

Recurring Seasonal Dynamics of Microbial Communities in Stream Habitats

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Recurring seasonal patterns of microbial distribution and abundance in three third-order temperate streams within the southeast Pennsylvania Piedmont were observed over 4 years. Populations associated with streambed sediments and rocks (epilithon) were identified using terminal restriction length polymorphism (tRFLP) and sequencing of 16S rRNA genes selectively amplified with primers for the bacterial domain. Analyses of the relative magnitudes of tRFLP peak areas by using nonmetric multidimensional scaling resolved clear seasonal trends in epilithic and sediment populations. Oscillations between two dominant groups of epilithic genotypes, explaining 86% of the seasonal variation in the data set, were correlated with temperature and dissolved organic carbon. Sequences affiliated with epilithic phototrophs (cyanobacteria and diatom chloroplasts), a *Rhodospirillum rubrum* sp., and a *Bacillus* species clustered in the summer, whereas sequences most closely related to “*Betaproteobacteria*” (putative *Burkholderia* sp.), and a putative cyanobacterium clustered in the fall/spring. The sediment genotypes also clustered into two groups, and these explained 85% of seasonal variation but correlated only with temperature. A summer tRFLP pattern was characterized by prevalence of “*Betaproteobacteria*,” “*Gammaproteobacteria*,” and a *Bacillus* sp., whereas the winter/spring pattern was characterized by phylotypes most closely related to “*Firmicutes*,” “*Gammaproteobacteria*,” and “*Nitrospirae*.” A close association between these headwater streams and their watersheds was suggested by the recovery of sequences related to microbial populations provisionally attributed to not only freshwaters but also terrestrial habitats.

Headwater streams are a primary link between aquatic and terrestrial ecosystems, transforming and exporting terrestrially derived materials and also contributing to export through autochthonous primary production. Complex communities of macro- and microbiota distributed among different stream habitats primarily mediate these transformations. The trophic structures of stream macrobiota and phototrophic eukaryotic microbiota have been intensively studied as contributors to production and energy flow in lotic systems (26). In contrast, although microorganisms exert significant, if not major, control on system chemistry and the processing of terrestrial inputs, there is little understanding of differences in lotic microbial population structure among biomes, population similarity among streams within a biome, or changes in structure associated with seasonal oscillations in chemical and physical parameters. This information is essential for developing a more complete description of the trophic structure and biogeochemistry of streams, one that more fully incorporates their relationship to the proximal terrestrial system (11, 12, 16, 47, 48).

Microbial communities in the temperate deciduous forest biome are exposed to seasonal changes in their chemical and physical environments. Some seasonal changes are constrained, such as variation in light and temperature, and their influence on the structure and activity of aquatic microbial communities is reasonably well documented (4, 9, 30, 46, 51, 67). Other variables, including variations in flow and terrestrial runoff associated with storms (22, 39), are only partly deter-

mined by season, as seasonal differences in storm frequencies are constrained by regional climatic patterns. Many variables that directly influence stream microbiota have not been circumscribed. For example, how the quantity and quality of allochthonous and autochthonous substrates (e.g., dissolved organic carbon [DOC] and particulate organic carbon [POC]) vary, both seasonally and between the major stream habitats characterized by sediments in pools and rocks in riffles, is very poorly understood.

Headwater streams experience seasonal changes in the quantity and quality of organic substrates originating from multiple sources, including leaf litter (38, 40) and algal blooms occurring prior to canopy closure (30, 31). Habitat differences in organic resources and sheer stress exist as well. For example, high depositional loads of POC and upwelling hyporheic zone DOC tend to be associated with sediment habitats (5, 54), whereas algal biomass and associated exudates are more consistently present within rock-associated epilithic biofilms (30, 31). Varying flows alter the sediment system through erosion and transport, redistributing the attached bacteria and likely exposing them to different environmental conditions. Although the epilithic community is not as prone to translocation, differences in hydrodynamics are known to influence the architecture, composition, and activity of epilithic multiphylum biofilms (7, 25), altering nutrient storage, particle capture, and solute uptake (6).

To better understand how biotic and abiotic variables influence the structure of stream microbiota, we conducted a long-term comparative study of three streams within the same deciduous biome. Our primary objective was to determine whether microbial populations were conserved over annual cycles. By restricting our replicate study sites to neighboring

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streams within a single biome, we constrained some controlling environmental variables, including climate, topography, underlying geology, inorganic water chemistry, and terrestrial vegetation.

Sediment and epilithic populations were characterized by molecular inspection, using both fingerprinting and comparative sequencing of the 16S rRNA genes. Seasonal variation in population structure was determined using terminal restriction fragment length polymorphisms (tRFLP), a fingerprinting technique developed for the rapid assessment of microbial community structure (35). Near-complete sequences were determined for those clones corresponding to the seasonally abundant restriction fragments. We observed a recurring seasonal pattern of microbial population abundance and distribution in all three streams over four separate years. Multivariate statistical analysis revealed a correlation of populations with seasonal changes in temperature and DOC concentration.

MATERIALS AND METHODS

Study sites. Three streams of similar size (White Clay Creek, Birch Run, and West's Creek) in rural watersheds of the southeast Pennsylvania Piedmont (centered around 39°54'0"N, 75°50'24"W) were sampled for analyses of water chemistry and microbial communities. These study watersheds have been logged periodically during the past 350 years, but the sections of all three streams selected for sampling have intact riparian woodlands typical of the temperate Eastern deciduous forest. All three streams are part of the Delaware River drainage and are within 20 km or less of each other.

Experimental design. Streambed substrata, rocks and sediments, were sampled seasonally over 4 years (seven dates between November 1999 and June 2002) from White Clay Creek and over 2 years (four dates between January 2001 and May 2002) from Birch Run and West's Creek to assess seasonal patterns in bacterial community structure by tRFLP and sequence analyses of 16S rRNA genes recovered by PCR amplification with primers selective for the bacterial domain. White Clay Creek clone libraries were constructed from amplification of DNAs recovered from multiple sediment samples collected over multiple seasons or from multiple epilithic samples collected on a single day in June. The molecular data were related to the temperature and chemistry of water samples collected concurrently. For analyses of seasonal patterns, samples were a posteriori grouped by season (winter, January; spring, April and May; summer, June; and fall, October and November).

Sampling of benthic microbial communities. Bacteria attached to streambed substrata were collected in triplicate composite samples from rocks in riffles (epilithon) and surface sediments in runs. Submerged rocks were removed from the streambed, and a 60-mm-diameter region of surface was circumscribed using a polyvinyl chloride (PVC) ring sealed with a gasket of Mortite clay. The circumscribed surface area was then scraped with an Exacto knife, recovering the liberated epilithic material by irrigation with 10 ml of unfiltered stream water. Each epilithic composite consisted of scrapings from five circumscribed surfaces and a total of 10 ml of irrigation water used repeatedly, which were combined in a grinding tube, homogenized for 1 min with a pestle, aliquoted into three vials, and flash frozen in liquid N₂. This procedure was repeated two more times for a total of nine vials from each sampling date, representing three composite epilithon samples and 15 scrapings. Assuming that 10 ml of stream water used for irrigation contains 2×10^6 bacteria (32), we estimate that this would contribute 2 to 4 orders of magnitude fewer bacteria than the total cells recovered from a composite rock sample. Composite sediment cores were sampled with a 60-mm-diameter, 1-cm-deep PVC ring that was pushed into surface sediments and lifted out of the stream with minimal disturbance by placing a Plexiglas sheet above and below the ring. The upper approximately 3 mm of sediment from three replicate cores were combined in a Whirl Pak bag with a metal spatula, aliquoted into three vials, and flash frozen in liquid N₂. This procedure was repeated two more times for a total of nine vials from three composite sediment samples and nine cores. Vials from different composite sediment or epilithon samples were treated separately for DNA extraction and tRFLP analysis. Thus, the variability among the composite samples collected on a given date was taken into account. Samples were taken in November 1999, June 2000, October 2000, January 2001, April 2001, May 2002, and June 2002.

Environmental variables. The temperature and discharge of White Clay Creek were continuously monitored. Water temperature was measured with an Onset Optic StowAway probe. Stage height was monitored at a gauging station with a type A model 71 horizontal float recorder. Continuous data records were reduced to mean daily values for use as discrete variables in correlation analyses. Daily samples of White Clay Creek water were collected for DOC concentration analyses. When benthic samples were collected, temperature was measured with a hand-held field thermometer and water collected for analyses of DOC, anions, cations, and conductivity. Samples for DOC analyses were collected in borosilicate glassware that had been rendered organic C free by combustion (500°C, 6 h) and then filtered through glass-fiber filters (Whatman GF/F) with a syringe and syringe-type filter holder (29). DOC concentration was determined by Pt-catalyzed persulfate oxidation with either an OI 700 or OI 1010 total organic carbon analyzer (29). Anions and cations were measured with a Dionex DX 500 ion chromatography system equipped with an ED40 electrochemical detector after passing each sample through a sterile 0.22- μ m Pall Gelman HF Tuffryn Acrodisc filter. Conductivity was determined with a YSI model 32 conductance meter, and pH was measured with a Fisher Scientific pH probe and meter.

DNA extraction. Approximately 0.5 g of each sample was collected in a 2-ml microcentrifuge tube containing 0.5 g of prebaked (80°C for at least 2 h) zirconium beads (0.1-mm diameter; Biospec). Phosphate and MT buffers from the Fast DNA spin kit for soils (Qbiogene, Carlsbad, CA) were added to the sample tubes. The samples were processed in a Bio101 FastPrep (Qbiogene, Carlsbad, CA) at speed 4.5 for 15 seconds (two times) and placed on ice for 1 min between mechanical disruptions. The samples were centrifuged at 4°C at 15,000 \times g for 5 min. Supernatants were placed into new tubes, and samples were processed according to the manufacturer's protocol by elution in 50 μ l diethylpyrocabonate (Ambion, Austin, Texas)-treated water and stored at -80°C. To evaluate quality, the DNA was resolved on 0.8% (wt/vol) high-melt agarose gels with 1 \times Tris-acetate-EDTA buffer and visualized by ethidium bromide staining (43).

tRFLP analysis. Bacterial 16S rRNA gene sequences were amplified using primers S-D-Bact-1512-a-A-21 and S-D-Bact-008-a-S-17 (33). Primer S-D-Bact-008-a-S-17 was end labeled with 6-carboxyfluorescein on the 5' end. Each 50- μ l PCR mixture contained primers at 0.7 μ M, 10 μ M Tris-HCl (pH 8.8), 50 μ M KCl, 1.5 μ M MgCl₂, 25 μ M of each deoxynucleoside triphosphate (Invitrogen, Carlsbad, CA), 25 μ g of bovine serum albumin, and 2.5 U *Taq* DNA polymerase (Invitrogen, Carlsbad, CA). Each reaction mix was incubated on a PTC-100 thermal cycler (MJ Research, Waltham, MA), using the following "touchdown" parameters: initial denaturation at 94°C for 1 min, followed by four cycles of denaturation at 94°C for 30 seconds, annealing at 65°C for 30 seconds, and extension at 72°C for 30 seconds. The annealing temperature for the reaction was subsequently reduced by 1°C per cycle until a 50°C annealing temperature was reached, cycled was continued five more times at a 50°C annealing temperature, and the reaction was terminated with a final extension at 72°C for 5 min. The PCR products were visualized on an 0.8% (wt/vol) high-melt agarose gel stained with ethidium bromide and quantified using a quantitative DNA ladder (Invitrogen, Carlsbad, CA) and NIH image version 1.63 (<http://rsb.info.nih.gov/niimage>).

PCR products, derived from environmental samples or clones, were digested overnight in the dark at 37°C using 10 U of a tetrameric restriction enzyme (HaeIII) in a standard restriction buffer (One-Phor-All Plus; Amersham-Pharmacia). Digested samples were ethanol precipitated (43), dried, and analyzed using an ABI 377 DNA sequencer (Applied Biosystems, Inc., Fremont, CA) operated by the OSU Center for Genomic Research and Biotechnology. At least 100 fmol of each digested sample was loaded onto a 4% polyacrylamide gel for fragment analysis in Gene Scan mode. The Genescan 500 6-carboxytetramethylrhodamine size standard (HaeIII) was added to each sample lane. ASCII files of electropherograms were analyzed using Dax analysis software (van Mierlo Inc., Holland). Peaks representing tRFLP fragments were measured as integrated peak areas. Peaks less than 2 base pairs apart were binned and normalized using the sum of total peak area. Peaks less than 1% of the total area or found in fewer than three replicate profiles were excluded from further analysis. The assignment of clones to specific peaks was made using Fragment Finder (www.ce.stahl.washington.edu) and analysis of clones as described above.

Environmental 16S rRNA gene sequences. Bacterial 16S rRNA gene sequences were amplified from sediment and epilithic DNA extracts by using primers S-D-Bact-008-a-S-17 (33) and S-D-Bact-1512-a-A-21 (33). PCR products were purified using a gel kit (QIAGEN, Valencia, CA) and cloned into the pGEM-T easy vector (Promega, Madison, Wisconsin) according to the manufacturer's protocol. Transformants were randomly selected and inoculated into 100 μ l of LB broth with 100 μ g ml⁻¹ ampicillin in 96-well microtiter plates and incubated overnight at 37°C. For sediment samples, five clone libraries were constructed from DNA extracted from triplicate composite samples collected in

TABLE 1. Seasonal values of chemical and physical parameters for study streams^a

Stream	Date	Concn (mg/liter) ^b							Conductivity (μS/cm)	pH	Temp (°C)	DOC (mg/liter)	
		Cl	NO ₃ N	SO ₄	Ca	Mg	K	Na					
Birch Run	05 June 2000	24.1	12.7	17.6	11.1	7.0	1.0	5.5	171	6.92	— ^c	1.02 (0.05)	
	28 Jul 2000	17.4	7.4	11.9	11.1	6.6	1.1	5.3	168	7.57	—	1.30 (0.01)	
	23 Aug. 2000	6.9	3.6	5.1	11.8	7.3	1.2	6.0	172	7.09	—	1.00 (0.02)	
	21 Sep. 2000	7.0	3.3	5.2	11.6	6.9	1.5	5.9	151	7.41	16.7	1.29 (0.04)	
	20 Oct. 2000	7.2	3.6	5.4	12.0	7.1	1.7	5.8	163	7.29	9.0	1.65 (0.01)	
	16 Nov. 2000	7.3	3.8	5.5	11.6	7.0	1.5	5.7	164	7.44	4.5	1.14 (0.01)	
	05 Dec. 2000	7.5	4.5	5.8	11.5	7.0	1.3	5.7	165	7.43	1.2	0.80 (0.04)	
	18 Jan. 2001	8.0	4.5	6.3	12.1	7.2	1.5	5.8	175	7.15	2.0	0.86 (0.01)	
	20 Feb. 2001	—	—	—	—	—	—	—	—	—	3.1	0.91 (0.01)	
	14 Mar. 2001	19.5	7.8	12.1	6.0	3.7	0.7	3.4	173	6.76	5.7	1.24 (0.11)	
	17 Apr. 2001	20.0	7.7	12.3	10.9	6.7	1.2	5.0	167	8.15	7.4	1.21 (0.02)	
	18 May 2001	21.6	8.9	11.3	12.5	8.2	1.1	9.7	176	6.78	11.8	0.84 (0.01)	
	White Clay Creek	01 Dec. 1999	9.3	3.7	16	18	8.4	2	5.5	221	7.51	4.3	1.30 (0.01)
		31 May 2000	13.2	5.8	28.6	23.7	8.3	1.4	5.7	212	7.70	12.0	1.70 (0.04)
23 Aug. 2000		11.5	4.4	20.7	20.9	7.7	1.8	5.8	210	8.10	16.1	—	
29 Sep. 2000		11.9	4.4	22.0	22.0	7.9	2.2	5.9	218	7.71	10.3	1.56 (0.01)	
23 Oct. 2000		12.3	4.4	21.3	22.3	8.0	2.3	5.9	176	7.54	11.0	1.74 (0.02)	
16 Nov. 2000		12.7	4.5	22.1	22.1	7.8	2.0	5.7	180	8.34	6.2	1.54 (0.02)	
05 Dec. 2000		12.3	5.2	22.7	21.9	7.9	1.9	5.7	195	7.63	2.3	1.06 (0.03)	
17 Jan. 2001		13.2	4.9	24.0	21.2	7.8	2.3	5.7	216	7.34	2.4	1.64 (0.01)	
20 Feb. 2001		—	—	—	—	—	—	—	—	—	6.1	1.12 (0.02)	
12 Mar. 2001		10.9	4.5	18.5	21.0	7.8	1.8	4.9	215	6.52	8.3	1.14 (0.02)	
20 Apr. 2001		11.9	4.1	17.5	12.1	4.7	0.9	3.1	200	7.95	8.0	1.45 (0.07)	
24 May 2001		11.9	4.0	17.2	25.0	8.0	1.6	6.0	223	6.74	13.4	1.92 (0.04)	
03 Apr. 2002		8.8	3.4	16	21.7	8.9	2.3	5.7	227	7.93	12.3	1.42 (0.01)	
West's Creek		05 June 2000	6.6	2.8	42.1	13.6	4.6	1.8	5.2	148	7.03	—	1.73 (0.02)
	28 July 2000	4.8	1.5	28.5	13.2	4.5	1.6	4.9	153	7.10	—	2.32 (0.02)	
	23 Aug. 2000	5.3	1.6	32.9	14.8	5.4	2.6	5.6	150	7.41	—	1.40 (0.01)	
	21 Sep. 2000	5.7	1.2	30.6	13.9	4.6	2.6	5.4	130	8.57	16.2	2.22 (0.01)	
	20 Oct. 2000	5.5	1.2	32.9	13.7	4.8	2.8	5.6	123	8.03	9.1	1.83 (0.02)	
	16 Nov. 2000	5.9	1.5	35.0	13.2	4.0	2.2	4.9	133	7.78	5.5	1.60 (0.02)	
	05 Dec. 2000	5.9	2.2	35.8	13.1	4.7	2.4	5.3	144	7.49	1.6	1.32 (0.06)	
	18 Jan. 2001	6.0	2.2	35.0	12.5	4.2	2.5	4.9	142	7.30	1.6	1.50 (0.01)	
	20 Feb. 2001	—	—	—	—	—	—	—	—	—	4.1	1.62 (0.06)	
	14 Mar. 2001	5.5	1.8	27.1	13.6	4.6	2.4	4.4	150	6.70	5.5	1.92 (0.04)	
	17 Apr. 2001	6.1	1.7	26.3	13.3	4.6	2.1	4.1	148	8.25	7.9	2.02 (0.03)	
	18 May 2001	5.6	1.9	25.9	15.8	5.6	2.1	6.1	152	6.73	12.4	1.56 (0.01)	

^a Values represent results for single samples, except for DOC values, which are the means (standard deviations) for three replicate.
^b The analytical precisions of anions and cations, based on the coefficient of variation of replicate analyses, were 0.68 and 2.40, respectively.
^c —, no data.

June 2000 (WCC77A, WCC77B, and WCC77C), June 2002 (WCC54D), and January 2001 (WCC60E). A clone library representing the epilithic community was constructed from composite samples taken in June 2002 (WCC54F). Plasmids were prepared from overnight cultures of clones grown in LB on a RevPrep Orbit (GeneMachines, San Carlos, CA). One hundred sixty clones from the sediment clone libraries and 16 clones from the epilithic library were initially characterized by partial sequence determination (approximately 700 bp from the 5' terminus) using an ABI373 sequencer (Applied Biosystems, Fremont, CA), dye terminator chemistry, and the 700r primer (63).

Phylogenetic inference. Sequences were inspected using Sequencher version 4.0 (Gene Codes, Ann Arbor, MI), and BLAST version 2.0 was used to identify closely related sequences. Preliminary alignment of the sequences was done using the sequence editor and Fast Align in ARB (35a), and all final alignments were checked manually (33). Representatives from each group were sequenced entirely, using small-subunit-rRNA-specific primers 700R and 700F (63). Chimeric sequences were identified using the CHECK CHIMERA utility of the Ribosomal Database Project (36). Regions with uncertain alignment were not used for phylogeny inference. Phylogenetic relationships were inferred by neighbor joining with Kimura two-parameter genetic distances (2:1 transition/transversion ratio), with bootstrap proportions calculated by neighbor-joining and maximum parsimony using PAUP (version 4.0) (62). In all cases, bootstrap proportions were calculated from 100 resampled data sets.

Statistical analysis. Nonmetric multidimensional scaling (NMS) was used to compare tRFLP fragment compositions among different sampling dates after Sorensen's distance was calculated. PC-ORD version 4 was used to perform all multivariate analyses (37). The raw data set consisted of 66 samples made up of 32 fragment lengths, or operational taxonomic units (OTUs). To reduce skew, the square root of the relative percent area was arcsine transformed (55). NMS was used because it does not require the assumption of linearity between variables as in principle-component analysis (37). NMS is an ordination method based upon an iterative optimization procedure that minimizes ranked distances between the original data set and ordination space. For the ordination, the autopilot option was set to slow and thorough. Choosing the axis that minimizes stress and maximizes the interpretation of the data assessed the amount of variability explained in the data set. Stress, an indicator of goodness of fit, measured the inverse of the fit between the original data matrix and the reduced-dimension ordination matrices. Stress values of between 0 and 15 give a good approximation of the data in multivariable space with a low risk of drawing false inferences (37). A plot between the final stress and the number of iterations was used to assess the stability of the solutions. Monte Carlo tests identified the dimensions that gave solutions that were significantly different than those due to random chance. The proportion of the variance of the distances between the original matrix and the ordination space explained was described for each axis. A joint plot was generated to show the relationship between environmental vari-

ables from each stream (Table 1) and ordination scores from the NMS analysis (37). The angle and length of the vector indicates the direction and strength of the relationship. The length of the vector is proportional to a function of the r^2 values for the two axes, and the angle is calculated as the arc cosine of the correlation of the variable with the horizontal axis. The vectors radiate from the centroid of the ordination scores, and orthogonal rotation was used to rotate the axes 90° for ease of visualization.

Seasonal differences in White Clay Creek base flow DOC concentrations from the period from November 1999 through June 2002 were analyzed by analysis of variance, followed by Tukey's Studentized range test, which controls the type I experiment-wise error rate (55). Significant differences were assessed at an α error level of $P = 0.05$.

Nucleotide sequence accession numbers. Sequence data have been submitted to the GenBank databases under accession numbers DQ310736 to DQ310758.

RESULTS

Annual and seasonal trends in environmental variables.

Annual trends in concentrations of cations and anions, pH, or conductivity were not discernible (Table 1). Water temperature ranged from 0 to 22°C over the 4 years, with clear annual trends typical of temperate environments (Table 1). Although seasonal trends for DOC were variable, significantly lower base flow DOC concentrations (milligrams of C per liter) were found in the winter (1.27 ± 0.27 [mean \pm standard deviations]; $n = 120$) and significantly higher concentrations were found in the summer (1.70 ± 0.29 ; $n = 102$), with intermediate concentrations in the spring (1.54 ± 0.31 ; $n = 146$) and fall (1.51 ± 0.27 ; $n = 120$) (Table 1). DOC concentrations associated with storm flows occurred at all seasons and ranged from 2 to 18 mg C/liter.

Phylogenetic relationships. The clone libraries from the sediment community in White Clay Creek included both bacteria and diatom plastid sequences (Fig. 1; Table 2). Approximately 92% of the more abundant phylotypes in the sediment libraries were identified as calculated using Good's estimator (C) (21). Recovered sequences from both the sediment and epilithic communities were matched, where possible, to the sequences from the most closely related uncultured and cultured relatives, which were typically retrieved from either terrestrial or freshwater aquatic systems, including soils (34), lake water and sediments (57, 63, 64, 66), a riverine biofilm (19), Arctic sea ice (8), sphagnum bog (52), a phosphorus-removing bioreactor (15), oak leaves (59), and a drinking water distribution system (65) (Table 2). Functionally, some of the cyanobacterial sequences were most closely related to non-nitrogen-fixing species such as *Phormidium subfuscum* (12a) and some organo-heterotroph sequences were most closely related to species such as *Burkholderia* sp. (57) and *Dendrosporobacter quercicolus* (59), which are known to metabolize a wide range of carbon compounds (Table 2). Table 2 also contains the number of sequences for each clone, the size of the sequence, GenBank descriptors, the habitat where the sequence was retrieved, taxons of the cultured species, accession numbers, and percent similarity. The distribution of sediment-derived sequences among phyla was as follows: cyanobacteria, 40%; "*Betaproteobacteria*," 12%; "*Gammaproteobacteria*," 9%; "*Firmicutes*," 7%; "*Gemmatimonadetes* phylum nov.," 5%; "*Nitrospirae*," 5%; "*Bacteroidetes*," 5%; "*Acidobacteria*," 6%; "*Planctomycetes*," 3%; "*Alphaproteobacteria*," 3%; "*Actinobacteria*," 3%; and "*Deltaproteobacteria*," 1.2%. The distribution of cloned sequences among phyla derived from the epilithic community

was as follows: cyanobacteria, 25%; "*Betaproteobacteria*," 38%; "*Gammaproteobacteria*," 25%; and "*Bacteroidetes*," 12%.

Statistical analyses. NMS ordination of tRFLP patterns showed that distinct communities were associated with distinct seasonal patterns over annual cycles within the epilithic and sediment habitats (Fig. 2 and 3). Multivariate statistical analysis resolved seasonally episodic shifts of populations in each of the three streams that were correlated with seasonal changes in DOC and temperature (Fig. 2 and 3). Shifts in tRFLP peaks representing specific bacterial clones were correlated with changes in the seasons, as discussed below.

In the epilithic community, arcsine transformation reduced the skew in samples by 75%. After 75 iterations, the stress of the final solution was 12.22 and stable (Table 3). The final solution showed that two axes explained a cumulative variation in the data set of 86%. The seasonal variation in the epilithic community was illustrated by the correlation of OTUs with the ordination axes (Table 4). Samples taken in the spring and fall were separated from summer samples along axis 1, while fall and spring samples were separated along axis 2 (Fig. 2). A 216-bp peak fragment (designated peak 216) negatively correlated with axis 1 was attributed to the "*Betaproteobacteria*" (putative *Burkholderia* sp.) and an uncultured member of the "*Gammaproteobacteria*" (Tables 2 and 4). Peaks 190 (unidentified sequence) (Table 4), 293 (putative *Phormidium subfuscum*) (Tables 2 and 4), 315 (putative *Rhodospirillum rubrum* [20]) (Tables 2 and 4), 375 (putative *Navicula salincola* [41]) (Table 4), and 400 (unidentified sequence type) (Table 4) were positively correlated with axis 1, forming the summer data cluster ($r > 0.5$). Axis 2 showed a positive correlation with a peak at 226 bp (Fig. 2), associated with the fall cluster. Peak 226 was most similar to an uncultured cyanobacterium (Tables 2 and 4; Fig. 1).

Seasonal differences in terminal restriction fragments recovered from the sediment community were also identified using NMS ordination (Fig. 3). Transformation of the original data set using arcsine transformation of the relative percent area reduced the skew in samples by 85% for OTUs. After 75 iterations, the stress of the final solution was 9.54 and stable (Table 3). The final solution showed that two axes explained a cumulative variation in the data set of 85% (Fig. 3). Axis 1 separated most of the streams by season. Axis 2 separated Birch Run summer samples from White Clay Creek and West's Creek (Fig. 3). The tRFLP peaks most positively correlated with axis 1 were at 194 bp, 200 bp, and 315 bp. Peak 194 was affiliated with an uncultured member of the "*Betaproteobacteria*" (Tables 2 and 4). Peak 200 corresponded with sequences affiliated with "*Gammaproteobacteria*" (putative *Nevskia ramosa* [19]) (Table 4; Fig. 1). Peak 315 was also positively correlated with axis 1 and corresponded to a putative *Variovorax* sp. (18a) (Table 4; Fig. 1). Peak 327, negatively correlated with axis 1 (Table 4), was affiliated with a cloned sequence closely related to *Nitrospira moscoviensis* (17). Axis 2 showed a positive correlation with 204- and 256-bp fragments and a negative correlation with the 213-bp fragment (unidentified sequence) (Table 4). Fragment 204 was associated with a *Dendrosporobacter* sp. (59) (Table 4), and fragment 256 is similar to the sequence determined for an isolate from Australian soils affiliated with the "*Gammaproteobacteria*" (Fig. 1; Table 2) (44).

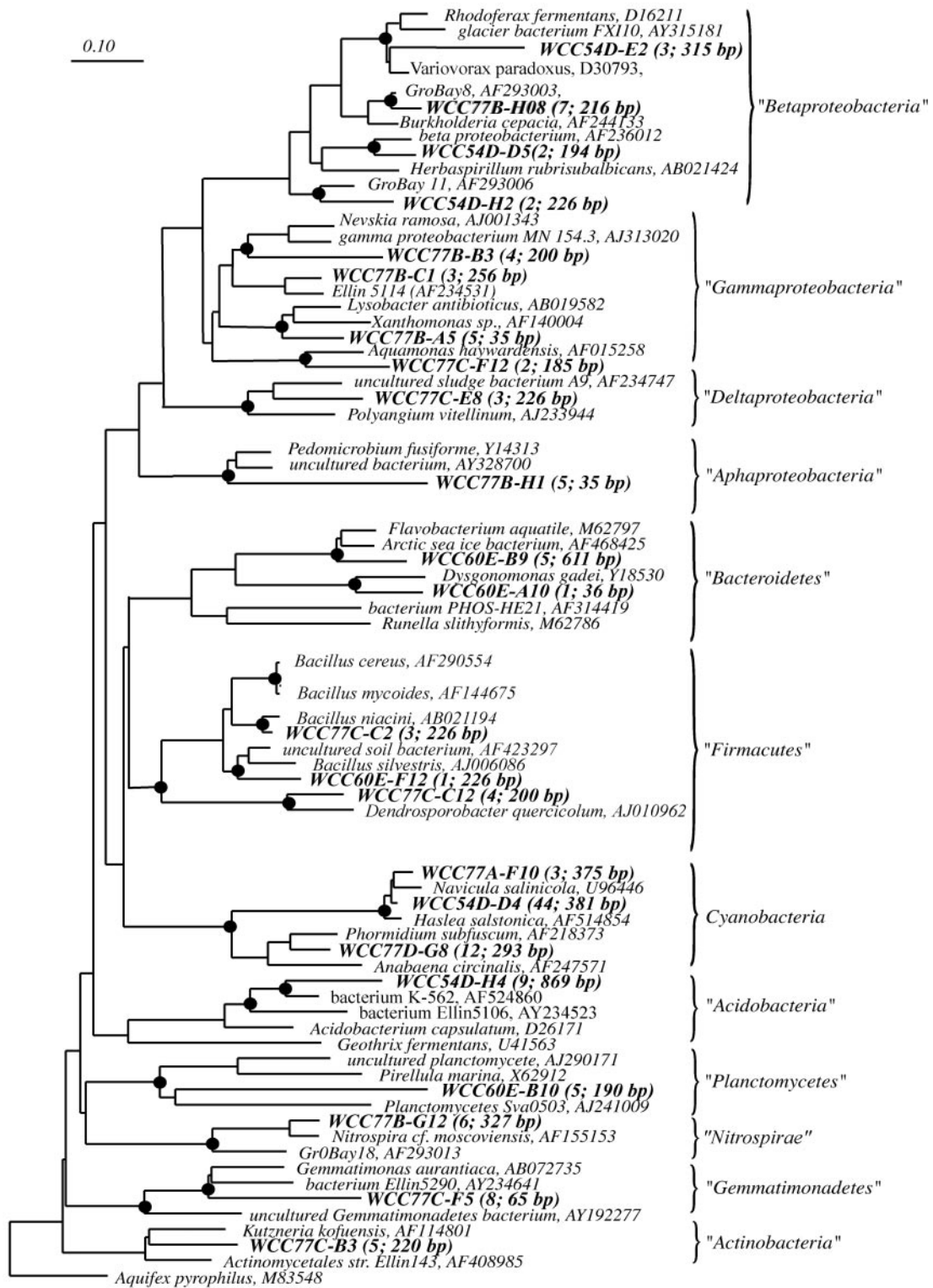


FIG. 1. Phylogenetic relationships of partial 16S rRNA gene sequences recovered from four clone libraries developed for White Clay Creek. The tree was inferred using the neighbor-joining algorithm, with the Kimura two-parameter correction factor. Bootstrap values of greater than 97 are shown on nodes (●). The tree is based on 551 positions.

TABLE 2. Phylogenetic affiliation of clones amplified from White Clay Creek sediment and epilithon

Environment	Phylogenetic affiliation	Representative clone	No.	tRFLP (bp)	Closest uncultured relative				Closest relative		
					GenBank descriptor	Accession no.	Similarity (%)	Habitat	Taxon	Accession no.	Similarity (%)
Sediment	Chloroplasts	WCC77A-F10	3	375					<i>N. salincola</i>	U96446	98
		WCC54D-D4	44	381					<i>H. salstonica</i>	AF514854	98
	Cyanobacteria "Betaproteobacteria"	WCC77D-G8	12	293					<i>P. subfuscum</i>	AF218373	96
		WCC77B-H8	7	216	Gro Bay 8	AF293003	99	Sediment	<i>B. cepacia</i>	AF244133	97
		WCC54D-E2	7	315	Glacier bacteria	AY315181	97	Sediment	<i>V. paradoxus</i>	D30793	95
		WCC54D-H2	2	226	Gro Bay 11	AF293006	97	Sediment	<i>H. rubrisubalbicans</i>	AB021424	93
		WCC54D-D5	2	194	Betaprot 55	AF236012	96				
	"Gammaproteobacteria"	WCC77B-A5	5	36					<i>L. antibioticus</i>	AB019582	94
		WCC77B-B3	4	200	γ -Proteobacteria	AJ313020	96	Biofilm	<i>N. ramosa</i>	AJ001343	96
		WCC77C-F12	2	185	Uncult. Bact.	AF015258	94		<i>A. haywardensis</i>	AF01528	96
		WCC77B-C1	3	256	Ellin 5114	AY234531	97	Soil			
	"Firmicutes"	WCC77C-C2	3	226	Uncult. Bact.	AJ000982	99	Soil	<i>B. niacini</i>	AB021194	99
		WCC77C-C12	4	204					<i>D. quercicolus</i>	AJ010962	92
		WCC60E-F12	1	226	110-114		95	Bog	<i>B. silvertris</i>	AJ006086	96
	"Acidobacteria"	WCC54D-H4	9	869	K-5b2	AF524860	96	Bog	<i>A. capsulatum</i>	D26171	96
	"Alphaproteobacteria"	WCC77B-H1	5	36	DSSF78	AY328700	95	Freshwater			
	"Planctomycetes"	WCC60E-B10	5	190	DSP08	AJ290171	93	Biofilm			
	"Bacteroidetes"	WCC60E-B9	5	611	ARK10169	AF468425	97	Sea ice	<i>F. psychrophilum</i>	AB078060	97
		WCC60E-A10	1	36					<i>D. gadei</i>	Y18530	95
	"Gemmatimonadetes"	WCC77C-F5	8	65	Ellin 5290	AY234641	94	Soil	<i>G. aurantiaca</i>	AB072735	92
"Nitrospirae"	WCC77B-G12	8	327	GroBAY8	AF293013	99	Sdiment	<i>N. moscoviensis</i>	AF155153	97	
"Actinobacteria" "Deltaproteobacteria"	WCC77C-B3	5	220	Ellin 143	AF408985	92	Soil	<i>K. kofuensis</i>	AF114801	97	
	WCC77C-E8	2	226					<i>P. vitellum</i>	AJ233944	93	
Epilithon	Chloroplasts	WCC54F-E8	1	375	Clone 182up	AY212634	97	Freshwater	<i>N. salincola</i>	U96446	98
	Cyanobacteria	WCC54F-H8	2	293	Clone FreP02	AY541578	98	Freshwater	<i>P. subfuscum</i>	AF218373	98
		WCC54F-D7	1	226	<i>Gloeotheca</i> sp. strain KO68DGA	AB067580	91	Seawater	<i>P. mucicola</i>	AB003165	86
	"Betaproteobacteria"	WCC54F-C6	1	195	Clone B3NR69D21	AY957936	97	Biofilm	<i>A. temperens</i>	AF078766	100
		WCC54F-G7	1	216					<i>B. cepacia</i>	AF244133	94
		WCC54F-A8	2	315	GKS2-77	AJ 290040	93	Freshwater	<i>R. ferrireducens</i>	AF435948	99
		WCC54F-D5	1	195	A0837	AF236012	96				
		WCC54F-B7	1	71	<i>Thiothrix</i> sp.	AF148516	96	Sludge	<i>T. ramosa</i>	AF32940	93
	"Gammaproteobacteria"	WCC54F-A5	1	36	Clone T92	Z93990	100	Sludge	<i>L. antibioticus</i>	AB019582	94
		WCC54F-G6	1	195	Clone T92	Z93990	96	Sludge			
		WCC54F-F5	2	216	Clone T92	Z93990	96	Sludge			
	"Bacteroidetes"	WCC54F-F8	1	36	N9-24	AY394634	88	Parasite	<i>F. columnariae</i>	AB015481	86

The ordination was rotated orthogonally by 90° to maximize the correlation coefficient for temperature on axis 1 in both data sets (Fig. 2 and 3). Both DOC and temperature were correlated with axis 1 in the epilithon data set ($r > 0.5$). Temperature was positively correlated with axis 1 in the sediment community ($r > 0.5$).

DISCUSSION

Our study has established recurring oscillations of multiple bacterial populations within streambed communities through several annual cycles. These observations extend shorter-term studies of seasonal variation in population structure of algae (23), fungi (28), and bacteria in aquatic systems (53). Several

studies have reported seasonal shifts in bacterial communities over an annual cycle (11, 12, 14, 18, 47), and one study described a recurring seasonal pattern of microbial functional groups in streambed sediments over a 3-year period (61). The oscillations we observed encompassed microbial populations of both apparent terrestrial and aquatic origins, as inferred by phylogenetic affiliation with microorganisms characteristic of those general habitat types. While our understanding of habitat attribution for bacterial species and autecology should be considered provisional, we suggest that the presence of terrestrial microbiota presumably reflects linkage between these streams and the terrestrial environment in which they are embedded.

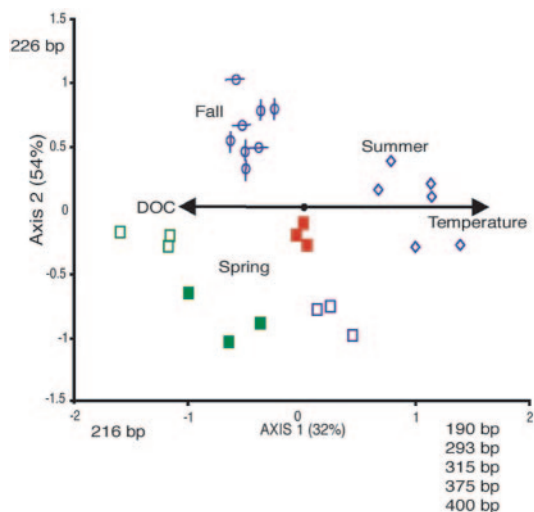


FIG. 2. NMS analysis of seasonal tRFLP patterns for streambed epilithon and correlation with environmental variables. Symbols represent streams (White Clay Creek, blue; West's Creek, green; Birch Run, red), seasons (spring, squares; summer, diamonds; fall, circles; winter, triangles), and years (1999, open symbol with vertical bar; 2000, open symbol with horizontal bar; 2001, solid symbol; 2002, open symbol).

The degree to which lotic ecosystems are integrated into the adjacent terrestrial environment is influenced by stream order, drainage density, and hydrologic exchanges between the two environments. A significant interaction was apparent in our analyses of these three third-order streams. For example, some of the White Clay Creek sediment clones were more closely related to soil *Actinobacter* spp. than to *Actinobacter* found in lakes (20, 63, 66) (Table 2; Fig. 1). Several clones affiliated with the "*Gemmatimonadetes*" were similar to sequences from Australian soil, as were some of the "*Gammaproteobacteria*" clones (Table 2; Fig. 1) (44). Similarly, clones and isolates from White Clay Creek included representatives of the "*Firmicutes*," which are common in soils and the deep subsurface but are generally not found in freshwater systems (Table 2; Fig. 1) (25). Feris et al. (18) also recovered clones similar to soil bacterial sequences from the hyporheic zones of small streams. This contrasts sharply with past censuses of bacterioplankton of lakes and large rivers, which identified few or no soil species (11, 20, 47, 66).

Primary production in headwater streams, dominated by algal photoautotrophs attached to surfaces (26), is known to respond to seasonal variation in inorganic nutrients and light availability, with the latter in large part determined by seasonal

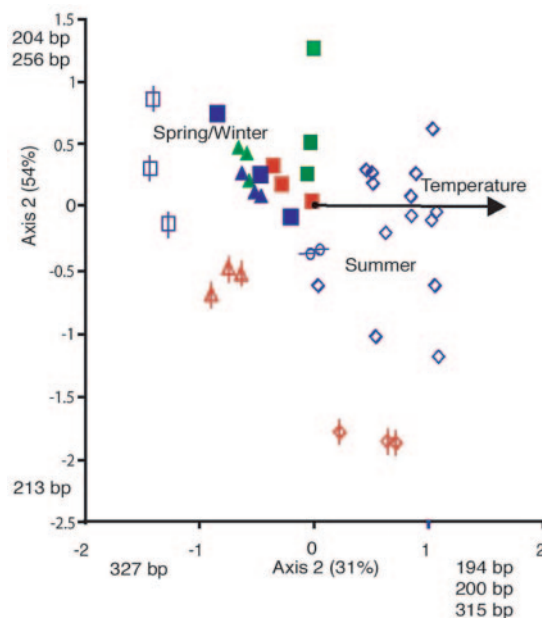


FIG. 3. NMS analysis of seasonal tRFLP patterns for streambed sediments and correlation with environmental variables. Symbols represent streams (White Clay Creek, blue; West's Creek, green; Birch Run, red), seasons (spring, squares; summer, diamonds; fall, circles; winter, triangles), and years (1999, open symbol with vertical bar; 2000, open symbol with horizontal bar; 2001, solid symbol; 2002, open symbol).

variation in the riparian zone tree canopy (31). In these three study streams, seasonal shifts in eukaryotic and prokaryotic autotrophic populations also covaried with temperature and DOC (Table 2; Fig. 2 and 3). Photoautotrophs were abundant in the clone library, corresponding to a dominant *Haslea*-like population (peak 379) and seasonally varying *Navicula* (peak 375) (41) (Table 2). Our molecular assessment of seasonal variation in *Navicula* is consistent with previous determinations based on frustule morphology (R. L. Vannote et al., unpublished data). *Haslea* spp. have been isolated throughout the year from temperate aquatic systems and have a broad temperature growth range (5 to 25°C) (1). The cyanobacterial sequences (*P. subfuscum*) were closely related to non-nitrogen-fixing sequence types from temperate lakes (41), possibly reflecting the high inorganic nitrogen concentrations (1.2 to 5.2 mg/liter nitrate N) in our study streams, which drain nearby croplands and pastures.

The correlation of temperature with changes in community structure of both the epilithic and sediment communities (Fig.

TABLE 3. Monte Carlo test of stress in relation to dimensionality, comparing 40 runs on the real data from the epilithic and sedimentary communities with 50 runs on randomized data

Environment	Axis	Stress in real data, 40 runs			Stress in randomized data, 50 runs			P
		Minimum	Mean	Maximum	Minimum	Mean	Maximum	
Sediment	1	25.57	41.58	55.89	42.27	51.16	55.90	0.019
	2	9.54	11.23	19.76	25.43	28.12	32.7	0.019
Epilithon	1	26.22	46.87	55.82	42.85	51.74	55.84	0.019
	2	12.22	13.95	27.27	25.47	27.90	32.28	0.019

TABLE 4. Pearson's correlation coefficients of tRFLP fragments, with taxonomic affiliations and ordination axis for each fragment

Environment	tRFLP (bp)	Axis	<i>r</i>	Taxonomic affiliation of representative clones ^a
Sediment	194	1	0.81	WCC54D-D5 (<i>Betaproteobacteria</i>) (Su)
	200	1	0.78	WCC77B-B3 (<i>Gammaproteobacteria</i>) (Su)
	315	1	0.81	WCC77D-E2 (<i>Comamonadaceae</i>) (Su)
	327	1	-0.80	WCC7B-G12 (<i>N. moscoviensis</i>) (W)
	204	2	0.74	WCC77C-C12 (<i>Firmicutes</i>) (W, Sp)
	213	2	-0.73	NC ^b (W, Sp)
	256	2	0.76	WCC77B-C1 (<i>Gammaproteobacteria</i>) (W, Sp)
Epilithon	190	1	0.78	NC
	216	1	-0.76	WCC54F-G07 (<i>Burkholderiaceae</i>) (Sp, F)
	293	1	0.82	WCC54F-H08 (cyanobacteria) (Su)
	315	1	0.79	WCC54F-A08 (<i>R. ferrireducens</i>) (Su)
	375	1	0.78	WCC54F-E08 (<i>Navicula salincola</i>) (Su)
	400	1	0.80	NC
	226	2	0.90	WCC54F-C06 (cyanobacteria) (F)

^a Su, summer; W, winter; Sp, spring; F, fall.

^b NC, no clone was recovered for the fragment.

2 and 3) is consistent with other studies of temperate ecosystems showing that changes in productivity (50) and community structure (61) are associated with temperature. In general, such correlations are observed when water temperatures remain below 20°C (49). The relationship between activity and temperature diminishes when temperatures rise above 20°C, possibly due to a shift towards more thermotolerant organisms or when other factors, such as substrate concentration, become limiting (51). This trend was also observed in past studies of White Clay Creek, where sediment and epilithic secondary productivities correlated positively with temperature at water temperatures below 20°C (30).

The correlation between DOC concentration and the community structure of the epilithic, but not the sediment, community suggests that sources of organic carbon supporting secondary productivity partition differently between these two streambed habitats (42, 54, 56). While the epilithic populations are generally limited to dissolved carbon in the bulk flow and endogenous carbon (DOC and POC derived from the biofilm), the sediment populations likely obtain additional resources from entrained POC and DOC upwelling from the hyporheic zone (5, 10, 30, 54). The appearance of both prokaryotic and eukaryotic primary producers in the epilithon during the summer months suggests a seasonal shift towards autochthonous carbon sources within this biofilm, as is also supported by prior estimates of autochthonous primary productivity in White Clay Creek (31). This shift would account for the seasonal appearance of heterotrophic populations in the epilithon that are negatively correlated with the bulk summer flow of DOC (Fig. 2). In contrast to the summer, there was a positive correlation between epilithic populations and bulk DOC during the spring/fall. This is the season of highest water column DOC concentrations, associated with peak litter fall and periods of vernal algal blooms prior to canopy closure (Fig. 2). The response of the epilithic microbial community to these seasonal DOC/POC inputs is evident in the spring/fall development of *Burkholderiaceae* (peak 216) (Fig. 2; Table 2) and is consistent with previous observations of *Burkholderiaceae* associated with decomposing leaf litter (24).

In contrast to the epilithic community, stream DOC did not

covary with the seasonal sediment tRFLP patterns (Fig. 3), indicating that metabolism by sediment bacteria is not directly linked to autochthonous primary production. For example, peak 204, which corresponds to a sequence related to an organoheterotroph isolated from oak leaves (*Dendrosporobacter quercicolus*) (59), was seasonally important in the spring/winter sediment community (Fig. 3; Table 4). Previous studies of White Clay Creek have shown that microbial production in the epilithic and sediment systems responds differently to seasonal fluctuations in DOC (30). Therefore, although up to half of the heterotrophic respiration in temperate stream sediments can be fueled by POC (54), seasonal variation in the flux of DOC from the hyporheic zone may be an important controlling variable (4).

The nitrogen cycle in headwater streams, as revealed by the distribution of nitrifiers, is also influenced by the dynamics of the terrestrial ecosystem (13). Seasonal changes in factors that influence nitrite oxidation and the distribution of nitrite oxidizers in sediments include ammonia concentrations, oxygen, and temperature (2, 3, 64). In White Clay Creek, a *Nitrospira*-like population (peak 327) was detected in the winter/spring cluster in the upper layers of the sediment (Fig. 3). While *Nitrospira*-like 16S rRNA gene sequences have been recovered from a variety of environments, including riverine sediments (45), this is the first indication of a seasonal distribution of sequences related to putative nitrite-oxidizing bacteria from a stream.

In conclusion, our data support an emerging picture in lotic ecology of stable seasonal oscillations in the community structures of two key stream habitats, the epilithon and sediment systems. Comparative analysis of 16S rRNA gene sequences suggests that the microorganisms resident in those two habitats are composed of a mixture of populations provisionally attributable to soil and aquatic environments. We do not know whether the terrestrial species are active components of the streambed community, contributing to nutrient cycling and the processing of organic matter. However, since much of the carbon and energy entering headwater streams is derived from plants in the terrestrial environment, it is reasonable to suggest that soil-derived species have the metabolic capabilities

needed to degrade organic carbon in the stream and that the streambed microbial communities are structured, in part, by the chemical signature generated from the specific plant communities associated with the terrestrial environment. The incorporation of active bacteria derived from soils into streambed microbial communities would provide a biological link between terrestrial and aquatic ecosystems that complements the hydrologic, chemical, and detrital links that we know are important to stream ecosystem structure and function (27, 58).

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