopeptidase according to Sekar et al. (2003). Hybridization with 5'-horseradish peroxidase (HPR)-labeled oligonucleotide probes, was carried out according to the protocol of Pernthaler et al. (2002) in 0.5-mL reaction vials filled up

with 300  $\mu\text{L}$  of hybridization buffer and the 5'-HPR-labeled probe at a final concentration of 0.5 ng  $\mu\text{L}^{-1}$ . Five different group-specific oligonucleotide probes (Thermo-Hybrid, Germany) were targeted to the domain *Bacteria* (EUB338), to  $\beta$ -*Proteobacteria* (BET42a) and its subgroup R-BT (R-BT065), to *Cytophaga*-like bacteria (CF319a), and to the class *Actinobacteria* (HGC69a). The proportion of formamide in the hybridization buffer was always 55% except for probe HGC69a (35%). Cells were hybridized at 35°C for at least 2 h and up to 4 h. After hybridization, the filter sections were washed at 37°C for 15 min in a prewarmed washing buffer containing 5 mmol  $\text{L}^{-1}$  ethylenediaminetetraacetic acid (pH 8), 20 mmol  $\text{L}^{-1}$  Tris-(Hydroxymethyl)-aminomethane hydrochloride (pH 7.5), 0.01% sodium dodecyl sulfate, and the appropriate amount of sodium chloride. The sections were then transferred to phosphate-buffered saline amended with Triton X-100 (PBS-T) for 10 min at room temperature. Amplification was carried out in the dark at 37°C for at least 15 min and up to 30 min in a reaction vial containing amplification buffer and tyramide-Alexa 488 in a 1:100 ratio. Thereafter, filters were washed for 10 min in PBS-T in the dark at room temperature, then rinsed with Milli-Q water, and finally dipped in 96% ethanol followed by air drying. Filters were stored frozen until they were processed for micro-autoradiography.

**Micro-autoradiography**—The microautoradiographic procedure we used is described in detail elsewhere (Teira et al. 2004). Briefly, hybridized filter sections were transferred onto slides coated with a molten Kodak NTB-2 photographic emulsion. Subsequently, slides were placed on a cold plate for a few minutes until the emulsion hardened. Slides exposure was carried out at 4°C for 24 h in light-tight boxes containing a drying agent. Optimum exposure time was determined empirically in a preliminary experiment. Development and fixation of the slides were done according to the specifications of the manufacturer. After fixation, the slides were rinsed in Milli-Q water for at least 2 min, then the filters were peeled off, and finally the cells were stained with an anti-fading solution containing 4',6-diamidino-2-phenylindole (DAPI) (final concentration of 1  $\mu\text{g mL}^{-1}$ ).

**Microscopy**—The slides were examined using a Zeiss Axioplan microscope equipped with a 100-W Hg lamp and Zeiss filter sets number 1 for DAPI and number 9 for Alexa 488. Silver grains around bacterial cells were observed using the transmission mode of the instrument. In the control samples, <0.6% of the cells were associated with three or more silver grains. Cells were counted in at least 20 randomly selected microscopic fields and for every field four different counts were recorded: (1) DAPI-positive cells, (2) probe-specific positive cells, (3) DAPI + autoradiography positive cells, and (4) probe specific + autoradiography positive cells. At least 350 DAPI-stained cells were counted per sample.

**Statistical analysis**—A two-way analysis of variance (ANOVA) was used to compare changes in the proportions

Table 1. Summary of the chemical and optical characteristics from DOM in the control and in the treatments at the beginning of the experiment. The DOC-specific absorption coefficient at 320 nm is expressed in  $\text{L mg DOC}^{-1} \text{m}^{-1}$ .\*

Treatment	TDP ( $\mu\text{g L}^{-1}$ )	TDN ( $\mu\text{g L}^{-1}$ )	DOC ( $\text{mg L}^{-1}$ )	C:N (molar)	C:P (molar)	$a^*_{320}$	$a_{250}:a_{365}$
Control	1.2	344	0.56	1.91	1,203	0.84	1.94
Algal	55.0	1,270	2.12	1.95	99.8	0.36	2.33
Soil	5.4	471	2.02	5.01	990	1.48	4.02

\* TDP, Total dissolved phosphorus; TDN, Total dissolved nitrogen; DOC, dissolved organic carbon; C:N, carbon:nitrogen ratio; C:P, carbon:phosphorus ratio;  $a^*_{320}$ , DOC-specific absorption coefficient at 320 nm;  $a_{250}:a_{365}$ , DOM absorption ratio between 250 nm and 365 nm.

of the bacterial groups detected by the respective oligonucleotide probes (expressed as a percentage of DAPI stained cells) at different times in the control and in the ALGAL and SOIL treatments. The pertinent post-hoc comparisons were made by the Holm-Sidak method with an overall significance level of 0.05.

## Results

**DOM chemical and optical characteristics**—Table 1 summarizes some chemical and optical characteristics of the DOM in the control and both treatments at the beginning of the experiment. Total dissolved nitrogen (TDN) and total dissolved phosphorus (TDP) concentrations in the ALGAL treatment were 1,270 and  $55 \mu\text{g L}^{-1}$ , respectively. TDN concentration was approximately three-fold higher than in the control and SOIL, whereas TDP was 10 times higher than in the SOIL treatment and 50 times higher than in the control. The carbon:nitrogen (C:N) molar ratios in the control and ALGAL treatment were  $\sim 1.9$  whereas the C:N ratio from SOIL DOM was 5. In the control and SOIL treatment, the carbon:phosphorus (C:P) ratio was high (1,203 and 990, respectively) and about one order of magnitude higher than in the ALGAL treatment (99.8). The DOC-specific absorption coefficient at 320 nm ( $a^*_{320}$ ) in the control was  $0.84 \text{ L mg DOC}^{-1} \text{m}^{-1}$ , whereas in the ALGAL and SOIL treatments it was 0.36 and  $1.48 \text{ L mg DOC}^{-1} \text{m}^{-1}$ , respectively. The DOM  $a_{250}:a_{365}$  ratio was low in the control and ALGAL treatment, indicating an important contribution of larger molecules. The  $a_{250}:a_{365}$  ratio in the SOIL treatment was twice as high as in the control. This ratio increased in all treatments at the end of the experiment (data not shown).

**DOC concentration**—DOC concentration (Fig. 1A) in GKS lake samples ranged from 0.28 to  $0.46 \text{ mg C L}^{-1}$ . In the control, DOC concentration slightly decreased during the incubation from  $0.56 \pm 0.05$  to  $0.47 \pm 0.04 \text{ mg C L}^{-1}$ . The DOC initial concentration in the SOIL ( $2.02 \pm 0.02 \text{ mg C L}^{-1}$ ) and ALGAL ( $2.12 \pm 0.02 \text{ mg C L}^{-1}$ ) treatments was ca. four times higher than in the control. DOC concentrations declined in both treatments reaching, after 5 days, a minimum of  $1.85 \pm 0.04 \text{ mg C L}^{-1}$  and  $1.85 \pm 0.03 \text{ mg C L}^{-1}$  in the ALGAL and SOIL treatment, respectively.

**Bacterial abundance and bacterial production**—Bacterial numbers (Fig. 1B) in lake samples collected at 2-m depth were fairly constant during the experiment ( $4.35 \pm 0.44 \times$

$10^5 \text{ cells mL}^{-1}$ ). Bacterial initial abundance in the treatments was  $\sim 10$  times lower than in the lake ( $\sim 0.58 \pm 0.09 \times 10^5 \text{ cells mL}^{-1}$ ). Bacterial abundance increased in all treatments and reached its maximum at the end of the experiment, i.e.,  $3.47 \pm 0.78 \times 10^5 \text{ cells mL}^{-1}$  in the control,  $12.2 \pm 0.61 \times 10^5 \text{ cells mL}^{-1}$  in the SOIL treatment, and  $25.9 \pm 1.60 \times 10^5 \text{ cells mL}^{-1}$  in the ALGAL treatment.

Similarly to bacterial numbers, bacterial production (Fig. 1C) was stable in GKS lake samples averaging  $2.52 \pm 0.79 \mu\text{g C L}^{-1} \text{d}^{-1}$ . In the control, bacterial production ranged from  $2.75 \mu\text{g C L}^{-1} \text{d}^{-1}$  to  $6.92 \mu\text{g C L}^{-1} \text{d}^{-1}$  at the end of the experiment. Bacterial production increased rapidly in the ALGAL treatment, reaching a maximum of  $155.2 \mu\text{g C L}^{-1} \text{d}^{-1}$  2 days after the addition of the algal extract. Afterwards, bacterial production dropped, getting close to initial values on the fourth day. In the SOIL treatment, the stimulation of bacterial production became apparent only 5 days after the addition of the soil extract and reached the maximum value of  $17.0 \mu\text{g C L}^{-1} \text{d}^{-1}$  on day 6.

**Changes in bacterial community composition and activity**—Between 85% and 90% of the GKS bacteria were detected with the *Eubacteria* probe EUB338. The GKS bacterial assemblage (Fig. 2) was dominated by members from the  $\beta$ -*Proteobacteria* group, i.e., the BET42a probe detected 50–60% of the DAPI-positive cells. The R-BT subgroup from  $\beta$ -*Proteobacteria* (probe R-BT065) represented between 20% and 30% of DAPI-stained cells and included about 40–60% of the  $\beta$ -*Proteobacteria* in the lake. *Actinobacteria* were the second dominant group, comprising 20–30% of DAPI counts. *Cytophaga*-like bacteria represented, on average, 10% of the DAPI counts. The bacterial community composition in lake samples did not exhibit major changes during the sampling period; neither did other microbial parameters measured. The percentage of active bacteria (Fig. 1D), as estimated by microautoradiography, also remained fairly constant ( $>70\%$  of DAPI counts).

The filtration step used to prepare the bacterial inoculum did not drastically change the relative abundance of the different bacterial groups as compared to the lake (Table 2). However we cannot exclude the possibility that some phylotypes were excluded by this procedure. Nevertheless, the initial bacterial assemblage composition and group contribution in the treatments at day 0 (i.e., 2 h after inoculation) did not significantly differ from that of the control (Table 3), indicating that the dilution used to set-up

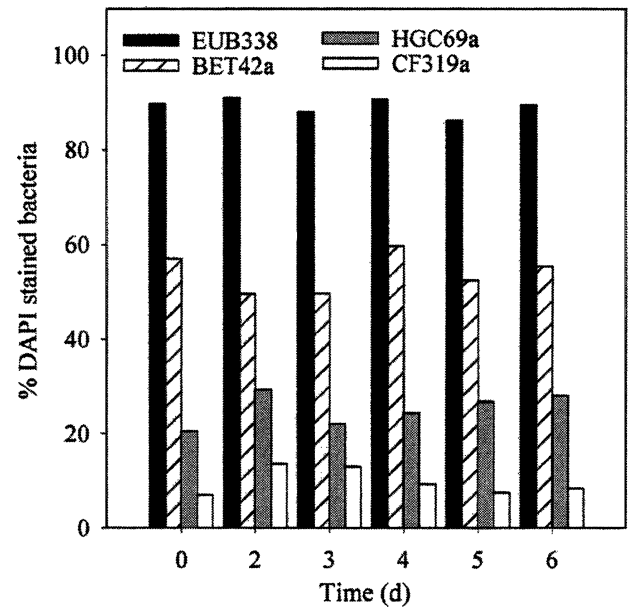
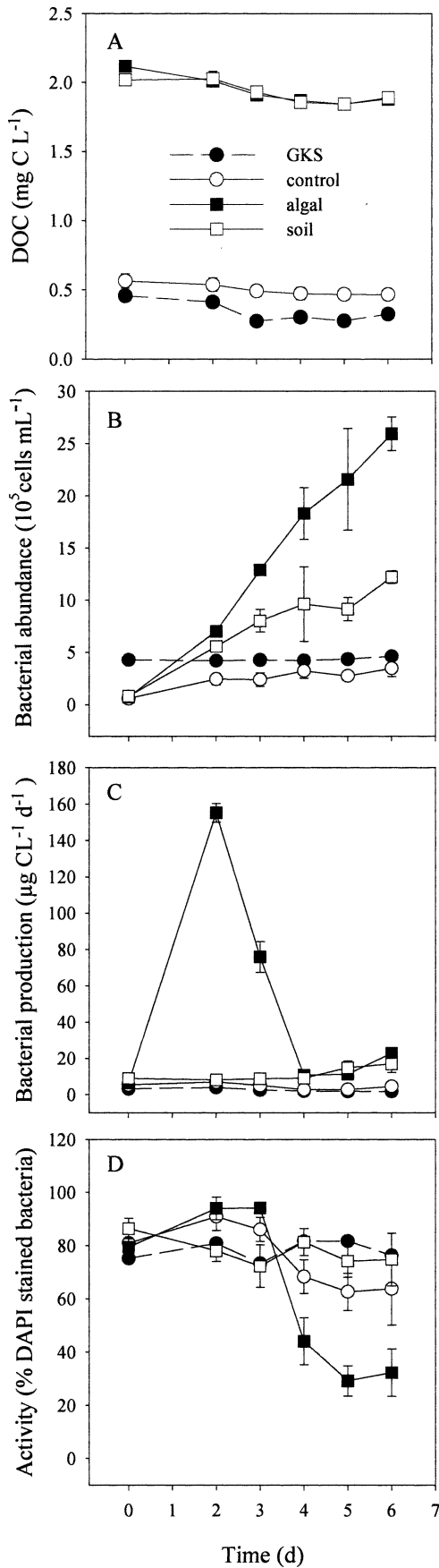


Fig. 2. Time course changes in the relative abundance of bacterial groups targeted by probes EUB338 (most bacteria), BET42a (*β-Proteobacteria*), HGC69a (*Actinobacteria*), and CF319a (*Cytophaga*-like bacteria) in GKS samples collected at 2-m depth.

the experiment affected equally all bacterial groups. In the control, the percentage of *β-Proteobacteria* appeared to slightly increase on day 2 (Fig. 3A); however, the post-hoc comparison indicated no significant differences in the development of the relative abundance of BET42a-detected cells with time (Table 3). The relative importance of the R-BT subgroup followed a very similar pattern to that of the total *β-Proteobacteria* (Fig. 3A). The relative abundance of this subgroup in the control remained fairly constant, the only significant difference (Table 3) was found when comparing the proportion of R-BT between day 2 and day 5, when a minimum of 20% was recorded. The percentage of *β-Proteobacteria* active in the control decreased continuously from day 0 to day 5, when only 50% were found active representing ~30% of DAPI-stained bacteria (Fig. 3A). The addition of the algal extract led to a significant increase (Table 3 and Fig. 3B) in the relative abundance of *β-Proteobacteria*, i.e., from 60% on day 0 to about 80% of DAPI counts from day 2 onward. Only in the last sampling day did their importance slightly decrease. Bacteria targeted by the R-BT065 probe represented ~60% of *β-Proteobacteria* and 35% of DAPI counts at day 0 (Fig. 3B). Two days after the addition of the ALGAL extract, this group increased significantly, representing

Fig. 1. Temporal changes of (A) DOC, (B) bacterial abundance, (C) bacterial production, and (D) activity in the different treatments, as well as at 2-m depth in GKS. Activity was assessed by micro-autoradiography using [<sup>3</sup>H]-L-leucine as substrate. Positive cells are expressed as the percentage of DAPI-stained bacteria. Values are the mean of three replicates, except for GKS where only one sample was analyzed. Error bars represent ± 1 SD.

Table 2. Relative contribution (percentage of DAPI-stained bacteria) of bacterial groups detected by probes BET42a (*β-Proteobacteria*), R-BT065 (R-BT group of *β-Proteobacteria*), HGC69a (*Actinobacteria*), and CF319a (*Cytophaga*-like bacteria) at day 0 in samples collected at 2-m depth in GKS and in the control and treatment bottles. Values are the mean of three replicates  $\pm$  SD, except for GKS where a single sample was collected.

Probe	GKS	Control	Algal	Soil
BET42a	57.1	57.7 $\pm$ 2.09	57.7 $\pm$ 1.28	53.7 $\pm$ 4.69
R-BT065	28.9	28.3 $\pm$ 4.90	34.4 $\pm$ 2.83	30.4 $\pm$ 4.70
HGC69a	20.4	19.2 $\pm$ 2.01	16.5 $\pm$ 4.18	20.0 $\pm$ 3.45
CF319a	7.02	4.84 $\pm$ 1.14	4.90 $\pm$ 1.24	7.05 $\pm$ 2.24

~70% of DAPI-stained bacteria on day 3. During the subsequent days, the importance of R-BT065-targeted bacteria decreased drastically, dropping below 20% of DAPI-stained cells. The percentage of active *β-Proteobacteria* followed a very similar pattern to that of the R-BT065 bacteria. The addition of the algal extract maintained high numbers of active *β-Proteobacteria* during the first 3 days, but then their activity decreased to a minimum (<20% of DAPI counts) on day 6. The addition of the SOIL extract did not induce drastic changes in the abundance of *β-Proteobacteria* (Fig. 3C). Their relative contribution to total bacterial counts ranged between 50% and 60% of DAPI counts. On average, the R-BT065 probe detected ~23% of DAPI-stained cells in the SOIL treatment, without any significant changes in the temporal evolution of this subgroup (Table 3). The percentage of active *β-Proteobacteria* was also relatively constant and represented, on average, 50% of DAPI counts (Fig. 3C).

The percentage of *Actinobacteria* increased significantly in the control (Fig. 3D and Table 3), reaching 28% of DAPI counts after 3 days. Afterward, their proportion decreased and dropped below 10% at the end of the experiment. The relative importance of *Actinobacteria* diminished significantly after the addition of the algal extract and remained low (~5% of total counts) until day 6 when it recovered to the initial value (Fig. 3E). In the SOIL treatment, the proportion of *Actinobacteria* remained fairly stable during the experiment and ranged between 15% and 23% (Fig. 3F). Regardless of their relative abundance in the different treatments, most of the *Actinobacteria* detected were active (70–95%), and their contribution to the bulk bacterial activity followed a similar pattern to their contribution to total abundance.

Although *Cytophaga*-like bacteria were numerically the third bacterial group, they only represented a minor fraction of the total DAPI counts. In the control, their relative abundance remained low (~5% of DAPI counts) until the fifth day when they reached 10% of the DAPI-stained bacteria (Fig. 3G). The dynamics of *Cytophaga*-like bacteria in the ALGAL treatment (Fig. 3H and Table 3) followed a similar pattern as in the control, i.e., their relative abundance was low until the end of the experiment when it exceeded 10% of DAPI counts. In the SOIL treatment, *Cytophaga*-like bacteria represented a constant fraction of the bacterial community throughout the experiment, averaging ~10% of DAPI counts (Fig. 3I). The contribution of this group to bacterial activity as estimated by micro-autoradiography was generally very low (~1–2% of DAPI-stained cells).

## Discussion

Our study aimed to assess the influence of different DOM sources on the community structure and activity of the bacterial assemblage of an alpine lake. The bacterial community of GKS has been the study subject of previous work (Alfreider et al. 1996; Pernthaler et al. 1998; Glöckner et al. 2000). Nevertheless, this is the first time that its short-term variability was followed and that changes in its composition and activity were simultaneously studied. The composition of the bacterial assemblage of GKS showed a remarkable stability during the study period. Bacterial numbers and production, as well as the percentage of active bacterial cells also reflected this stability. This is in accordance with previous findings by Šimek et al. (2001), who also found a remarkable constancy in the bacterial

Table 3. Results of the post-hoc all pairwise multiple comparison tests for the relative abundance of *β-Proteobacteria* (BET42a), R-BT subgroup of *β-Proteobacteria* (R-BT065), *Actinobacteria* (HGC69a), and *Cytophaga*-like bacteria (CF319a). Results are expressed by a code combining numbers and letters. Different numbers are used to indicate a significant difference in factor time within a given treatment. Different letters indicate a significant difference among treatments at a given time. Comparisons were done by the Holm-Sidak method with an overall significance level of 0.05.

Probe	Control Time (d)						Algal Time (d)						Soil Time (d)					
	0	2	3	4	5	6	0	2	3	4	5	6	0	2	3	4	5	6
BET42a	0a	0a	0a	0a	0a	0a	0a	1a	1b	1b	1b	0b	0a	01b	0a	0a	02ab	0ab
R-BT065	0a	01a	0a	0a	02a	0a	0a	1b	1b	2a	2a	2a	0a	0a	0a	0a	0a	0a
HGC69a	0a	01a	1a	01a	2a	2a	0a	12b	2b	12b	12a	01a	0a	0c	0c	0a	1a	0b
CF319a	0a	0a	0a	0a	0a	0a	01a	0a	0a	01a	01a	1a	0a	0b	0a	0a	0a	0a

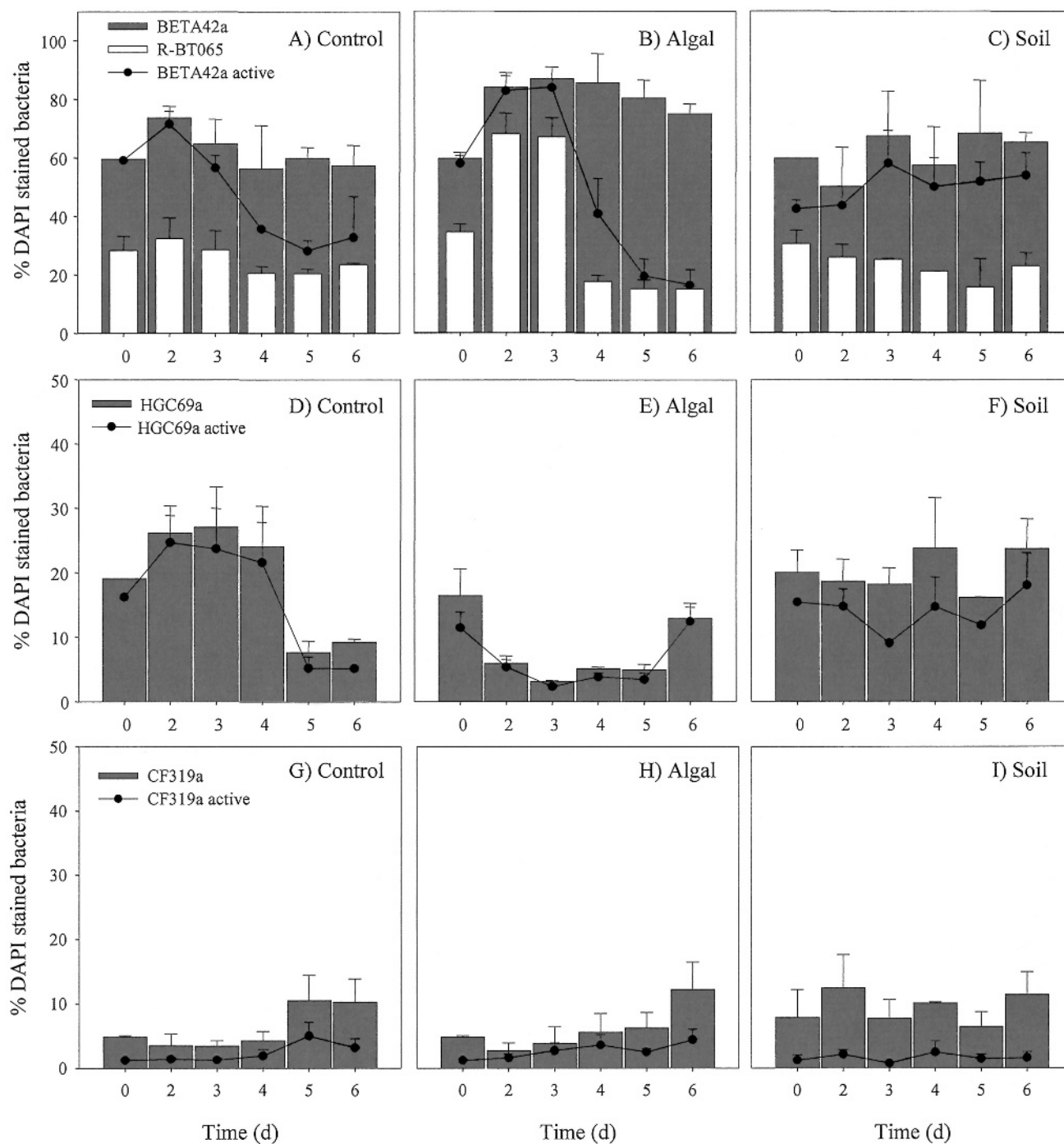


Fig. 3. Time course changes in the relative abundance of (A–C)  $\beta$ -Proteobacteria (probe BET42a) and R-BT subgroup of  $\beta$ -Proteobacteria (probe R-BT065); (D–F) Actinobacteria (probe HGC69a), and (G–I) Cytophaga-like bacteria (probe C319a) in the control and algal- and soil-derived DOM treatments. Temporal changes in the proportion of active bacteria (micro-autoradiography using [ $^3$ H]-L-leucine as substrate) for each group, control, and treatments are also included (solid line). Values are expressed as the percentage of DAPI-stained bacteria and represent the mean of three replicates  $\pm$  1 SD.

community composition in a mesoeutrophic reservoir during a few days sampling period. Thus, this stable background can be used as a reference against which the outcome of our experimental manipulations can be compared.

We used large volume dilution cultures to examine the effects of DOM source in the structure and activity of bacteria. Dilution cultures were originally used as a way to study in situ growth rates of aquatic bacteria (Kirchman et al. 1982; Ammerman et al. 1984). However, it has been

shown that the dilution factor used can affect the dynamics of the bacterial community, even if a similar bacterial biomass is achieved (Fuchs et al. 2000). We did not estimate bacterial growth rates from our dilution cultures; however, only small changes in bacterial community composition across time were observed in the control (Table 3). Consequently, we are confident that the observed changes in the bacterial assemblage reflected mainly the effect of DOM manipulation.

One important result of our study is that the DOM source was a main factor in structuring the bacterial community of this alpine lake (Fig. 3). The influence of the DOM origin was reflected not only by changes in bacterial production rates and activity, but also by changes in the proportion of the different bacterial groups considered. The addition of the algal lysate, presumably rich in labile organic compounds but definitely rich in nitrogen and very rich in phosphorus, stimulated extremely the bacterial production as compared to the control and to the SOIL treatment. Results from a previous study suggest that prokaryotic communities living in nutrient-poor environments are more sensitive to bottom-up regulation (Sanders et al. 1992). Our results confirm this hypothesis because removal of predators in the control did not induce substantial changes in bacterial community composition or in production rates. Furthermore, when considering the C:N:P ratio of the DOM (Table 1) it seems that phosphorus was limiting bacterial growth in GKS. For example, the C:P ratio in the control and SOIL treatment (1,203 and 990, respectively) was far above C:P ratios usually found in bacteria of ca. 50 (Kirchman 2000). Consequently, the algal-derived DOM having a lower C:P ratio (99.8) could have alleviated bacterial growth limitation. Our measurements of phosphorus cannot resolve the contribution of organic and inorganic forms; however, water-soluble soil extracts are usually dominated by phosphorus in organic form (Turner et al. 2002). Thus, it would be interesting to test the effect of inorganic and organic nutrient forms on composition and group-specific activity of the bacterial community. Furthermore, our results suggest that DOC concentration alone is a poor predictor of the outcome of such manipulations. For example, a similar DOC enrichment factor in the SOIL treatment did not translate into similar bacterial production rates as in the ALGAL treatment.

The increase in DOC concentration from soil origin did not favor any particular bacterial group at our level of taxonomical resolution, as they maintained very similar proportions to the ones naturally occurring in GKS during summer. Considering that soil-derived DOM is supposed to be more recalcitrant than algal-derived DOM, one hypothesis is that bacteria need to acclimate before using those compounds. This acclimation could be physiological (i.e., the appropriate enzymes would be induced within the phylotypes already present) or caused by changes in the bacterial community composition (i.e., the phylotypes harboring the adequate enzymatic activities or transport systems would be in advantage and overgrow the phylotypes lacking them). Some previous studies indicate that no single bacterial species can express all ectoenzymes with

high activity (Martinez et al. 1996), thus variations in enzymatic activity are probably linked to changes within the bacterial community (Arrieta and Herndl 2002; Kirchman et al. 2004). The lack of changes at the group level in the bacterial assemblage of GKS after the addition of soil-derived DOM does not exclude changes at the species level that could not be detected by the methodology used. Nevertheless, it suggests that all taxonomic groups present included species with appropriate ectoenzymes.

The increase in production rates following the addition of algal-derived DOM was coincident with an increase in the proportion of  $\beta$ -*Proteobacteria* in that treatment. Although  $\beta$ -*Proteobacteria* was already the prevalent bacterial group (50–60% of DAPI counts) in the lake samples, in the control and in the SOIL treatment, the addition of algal-derived DOM led to an almost complete dominance of the bacterial assemblage by them (75–87%). This suggests that some bacterial populations belonging to this group were able to rapidly grow and to outcompete other bacterial groups present. The importance of  $\beta$ -*Proteobacteria* within freshwater bacterial assemblages is a common observation (Glöckner et al. 1999). Previous studies in GKS (Glöckner et al. 2000) and in the mesoeutrophic Římov Reservoir, Czech Republic (Hornák et al. 2005), have shown high percentages of this group within the bacterial assemblage. In our study, the increase in the proportion of  $\beta$ -*Proteobacteria* in the ALGAL treatment can be largely attributed to the increase in the relative abundance of the subgroup R-BT of  $\beta$ -*Proteobacteria*. The percentage of R-BT065-positive cells rose from ~30% to 67% of DAPI counts in only 2 days (Fig. 3B). Until now only few studies (Šimek et al. 2003; Hornák et al. 2005; Šimek et al. 2005), mainly concerned with Římov Reservoir, have taken into account the ecology of this subgroup of  $\beta$ -*Proteobacteria*. From those studies, it appears that the R-BT cluster is a very dynamic component of the bacterioplankton in that reservoir, reacting very rapidly to experimental manipulations of both grazing pressure and resource availability. Šimek et al. (2005) defined the behavior of this cluster as a true example of an opportunistic strategy. This group seems to be composed by one or more fast growing species that respond rapidly to natural perturbations or experimental manipulations. Our results extend the conclusions of that study to a different system, an oligotrophic alpine lake, and to the manipulation of the DOM source. In fact, when the data from all treatments in our study were pooled together, we found that 82% and 59% of the variability in bacterial production and in the proportion of active bacteria, respectively, could be explained by changes in the R-BT subgroup of  $\beta$ -*Proteobacteria* (Table 4). These results underline the key role of this bacterial subgroup in this alpine lake.

Another striking feature in the ALGAL treatment was the decrease in the relative abundance of *Actinobacteria* (Fig. 3E). The study of Burkert et al. (2003) in Lake Fuchskuhle, Germany, found a similar decline in the proportion of *Actinobacteria* following changes in substrate availability, which promoted the growth of a  $\beta$ -*Proteobacteria* subgroup (Beta II). Although members of the class *Actinobacteria* appear to be a consistent component of the

Table 4. Relationships between the percentages of  $\beta$ -*Proteobacteria* (cells detected by either the probe BET42a or R-BT065) and of *Actinobacteria* (HGC69a) and some basic bacterial parameters in all treatments. The proportions of bacteria detected either by probes or by micro-autoradiography (i.e., active bacteria) are expressed as percentage of DAPI-stained bacteria. For all comparisons ( $n = 54$ ).\*

Parameter	% BET42a		% R-BT065		% HGC69a	
	Correlation coefficient ( $r$ )	Associated probability ( $p$ )	Correlation coefficient ( $r$ )	Associated probability ( $p$ )	Correlation coefficient ( $r$ )	Associated probability ( $p$ )
Abundance	0.207	n.s.	-0.202	n. s.	-0.251	n.s.
Bacterial production	0.446	< 0.001	0.816	< 0.0001	-0.416	< 0.001
% Bacteria active	0.071	n.s.	0.588	< 0.0001	0.180	n.s.

\* n.s., nonsignificant.

freshwater bacterioplankton (Glöckner et al. 2000; Hahn et al. 2003; Warnecke et al. 2004), it seems that they are also easily outcompeted by other bacterial groups and particularly by fast-growing members of  $\beta$ -*Proteobacteria*. Despite their ubiquitous presence in freshwater systems, relatively little is known about their role. Furthermore, it has been hypothesized that actinobacterial cells found in freshwaters could be resting stages of soil microorganisms. However, a recent study has shown that *Actinobacteria* from several mountain lakes, including GKS, are able to synthesize DNA de novo (Warnecke et al. 2005). Furthermore, our results from micro-autoradiography show for the first time that a high proportion of actinobacterial cells were able to use leucine as substrate. Considering data from all treatments, the actinobacterial group was negatively correlated with bacterial production and showed no particular relation with activity (Table 4). Nevertheless, a high and constant proportion of *Actinobacteria* were active (70–95%) during the experiment, regardless of the treatment. Thus, the absence of a significant relationship with activity might reflect their relative low abundance. We also found a highly significant negative correlation between *Actinobacteria* and  $\beta$ -*Proteobacteria* ( $r = -0.56$ ;  $p < 0.001$ ). As already stated, this result suggests that *Actinobacteria* and  $\beta$ -*Proteobacteria* exploit similar resources but the latter displace the actinobacterial group by exhibiting higher growth rates. This is coincident with the observation that in GKS, there is a temporal segregation in the maximum abundance of these two groups. Thus, in a previous seasonal study, *Actinobacteria* were found to reach a maximum in April, whereas  $\beta$ -*Proteobacteria* did so in September (Glöckner et al. 2000). It has also been shown that *Actinobacteria* are favored under high bacterivory pressure (Šimek et al. 2005) because their usually small size offers a refuge against predation (Hahn et al. 2003).

*Cytophaga*-like bacteria are common members of freshwater as well as of marine bacterial assemblages (Kirchman 2002). Yet, in the present study no correlation was found among members of *Cytophaga*-like bacteria and any of the bacterial parameters we examined, making it difficult to determine their role in the experiments. *Cytophaga*-like bacteria seem to play a role in the degradation of high-molecular-weight DOM in aquatic ecosystems (Kirchman 2002). However, the addition of the algal lysate presumably rich in labile proteins and polysaccharides did not favor *Cytophaga*-like bacteria. Soil-derived DOM maintained, on average, higher proportions of this group (~10%) than the

algal-derived one (~5%), suggesting that this bacterial group might be more competitive in GKS when less labile organic compounds are dominant. The increase of the relative abundance of this group at the end of the experiment in the control and ALGAL treatment supports the hypothesis that *Cytophaga*-like bacteria might be more successful in using less labile DOM. Yokokawa et al. (2004) found that growth rates of *Cytophaga*-like bacteria at a river station of the Delaware estuary are usually lower than those of  $\beta$ -*Proteobacteria*. Šimek et al. (2001) also found that *Cytophaga*-like bacteria grow slower than members of  $\beta$ -*Proteobacteria*. However, Jürgens et al. (1999) found that *Cytophaga*-like bacteria grow twice as fast as members of the  $\beta$ -*Proteobacteria*. Our data on activity measurements based on micro-autoradiography using [ $^3\text{H}$ ]-L-leucine as substrate suggest that *Cytophaga*-like bacteria were the least active group. However, it has been shown that members of this group are not specialized in incorporating amino acids (Cottrell and Kirchman 2000). Thus, micro-autoradiography using amino acids is probably not the most suitable method to assess the specific activity of this group.

A simple balance calculation based on bacterial carbon production rates during the experiment showed that in the ALGAL treatment 0.26 mg L<sup>-1</sup> of carbon were produced, in contrast to 0.05 mg L<sup>-1</sup> in the SOIL treatment. Based on the measured decrease in DOC concentration in our microcosms, we estimated 0.27 mg L<sup>-1</sup> of organic carbon was used in the ALGAL treatment compared to 0.17 mg L<sup>-1</sup> in the SOIL treatment. Although these estimates could be subject to a certain error and we cannot exclude the possibility that some carbon could have been released into the dissolved fraction because of viral lysis (but see Hofer and Sommaruga 2001), it seems that bacteria did use more efficiently the algal-derived DOM than the soil-derived one. This implies that a higher fraction of carbon was lost through bacterial respiration when terrestrially-derived DOM dominated. The different nutrient content and particularly the higher phosphorus concentration of the algal-derived DOM might help to explain the observed trends in DOM utilization efficiency. Smith and Prairie (2004) showed that changes in bacterial growth efficiency along a trophic gradient are positively related to total phosphorus concentrations whereas no relationship with bulk DOC could be found.

Finally, considering our results in connection to the climatic warming observed in the Alps, we suggest that an

increase in the export of soil-derived DOM having its current chemical composition will not substantially modify the composition of alpine lake bacterial assemblages at the group level. However, it might affect bacterial growth efficiency and lead to a more inefficient utilization of DOM in those ecosystems. Alternatively, in a warmer climate, higher decomposition rates of terrestrial vegetation may lead to an increase in the nutrient load to the lakes. This process will have similar effects, though at different time scales, to those caused by shorter periods of lake ice-cover, namely, an increase in phytoplankton productivity and autochthonous DOM supply (Pechlaner 1971, Hickman and Reasoner 1994, Sommaruga et al. 1999) and a change in bacterial activity and community structure.

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