9-2007

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Citation Details

Noah Fierer, Jennifer L. Morse, Sean T. Berthrong, Emily S. Bernhardt, and Robert B. Jackson 2007. Environmental Controls on the Landscape-Scale Biogeography of Stream Bacterial Communities. Ecology 2162. http://dx.doi.org/10.1890/06-1746.1

Ecology, 88(9), 2007, pp. 2162–2173

ENVIRONMENTAL CONTROLS ON THE LANDSCAPE-SCALE BIOGEOGRAPHY OF STREAM BACTERIAL COMMUNITIES

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Abstract. We determined the biogeographical distributions of stream bacteria and the biogeochemical factors that best explained heterogeneity for 23 locations within the Hubbard Brook watershed, a 3000-ha forested watershed in New Hampshire, USA. Our goal was to assess the factor, or set of factors, responsible for generating the biogeographical patterns exhibited by microorganisms at the landscape scale. We used DNA fingerprinting to characterize bacteria inhabiting fine benthic organic matter (FBOM) because of their important influence on stream nutrient dynamics. Across the watershed, streams of similar pH had similar FBOM bacterial communities. Streamwater pH was the single variable most strongly correlated with the relative distance between communities (Spearman's $\rho = 0.66$, $P < 0.001$) although there were other contributing factors, including the quality of the fine benthic organic matter and the amount of dissolved organic carbon and nitrogen in the stream water ($P < 0.05$ for each). There was no evidence of an effect of geographic distance on bacterial community composition, suggesting that dispersal limitation has little influence on the observed biogeographical patterns in streams across this landscape. Cloning and sequencing of small-subunit rRNA genes confirmed the DNA fingerprinting results and revealed strong shifts among bacterial groups along the pH gradient. With an increase in streamwater pH, the abundance of acidobacteria in the FBOM bacterial community decreased (from 71% to 38%), and the abundance of proteobacteria increased (from 11% to 47%). Together these results suggest that microorganisms, like "macro"-organisms, do exhibit biogeographical patterns at the landscape scale and that these patterns may be predictable based on biogeochemical factors.

Key words: 16S rRNA genes; bacteria; Hubbard Brook, New Hampshire, USA; microbial biogeography; microbial diversity; pH; stream benthic organic matter; terminal-restriction fragment length polymorphism; T-RFLP.

Introduction

For centuries scientists have known that microorganisms are abundant and ubiquitous on Earth. More recently we have learned that many natural environments host a remarkable diversity of microorganisms, the vast majority of which have not been cultivated in the laboratory (Floyd et al. 2005). With molecular methods of microbial community analysis, we are now able to survey the full extent of microbial diversity in individual environments and we can study microbial biogeography, the distribution of this microbial diversity across space. Microorganisms often exhibit predictable biogeographical patterns (Hughes-Martiny et al. 2006), but these patterns are not necessarily the same as those observed in better-studied taxa such as plants and animals (Fierer and Jackson 2006).

Manuscript received 18 October 2006; revised 8 February 2007; accepted 23 February 2007. Corresponding Editor: S. Findlay.

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In the early 20th century, microbial biogeography was summarized by the statement "everything is everywhere, the environment selects," also known as the "Baas-Becking hypothesis" (Beijerinck 1913, Baas-Becking 1934). We now know that microbial communities can exhibit spatial variability at scales ranging from millimeters to thousands of kilometers (Hughes-Martiny et al. 2006), and we know that a large number of biotic and abiotic environmental characteristics are capable of influencing microbial community composition in natural environments (Horner-Devine et al. 2004*b*). However, we do not know if "everything" really is "everywhere" nor do we know the factor, or set of factors, responsible for generating the biogeographical patterns exhibited by microorganisms. In other words, we often lack specific information on which aspect of "the environment" is responsible for the spatial patterns.

For this study, we focused on the bacterial communities inhabiting fine benthic organic matter (FBOM) in headwater streams of the Hubbard Brook watershed (New Hampshire, USA), a site that provides large gradients in biogeochemical properties. We have chosen

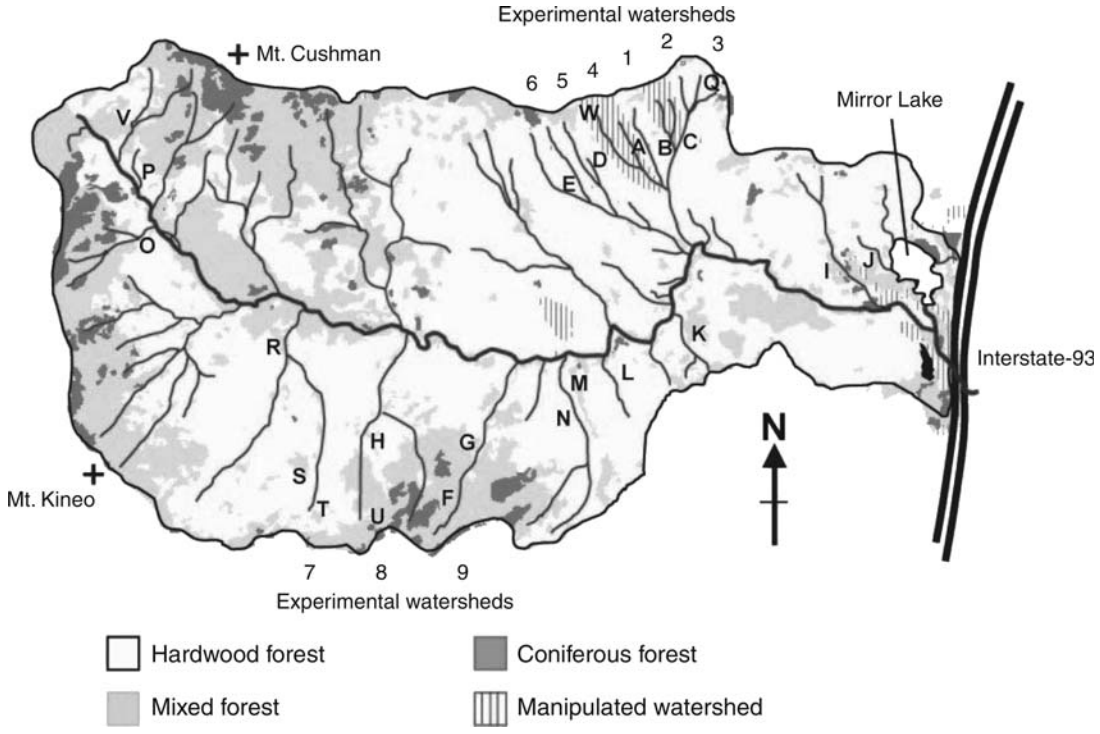

FIG. 1. Map of the Hubbard Brook watershed (New Hampshire, USA) with the sampling sites indicated (map adapted from figures presented in Likens and Buso [2006]).

to focus on FBOM communities because FBOM is the primary reservoir of organic nutrients (C, N, and P) in forested headwater streams and because the microbial processing of FBOM largely controls the carbon and nutrient budgets within these streams (Sinsabaugh et al. 1992, Webster and Meyer 1997, Bonin et al. 2000). Although fungi are the dominant microorganisms on submerged wood and leaves (Baldy et al. 1995, Hieber and Gessner 2002, Gulis and Suberkropp 2003), bacteria are the most abundant microorganisms on FBOM, and bacteria are likely to mediate the release of soluble nutrients from FBOM into the stream channel (Ellis et al. 1998, Findlay et al. 2002). Previous studies of microbial communities on benthic organic matter have largely focused on total bacterial or fungal biomass levels and general microbial processes (Sinsabaugh et al. 1992, Bonin et al. 2000, Findlay et al. 2002, Gulis and Suberkropp 2003, Stelzer et al. 2003). Only a few studies (e.g., Fazi et al. 2005) have directly examined the structure and composition of FBOM bacterial communities. The importance of "macro"-bial biodiversity in the functioning of benthic ecosystems is well recognized (Covich et al. 2004, Solan et al. 2004) and we expect that the same holds true for microbial biodiversity. If this is the case, the characterization of FBOM microbial communities is likely to enhance our understanding of within-stream heterotrophic activity and its effects on stream metabolism.

We assess FBOM bacterial communities in forested streams, describe the landscape-level spatial heterogeneity in these communities, and identify the environmental factor, or factors, that best explain the observed biogeographical patterns. From previous research on aquatic microbial communities, we expected stream-water chemistry and the characteristics of the organic-matter inputs to have the most important influence on the composition of FBOM bacterial communities (van Hannen et al. 1999, Crump et al. 2003, Eiler et al. 2003, Fazi et al. 2005, Yannarell and Triplett 2005, Findlay and Sinsabaugh 2006, Judd et al. 2006, Mille-Lindblom et al. 2006). For this reason, we chose to conduct this study at Hubbard Brook, a well-characterized watershed that is relatively small (~3000 ha), but contains streams with a range of water chemistries receiving different types and quantities of organic matter (Likens and Bormann 1995, Likens and Buso 2006). We combined several molecular approaches to survey FBOM bacterial communities throughout the Hubbard Brook drainage and measured streamwater and FBOM chemistry to examine the watershed-scale biogeography of FBOM bacterial communities and its likely causes.

METHODS

Sample collection

We collected fine benthic organic matter (FBOM) from 23 sites distributed across 17 streams within the Hubbard Brook watershed (Fig. 1) located in the White

TABLE 1. Stream and fine benthic organic matter (FBOM) characteristics at each of the sampling sites.

Sampling site†	Site elevation (m a.s.l.)‡	FBOM§		C mineralization rate ($\mu g\ C\text{-}CO_2 \cdot g^{-1} \cdot hr^{-1}$)	Microbial biomass ($\mu g\ C\text{-}CO_2 \cdot g^{-1} \cdot hr^{-1}$)||
		%N	%C		
A	506	0.18 (0.01)	3.3 (0.16)	1.5 (0.22)	26 (2.0)
B	534	0.25 (0.02)	4.2 (0.32)	2.0 (0.08)	63 (4.4)
C	555	0.34 (0.02)	6.5 (0.29)	2.5 (0.46)	67 (17)
D	474	0.14 (0.02)	3.0 (0.44)	1.9 (0.04)	35 (3.5)
E	573	0.30 (0.01)	5.5 (0.40)	2.3 (0.40)	52 (6.7)
F	820	0.81 (0.03)	14 (0.85)	3.7 (0.22)	46 (4.4)
G	485	0.16 (0.01)	3.0 (0.07)	1.2 (0.09)	14 (0.33)
H	602	0.22 (0.01)	4.9 (0.35)	2.2 (0.24)	22 (0.59)
I	285	0.23 (0.01)	4.7 (0.24)	4.2 (0.39)	44 (14)
J	270	0.30 (0.03)	5.7 (0.58)	3.2 (0.42)	33 (2.9)
K	383	0.13 (0.02)	2.5 (0.31)	0.90 (0.13)	12 (2.1)
L	389	0.20 (0.02)	3.9 (0.27)	2.6 (0.12)	22 (2.2)
M	389	0.21 (0.01)	3.5 (0.08)	1.4 (0.16)	22 (6.5)
N	561	0.30 (0.07)	5.9 (1.6)	1.7 (0.55)	51 (12)
O	569	0.24 (0.01)	5.2 (0.29)	2.0 (0.08)	33 (3.7)
P	537	0.21 (0.01)	3.7 (0.02)	1.3 (0.04)	17 (4.4)
Q	555	0.44 (0.01)	7.0 (0.22)	1.2 (0.39)	36 (5.8)
R	596	0.14 (0.01)	2.7 (0.22)	1.4 (0.19)	25 (4.9)
S	760	0.68 (0.05)	13 (1.4)	2.4 (0.24)	55 (4.2)
T	791	0.53 (0.05)	11 (1.1)	2.0 (0.26)	49 (12)
U	851	0.29 (0.01)	6.1 (0.18)	1.5 (0.16)	28 (2.1)
V	688	0.38 (0.02)	7.0 (0.5)	1.9 (0.14)	41 (6.9)
W	653	0.35 (0.03)	5.4 (0.53)	2.1 (0.06)	36 (4.9)

Note: Standard errors are indicated in parentheses.
† Site letters correspond to the site letters on Fig. 1.
‡ Meters above sea level.
§ All FBOM measurements are expressed per gram dry FBOM.
|| Microbial biomass was measured using the substrate induced respiration technique; hence, biomass is reported in terms of respiration rates.

Mountains of central New Hampshire, USA. Sites were selected to span a broad range of characteristics including streamwater chemistries, elevations, and catchment vegetation types (see also Likens and Buso 2006). We restricted our sampling to first- or second-order streams; locations and descriptions of the sampling sites can be found in Table 1 and Fig. 1.

All samples were collected within a three-day period at the end of September 2005. At each site, we sampled FBOM from depositional areas in the stream channel by collecting the top 3 cm of the FBOM using a plastic corer 3 cm in diameter. We collected 20–30 cores within a single 25-m reach of stream at each sampling site. Undecomposed leaves, wood, and other types of large organic detritus were uncommon in the FBOM samples and, when found, were manually removed from the samples. Samples were stored in sterile Whirlpak bags (Nasco, Fort Atkinson, Wisconsin, USA) and kept on ice during transport back to the laboratory. All cores from a site were homogenized together, and subsamples were stored at −80°C and 4°C for different analyses. A water sample (125 mL) was collected at the top of each stream reach at the time of FBOM sample collection. The streamwater sample was filtered on site through a Whatman GF/F filter for subsequent analysis of pH, dissolved organic carbon (DOC), total dissolved nitrogen (TDN), and dissolved inorganic ions (ammonium, nitrate, and phosphate).

Analyses of FBOM and streamwater samples

All FBOM analyses were conducted in triplicate within 10 days of sample collection and all samples were stored at 4°C prior to analysis. Total carbon and nitrogen concentrations of FBOM samples were determined using a Carlo Erba Elantech Model NC2100 elemental analyzer (ThermoQuest Italia, Milan, Italy) with combustion at 900°C after drying samples for 48 h at 110°C. Extractable NH_4^+ and NO_3^- concentrations were measured by extracting FBOM samples with 2mol/L KCl, filtering through KCl-rinsed Whatman number-1 filters, and analyzing the extracts on a Lachat QuikChem 8500 flow-injection analyzer (Hach Company, Loveland, Colorado, USA). We measured C mineralization rates (abbreviated as C_{min} rates) over a 10-d period in order to compare the potential rates of microbial activity between samples. FBOM samples (4–5 g wet mass) were adjusted to 3 g H_2O/g dry FBOM and incubated in 20-mL borosilicate-glass vials equipped with 0.125-cm Teflon-silicone septa at 22°C in a dark growth chamber. In order to avoid anaerobiosis, samples were left uncapped, and air was constantly circulated in the chamber. CO_2 production rates were estimated by conducting a series of 24-h sealed, static incubations at regular intervals during the 10-d period. Headspace CO_2 concentrations were measured with a LI-COR 6262 CO_2 gas analyzer (LI-COR, Lincoln, Nebraska, USA) with a 1-mL sample loop and a manual

TABLE 1. Extended.

Streamwater		
pH	Dissolved organic C (mg C/L)	Total dissolved N (mg N/L)
5.9	2.0	0.13
5.5	1.5	0.15
5.9	2.0	0.10
5.9	1.6	0.16
5.3	1.8	0.17
4.0	19	0.35
4.4	9.9	0.23
5.6	4.4	0.22
6.0	3.9	0.19
5.2	4.1	0.13
6.2	3.1	0.16
6.1	2.8	0.16
5.3	2.3	0.12
5.1	4.8	0.12
5.0	3.8	0.18
5.6	2.4	0.10
4.3	11	0.38
6.3	2.7	0.11
5.3	2.0	0.08
4.9	2.2	0.16
4.3	12	0.43
4.8	4.2	0.16
5.2	2.2	0.15

injection valve, using CO_2-free air as the carrier gas. During the course of the incubation, headspace CO_2 concentrations never exceeded 2%. Microbial biomass was measured using the substrate induced-respiration method described in Fierer et al. (2003). Briefly, FBOM samples were amended with an autolyzed yeast-extract solution and CO_2 production rates were measured over a 4-h period. Since the substrate supply is essentially unlimited and the incubations are of short duration, the amount of microbial biomass in the FBOM samples should be directly related to the measured rates of CO_2 production (Stenstrom et al. 1998).

Streamwater pH was measured in the laboratory on a Thermo Electron Orion 250Aplus portable pH meter (ThermoElectron Corporation, Waltham, Massachusetts, USA). Dissolved organic carbon (DOC) and total dissolved nitrogen concentrations in the streamwater samples were measured on a Shimadzu TOC-V total carbon analyzer with a TNM-1 nitrogen module (Shimadzu Scientific Instruments, Columbia, Maryland, USA). Streamwater NO_3^- and PO_4^{3-} concentrations were measured using a Dionex ICS-2000 ion chromatograph with an AS-18 column (Dionex Corporation, Sunnyvale, California, USA). PO_4^{3-} concentrations are not reported here because they were below the limit of detection in all stream samples. Streamwater NH_4^+ concentrations were determined fluorometrically using a Turner Designs 10-AU field fluorometer with NH_4^+ optical kit (Turner Designs, Sunnyvale, California, USA) following the method described by Holmes et al. (1999).

Microbial community analyses

All FBOM samples were stored at −80°C prior to DNA extraction. DNA was extracted from triplicate 2–3 g (wet mass) subsamples of each FBOM sample using the MoBio PowerSoil DNA extraction kit (MO BIO Laboratories, Carlsbad, California, USA). DNA concentrations were quantified by PicoGreen fluorometry (Invitrogen, Carlsbad, California, USA).

In order to compare bacterial communities from all 23 FBOM samples, we analyzed terminal restriction fragment length polymorphisms (T-RFLPs) from amplified bacterial 16S rRNA genes. When applied to complex communities, this fingerprinting approach underestimates total diversity and does not permit the phylogenetic identification of specific bacteria because species can share similar phylotypes (restriction fragments) and only a limited number of bands can be resolved per gel (Dunbar et al. 2001). However, the technique is appropriate for comparing overall levels of similarity between bacterial communities (Liu et al. 1997, Lukow et al. 2000, Osborn et al. 2000, Fierer and Jackson 2006).

The T-RFLP procedure followed the protocol described in Fierer and Jackson (2006). Briefly, we amplified bacterial 16S rRNA genes with the universal bacterial primer set Bac8f (5′-AGAGTTTGATCCTGGCTCAG-3′, HEX labeled) and Univ1492r (5′-GGTTACCTTGTTACGACTT-3′) (Reysenbach and Pace 1995) using the HotStarTaq Master Mix (Qiagen, Valencia, California, USA). Each of the 35 PCR (polymerase chain reaction) cycles consisted of 60 s at 94°C, 30 s at 50°C, and 60 s at 72°C. Triplicate DNA subsamples were amplified independently (four 50-μL reactions per subsample) and the amplicons from all 12 reactions were pooled together and purified using a Qiaquick PCR purification kit (Qiagen). After size verification by agarose gel electrophoresis, PCR products were digested in separate reactions using *Hha*I and *Rsa*I restriction enzymes (New England Biolabs, Ipswich, Massachusetts, USA) with fragments separated by electrophoresis on an ABI Prism 3100 genetic analyzer using GeneScan analysis software (Applied Biosystems, Foster City, California, USA). The analysis and standardization of the T-RFLP profiles was conducted as described in Dunbar et al. (2001). Only those restriction fragments in a particular sequencing run between 50 and 600 bp in length that had a standardized fluorescence >1% of the total fluorescence for that sample were included in the analyses. Any fragment unique to only one FBOM sample was not included in the analyses.

Based on the T-RFLP results, we identified three major types of FBOM bacterial communities and selected one FBOM sample that was representative of each community type for more detailed phylogenetic analyses (samples from sites E, G, and K, Fig. 1). We used a cloning and sequencing approach to characterize the bacterial communities in each of these three FBOM

samples and to identify which bacterial groups are likely to account for the differences between the three major categories of FBOM bacterial communities. For each of the three FBOM samples, triplicate DNA subsamples were amplified (four 25-μL reactions per subsample) using the primers and PCR conditions described above. Amplicons from each sample were pooled together, cleaned using the Qiaquick PCR purification kit (Qiagen), and one clone library was constructed per sample using the TOPO TA Cloning for Sequencing kit (Invitrogen). Clones were picked and unidirectionally sequenced following standard protocols (Agencourt Bioscience Corporation, Beverly, Massachusetts, USA).

Sequences were trimmed at conserved motifs, screened for chimeras with the Ribosomal Database Project ChimeraCheck program (*available online*),[6] and aligned against the Greengenes database (DeSantis et al. 2006*b*) using the NAST aligner (DeSantis et al. 2006*a*). Sequences were classified into taxonomic groups using the Greengenes "classify" utility (*available online*).[7] Only those sequences sharing >85% similarity to reference sequences over a 500 bp (base-pair) region were classified. We used the phylogenetic test (Martin 2002) with pairwise neighbor-joining trees constructed in PAUP version 4.0 (Swofford 2002) to determine if the three bacterial communities were significantly different from one another based on the clone-library results. Distances between communities (D, where $D = 0$ indicates that two communities are identical) were estimated by measuring the pairwise minimum evolution scores between phylogenies (Nemergut et al. 2005). To compare bacterial richness and evenness qualitatively between communities, we used FastGroupII (Yu et al. 2006) to perform rarefaction analyses with libraries dereplicated at the ≥97% sequence similarity level (the "species" level as per convention (Rossello-Mora and Amann 2001).

Statistical analyses

All multivariate analyses were conducted using PRIMER version 5 (Primer-E 2005). For ordination of the T-RFLP data, we used a nonmetric multidimensional scaling (NMDS) approach with phylotype length and log-transformed proportional abundance from both restriction enzymes as the input data. Our results were very similar if we used presence–absence data instead of the proportional abundances of phylotypes (data not shown). The NMDS ordination technique is well-suited for T-RFLP data since it makes few assumptions about the form of the data (Rees et al. 2004). Similarities between samples were calculated using the Bray-Curtis distance metric (Legendre and Legendre 1998), which has been recommended for T-RFLP data sets (Rees et al. 2004). We performed Mantel tests (the RELATE or BIO-ENV routines in PRIMER [Primer-E 2005]) to determine which sample or site characteristics were most closely associated with the patterns of similarity between bacterial communities (Clarke and Warwick 2001). All Mantel tests were conducted with Monte Carlo tests (1000 randomized runs) to determine significance with a Pearson correction for multiple comparisons. For the environmental variables included in the Mantel tests (with the exception of geographic distance), similarity matrices were calculated from normalized Euclidean distances (Clarke and Warwick 2001). We used partial Mantel tests to examine the influence of geographic distance (straight-line distance between sampling sites) and stream distance (estimated distance along a stream channel) on community composition with variation in environmental attributes held constant. To examine correlations between individual environmental characteristics and diversity indices we used SYSTAT (Systat Software 2004).

Results

Streamwater and FBOM characteristics

Sampling sites encompassed a wide range of stream and FBOM (fine benthic organic matter) characteristics across the 3000-ha watershed, although the streams were all fairly oligotrophic and acidic. Streamwater pHs ranged from 4 to 6.3, streamwater DOC (dissolved organic carbon) concentrations ranged from 1.5 to 19 mg/L, with FBOM C mineralization rates and microbial biomass varying by factors of 4 and 6, respectively, across all samples (Table 1). Sites at higher elevations generally had higher organic-carbon concentrations in FBOM than sites at lower elevations (Table 1) but FBOM C:N ratios were relatively invariant across the sites (19 ± 2 [mean ± SE]). Streamwater DOC and dissolved organic nitrogen (DON) concentrations were strongly correlated (Table 2) and the majority of the dissolved N was organic with inorganic N accounting for only 10–20% of the total dissolved nitrogen (data not shown). Streamwater pH was significantly correlated with streamwater DOC and DON concentrations (Table 2); those sites with pH <4.5 (sites F, G, Q, and U) had particularly high DOC and total dissolved nitrogen (TDN) concentrations (Table 1). Likewise, streamwater pH was inversely related to FBOM C and N concentrations, but these correlations were weak and not statistically significant (Table 2). Mantel tests show that the geographic distance between sites was not significantly correlated with the degree of similarity in any of the measured environmental variables listed in Table 2 (Spearman's $\rho = -0.2$ to 0.1, $P > 0.3$ in all cases).

Comparison of FBOM bacterial communities with T-RFLP

Based on the T-RFLP (terminal-restriction fragment length polymorphism) analysis of similarity, the 23 FBOM communities fell into at least three general clusters (Fig. 2). These three clusters were evident when between-sample differences in bacterial community

[6] ⟨http://rdp.cme.msu.edu⟩
[7] ⟨http://greengenes.lbl.gov/cgi-bin/nph-classify.cgi⟩

TABLE 2. Correlations (Pearson's r) between the 10 measured fine benthic organic matter (FBOM) and streamwater characteristics across all 23 sites.

Streamwater characteristics	Stream-water pH	Site elevation	$\%N^{lt}$	$\%C^{lt}$	C:N	C_{min} rate	Microbial biomass	Extractable $N_{inorganic}$	Streamwater DOC^{lt}	Streamwater DON^{lt}
Site elevation	−0.56									
$\%N^{lt}$	−0.63	0.62								
$\%C^{lt}$	−0.60	**0.65**$^{0.08}$	**−0.98**$^{<0.01}$							
C:N	0.19	0.06	−0.21	0.00						
C_{min} rate	−0.01	−0.10	0.43	0.47	0.17					
Microbial biomass	−0.11	0.31	0.62	0.62	−0.07	0.45				
Extractable $N_{inorganic}$	0.14	−0.10	0.37	0.35	−0.15	0.52	0.40			
Streamwater DOC^{lt}	**−0.73**$^{0.01}$	0.27	0.30	0.30	−0.01	0.12	−0.23	−0.26		
Streamwater DON^{lt}	**−0.67**$^{0.05}$	0.26	0.29	0.25	−0.05	0.09	−0.12	−0.28	**0.88**$^{<0.01}$	
Streamwater DIN	−0.36	0.54	0.04	0.10	0.38	−0.13	−0.11	−0.20	0.33	0.41

Notes: DOC, DON, DIN, and pH measurements were measured on streamwater samples collected at the sampling site; all other characteristics were measured on the collected FBOM samples. Variables with superscript "lt" were log-transformed prior to determining correlations. Values in boldface type indicate correlations with Bonferonni-corrected probabilities <0.1, with P values indicated in superscript. Abbreviations: C_{min} rate, C mineralization rate measured with incubated FBOM samples; DOC, dissolved organic carbon; DON, dissolved organic nitrogen; DIN, dissolved inorganic N ($NH_4^+ + NO_3^-$).

composition were visualized by either NMDS ordination (Fig. 2) or hierarchical clustering (results not shown). Using an analysis of similarity procedure (ANOSIM in PRIMER [Clarke and Warwick 2001]), we observed that the three clusters of samples shown in Fig. 2 are significantly different from one another (Global $R = 0.92$; $P < 0.02$ for all three pairwise comparisons). The three clusters closely correspond to groups of FBOM samples from sites with similar streamwater pH values. However, because pH is correlated with other variables (Table 2), streamwater pH could be either directly or indirectly related to the observed clustering.

We used Mantel tests (see *Methods: Statistical analyses*, above) to determine which of the measured FBOM, streamwater, or site characteristics had the most important influence on patterns of bacterial community composition. The qualitative observation that streamwater pH was closely related to the structure of FBOM bacterial communities (see Fig. 2) was confirmed by the

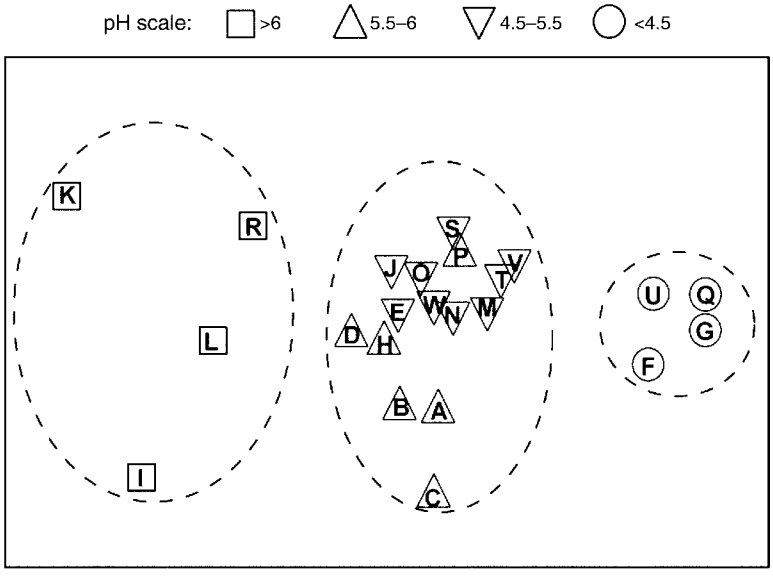

FIG. 2. NMDS (nonmetric multidimensional scaling) ordination of FBOM (fine benthic organic matter) bacterial communities; letters correspond to the sampling sites listed in Table 1 and Fig. 1. Points that are close together represent FBOM samples with similar bacterial community composition (based on the T-RFLP [terminal restriction fragment length polymorphism] method). Each symbol shape represents samples from sites with similar streamwater pH, and the dashed ovals show the three clusters of bacterial community types (see *Results: Comparison of FBOM bacterial communities with T-RFLP*). The stress value of this ordination is 0.1, which indicates that the ordination provides a good representation of the overall structure of the data set (Clarke and Warwick 2001). No scales are shown on the axes because there are no meaningful absolute units for the axes of MDS plots (Clarke and Warwick 2001); the relative distances between points represent the rank order of dissimilarities between samples.

Mantel tests, where pH was the single variable most strongly associated with bacterial community composition. As mentioned above, streamwater pH was also correlated with streamwater DON and DOC and both of these variables were also correlated with bacterial community composition, but not as strongly as streamwater pH (Table 3). FBOM C mineralization rate, a measure of potential microbial activity, was also significantly correlated with bacterial community composition. FBOM C mineralization is independent of streamwater pH (Table 2), but the consideration of both C mineralization and streamwater pH provides only a marginal improvement in the ability to predict the degree of similarity in FBOM bacterial communities (Table 3).

The number of phylotypes (restriction fragments) per sample ranged from 32 to 69 (mean: 55), but there was no significant correlation between the phylotype richness and any of the measured variables listed in Table 3 (Bonferroni-corrected $P > 0.5$ in all cases). Likewise, there was no correlation between the values for Simpson's diversity index (Magurran 2004) and any of the measured variables ($P > 0.5$ in all cases).

Comparison of FBOM bacterial communities with clone libraries

Based on the T-RFLP results, we chose one community from each general group (Fig. 2) for cloning and sequencing in order to characterize FBOM bacterial communities and identify which specific phylogenetic groups may have been responsible for the observed shifts in overall bacterial community composition. Clone libraries were constructed for samples E (pH 5.3), G (pH 4.4), and K (pH 6.2) with 75–85 clones sequenced per library. Analysis of the clone libraries using the phylogenetic (P) test (Martin 2002) indicates that the bacterial communities in samples G and K were significantly different from one another ($D = 0.74$, $P < 0.001$), but sample E was not statistically different from either sample G ($D = 0.66$, $P = 0.12$) or sample K ($D = 0.67$, $P = 0.07$). The relative differences in the distances between libraries (D) were roughly proportional to the relative distances between samples as determined by the T-RFLP analyses (Fig. 2).

The rarefaction curves generated by dereplicating libraries at the $\geq$97% sequence-similarity level were nearly identical for all three libraries (data not shown) suggesting that, at this level of phylogenetic resolution (the "species" level), bacterial community richness and evenness are similar across all three communities. Each of the communities was highly species rich; the ratio of unique species to the number of clones analyzed ranged from 0.84 to 0.86 across the three libraries. Since the rarefaction curves were nearly linear (data not shown), we know that we did not sample the full extent of bacterial species richness within each of the communities. In fact, we did not find the same species of bacteria at more than one site, suggesting that, across the entire watershed, the total species richness of bacteria inhabiting FBOM must be very high.

TABLE 3. Correlations between bacterial community composition, as measured by T-RFLP (terminal restriction fragment length polymorphism), and measured FBOM (fine benthic organic matter), streamwater, and site characteristics.

Variable†	ρ‡
Single variable	
Streamwater pH	0.66***
FBOM C mineralization rate, C_{min}	0.42*
Streamwater DON	0.41*
Streamwater DOC	0.36*
Site elevation	0.16
FBOM %C	0.09
SIR biomass	0.08
FBOM %N	0.08
FBOM extractable $N_{inorganic}$	0.06
Site location§	0.05
Streamwater DIN	−0.06
FBOM C:N ratio	−0.14
Multiple variables	
pH + C_{min}	0.72***
pH + C_{min} + DOC	0.71***
pH + C_{min} + streamwater DON	0.69***

* $P < 0.05$; *** $P < 0.001$.

† Abbreviations: DIN, dissolved inorganic nitrogen; DOC, dissolved organic carbon; DON, dissolved organic nitrogen; SIR, substrate-induced respiration.

‡ The Spearmen rank ρ values indicate rank correlation between the matrix of bacterial community similarity and the similarity matrix generated from a single variable or multiple variables together. Asterisks indicate those correlations that are statistically significant based on 1000 random permutations. We only show the ρ values for those combinations of variables that yielded ρ values greater than that of pH alone.

§ Site location represents a matrix of geographic distance between sites generated by calculating rectangular coordinates from measured latitude and longitude values for each sampling location.

Acidobacteria were the most abundant group of bacteria in the FBOM samples, accounting for 71%, 58%, and 38% of the identified clones in samples G, E, and K, respectively (Fig. 3). The FBOM Acidobacteria were not monophyletic; in each of the three samples we found clones similar to representative sequences from most of the major subdivisions of Acidobacteria (Hugenholtz et al. 1998, DeSantis et al. 2006*b*). Proteobacteria (which includes the α, β, γ, and δ subgroups) were also abundant, accounting for 11%, 18%, and 47% of the identified clones in samples G, E, and K, respectively (Fig. 3). Each of the other identified bacterial groups accounted for <6% of the clones in any given sample (Fig. 3). There were marked differences in the relative abundances of the bacterial groups between samples. In particular, sample G (the low-pH FBOM sample) had more Acidobacteria and fewer Proteobacteria than samples E or K. The β and γ subgroups of Proteobacteria accounted for 21% and 10%, respectively, of the clones in Sample K (the high-pH FBOM sample) but β-proteobacteria were absent and γ-proteobacteria were rare (only 1% relative abundance) in sample G (Fig. 3).

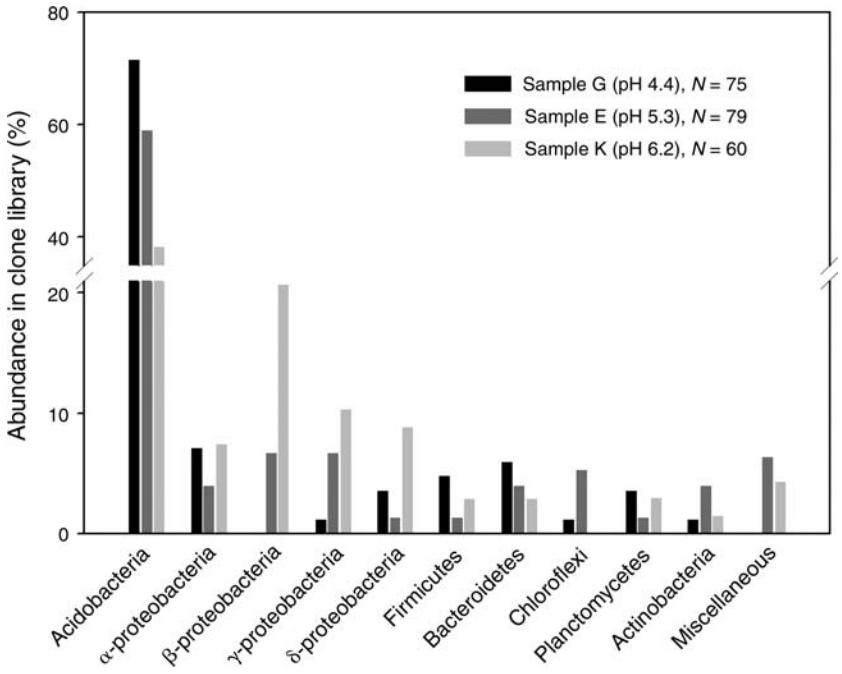

FIG. 3. Relative abundances of major groups of bacteria in each of the three samples from which clone libraries were constructed. In the key, *N* is the number of sequences included in the analyses (see *Results: Comparison of FBOM bacterial communities with T-RFLP*). The "miscellaneous" category largely consisted of clones identified as either Verrucomicrobia or Ktedobacteria.

DISCUSSION

Bacterial communities and pH

Recent literature has focused on the overwhelming complexity of microbial communities, highlighting the incredible diversity of microorganisms in individual samples and the high degree of temporal and spatial variability in microbial community composition (Morris et al. 2002, Horner-Devine et al. 2004*b*, Tringe et al. 2005). Therefore, the appearance of predictable patterns in microbial biogeography, even in an environment as heterogeneous as the stream benthos, suggests strong regulatory factors. Although bacterial communities can be influenced by a wide variety of biotic and abiotic factors, we found that a single variable, streamwater pH, could predict much of the variability in fine benthic organic matter (FBOM) bacterial community composition across the Hubbard Brook watershed. Of course, we cannot conclude that pH, as an independent variable, drives bacterial community composition. Streamwater pH can be influenced by, and is associated with, a number of factors, including watershed geology, vegetation, hydrology, and soil (Likens and Bormann 1995, Likens and Buso 2006). Stream pH may simply be one variable that effectively integrates a number of measured and unmeasured stream attributes that influence bacterial community composition. For example, we observed a significant correlation between streamwater pH and dissolved organic carbon (DOC) levels (Table 2), and previous work (Eiler et al. 2003) has shown that DOC concentrations can also have an important influence on bacterial community composition. Likewise, it is possible that bacterial communities themselves are unaffected by differences in streamwater pH, and the observed changes are a result of pH-induced changes in other stream biota or processes that may have an indirect influence on bacterial community composition. With a biogeographical study such as this, we cannot identify with certainty the mechanism(s) responsible for producing the relationship between pH and the observed biogeographical patterns.

These caveats aside, we do know that pH often has a direct influence on bacterial communities (Madigan et al. 1997) and FBOM bacterial communities are not unique in appearing to be structured by pH. A strong link between pH and aquatic microbial communities has been observed in a number of other studies (Methé and Zehr 1999, Hornstrom 2002, Lindström et al. 2005, Yannarell and Triplett 2005, Telford et al. 2006). Significant correlations between microbial community composition and pH have also been observed in non-aquatic habitats (Pennanen et al. 1998, Bååth and Anderson 2003, Kennedy et al. 2004, Fierer and Jackson 2006), suggesting that pH can often be a good predictor of bacterial community composition within a given habitat. Even though all of the sampled streams are fairly acidic (Table 1), the results presented here lend support to the universality of pH as one of the primary factors associated with, and perhaps directly causing, the biogeographical patterns exhibited by microorganisms.

The diversity and composition of FBOM communities

We know of no previous studies that have comprehensively characterized FBOM bacterial communities. However, we expected FBOM bacterial communities, like communities in other aquatic benthic environments, to be highly diverse. This hypothesis was confirmed by the clone-library results; very few species were observed more than once in a given clone library (indicating a high local, or α, diversity), no species was observed in more than one library (indicating a high landscape-level, or β, diversity), and a variety of distinct, major bacterial lineages were found in each community (Fig. 3). Without more sampling, we can not use the clone-library results to compare total levels of species richness between samples. Likewise, the T-RFLP (terminal-restriction fragment length polymorphism) method does not provide an absolute measure of bacterial diversity (Dunbar et al. 2001), but it can be used to compare relative levels of diversity between samples by looking at the number and abundances of distinct phylotypes (Liu et al. 1997, Fierer and Jackson 2006). We observed no correlations between T-RFLP diversity and any of the measured environmental variables, a finding in direct contrast to other studies, which have observed changes in overall bacterial diversity across environmental gradients (Horner-Devine et al. 2004*b*, Fierer and Jackson 2006). Either we have not measured the appropriate environmental variables, diversity is a function of a suite of interacting variables, or bacterial diversity in FBOM is driven by stochastic factors that are not directly related to environmental characteristics of the habitat.

Due to a paucity of similar studies, we cannot directly compare our clone-library results with previous results. Fazi et al. (2005) examined FBOM bacterial communities with a probe-based technique, quantifying the abundances of four different groups of bacteria (α-, β-, γ-proteobacteria, and Cytophaga-Flavobacterium). Although differences in methodologies make direct comparisons difficult, Fazi et al. (2005) also found that Proteobacteria are not the dominant group of bacteria in FBOM communities, confirming our results (Fig. 3). There are a number of lines of evidence suggesting that the FBOM bacterial communities are distinct from other stream bacterial communities that have been surveyed to date, but the streams included in our study do not represent the full spectrum of aquatic environments, as all of the streams are relatively oligotrophic and acidic (Table 1). The bacterial communities found in various stream habitats (water column, biofilms, and sediments) tend to be dominated by members of the Actinobacteria, α-proteobacteria, β-proteobacteria, and Bacteroidetes groups, with few, if any, Acidobacteria (Brummer et al. 2000, Sekiguchi et al. 2002, Zwart et al. 2002, Feris et al. 2003, Hullar et al. 2006). Acidobacteria were very abundant in the FBOM samples (Fig. 3) but Acidobacteria appear to be rare in most aquatic environments (Hugenholtz et al. 1998). In general, the Acidobacteria group is most commonly associated with soil (Kuske et al. 1997, Hugenholtz et al. 1998, Buckley and Schmidt 2002, Lipson and Schmidt 2004, Fierer et al. 2007) and the majority of acidobacterial sequences deposited in GenBank (*available online*)[8] have originated from soil samples. Based on this evidence, we can tentatively conclude that FBOM bacterial communities are distinct from the communities found in other aquatic habitats and that FBOM communities appear to be similar, at a coarse level of phylogenetic resolution, with the bacterial communities found in soil. This similarity between FBOM and soil communities may be a result of convergence, or FBOM communities may be derived, wholly or in part, from soil bacterial communities.

FBOM microbial communities and processes

Although changes in streamwater pH have been shown to influence rates of microbial decomposition within streams (Mulholland et al. 1992, Dangles et al. 2004), we observed no significant correlation between microbial CO_2 production (C mineralization rate) and pH, or any of the other individual, measured variables (Table 2). However, we did observe a significant correlation between FBOM mineralization rates and bacterial community composition (Table 3). Of course, this does not suggest that bacterial community composition is the primary factor influencing rates of microbial C mineralization. However, it does suggest that there are direct or indirect linkages between bacterial community composition and function in streams. A similar conclusion was reached by Findlay and Sinsabaugh (2006) in their study of stream biofilm communities. While microbial community composition is likely to regulate microbial processes at some level, we cannot assess the direct linkages between bacterial community structure and stream processes with observational studies and the analyses of apparent correlations. Instead, understanding the direct relationships between microbial communities and processes requires more detailed, experimental studies, such as those described in Cavigelli and Robertson (2000) and Fernandez et al. (2000).

Is "everything everywhere"?

While this study was not designed to directly test the Baas-Becking hypothesis (Beijerinck 1913, Baas-Becking 1934), our results do provide anecdotal evidence in support of the hypothesis because there was no apparent relationship between geographic distance and community similarity at the landscape scale (Table 3). Even if we controlled for the effect of environmental similarity (with a partial Mantel test), we still failed to observe a significant influence of geographic (straight-line) distance between sites on bacterial community composition ($P > 0.4$). Likewise, if we only examine pairs of samples collected at different locations within the same stream

[8] ⟨http://www.ncbi.nlm.nih.gov⟩

(Fig. 1) and control for the effect of environmental similarity (using a partial Mantel test), there was no significant correlation between stream distance (estimated distance between two sites along a stream channel) and bacterial community composition ($P > 0.5$). FBOM bacterial community composition is apparently best predicted by environmental characteristics, not geographic distance or distance along an individual stream channel.

In this landscape, dispersal rates do not seem to be an important factor regulating bacterial community composition. While we cannot estimate specific rates of microbial dispersal, our results do suggest that dispersal rates are neither very high nor very low. If dispersal rates were very high, all of the communities would be relatively similar and we would not have observed the apparent local adaptation of bacterial communities to their environmental conditions. Likewise, if dispersal rates were very low, we would expect a stronger influence of geographic location on bacterial community composition.

Green and Bohannan (2006) (see also Green et al. [2004]) have argued that microorganisms are not cosmopolitan and that geographic distance (even at regional and landscape scales) often has an important influence on microbial community composition. However, in this study, environmental factors, not distance per se, appear to be the primary drivers of bacterial community composition, a pattern also observed in stream biofilm communities (Findlay and Sinsabaugh 2006) and in bacterial communities from other environments (Horner-Devine et al. 2004*a*, Fierer and Jackson 2006). We would argue that the apparent linkages between geographic distance and community composition, as highlighted in Green and Bohannan (2006), may often be related to an inherent (but unmeasured) correlation between geographic distance and the environmental "distance" between samples. For example, Reche et al. (2005) conclude that the geographic distance between lakes has a larger influence on lake bacterial communities than the environmental attributes of the lakes. However, their conclusion was based on the measurement of only a handful of environmental characteristics (not including lake pH). Likewise, Green et al. (2004) based their conclusions on the inferred, but unmeasured, environmental characteristics of the samples included in their analysis. A correlation between geographic distance and community distance does not disprove the Baas-Becking hypothesis unless differences in the environmental attributes of the microbial habitats have been thoroughly characterized and accounted for.

Conclusions

We found that FBOM bacterial communities exhibited clear biogeographical patterns within the Hubbard Brook watershed as streams with distinct pH levels had distinct bacterial communities. Although proposed more than a century ago, Baas-Becking's hypothesis "everything is everywhere, the environment selects" (Baas-Becking 1934) remains a useful framework for studying microbial biogeography. The composition of microbial communities, like "macro"-bial communities, is not likely to be a product of random, stochastic events; if environmental characteristics are appropriately measured, the spatial distribution of bacterial communities can be predicted.

Acknowledgments

We thank Gene Likens, Don Buso, Ian Helm, and other personnel at the USDA Forest Service for providing access to the HBEF watersheds. In addition, we thank Heather Hamilton for assistance with the construction of clone libraries and Ryan Jones for help with the phylogenetic analyses. Craig Nelson and two anonymous reviewers provided valuable comments on previous drafts of this manuscript. This work was supported by grants from NSF to N. Fierer and grants from the Andrew W. Mellon Foundation and NSF to R. B. Jackson.

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