VARIATION IN DISSOLVED ORGANIC MATTER CONTROLS BACTERIAL PRODUCTION AND COMMUNITY COMPOSITION

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Abstract. An ongoing debate in ecology revolves around how species composition and ecosystem function are related. To address the mechanistic controls of this relationship, we manipulated the composition of dissolved organic matter (DOM) fed to aquatic bacteria to determine effects on both bacterial activity and community composition. Sites along terrestrial to aquatic flow paths were chosen to simulate movement of DOM through catchments, and DOM was fed to downslope and control bacterial communities. Bacterial production was measured, and DOM chemistry and bacterial community composition (using denaturing gradient gel electrophoresis of 16S rRNA genes) were characterized following incubations. Bacterial production, dissolved organic carbon (DOC)-specific bacterial production, and DOC consumption were greatest in mesocosms fed soil water DOM; soil water DOM enhanced lake and stream bacterial production by 320–670% relative to lake and stream controls. Stream DOM added to lake bacteria depressed bacterial production relative to lake controls in the early season (−78%) but not the mid-season experiment. Addition of upslope DOM to stream and lake bacterial communities resulted in significant changes in bacterial community composition relative to controls. In four of five DOM treatments, the bacterial community composition converged to the DOM source community regardless of the initial inoculum. These results demonstrate that shifts in the supply of natural DOM were followed by changes in both bacterial production and community composition, suggesting that changes in function are likely predicated on at least an initial change in the community composition. The results indicate that variation in DOM composition of soil and surface waters influences bacterial community dynamics and controls rates of carbon processing in set patterns across the landscape.

Key words: bacterial community composition; DGGE; DOC; DOM bioavailability; ecosystem; landscape; terrestrial DOM.

INTRODUCTION

A fundamental goal in ecosystem ecology is to understand the interaction between biogeochemical processes and biological community composition across the landscape. It is clear that strong links exist between community composition and biogeochemical cycles, and the roles of particular species or assemblages of species in controlling ecosystem functions such as nitrogen (N) fixation, N mineralization, decomposition, and primary production have been demonstrated in many studies, especially with respect to the role of biodiversity (Naeem et al. 1994, Tilman et al. 1997, Loreau and Hector 2001). The nature of this interaction, however, is potentially bidirectional, and strong effects of biogeochemistry (e.g., soil parent material or nutrient availability) on species composition and ecosystem function have also been documented (Schindler et al. 1974, Shaver and

Chapin 1991, Carpenter et al. 1992, Chadwick et al. 1999).

A recent review of the biodiversity–ecosystem function debate highlights the need to integrate these perspectives by examining the full structure of ecological communities in relation to environmental and biotic controls on ecosystem properties (Hooper et al. 2005). For example, increased plant diversity appears to influence the activity and community composition of soil microbes (Stephan et al. 2000, Zak et al. 2003), although interactions and feedbacks among trophic levels and between above and belowground systems complicate simple generalizations between diversity and ecosystem function (Wardle et al. 2004, Patra et al. 2005). Although the exact mechanisms responsible for these influences on microbes are unclear, it may be due to a greater range of resource diversity (i.e., biochemical compounds from plants) (Zak et al. 2003), which is consistent with studies showing that variations in dissolved organic matter (DOM) composition can alter aquatic bacterial communities (Pinhassi et al. 1999, Findlay et al. 2003). While it is clear that environmental drivers (e.g., DOM, climate, or nutrients) can change both species composition and ecosystem function (e.g.,

bacterial activity), the specific mechanistic pathways of change are poorly documented and questions remain; for example, are changes in community composition a prerequisite for functional responses?

Microbes, in particular, are important mediators of biogeochemical fluxes, and the presence and activity of specific groups can influence biogeochemical processes (e.g., nitrogen fixation, lignin decomposition). However, we know little about how the broad diversity of microorganisms impacts biogeochemical cycling, or how this diversity may affect more general biogeochemical processes such as C metabolism. DOM chemistry has been shown to control bacterial activity (e.g., Amon and Benner 1996, Sun et al. 1997, Benner 2003), and the metabolism of specific compounds has been linked to specific bacterial groups (Cottrell and Kirchman 2000). This linkage suggests that the diversity of C substrates in natural environments has the potential to select for particular groups of microorganisms, because different microbes have the ability to use different C substrates (e.g., Zak et al. 2003). Whether microbial species composition is a strong driver of biogeochemical processes, or whether biogeochemistry selects for particular microbial assemblages, may differ spatially, temporally, and with respect to particular biogeochemical cycles.

From a catchment perspective, the interaction between DOM composition, microbial community composition, and microbial activity has important implications on DOM movement and processing along hydrological flow paths. In heterogeneous catchments, distinct patterns in both DOM composition and microbial community composition can exist among vegetation types and along flow paths from uplands to riparian zones to nearshore habitats (e.g., Nubel et al. 1999, Myers et al. 2001, Judd and Kling 2002). Whether shifts in the supply and composition of upslope DOM to microbes influences the processing of DOM as it moves through catchments will depend on the time scale of microbial response to new DOM sources. The response may be short-term and physiological in nature, or it also may involve changes in community composition. To investigate these responses, we set up experiments to test the hypothesis that upslope DOM supply controls microbial activity through changes in microbial community composition. We manipulated the composition of DOM fed to aquatic bacteria in mesocosm experiments by using soil and stream water along terrestrial to aquatic flow paths to determine the effects on both bacterial activity and community composition.

METHODS

Study site

Samples were collected along a terrestrial-aquatic transect in a small arctic tundra sub-catchment surrounding Toolik Lake (68°38′00″ N, 149°36′15″ W; see Plate 1; see also O'Brien et al. 1997), the site of the Arctic LTER located in the Northern foothills of the Brooks Range, Alaska, USA. Arctic tundra is ideally suited for such a study for several reasons: (1) there are distinct vegetative communities across the landscape with consistent differences in microbial community composition (Judd 2004) and DOM bioavailability (Judd and Kling 2002); (2) water flow is confined to a thick organic horizon due to the underlying permafrost, and the substantial movement of DOM from land to water is similar to this important flux in other systems; and (3) terrestrial DOM is the major source of C for aquatic bacteria and is important to overall rates of ecosystem C cycling.

An inlet stream to the south of the lake drains several lakes in the catchment and provides ~70% of water inputs to the lake (Kling et al. 2000). A small primary stream draining a 1.5-ha subcatchment of tussock tundra supplies an additional 9%. The remainder is supplied by similar small streams and ground water. Tussock tundra covers the hillslopes in the catchment and is dominated by the tussock-forming *Eriophorum vaginatum*. Soils are often moist, but water content fluctuates with snow melt and rainfall. Wet sedge tundra occurs at lower elevations where soils are nearly continuously saturated. The vegetation is dominated mainly by *E. angustifolium*, *Carex rotundata*, and *C. chordorrhiza* (see Shaver et al. 1998). Sites were chosen based on differences in elevation and vegetation to represent different sources of DOM.

Sample collection

Water samples were collected from Toolik Lake at 0.1 m depth in the deepest basin, and stream samples were collected from the shore about 5 m upstream from the lake inlet. Soil water samples were collected by inserting a steel needle into the soil and withdrawing water through an attached syringe. Within a site, water was drawn from 10 randomly chosen locations and pooled. Surface and soil waters were divided into two fractions: an inoculum and particle-free DOM. The inoculum was prepared by filtering 2 L of each sample through 1.0-μm nitrocellulose filters (Millipore, Billerica, Massachusetts, USA) to remove flagellate grazers. The remaining water was filtered using a small peristaltic pump through 0.22-μm filter cartridges, which removed particles >0.22 μm and virtually all microbes (but not particles <0.22 μm, such as viruses). This fraction served as the DOM source. The absence of flagellates in the inoculum and particles and microbes in the DOM fraction was confirmed by cell counts of DAPI-stained samples using an epifluorescence microscope.

Differences in inorganic nutrient levels among DOM sources are not likely to influence bacterial production (BP) because Toolik Lake bacteria are strongly limited by DOM and not inorganic nutrients (Kling 1995). Phosphate levels are not significantly different among lake, lake inlet, tussock, and wet sedge soil waters, but ammonium concentrations are higher in wet sedge soil waters (LTER data not shown). Approximately every

PLATE 1. A stream near Toolik Lake (Alaska, USA) during the spring melt. Large amounts of DOM (dissolved organic matter) are transferred from land to water in arctic tundra ecosystems. Photo credit: G. W. Kling.

fourth day, subsamples were taken from mesocosms and examined for the presence of flagellate grazers. No flagellates were observed in any of the mesocosms.

Experimental setup

To determine the effect of DOM on specific bacterial communities from different sources along the terrestrial to aquatic flow paths, mesocosm factorial experiments were conducted twice in 2002 (ice-free season). DOM was added to a bacterial inoculum in a $9 + 1$ (respectively) mixture. DOM chemistry and bacterial community composition were characterized at the beginning and end of the experiments, and BP was measured throughout. Any differences in bacterial concentration among source inocula were ignored because the long incubation time and 10-fold dilution would minimize the final impacts of these initial differences. Our results confirm that initial bacterial concentration was unimportant; there was no difference in BP among wet sedge soil water and lake water fed the same DOM.

Treatments were selected so that upslope DOM was added to downslope microbial communities (Fig. 1). Experiment 1 (samples collected 23 June 2002, 13 days after ice-off) examined two hydrological flow paths into Toolik Lake: the small primary stream and water from lake-fringing wet sedge habitat soil water, and consisted of two treatments each with three replicate mesocosms: (i) addition of lake bacteria to wet sedge soil water DOM (indicated as L/W to represent the lake bacteria inoculum and wet sedge DOM); (ii) lake bacteria added to small stream DOM (L/S); and three controls of (iii) wet sedge bacteria added to wet sedge DOM (W/W); (iv) stream bacteria added to stream DOM (S/S); and (v) lake bacteria added to lake water DOM (L/L). Experiment 2 (samples collected 25 July 2002) examined the flow of DOM from tussock soils to the small stream to Toolik Lake, and included three treatments: the addition of (i) stream and (ii) lake bacteria to tussock soil water DOM (S/T) and (L/T); and (iii) lake bacteria to stream DOM (L/S), and three controls: (iv) T/T, (v) S/S, and (vi) L/L. Because BP measured in the first experiment was very similar among the three replicates, only two replicates were used in the second experiment (L/T had only one replicate and was not included in statistical analyses). Mesocosms were kept in the dark at 4°C. Experiments 1 and 2 were terminated after 23 and 18 days, respectively. Because the control mesocosms account for the influence of experimental artifacts, our experiments are well suited to test general hypotheses about the role of DOM chemistry in controlling BP and bacterial community composition.

DOM characterization

Dissolved organic carbon (DOC) concentration, phenolic concentration (Waterman and Mole 1994), and reducing sugar concentration (Nelson 1944) were
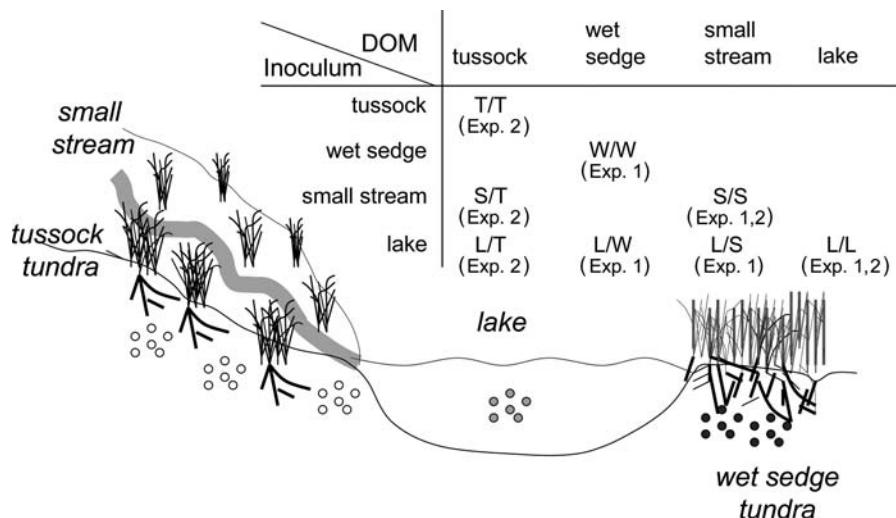
F IG. 1. Sampling locations, dissolved organic matter (DOM) manipulations, and abbreviations in experimental mesocosms. DOM was added to an inoculum so that treatment combinations simulated natural DOM flow paths. Letters indicate site abbreviations (see Table 1) in the order "inoculum/DOM." Controls (diagonal) received local DOM, and treatments received upslope DOM. The experiment was performed twice: Exp. 1, 23 June–18 July; Exp. 2, 25 July–12 August. In the drawing, small white, gray, and black circles represent distinct microbial communities at each site.

measured on initial and final samples. Spectral absorbance at wavelengths between 220 and 400 nm (integrated over 10-nm bins) was measured in a 5-cm quartz cuvette in a spectrophotometer. Absorbance in this range indicates the presence of chromophores and has been correlated with terrestrial humic material (McKnight and Aiken 1998). All water samples for chemical analysis were filtered through GF/F filters. Samples for DOC were acidified to pH 3 and were stored in the dark at 4°C until analyzed on a Shimadzu TOC 5000 (Shimadzu Scientific Instruments, Columbia, Maryland, USA) using a platinum catalyzed high-temperature combustion to CO_2 and infrared detection. Potential phenol oxidase and peroxidase activities were measured using L-3,4-dihydroxyphenylalanine (L-DOPA) as a substrate (Sinsabaugh et al. 1992). Two analytical replicates were run for each assay. Tubes were incubated at 22°C until a color change occurred (generally 2 h), and the change in absorbance relative to a blank control (no L-DOPA added) was measured at 460 nm on a spectrophotometer.

Bacterial production

Bacterial production was measured on initial inoculum samples and on mesocosm subsamples every two to three days using incorporation of ^{14}C-labeled leucine (30 nmol/L final concentration; Simon and Azam 1989). Three 10-mL subsamples were removed from each mesocosm (three analytical replicates), the radio tracer was added, and subsamples were incubated at 4°C in the dark. Incubations were terminated after three hours with 5% tri-chloroacetic acid (TCA). TCA precipitated molecules were collected on 0.2 μm pore size Millipore nitrocellulose filters and rinsed two additional times with

ice cold 5% TCA. Filters were placed in 7-mL scintillation vials and made transparent with 1-mL of methyl-Cellusolve. Scintisafe scintillation cocktail (6 mL; Fisher Scientific; Fairlawn, New Jersey, USA) was added, and vials were stored in the dark for 24 h, then assayed on a scintillation counter. Bacterial carbon production was calculated using theoretical conversion factors (Simon and Azam 1989) assuming an isotope dilution factor of 2.0 (Kirchman 1993).

Bacterial community composition

Bacterial community composition was assessed using denaturing gradient gel electrophoresis (DGGE) following the methods of Crump et al. (2003). We sampled the bacterial community at the beginning (each inoculum fixed within two hours of collection) and end of the experiment by filtering 500 mL of water from each mesocosm through 0.2 μm pore size Sterivex filters (Millipore, Burlington, Massachusetts, USA). DNA was extracted from samples and bacterial-specific 16S rRNA genes were amplified using polymerase chain reaction (PCR; Saiki et al. 1988) under carefully controlled conditions to minimize PCR bias (see Crump et al. 2003 for specific conditions). PCR amplification used primer 357f(g+c)(5′-CGCCCGCCGCGCCCCGCGCCCGGCCCGCCGC-CCCCGCCCCCCTACGGGAGGCAGCAG-3′), which contains a GC clamp and is specific for most bacteria, and universal primer 519r (5′-ACCGCGGCTG CTGGCAC-3′). We minimized the problem of under- or over-amplification of particular gene sequences (Suzuki and Giovanoni 1996) by carefully controlling the reaction parameters (Polz and Cavanaugh 1998).

DGGE procedures followed Muyzer et al. (1993). Acrylamide (8%) gels were prepared with 30–60% denaturing gradient. Samples from each experiment were run on separate gels, and a third gel was used to compare samples between experiments. Each gel had five lanes of standards from the Plum Island Sound Estuary (Crump et al. 2004). Magnified sections of the gel were photographed with a ChemImagerTM 4000 imaging system (Alpha Innotech, San Leandro, California, USA), and a complete image was reconstructed using Photoshop (Adobe, San Jose, California, USA). The height of the bands in the gel was determined based on the vertical position of bands from the reference ladder. The total number of vertical positions of bands was identified and standardized to the reference ladder, and bands from each sample were scored as present or absent at each position using the GelCompar software package (Applied Maths, Kortrijk, Belgium).

Data analysis

A principal-components analysis (SAS 8.2, 2003) was used to determine overall differences in DOM chemical composition among sources using the variables DOC concentration, phenolic concentration, reducing sugar concentration, integrated spectral absorbance, and potential phenol oxidase and peroxidase activities. To compare DOM bioavailability among sources differing in DOC concentration, we calculated DOC-specific BP using peak BP values during the incubations (note: peak BP for the stream control in experiment 2 was taken on day 7, rather than day 14, which appears to be anomalously high). Integrated BP was calculated over the first 15 days of each experiment. Analysis of variance (ANOVA; SPSS 10.0; SPSS, Chicago, Illinois, USA) was used to test differences in mean BP and DOC-specific BP (bioavailability) among treatments. The percentage of change in band number for a given treatment was calculated as the average difference in band number between the inoculum and the final sample, divided by inoculum band number.

The effect of DOM on bacterial community composition was assessed both quantitatively and qualitatively. To test the hypothesis that DOM chemistry controls bacterial community composition, we used multi-response permutation procedure (MRPP; Mielke 1984; experiment 1 only, due to lower replication in experiment 2). To visualize and evaluate the direction of changes in community composition, we used multi-dimensional scaling (MDS). The binary matrix of presence–absence data from gel banding patterns was used to calculate a distance matrix (Dice) for all final bacterial community pairs (i.e., initial inoculum samples were excluded; $N = 15$), and the distance matrix was analyzed using the MDS module in Statistica (StatSoft, Inc., Tulsa, Oklahoma, USA). The graphical representation of this analysis results in samples with similar banding patterns grouped close to one another.

To assess the magnitude of directional shifts in community composition, we compared banding pattern similarity between mesocosm pairs of treatments and controls (same inoculum and DOM source) with the same (1) inoculum and (2) DOM as the treatment. Similarity was measured as $1/d$, where d is the distance (or dissimilarity) between mesocosms pairs. The percentage of shared bands was calculated as the average number of bands shared between treatment and control mesocosms, divided by the average number of bands in the control mesocosms.

RESULTS

DOM chemistry and bacterial activity

In general, DOC concentrations, BP, and DOM bioavailability were significantly greater in soil water than in lake and stream water (ANOVA, $P < 0.05$; Table 1, Figs. 1 and 2). Lake water DOC concentrations were similar in both experiments, but stream DOC concentrations were lower in experiment 2 than in experiment 1. In experiment 2, there was greater variation in DOC consumption rates than in experiment 1, and DOC consumption accounted for a smaller fraction (~0–9%) of the original DOC pool (Table 1). Soil water DOM enhanced integrated BP in lake and stream water inocula by 320–670% relative to controls over the first 15 days (Table 1). In experiment 1, there was no significant difference between the wet sedge control (W/W) and the lake bacteria fed wet sedge DOM treatment (L/W), indicating that bioavailability was independent of the initial bacterial community. In experiment 2, addition of soil DOM also enhanced lake (L/T) and stream (S/T) bacterial activity, but BP in these treatments was lower than in the tussock control (T/T) mesocosms (ANOVA; $P < 0.05$). Stream water DOM addition to lake bacteria did not significantly change BP relative to lake controls in either experiment, but in experiment 1, the trend was still similar to soil water DOM treatments; addition of stream DOM depressed lake BP so that it was more similar to stream controls. In experiment 2, there was virtually no difference in BP between stream and lake controls (Fig. 2). These patterns were the same for integrated BP, peak BP, and DOC-specific BP.

Differences in BP among treatments were not due to differences in DOC concentration, indicating that the chemistry of each source differed in various compounds important as substrates for bacteria. For example, DOC-specific BP (bioavailability) was significantly higher on soil water DOM than on lake or stream DOM (ANOVA tests, Fig. 3). In the first experiment, DOM bioavailability was independent of the starting bacterial inoculum (Fig. 3A), but in the second experiment, bioavailability of tussock soil water DOM was significantly greater in control than in treatment mesocosms (Fig. 3B). Our chemical characterization verified these distinctions based on various compounds representative of natural DOM composition (Table 1). However, none

TABLE 1. Average DOM (dissolved organic matter) chemical characteristics, consumption rates, and bacterial activity in experimental mesocosms.

	Experiment 1					Experiment 2					
	Controls					Controls					
Fraction	WS/WS	S/S	L/L	L/WS	L/S	T/T	S/S	L/L	S/T	L/S	L/T
DOC (dissolved organic carbon)											
Initial (μmol C/L)	593	683	445	594	664	856	635	470	839	618	822
Used (%)	9.2	2.9	4.3	12.2	3.2	6.5	−0.4	0.9	8.8	−0.2	9.9
	(1.5)	(1.8)	(0.4)	(1.0)	(1.0)	(2.2)	(3.3)	(0.1)	(2.0)	(0.9)	
Phenolics											
Initial (μmol C/L)	237	233	108	211	206	591	209	138	579	203	574
Used (%)	−8.9	−28.3	−5.6	−11.8	−17.5	48.9	−4.8	−8.0	48.4	−5.4	48.1
	(0.3)	(1.1)	(0.3)	(1.5)	(0.9)	(0.2)	(0.9)	(1.1)	(0.2)	(0.3)	(0.0)
Reducing sugars											
Initial (μmol C/L)	13	13.9	10.1	13	13.9						
Used (%)	36.9	41.7	9.9	14.6	39.6						
	(24.6)	(18.0)	(8.9)	(0.8)	(9.4)						
Absorbance (AU × nm)	137	137	66	130	130	156	129	72	153	123	148
Enzyme activity (nmol·mL^{-1}·h^{-1})											
Phenol oxidase	23.69	3.17	0.12	23.16	2.73	5.29	6.79	3.89	5.35	6.5	5.06
Phenol oxidase (end)	12.2	8.6	1.8	9.2	2.2	2.9	2.0	1.7	2.7	2	2.5
	(2.9)	(2.9)	(1.6)	(1.6)	(2.2)	(0.0)	(0.1)	(0.0)	(0.1)	(0.1)	(0.2)
Peroxidase	14.7	7.2	8.5	14.0	7.2	60.4	1.0	1.0	55.3	0.8	55.2
Peroxidase (end)	11.8	2.4	1.9	6.7	4.2	20.3	1.9	5.0	9.4	2.4	7.0
	(0.1)	(0.1)	(0.1)	(2.0)	(0.2)	(2.9)	(0.3)	(2.6)	(0.7)	(0.8)	
Bacterial production, BP (μg C/L)	638	100	152	640	32	1358	154	86	826	88	662
	(136)	(6)	(26)	(12)	(2)	(56)	(1)	(1)	(30)	(8)	

Notes: Controls and treatments are indicated as "Inoculum/DOM" (WS, wet sedge; S, stream; L, lake; T, tussock). Initial concentrations (in μmol C/L) of DOC, phenolics, reducing sugars, and absorbance$_{400-220}$ are the result of mixing the inoculum (10%) and the DOM. Percentage DOC consumption ["used (%)"] was calculated based on differences between concentrations at the beginning and end of each experiment. Data shown include average potential phenol oxidase and peroxidase activities in initial DOM and inoculum mixture and at the end of the experiment, and integrated BP. Standard errors for end values of triplicate mesocosms are shown in parentheses.

of the broad classes of DOM measured (reducing sugars, phenolics, and absorbance characteristics) alone or in combination explained bacterial responses. A principal-components analysis (PCA) on all chemical parameters (experiment 1) shows that lake, stream, and wet sedge soil water have distinct chemical compositions (Appendix: Fig. A1). The first three PC axes explained 44%, 26%, and 18% of the total variance.

Specific DOM compounds and enzyme activities also varied among sites (Table 1). Phenolic concentrations and absorbance characteristics were highest in tussock soil water and decreased with site elevation. Phenolic concentrations increased over the course of the experiment in most mesocosms, but decreased in the second experiment in all treatments receiving soil water (tussock) DOM. Reducing sugars started out as ~2% of the DOM pool and were reduced by ~1% during the experiment; concentrations were highest in stream water and wet sedge soil water, and were slightly lower in lake water. In general, oxidative enzyme activities were greatest in soil waters; phenol oxidase activities were greatest in wet sedge soil water and peroxidase activities were greatest in tussock soil waters. Potential phenol oxidase activities declined over the course of the

experiment, while peroxidase activities increased in all mesocosms except those receiving tussock soil water.

Bacterial community composition

In four of five cases, bacterial community composition in treatment mesocosms shifted after the addition of downslope DOM to become more similar to the community from which the DOM originated (Fig. 4). Compared to the BP results (Figs. 1 and 2), shifts in community composition were less complete (i.e., at the end of the experiment, treatment and DOM control communities were more similar than initially, but still distinct). MDS analysis revealed shifts in community composition over the course of the experiment in both controls and treatments (Fig. 4); however, changes in controls relative to the initial inoculum were similar in all replicates. An MRPP analysis revealed that the final bacterial community composition was distinct for each group of replicate mesocosms ($P < 0.05$).

Eighty-one and 124 DGGE bands were identified in experiments one and two, respectively. In general, band richness decreased with catchment elevation (Table 2). In experiment 1, L/S treatments had significantly more bands in common with DOM controls (S/S, 48%) than
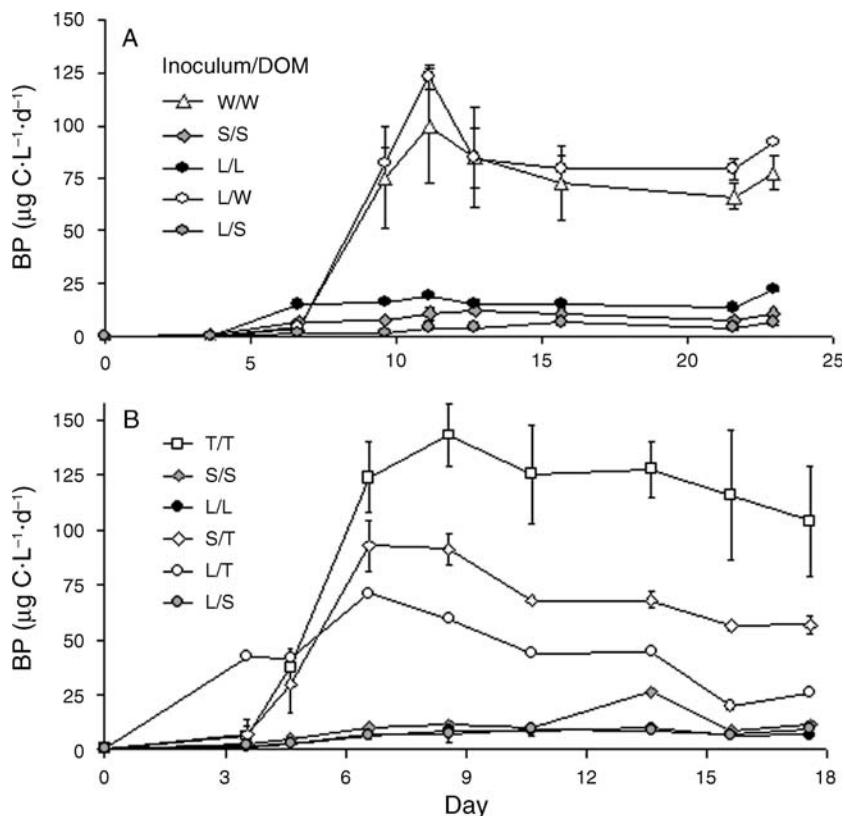
FIG. 2. Bacterial production (BP) over time (mean ± SE) in experimental and control mesocosms from (A) experiment 1 ($N = 3$) and (B) experiment 2 ($N = 2$). Symbol shape indicates inoculum (W, wet sedge; T, tussock; S, stream; L, lake), and symbol color indicates DOM source (white, soil water [for W or T]; gray, stream; black, lake).

with the inoculum controls (L/L, 33%, $P < 0.001$), while L/W treatments were equally similar to both W/W and L/L controls (38%). In contrast, treatments were significantly ($P < 0.01$) more similar to the inoculum controls in two of the three cases in experiment 2, indicating that shifts in community composition were less complete in these experiments.

Similarity in bacterial community composition between treatments and DOM controls (same DOM) were higher than expected based on the null hypothesis that similarity would be high for treatments and inoculum controls (same initial bacterial community), but low for treatments and DOM controls. Treatments were at least equally similar to mesocosms which shared the same DOM but not the same starting bacterial community, based on inverse distance values, and shifts were greater in experiment 1 than in experiment 2 (Fig. 5).

DISCUSSION

These results add to recent literature indicating that environmental drivers, such as DOM supply or nutrients, control bacterial processing and function through shifts in community composition (e.g., Gasol et al. 2002, Burkert et al. 2003, Findlay et al. 2003, Kirchman et al. 2004). The novel demonstration here is that the effects of DOM chemistry on a functional

response (bacterial productivity) were indirect and were mediated through shifts in bacterial community structure. The specific direction of community change (i.e., in the direction of the DOM source community) suggests that these aquatic bacterial communities contain a nearly full complement of functional groups with respect to C processing, and C supply and character selects for different groups over relatively short time scales. Our results also demonstrate that the unique DOM chemistry at different sites (ecosystems) within this catchment is a strong driver of the composition and functioning of downstream bacterial communities. In turn, we propose that the spatial arrangement of ecosystems and the connecting hydrological flow paths will influence bacterial metabolism and DOM processing at the catchment scale.

The strong relationship between DOM composition and BP appears to be mediated by shifts in bacterial community composition. While our characterization of the DOM pool revealed distinct chemistries among DOM sources (Table 1; Appendix: Fig. A1), these somewhat coarse chemical measurements were not very informative with respect to DOM bioavailability. At the end of the experiments, bacterial community composition in treatment mesocosms was always significantly different from inoculum controls, and, in four of five
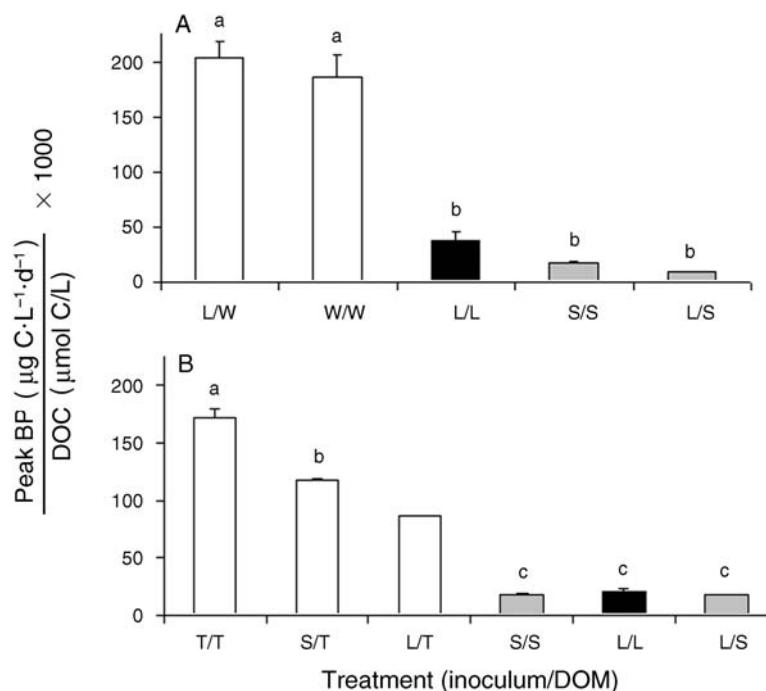
FIG. 3.   DOM bioavailability in experimental mesocosms as peak BP (μg C·L^{-1}·d^{-1}) per unit DOC (μmol C/L) $\times$ 1000 of each treatment in (A) experiment 1 ($N = 3$) and (B) experiment 2 ($N = 2$). Treatments are indicated as inoculum/DOM (L, lake; S, stream; W, wet sedge; T, tussock), and bar color indicates the source of the DOM (black, lake; gray, stream; white, soil water). Different letters above the bars indicate significant differences ($P < 0.05$) in DOM bioavailability (note, treatment L/T had only one replicate and was excluded from the ANOVA).

treatments the composition was more similar to the community from which the DOM originated than to the community from which the inoculum originated (Fig. 4; note that these comparisons are made with the control communities at the end of the experiment, thus eliminating any potential bias due to natural drift of community composition during the experiment). Bacterial community composition was more similar between pairs of mesocosms with the same DOM (but not necessarily the same inoculum) than between pairs with the same inoculum (but not necessarily the same DOM) in experiment 1 (Fig. 5), indicating that DOM was a stronger control than the starting community composition. Responses in BP and community composition to upslope DOM were also observed in experiment 2. Interestingly, however, the initial community composition constrained these responses somewhat (Figs. 1B and 3B). In both experiments, the magnitude of change in BP was proportional to the shift in community composition. For example, both the transition from the initial to DOM source community (Fig. 4) and the shift in BP (Fig. 2) were less complete in experiment 2. Although DGGE is excellent at characterizing the phylogenetic community composition of numerically dominant, and therefore competitively successful, bacteria, it is unclear exactly how many and which bacterial groups are contributing to functional responses. Sequencing new bands that appear in DGGE gels when

upslope DOM is added, in conjunction with techniques that identify active members (e.g., Radajewski et al. 2000, Torsvik and Ovreas 2002), will help in the next step of determining which specific members of the bacterial community are important in responding to changes in DOM supply.

The degree to which these results may be generalized across scales or to other systems will depend on system-specific factors such as variation in DOM supply, residence time of water and DOM, and previous exposure to DOM source. For example, stream biofilm communities, which experience variable DOM supply and relatively short water residence times, have been shown to respond to changes in DOM supply (Kaplan and Newbold 1995, Findlay et al. 2003). Some studies, however, note only weak shifts in bacterial community composition in response to a new DOM source (e.g., Langenheder et al. 2005). Strong shifts in community composition may be dependant on a dormant "seed bank" of species able to use the new DOM compounds. This species bank may be lacking or less effective (and therefore show only a weak response) if the bacterial inoculum has not had previous contact with the DOM source. This may explain why the addition of DOM from different catchments could result in only weak shifts in bacterial community composition (Langenheder et al. 2005). Thus there may be spatial boundaries within which "all species are everywhere" in terms of a given
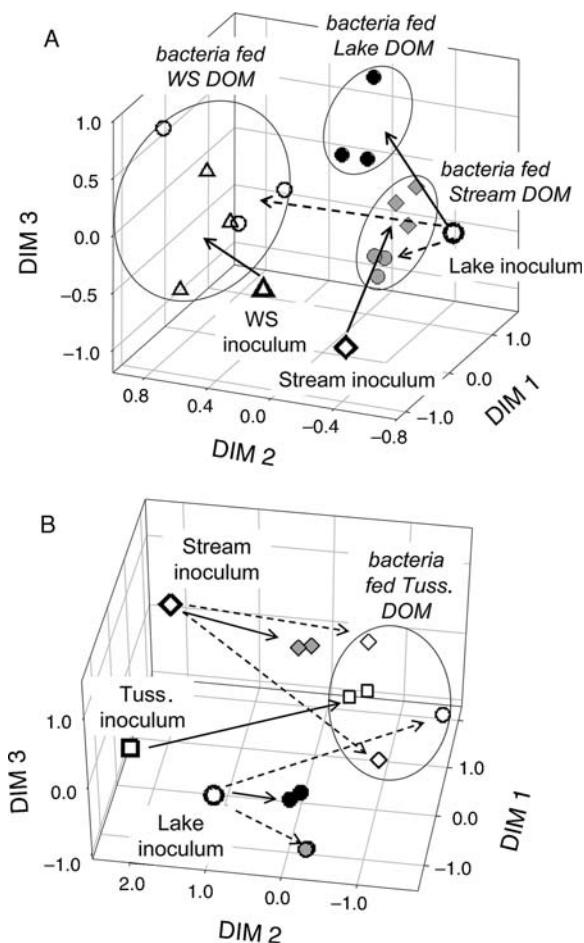
FIG. 4. Changes in bacterial community composition in experimental and control mesocosms: (A) experiment 1; (B) experiment 2. Multidimensional scaling (MDS; DIM is dimension) shows the direction of change in bacterial community composition from the beginning (initial inoculum, large symbols at arrow tail) to the end (small symbols at arrow head; (23 days for experiment 1; 18 days for experiment 2). MDS represents the Dice distance matrix based on band presence–absence data. Symbol shape represents the source of the bacterial inoculum (circle, lake; diamond, stream; square, soil water [SW]), and symbol shade indicates the DOM source (black, lake; gray, stream; white, soil water (A, wet sedge; B, tussock). MDS stress values were (A) 0.10 and (B) 0.07. Note that the "control" community for comparisons was the ending, not the beginning, community.

functional response, as was found in our tundra ecosystems, as well as spatial boundaries beyond which the effective functional response of the community is limited by community composition. Also potentially important, but not addressed in our study, are changes in environmental conditions along flow paths. For example, changes in temperature (Weston and Joye 2005) or oxygen status may limit the extent to which biogeochemical redundancy is expressed.

Differences in the magnitude of response of lake bacteria to wet sedge soil DOM (experiment 1) and

tussock tundra soil DOM (experiment 2; i.e., complete vs. incomplete shifts in BP of treatments compared to their inoculum controls) may be due to differences in DOM chemical characteristics and the topographical locations of the two sources. For example, lake bacteria may be better adapted to use wet sedge DOM, because DOM from wet sedge meadows feeds directly into lakes. In contrast, much, if not all, of upland tussock soil water DOM is channeled through riparian zones and streams where processing by biofilms may be important in altering its composition and bioavailability before reaching a lake. Alternatively, seasonal changes in the lake bacterial community may be responsible for differing responses to soil water DOM inputs. Later in the season, lake communities may be adapted to use stream and terrestrial sources of DOM, while early season communities may be adapted for winter (in-lake) sources of DOM. Our first experiment was conducted 13 days after lake ice-off, while soil thaw depth was thin and the contact of runoff with soil materials was reduced. Thus the initial lake inocula were likely least adapted to soil DOM, and greater changes in community composition were required for functional responses to soil water DOM sources. In fact, cyclical annual changes in bacterial communities have been observed in this arctic tundra lake (Crump et al. 2003), and in other ecosystems (Schadt et al. 2003), and these changes are hypothesized to be due to seasonal shifts in DOM supply rate and composition. Our data cannot be used to assign a relative importance of these two explanations, although both should be considered when determining the mechanisms of response of aquatic bacteria to terrestrial inputs of DOM.

A possible alternative mechanism for the observed shifts in bacterial community composition is the selection against groups targeted by viruses present in the "foreign" upslope DOM fraction. Recent work has shown that the abundance (e.g., Schwalback et al. 2004, Winter et al. 2004) and habitat source (Sano et al. 2004) of viruses might influence bacterial community composition in some (but certainly not all) cases. However, neither the "phage kills winner" paradigm (Thingstad 2000) nor a scenario in which differences in viral specificity among habitats results in adaptation of local bacterial communities to local viruses is sufficient to explain why bacterial communities changed to become more similar to source DOM communities. Furthermore, the strong enhancement effect of soil water DOM on lake bacterial activity suggests that shifts in community composition which facilitate uptake of the new carbon source, rather than shifts due to viral infection, is the more likely mechanism of change. At this point, the available evidence suggests that resource (DOM) rather than phage infection is the dominant driver shaping bacterial community composition in these experiments.

In contrast to studies of diversity-function relationships in plants and animals, our results suggest that

TABLE 2. Characterization of bacterial community diversity for treatment and control mesocosms in experiments 1 and 2 as given by average band richness (number of bands) from DGGE.

Inoc/DOM	Band number ($t = 0$)	Band number (end)	Change (%)	Shared, inoc. (%)	Shared, DOM (%)
Experiment 1					
WS/WS	33	23.7 (2.3)	−46		
S/S	30	23.7 (3.3)	−22		
L/L	26	14 (1.5)	−28		
L/WS	26	35.7 (0.3)	+37	39 (4.0)	38 (2.7)
L/S	26	28.7 (2.3)	+11	33 (2.9)	48 (0.6)
Experiment 2					
T/T	32	30.0 (0.7)	+18		
S/S	33	32.5 (0.5)	−2		
L/L	34	40.0 (1.0)	−6		
S/T	33	28.5 (0.5)	−11	62 (2.7)	39 (1.7)
L/S	34	29.5 (2.5)	−12	75 (3.4)	42 (0.9)
L/T	34	24.0	−6	45 (0.9)	32 (2.9)

Notes: Treatments and controls are indicated by letters as in Table 1. Band richness at the beginning ($t = 0$) and end of the experiment (with standard error in parentheses) is shown with the percentage change in band number from the beginning of the experiment to the end and percentage of bands shared between treatments and inoculum (inoc.) and DOM controls.

overall diversity may be a meaningless concept with respect to microbial function if "everyone is everywhere" (at least in terms of functional groups, and albeit some groups at very low numbers). Because no new "species" (i.e., taxonomic units) were introduced in the experiments, and those "species" that came to dominance in any one community were present in all communities at low to undetectable numbers (DGGE is generally considered reliable at detecting groups which make up 1% or more of the total community [Muyzer et al. 1993]), then the simple number of observable "species" (alpha diversity) will relate poorly to measures of function in part because beta diversity appears to be low. Overall, we found no relationship between either BP or DOC-specific BP and initial or final DGGE band richness (Table 2). In fact, the relationship was negative in the second experiment, suggesting that at our current detection ability, measures of alpha diversity may be less informative than diversity measures that include information on population sizes of microbes.

Our results highlight that DOM processing across the landscape is dependent on spatial patterns of DOM composition, and that the delivery of this DOM to downslope microbes coincides with the time scale of adaptation and change in bacterial community composition. The implication of these results is that bacterial communities contained a full, or nearly full, complement of functional groups with respect to one aspect of DOM processing (BP), and that upslope DOM sources "selected" for different groups over short time scales (within the initial ten-day growth phase, Fig. 2). While experiments are needed to test whether the attached soil and stream-bed microbial communities, responsible for the majority of DOM processing, respond to changes in DOM composition in a manner similar to the soil water, stream water, and lake communities observed in our study, we hypothesize that DOM composition drives the structure and function of these communities as well. Although we measured only one aspect of bacterial

function related to C processing (BP), we hypothesize that other functions carried out by a broad diversity of microbial groups (e.g., respiration, N mineralization) might exhibit a greater degree of functional redundancy across the landscape than those carried out by more specific groups (e.g., denitrification, methanogenesis, etc.). Alternatively, it may be that functional redundancy of a given bacterial process is positively related to the degree to which communities experience fluctuations in environmental drivers. In our case, seasonal and hydrologically driven changes in DOM maintain functional redundancy. Fluctuations in other factors, such as redox condition or substrate availability, may maintain functional redundancy of other biogeochemical processes.

If shifting bacterial community composition in response to "new" DOM occurs at various points on the landscape where DOM and community composition
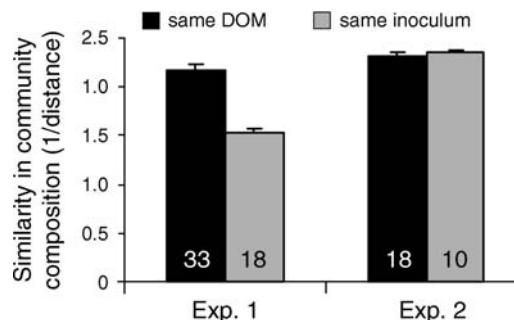
FIG. 5. Similarity (mean + SE) among mesocosms with common DOM (black) or common bacteria (gray). Distances were generated from a Dice distance matrix of DGGE (denaturing gradient gel electrophoresis) banding patterns. Bars represent the average inverse distance between mesocosm pairs grown on the same DOM source (but not necessarily the same starting bacterial inoculum; black) and pairs containing the same starting bacterial community (but not necessarily grown on the same DOM source; gray). *N* (the number of paired mesocosms) is indicated inside the bars.

differ, then the magnitude of functional changes will depend on the contact time with "new" DOM, which will be largely controlled by hydrology, versus the time scale of community adaptation. For example, at high flow, contact time may be insufficient for changes in community composition and functional responses (altered DOM processing), resulting in pulses of highly bioavailable terrestrial DOM which can alter aquatic bacterial composition and function. In instances where aquatic bacterial activity is increased by the DOM introduced in spring floods, as observed in streams, lakes, and estuaries (Kling 1995, Michaelson et al. 1998, Wikner et al. 1999), the bacterial community composition should show a concurrent, if not prior, shift. Overall, our results imply that bacterial community dynamics and physiology, landscape patterns of DOM consumption, and ecosystem C budgets may be more tightly linked than previously thought.

LITERATURE CITED

Amon, R. M. W., and R. Benner. 1996. Bacterial utilization of different size classes of dissolved organic matter. Limnology and Oceanography **41**:41–51.

Benner, R. 2003. Molecular indicators of the bioavailability of dissolved organic matter. Pages 121–137 *in* S. E. G. Findlay and R. L. Sinsabaugh, editors. Aquatic ecosystems: interactivity of dissolved organic matter. Academic Press, San Diego, California, USA.

Burkert, U., F. Warnecke, D. Babenzien, E. Zwirnmann, and J. Pernthaler. 2003. Members of a readily enriched β-proteobacterial clade are common in surface waters of a humic lake. Applied and Environmental Microbiology **69**: 6550–6559.

Carpenter, S. R., K. L. Cottingham, and D. E. Schindler. 1992. Biotic feedbacks in lake phosphorus cycles. Trends in Ecology and Evolution **1**:332–336.

Chadwick, O. A., L. A. Derry, P. M. Vitousek, B. J. Huebert, and L. O. Hedin. 1999. Changing sources of nutrients during four million years of ecosystem development. Nature **397**: 491–497.

Cottrell, M. T., and D. L. Kirchman. 2000. Natural assemblages of marine proteobacteria and members of the Cytophaga-Flavobacter cluster consuming low- and high-molecular-weight dissolved organic matter. Applied and Environmental Microbiology **66**:1692–1697.

Crump, B. C., C. S. Hopkinson, M. L. Sogin, and J. E. Hobbie. 2004. Microbial biogeography along an estuarine salinity gradient: combined influences of bacterial growth and residence time. Applied and Environmental Microbiology **70**:1494–1505.

Crump, B. C., G. W. Kling, M. Bahr, and J. E. Hobbie. 2003. Bacterioplankton community shifts in an arctic lake correlate with seasonal changes in organic matter source. Applied and Environmental Microbiology **69**:2253–2268.

Findlay, S. E. G., R. L. Sinsabaugh, and M. Hoostal. 2003. Metabolic and structural response of hyporheic microbial communities to variations in supply of dissolved organic matter. Limnology and Oceanography **48**:1608–1617.

Gasol, J. M., M. Comerma, J. C. Garcia, J. Armengol, E. O. Casamayor, P. Kojecka, and K. Simek. 2002. A transplant experiment to identify the factors controlling bacterial abundance, activity, production, and community composition. Limnology and Oceanography **47**:62–77.

Hooper, D. U., et al. 2005. Effects of biodiversity on ecosystem functioning: a consensus of current knowledge. Ecological Monographs **75**:3–35.

Judd, K. E. 2004. Dissolved organic matter dynamics in an arctic tundra catchment: linking DOM chemistry, bioavailability and microbial community composition. Dissertation. University of Michigan, Ann Arbor, Michigan, USA.

Judd, K. E., and G. W. Kling. 2002. Production and export of dissolved C in arctic tundra mesocosms: the roles of vegetation and water flow. Biogeochemistry **60**:213–234.

Kaplan, L. A., and J. D. Newbold. 1995. Measurement of streamwater biodegradable dissolved organic carbon with a plug-flow bioreactor. Water Research **29**:2696–2796.

Kirchman, D. L. 1993. Leucine incorporation as a measure of biomass production by heterotrophic bacteria. Pages 509–512 *in* P. F. Kemp, B. F. Sherr, E. B. Sherr, and J. J. Cole, editors. Handbook of methods in aquatic microbial ecology. Lewis Publishers, Boca Raton, Florida, USA.

Kirchman, D. L., A. I. Dittel, S. E. G. Findlay, and D. Fischer. 2004. Changes in bacterial activity and community structure in response to dissolved organic matter in the Hudson River, New York. Aquatic Microbial Ecology **35**:243–257.

Kling, G. W. 1995. Land–water linkages: the influence of terrestrial diversity on aquatic systems. Pages 297–310 *in* F. S. Chapin and C. Korner, editors. Arctic and alpine biodiversity; patterns, causes, and ecosystem consequences. Springer-Verlag, New York, New York, USA.

Kling, G. W., G. W. Kipphut, M. M. Miller, and W. J. O'Brien. 2000. Integration of lakes and streams in a landscape perspective: the importance of material processing on spatial patterns and temporal coherence. Freshwater Biology **43**: 477–497.

Langenheder, S., E. S. Lindstrom, and L. J. Tranvik. 2005. Weak coupling between community composition and functioning of aquatic bacteria. Limnology and Oceanography **50**:957–967.

Loreau, M., and A. Hector. 2001. Partitioning selection and complementarity in biodiversity experiments. Nature **412**:72–76.

McKnight, D. M., and G. R. Aiken. 1998. Sources and age of aquatic humus. Pages 93–105 *in* D. O. Hessen and L. J. Tranvik, editors. Aquatic humic substances: ecology and biogeochemistry. Ecological studies. Springer-Verlag, Berlin, Germany.

Michaelson, G. J., C. L. Ping, G. W. Kling, and J. E. Hobbie. 1998. The character and bioactivity of dissolved organic matter at thaw and in the spring runoff waters of the arctic tundra north slope, Alaska. Journal of Geophysical Research-Atmospheres **103**:28939–28946.

Mielke, P. W., Jr. 1984. Meteorological applications of permutation techniques based on distance functions. Pages 813–830 *in* P. R. Krishnaiah and P. K. Sen, editors. Nonparametric methods: handbook of statistics. Volume 4. Elsevier Science Publishers, Amsterdam, The Netherlands.

Muyzer, G., E. C. de Waal, and A. G. Uitterlinden. 1993. Profiling of complex microbial-populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S ribosomal-RNA. Applied and Environmental Microbiology **59**:695–700.

Myers, R. T., D. R. Zak, D. C. White, and A. Peacock. 2001. Landscape-level patterns of microbial community composition and substrate use in upland forest ecosystems. Soil Sciences Society of America Journal **65**:359–367.

Naeem, S., L. J. Thompson, S. P. Lawler, J. H. Lawton, and R. M. Woodfin. 1994. Declining biodiversity can alter the performance of ecosystems. Nature 368:734–737.

Nelson, N. 1944. A photometric adaptation of the Somogyi method for the determination of glucose. Journal of Biological Chemistry 153:375–380.

Nubel, U., F. Garcia-Pichel, M. Kuhl, and G. Muyzer. 1999. Quantifying microbial diversity: Morphotypes, 16S rRNA genes, and carotenoids of oxygenic phototrophs in microbial mats. Applied and Environmental Microbiology 65:422–430.

O'Brien, W. J., M. Bahr, A. E. Hersey, J. E. Hobbie, G. W. Kipphut, G. W. Kling, H. Kling, M. McDonald, M. C. Miller, P. Rublee, and J. R. Vestal. 1997. The limnology of Toolik Lake. Pages 61–106 *in* A. M. Milner and M. W. Oswood, editors. Freshwaters of Alaska. Springer-Verlag, New York, New York, USA.

Patra, A. K., et al. 2005. Effects of grazing on microbial functional groups involved in soil N dynamics. Ecological Monographs 75:65–80.

Pinhassi, J., F. Azam, J. Hemphala, R. A. Long, J. Martinez, U. L. Zweifel, and A. Hagstrom. 1999. Coupling between bacterioplankton species composition, population dynamics, and organic matter degradation. Aquatic Microbial Ecology 17:13–26.

Polz, M. F., and C. M. Cavanaugh. 1998. Bias in template-to-product ratios in multitemplate PCR. Applied and Environmental Microbiology 64:3724–3730.

Radajewski, S., P. Ineson, N. R. Parekh, and J. C. Murrell. 2000. Stable-isotope probing as a tool in microbial ecology. Nature 403:646–648.

Saiki, R. K., D. H. Gelfand, S. J. Stoffel, S. J. Scharf, R. Higuchi, G. T. Horn, K. B. Mullis, and H. A. Erlich. 1988. Primer-directed enzymatic amplification of DNA with thermostable DNA polymerase. Science 239:487–491.

Sano, E., S. Carlson, L. Wegley, and F. Rohwer. 2004. Movement of viruses between biomes. Applied and Environmental Microbiology 70:5842–5846.

SAS Institute. 2003. SAS 8. 2. SAS Institute, Cary, North Carolina, USA.

Schadt, C. W., A. P. Martin, D. A. Lipson, and S. K. Schmidt. 2003. Seasonal dynamics of previously unknown fungal lineages in tundra soils. Science 301:1359–1362.

Schindler, D. W. 1974. Eutrophication and recovery in experimental lakes: implications for lake management. Science 184:897–899.

Schwalbach, M. S., I. Hewson, and J. A. Fuhrman. 2004. Viral effects on bacterial community composition in marine plankton microcosms. Aquatic Microbial Ecology 34:117–127.

Shaver, G. R., and F. S. Chapin. 1991. Production-biomass relationships and element cycling in contrasting arctic vegetation types. Ecological Monographs 61:1–31.

Shaver, G. R., L. C. Johnson, D. H. Cades, G. Murray, J. A. Laundre, E. B. Rastetter, K. J. Nadelhoffer, and A. E.

Giblin. 1998. Biomass and CO_2 flux in wet sedge tundras: Responses to nutrients, temperature, and light. Ecological Monographs 68:75–97.

Simon, M., and F. Azam. 1989. Protein content and protein synthesis rates of planktonic marine bacteria. Marine Ecology Progress Series 51:201–213.

Sinsabaugh, R. L., T. Weiland, and A. E. Linkins. 1992. Enzymatic and molecular analysis of microbial communities associated with lotic particulate organic matter. Freshwater Biology 28:393–404.

Stephan, A., A. H. Meyer, and B. Schmid. 2000. Plant diversity affects culturable soil bacteria in experimental grassland communities. Journal of Ecology 88:988–998.

Sun, L., E. M. Perdue, J. L. Meyer, and J. Weiss. 1997. Use of elemental composition to predict bioavailability of dissolved organic matter in a Georgia river. Limnology and Oceanography 42:714–721.

Suzuki, M. T., and S. J. Giovannoni. 1996. Bias caused by template annealing in the amplification of mixtures of 16S rRNA genes by PCR. Applied and Environmental Microbiology 62:625–630.

Thingstad, T. F. 2000. Elements of a theory for the mechanisms controlling abundance, diversity, and biogeochemical role of lytic bacterial viruses in aquatic systems. Limnology and Oceanography 45:1320–1328.

Tilman, D., J. Knops, D. Wedin, P. Reich, M. Ritchie, and E. Siemann. 1997. The influence of functional diversity and composition on ecosystem processes. Science 277:1300–1302.

Torsvik, V., and L. Orveas. 2002. Microbial diversity and function in soil: from genes to ecosystems. Current Opinion in Microbiology 5:240–245.

Wardle, D. A., R. D. Bardgett, J. N. Klironomos, H. Setala, W. H. van der Putten, and D. H. Wall. 2004. Ecological linkages between aboveground and belowground biota. Science 304: 1629–1633.

Waterman, P. G., and S. Mole. 1994. Analysis of phenolic plant metabolites. Blackwell Scientific Publications, Oxford, UK.

Weston, N. B., and S. B. Joye. 2005. Temperature-driven decoupling of key phases of organic matter degradation in marine sediments. Proceedings of the National Academy of Sciences (USA) 102:17036–17040.

Wikner, J., R. Cuadros, and M. Jansson. 1999. Differences in consumption of allochthonous DOC under limnic and estuarine conditions in a watershed. Aquatic Microbial Ecology 17:289–299.

Winter, C., A. Smit, G. J. Herndl, and M. G. Weinbauer. 2004. Impact of virioplankton on archaeal and bacterial community richness as assessed in seawater batch cultures. Applied and Environmental Microbiology 70:804–813.

Zak, D. R., W. H. Holmes, D. C. White, A. D. Peacock, and D. Tilman. 2003. Plant diversity, soil microbial communities and ecosystem function: are there any links? Ecology 84:2042–2050.

APPENDIX

Principal-components analysis of DOM chemical composition at the beginning and end of experiments (*Ecological Archives* E087-129-A1).