Microbial Ecology

Comparison of Bacterial Communities in New England *Sphagnum* Bogs Using Terminal Restriction Fragment Length Polymorphism (T-RFLP)

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Abstract

Wetlands are major sources of carbon dioxide, methane, and other greenhouse gases released during microbial degradation. Despite the fact that decomposition is mainly driven by bacteria and fungi, little is known about the taxonomic diversity of bacterial communities in wetlands, particularly *Sphagnum* bogs. To explore bacterial community composition, 24 bogs in Vermont and Massachusetts were censused for bacterial diversity at the surface (oxic) and 1 m (anoxic) regions. Bacterial diversity was characterized by a terminal restriction fragment length (T-RFLP) fingerprinting technique and a cloning strategy that targeted the 16S rRNA gene. T-RFLP analysis revealed a high level of diversity, and a canonical correspondence analysis demonstrated marked similarity among bogs, but consistent differences between surface and subsurface assemblages. 16S rDNA sequences derived from one of the sites showed high numbers of clones belonging to the Deltaproteobacteria group. Several other phyla were represented, as well as two Candidate Division-level taxonomic groups. These data suggest that bog microbial communities are complex, possibly stratified, and similar among multiple sites.

Introduction

Sphagnum bogs store one-third of the earth's carbon, and in New England they are responsible for 36% of methane emissions [10]. In spite of the importance of microorganisms to the decomposition of peatland vegetation [13, 21, 39, 40] and thus their effect on nutrient release, there has been little study of the taxonomic diversity of peatland microbial communities.

Bacterial numbers are relatively low in peat compared to aerated soils [30]. Bacterial density in high-latitude peatlands, including fens and bogs, peaks in spring and fall coinciding with seasonal nutrient pulses [10]. Total cell counts in bogs are rather constant (10^8–10^9 mL^{-1}), although metabolic activity can fluctuate [19, 41]. To date, bacterial diversity has been described from one *Sphagnum* bog, using only cultivation-based approaches [41]. Peatlands are known to have a wide variety of microbes capable of growing and metabolizing over a wide range of pH and growth conditions. Williams and Crawford [41] noted that bacterial genera in peatlands include *Bacillus*, *Pseudomonas*, *Achromobacter*, *Cytophaga*, *Micrococcus*, *Chromobacterium*, *Clostridium*, *Streptomyces*, *Actinomyces*, *Mycobacterium*, *Micromonospora*, and *Nocardia*. Such a diverse group of bacteria suggests a complex ecological web, with microbes specialized for many niches. Metabolic relationships, such as the one serving to maintain a controlling balance over the release of methane (methanotrophs and methanogens), have been indicated by targeted surveys [7, 10, 17]. Nevertheless, our knowledge of bacterial communities in bogs is limited to specific groups of bacteria from studies mostly targeting individual sites. To assess the ability of bogs to alter levels of greenhouse gases and to understand the microbial processes involved in them, we first need to address the lack of information on bacterial composition in this type of wetland.

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In this study, we characterized bacterial diversity within and among ombrotrophic *Sphagnum* bogs by sampling 24 New England bogs using rDNA-based techniques [terminal restriction fragment length (T-RFLP) [1, 2, 25, 26, 27] and sequencing]. Within each bog, we sampled both surface and anoxic subsurface microbial communities. Based solely on their shared oligotrophic conditions and acidic nature, we hypothesized that bacterial diversity might be relatively similar among bogs, but that surface and subsurface assemblages would be taxonomically distinct, reflecting the different abiotic conditions in these two microhabitats. Although watershed and land cover are additional variables potentially influencing community composition, the data supported the view that the studied sites are hydrologically isolated units (i.e., ombrotrophic) and that the potential variables of watershed or land cover had little or no effect on the bacterial composition.

Methods

Sample Collection. Liquid samples, with varying amounts of suspended particulate matter and therefore containing attached and unattached bacteria, were collected for bacterial enumeration and T-RFLP analysis from 24 different bogs throughout New England (Table 1) during a 2-and-1/2-week period in June of 2002. We also collected a single surface sample from Molly Bog, VT, in June of 2001 to create a clone library. These bogs have been classified elsewhere [11, 18] as acidic peatlands (mean pH = 4.97, range 4.03–6.37) with an active *Sphagnum* layer extending to a depth of 40 cm, with two of them also described as having anoxic waterlogged conditions beneath that depth [10]. Sampling locations within bogs were standardized to include areas with the highest density of Northern Pitcher Plants (*Sarracenea purpurea*) at the mat surface [18]. The presence of those carnivorous plants serve as an indicator for levels of Fe^{3+}, Na^{+}, Cl^{-}, SO_4^{-} [31], and general ombrotrophy [11]. Two samples were taken from each site, one from the top 15.24 cm of the peat mat and another from 1 m deep (representing oxic and anoxic zones, respectively). Analyses to establish the depth at which anoxic conditions are encountered in *Sphagnum* bogs were previously indicated (by sulfide and methane production, in addition to methanogen detection) at depths greater than 10 cm below the water surface [10, 16, 30, 37]. Samples were collected by inserting a closed-end, perforated pipe (1 in. diam.) into the mat. The sample was then collected using a hand pump, after flushing the length of plastic tubing with ddH_20. Samples ranged in texture and consistency from moderately humic and watery, to thick

Table 1. Location and code of all bog sites in the study

Site	*Code*	*Latitude*	*Longitude*	*Elevation (m asl)*[a]	*Bog area (m^2)*
Massachusetts					
Arcadia	AR	42.31	72.43	95	1,190
Bourne Hadley	BH	42.56	72.09	274	105,369
Black Pond	BP	42.18	70.81	45	9,679
Chokalog	CHO	42.03	71.67	152	7,422
Clayton	CLA	42.05	73.30	210	73,120
Hawley	HAW	42.58	72.89	543	36,813
Lily Pond Bog	LIL	42.44	72.84	468	56,559
Otis Bog	OT	42.23	73.06	491	89,208
Ponkapoag	PON	42.19	71.10	47	491,189
Quabbin	QU	42.42	72.22	175	6,706
Quag Pond	QP	42.57	71.96	335	40,447
Round Pond	RP	42.17	72.70	78	10,511
Snake Pond	SNP	41.41	70.61	17	
Shankpainter	SP	42.05	70.71	1	55,152
Swift River	SW	42.27	72.34	121	19,699
Lake Jones	WIN	42.69	72.00	323	84,235
Vermont					
Carmi	CAR	44.95	72.90	133	38,023
Chickering	CHI	44.32	72.48	362	38,081
Colchester	COL	44.55	73.28	30	623,284
Molly	MOL	44.50	72.64	236	8,852
Molly Soil	SOIL	44.50	72.64	236	
Moose	MOO	44.76	71.74	353	864,970
Peacham	PEA	44.29	72.24	468	576,732
Snake Mt.	SNM	44.06	73.27	313	248
Springfield	SP	43.33	72.51	158	435

[a]m asl: meters above sea level.

sludge. Surface samples tended to be less degraded and lighter in color with intact *Sphagnum* particulate, whereas 1 m samples were generally moderately to completely degraded, with a dark color, and lower water content. Subsamples (20 mL) were decanted into smaller tubes and fixed in the field with glutaraldehyde (final concentration 2.5%) for microscopic counts. A soil sample from outside the wetland, classified as a hemist histosol, was also taken at one site. All samples were kept on ice in the field, and stored at 4°C in the laboratory until DNA extraction, and all extractions were performed within 48 h of collection. Archival material was stored at −20°C.

Bacterial Abundance. We analyzed 59 samples. Four samples were not countable and were therefore omitted from the study, leaving a set of 28 surface and 27 one-meter samples. Prior to staining, we diluted fixed samples 5- to 40-fold for optimum counting The dilution was then vortexed for 1 min, sonicated on a Branson 5200 water bath sonicator for 1.5 min, and vortexed again for an additional minute. Samples were then filtered through a 100-μm (pore size) nytex membrane (Sefar America, Kansas City, MO, USA) and 1 mL was stained with 0.05% (v/v) SYBR Green II (Molecular Probes) for 15 min at 4°C. Stained preparations were then collected on 0.2-μm (pore size) black polycarbonate filters and mounted following the protocol of Fisher *et al.* [14]. Cell counts were measured at 1000× magnification on a Whipple grid using a Nikon E400 epifluorescence microscope. Ten randomly chosen fields were screened using two different filters (XF53 dual wavelength filter and XF100 from Omega Optical Inc., Brattleboro, VT, USA) to distinguish bacteria from background fluorescence.

DNA Extractions. Biomass in samples was pelleted by centrifugation in a Sorvall RC-5B centrifuge at 10,000 × *g* for 10 min. Total community DNA was extracted from 0.25 to 0.5 g of pellet material using the MoBio Ultraclean soil DNA extraction kit (MoBio, Solana Beach, CA, USA) using water in the final step. *Nitrosomonas europaea* (ATCC 19718) genomic DNA was used to produce an internal standard DNA (described below). *N. europaea* was grown on the ATCC-specified medium prior to genomic DNA extraction using a Wizard Genomic DNA purification kit (Promega, Madison, WI, USA).

PCR Amplification. Amplification of the 16S rRNA gene was performed with primers universal for the domain Bacteria: 8-27F labeled with 6′ carboxyfluorescein (6-FAM) and 1392-1407R [2] for T-RFLP, and S-D-Bact-1327 [42] for the clone library. PCR was carried out using 50 ng of DNA in a final volume of 50 μL, for 30 cycles, as described in [2]. PCR products (5 μL) were run on 0.8% agarose gels to verify amplification. Triplicate reactions were pooled, and DNA concentrated and cleaned using a QIAquick PCR purification kit (Qiagen, Valencia, CA, USA) prior to restriction digests.

An internal standard for T-RFLP analysis was made by amplifying the *amoA* gene of *N. europaea* (AMOF GGGGHTTYTACTGGTGGT and AMOR CCCCTCKGSAAAGCCTTCTTC) from genomic DNA using a tetrachloro-6-carboxy-fluorescein (TET) labeled forward primer. PCR product was cleaned as described above.

Cloning of 16S rRNA Gene. A clone library was constructed using DNA extracted from Molly Bog surface and 16S rRNA gene amplified using unlabeled forward primer. The clone library was generated by ligating PCR products into pGem-T-easy and transforming them into *E. coli* JM109 competent cells (Promega) according to the manufacturer's protocol. Transformants were picked and inoculated into a 96-well microtiter plate containing 100 μL LB with glycerol and ampicillin. The plate was incubated overnight at 37°C, then frozen on dry ice and shipped to The Institute for Genomic Research (TIGR; Rockville, MD, USA) for DNA extraction and sequencing.

Restriction Digests and T-RFLPs. Restriction digests contained 5 U of enzyme, 5 μL of labeled, purified PCR product, and 1 μL *Nitrosomonas* standard in a 20-μL total volume. Three different restriction enzymes were used separately (*Hha*I, *Hae*III, and *Msp*I). Reactions were performed at 37°C for 2 h followed by an inactivation step at 65°C for 10 min. To remove fluorescence-quenching salts, and to concentrate DNA, digests were ethanol precipitated along with Pellet Paint NF coprecipitant (Novagen, Inc., Madison, WI, USA) according to the manufacturer's protocol. Digested DNA was resuspended in 5 μL of water for fragment analysis. One microliter of each T-RFLP digest was mixed with 0.8 μL 500 bp *N*,*N*,*N*′,*N*′-tetramethyl-6-carboxyrhodamine (Tamra) standard (PE Biosystems, Foster City, CA, USA) and brought up to a 20-μL total volume in deionized formamide. Capillary electrophoresis was performed on an ABI Prism 310 Genetic Analyzer (PE Biosystems) and T-RFs were analyzed using Genescan (PE Biosystems).

TRFLP Analysis. Profiles were analyzed using Genescan and Genotyper programs (PE Biosystems). Parameters were set to exclude peaks under 50 fluorescent units and those smaller than 25 bp or larger than 600 bp. Internal *Nitrosomonas* standards for all samples were aligned using Genotyper and compared manually to assess loss during sample processing, digestion efficiency, and accuracy of size determinations. Loads were considered equal if the standard peak was 3000

(±1000) fluorescent units. Size determinations were considered accurate when within 0.5 bp of the predicted size. Profiles whose total fluorescence was below 10,000 units were rerun and aligned to verify peak reproducibility. Peak heights for most peaks were kept in the 50–300 fluorescent unit range with an average total fluorescence of 13,104 units. Profiles were exported and data were normalized to total peak height. T-RF data were exported and used to run the tRFLP Fragment Sorter Program (http://www.oardc.ohio–state.edu/trflpfragsort/default.htm) [26, 34]. The Fragment Sorter program takes into account the potential variability of T-RFs within individual taxa and matches sample data with those in the RDPII database to make assignments. This step reduced the apparent taxonomic complexity by compiling lists of three-enzyme rRNA fingerprints (ribotypes) from the T-RF peak data. Data sets for surface, 1 m, and total community were generated by combining multiple sample data and creating a three-enzyme ribotype master list with all the unique ribotypes present. Once generated, this list was used to create a presence/absence matrix for all the samples in the list.

Statistical Analysis. Data were organized as a binary presence–absence matrix, with rows representing ribotypes and columns representing sites. The entries indicate the presence (1) or absence (0) of a particular ribotype at a site. Canonical correspondence analysis (CCA) was used to analyze the presence/absence matrices created using SAS Version 8.02 Statistical Software (Cary, NC, USA). Correspondence scores were plotted by site, abbreviated as shown in Table 1, for the first two canonical dimensions.

Phylogenetic Analysis. All 16S rRNA gene sequences were imported into Sequencher Version 4.1.4 (Gene Codes, Ann Arbor, MI, USA) and contigs were created using sequences from the forward and reverse primers, eliminating primer and vector sequences. For clones whose forward and reverse sequence reads did not overlap, only the forward primer derived sequences were used. All sequences were compared to each other using the dirty assembly algorithm and realigner feature with 20% overlap and 90 minimum match percentage. Rarefaction curves were generated using Analytic Rarefaction version 1.3 (http://www.uga.edu/~strata/software/).

Fragsort, like other programs [23], uses the T-RF data to search sequences in a database [generated from the ribosomal database project (RDP)]. Although the program created a list of possible matching organisms classified to the phylum (and in many cases, genus) level, present in our samples, we compared the three-enzyme ribotypes with our clone library (Table 5). *In silico* digests were performed, using Sequencher, on all clones using all three enzymes. Generated patterns were then compared to observed TRFs found from the same site. Based on published data [22] and personal experience, we set a default gate of ±3 bp to take into consideration variations in fragment length assignments for clone library sequences. Searching was done manually, and in cases where

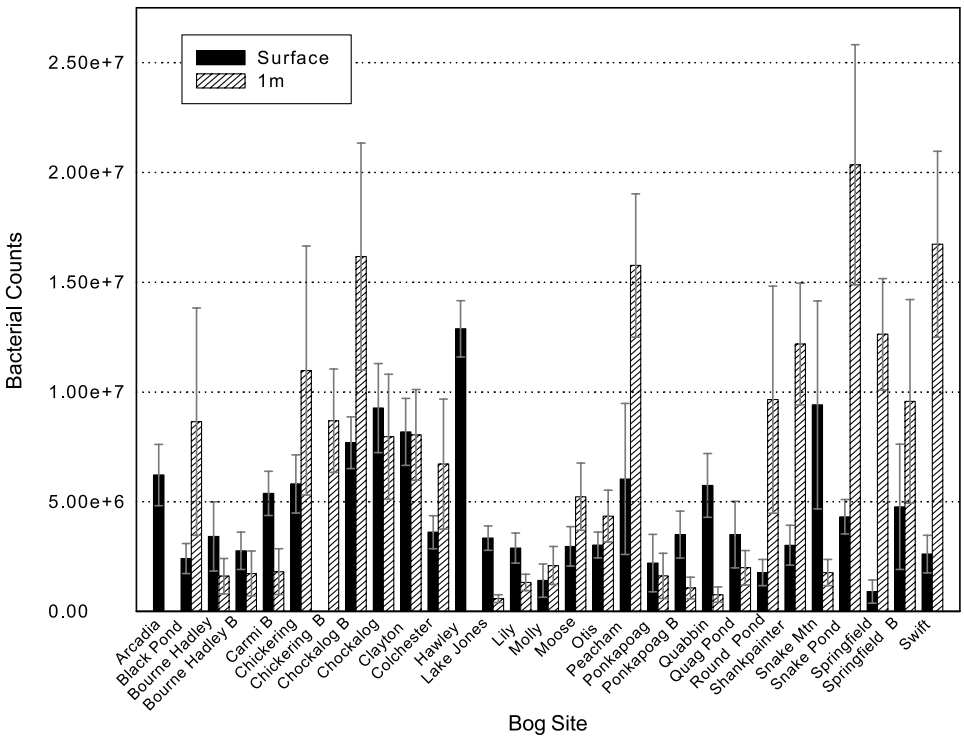

Figure 1. Bacterial abundance by site as estimated from direct counts using SYBR Green II.

Table 2. Two-way ANOVA of bacterial counts from surface and 1-m samples taken at 22 New England bogs

Source	*df*	*SS*	*MS*	*F ratio*	*P value*
Bog	21	5×10^{15}	2×20^{14}	1.172	0.360
Depth	1	9×10^{14}	9×10^{14}	4.746	0.041
Bog × Depth	21	4×10^{15}	2×10^{14}	29.926	<0.001
Error	396	2.543×10^{15}	6.42×10^{12}		
Total	439	1.222×10^{16}			

Bog and depth were random effects in the mode, and there was a significant Bog × Depth interaction.
df: degrees of freedom; SS: sum of squares; MS: mean square.

two of the enzymes fell within the ±3-bp gate and a third one fell outside by <2 bp, a wider gate was accepted.

Phylogenetic analyses were performed using a TIGR in-house pipeline (Dongying Wu, unpublished data) that automatically assigns taxonomic tags to environmental small subunit rRNA (SSU rRNA) sequences based on neighbor-joining phylogenetic trees. The 16S rDNA sequences obtained in this study were searched against the Ribosomal Database Project (RDP) rRNA database by BLASTN. Top matches, as well as selected matches within different score ranges, were aligned to the query sequence using CLUSTALW, and the alignments automatically trimmed using a Perl script based on alignment quality score for all the columns. A neighbor-joining phylogenetic tree based on the automatically trimmed alignments was constructed, and the taxonomy of the

Table 3. Taxonomic assignment for the most represented ribotypes at 1 m and surface

Ribotype fingerprint				*Percent*[a]		
HhaI[b]	*MspI*[b]	*HaeIII*[b]	*Taxonomic assignment*	*1 m*	*Surface*	*1 m+ surface*
60	148	203	Bacteria, Planctomycetes, Planctomycetacia, Planctomycetales, Planctomycetaceae, Pirellula	96	92	94
60	147	245	Bacteria, Firmicutes, Bacillales		85	75
90	159	202	Bacteria, Proteobacteria, Deltaproteobacteria, Myxococcales, Cystobacterineae, Myxococcaceae, Myxococcus		85	82
91	159	202	Bacteria, Proteobacteria, Alphaproteobacteria, environmental samples	88	85	84
60	449	201	Bacteria, Proteobacteria, environmental samples		81	
97	265	268	Bacteria, Proteobacteria, Epsilonproteobacteria, Campylobacterales, Helicobacteraceae, Helicobacter		81	75
97	266	269	Bacteria, Proteobacteria, Epsilonproteobacteria, Campylobacterales, Helicobacteraceae		81	75
60	159	202	Bacteria, Proteobacteria, Deltaproteobacteria, Myxococcales, Cystobacterineae	88	77	80
60	162	246	Bacteria, Proteobacteria, Deltaproteobacteria, environmental samples	80	77	78
60	438	192	Bacteria, Proteobacteria, Alphaproteobacteria		77	
92	161	404	Bacteria, Proteobacteria, Deltaproteobacteria, environmental samples		77	
98	267	270	Bacteria, Proteobacteria, Epsilonproteobacteria, Campylobacterales, Helicobacteraceae, Helicobacter		77	
98	268	271	Bacteria, Proteobacteria, Epsilonproteobacteria, Campylobacterales, Helicobacteraceae, Helicobacter		77	
60	160	247	Bacteria, Firmicutes, Bacillales, Bacillaceae, Virgibacillus	92		78
60	160	246	Bacteria, Firmicutes, Bacillales, Bacillaceae, Virgibacillus	84		76
90	159	202	Bacteria, Proteobacteria, Deltaproteobacteria, Myxococcales, Cystobacterineae, Myxococcaceae, Myxococcus	84		
94	163	202	Bacteria, Proteobacteria, Deltaproteobacteria, Desulfovibrionales, Desulfovibrionaceae, Desulfovibrio	80		

All samples included in the analysis.
[a] Percent was based on specific ribotypes found in individual sets.
[b] TRF in bp.

query sequence mapped to the taxonomy of the closest-branching RDP sequence in the midpoint-rooted neighbor-joining tree. This first-pass assignment was used to construct a second, higher-resolution tree using sequences related to the query sequence at the family level, and two nonfamily members were also included to serve as outgroups for tree rooting. The final taxonomic mapping of the query sequence was provided by the closest neighbor in the second tree.

Chemical Analysis. Water samples for chemical analysis were filtered in the field through glass fiber filter paper (Whatman GFF), preserved, and analyzed as follows: a subsample for Al, Ca, Cu, Fe, K, Mg, Mn, Na, Si, and Zn was acidified with nitric acid (final concentration 0.1 mol L^{-1}) and analyzed by inductively coupled plasma-atomic emission spectrometry (ICP-AES) using standard techniques; a subsample for NH_4-N and NO_3-N was preserved with sulfuric acid (to pH 2) and analyzed on a flow injection autoanalyzer using the Cd reduction method for NO_3-N and the salicylate–nitroprusside method for NH_4-N; a subsample for DOC and DON was frozen as soon as possible after sampling, and subsequently thawed and analyzed using the persulfite oxidation method; a separate subsample was frozen for subsequent analysis for SO_4 and Cl by ion chromatography, and soluble reactive P by the stannous chloride molybdate blue procedure. Plant samples were dried, ground and microwave-digested in nitric acid followed by ICP-AES determination of Ca, Mg, Fe, Cr, Cu, K, Al, Co, B, Cd, Mo, Mn, Na, Ni, P, Pb, S, and Zn. Plant C, H, and N were determined by elemental analyzer. All analyses were performed using standard methods and quality control, including blanks, duplicates, and NIST traceable standard materials.

Nucleotide Sequence Accession Numbers. The partial 16S rRNA sequences used in the analyses have been deposited in GenBank under accession numbers AY775458–AY775538.

Results

Bacterial Abundance in New England Bogs. Direct counts on fixed cells stained with SYBR green were between 10^6 and 10^7 with average counts of 4.6×10^6 for surface samples and 7.0×10^6 for 1 m samples (Fig. 1). Two-way ANOVA revealed a significant Bog × Depth interaction term (Fig. 1, Table 2), thus we analyzed each factor separately. Overall, there was significant variation in bacterial abundance across our 22 sites ($F_{21,418}$ = 12.572, $P < 0.0001$). Within a site, bacterial counts were significantly higher for 1 m samples than for surface samples (paired t test: mean difference = 2,798,847, t_{21} = 2.053, $P = 0.05$). The surface samples also had a lower overall variance than 1 m samples, which is noticeable for both grouped and individual sites (Fig. 1).

T-RFLP Analysis of Bacterial Community Similarity Among Bogs. Individual enzyme profiles for surface samples contained, on average, 55 different peaks throughout all enzymes, whereas 1 m profiles averaged 42. The three-enzyme presence/absence matrix generated from site data using the tRFLP Fragment Sorter Program identified 618 distinct three-enzyme ribotypes at 1 m, 753 at the surface, and 817 total ribotypes. There were 64 ribotypes unique to 1 m samples, 199 ribotypes unique to the surface samples, and 554 shared ribotypes. There were 148 ribotypes that were unique to only one bog

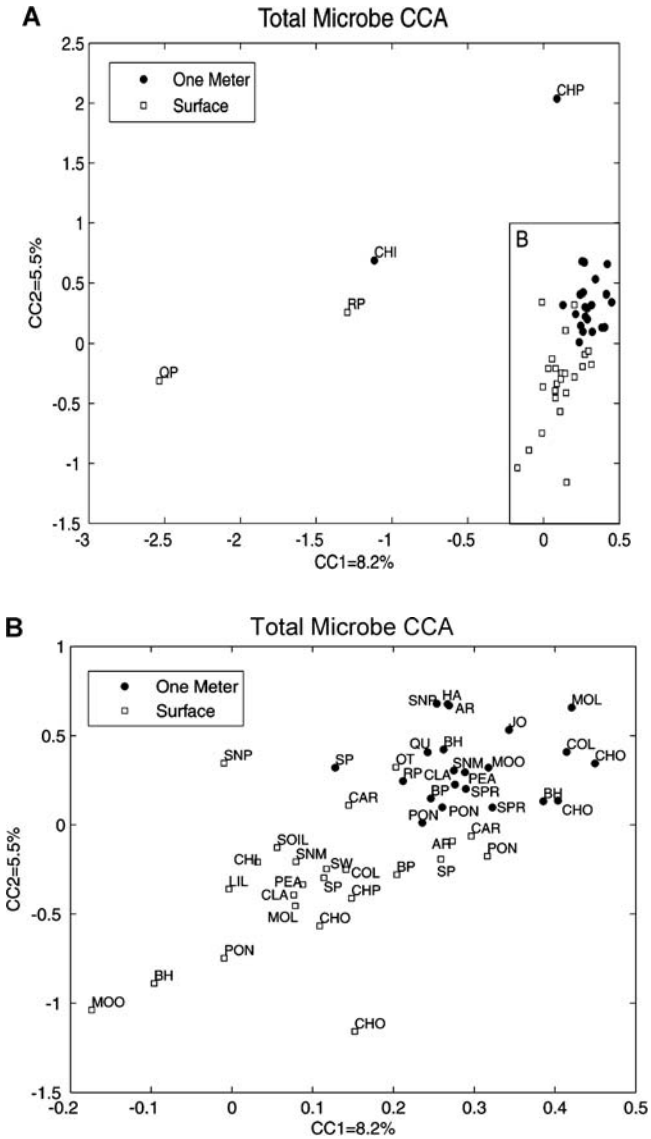

Figure 2. Canonical correspondence analysis of bog samples, based on ribotype data, showing clustering between samples of the same depth. Filled symbols represent 1 m samples, open squares represent surface samples. A. Full view. B. Detail.

location, averaging to approximately three unique ribotypes per site. In contrast, a single ribotype classified as a Planctomycetes was present in 46 of 49 (94%) samples (Table 3). Ribotypes present in more than 75% of the samples were classified based on their RDP library match (Table 3). In 1 m samples, four out of the eight most common ribotypes were assigned as Deltaproteobacteria. In surface profiles, the two major groups were Proteobacteria, with half belonging to the Delta and the other to the Epsilon subdivision. When data for both depths were analyzed together, 10 ribotypes were present in at least 75% of samples. Firmicutes and Deltaproteobacteria assignments were most frequently represented with three ribotypes each.

Statistical analysis of the ribotypes profiles showed that within the surface zone, the first two canonical dimensions (CC1 and CC2) described 19.72% of the data set variance, with a large number of sites clustering together. The close or tight clustering of all but two sites, Quag Pond (QP) and Round Pound (RP), indicates a high degree of similarity among bacterial communities in the surface samples from different bogs. The first two canonical dimensions for the 1 m samples accounted for 19.27% of the data set variance, with one outlier, Chickering Bog (CHI). The remaining sites clustered together, but were not as closely grouped as the surface samples. When all samples were analyzed together, the surface and 1 m samples constituted distinct clusters

Table 4. Taxonomic assignment of ribotypes contributing to separation of outlier sites

Ribotype fingerprint				
HhaI[a]	*MspI*[a]	*HaeIII*[a]	*Taxonomic assignment*	*Sites*
60	131	38	Bacteria, Proteobacteria, Alphaproteobacteria, environmental samples	QPs
80	437	38	Bacteria, Proteobacteria, Alphaproteobacteria, Rhodospirillales, Rhodospirillaceae, Rhodospirillum	QPs
81	162	38	Bacteria, Proteobacteria, Alphaproteobacteria, environmental samples	QPs
331	149	38	Bacteria, Proteobacteria, Alphaproteobacteria, Caulobacterales, Caulobacteraceae, Caulobacter	QPs
333	440	38	Bacteria, Proteobacteria, Alphaproteobacteria, Rhodospirillales, Rhodospirillaceae, Rhodospirillum	QPs
341	86	38	Bacteria, Proteobacteria, Alphaproteobacteria, Rhizobiales, Methylocystaceae, Terasakiella	QPs
341	448	38	Bacteria, Proteobacteria, Alphaproteobacteria, environmental samples	QPs
81	474	38	Bacteria, Bacteroidetes, Sphingobacteria, Sphingobacteriales, Flexibacteraceae, Flectobacillus	QPs
81	475	38	Bacteria, Bacteroidetes, Sphingobacteria, Sphingobacteriales, Flexibacteraceae, Flexibacter	QPs
66	487	216	Bacteria, Proteobacteria, Betaproteobacteria, Rhodocyclales, Rhodocyclaceae, Zoogloea, environmental samples	CHP1m
66	489	218	Bacteria, Proteobacteria, Betaproteobacteria, Rhodocyclales, Rhodocyclaceae, environmental samples	CHP1m
66	491	220	Bacteria, Proteobacteria, Betaproteobacteria, Burkholderiales, Oxalobacteraceae, Herbaspirillum	CHP1m
81	483	38	Bacteria, Proteobacteria, Deltaproteobacteria, Myxococcales, Nannocystinea, Nannocystaceae, Nannocystis	QPs
98	167	83	Bacteria, Proteobacteria, Deltaproteobacteria, Syntrophobacterales, Syntrophobacteraceae, Desulfacinum	CHP1m, CHI1m
149	289	199	Bacteria, Firmicutes, Clostridia, Clostridiales, Acidaminococcaceae, Acetonema	CHP1m
206	302	38	Bacteria, Proteobacteria, Gammaproteobacteria, Oceanospirillales, Oceanospirillum	QPs
210	460	38	Bacteria, Proteobacteria, Gammaproteobacteria, Xanthomonadales, Xanthomonadaceae, Stenotrophomonas	QPs

[a] TRF in bp.

(Fig. 2B). Although the first two canonical dimensions accounted for only 13.7% of the data set variance, vertical zones could be almost completely separated based on their bacterial ribotype profiles. The majority of this variance is accounted for by the separation of two surface samples in CC1 (QP and RP); and two other 1 m sites in CC2 [Chickering Pond (CHP) and Chickering (CHI)] (Fig. 2A). Even without these outliers, a detailed view of the plot (Fig. 2B) clearly shows the segregation of the microbial communities into two separate groups (1 m and surface).

Individual samples taken from a different area in the same bog were typically very similar (PON and SPR) at 1 m depth. Surface samples replicated in this manner exhibited more unique communities from within the same bog (CHO and PON), although they still clustered with their respective depth. The single soil sample, obtained near one of the bogs, clustered with bog surface samples.

Correlations between the bacterial communities and environmental data including water chemistry (pH, DOC, ammonium, DON, NO_3-N, SO_4, Cl, P-SR, Al, Ca, Cu, Fe, K, Mg, Mn, Na, Si, and Zn) and plant chemistry (C, H, N, Ca, Mg, Fe, Cr, Cu, K, Al, Co, B, Cd, Mo, Mn, Na, Ni, P, Pb, S, and Zn) for three different species (pitcher plant, leather leaf, and *Sphagnum*) were conducted. Vegetation data, from the bog and the surrounding forest, and geographical data including latitude, longitude, bog area, and elevation were also used in correlation analyses [18]. None of the tested parameters showed any significant correlation to specific ribotypes or general community structure. The only link between sample segregation and chemistry was Ca^{2+} levels. Two sites, Chickering (8.59 mg/L) and Colchester (8.24 mg/L), showed approximately 8-fold higher Ca^{2+} levels when compared to the average of all other bogs [0.98 mg/L, SD = 7 (excluding Chickering and Colchester)].

Based on the CCA plots generated (Fig. 2), ribotypes contributing to the majority of the variance among the data were identified (Table 4). Canonical dimensions showing the greatest variance shift depending on the data included. Limiting the set of ribotypes to those explaining the greatest variance among the complete data set, (i.e., those unique to Quag Pond and Chickering Bog samples) still gave an extensive list of candidate taxa. Because of the questionable nature of inferring physiology based on such assignments, greater confidence regarding habitat differences that explain the uniqueness of those bacterial communities may await direct characterization of metabolic potential. Nonetheless, this work provides an initial characterization of bog bacterial ecology that may be used in metering change and evaluating additional habitat characteristics for their impact on those systems.

Taxonomic Representation as Identified by 16S rDNA Sequences. A total of 75 clones were obtained and used for sequence determination and phylogenetic analysis. Ten sequences were identified as possible chimeras and discarded. The remaining sequences were dominated by Proteobacteria (36 clones/55%): [Delta (18/27%), Alpha (9/14%), Beta (7/11%), Gamma (2/3%)], followed by Firmicutes and Acidobacteria with seven representative clones (11%) each (Fig. 3). Two

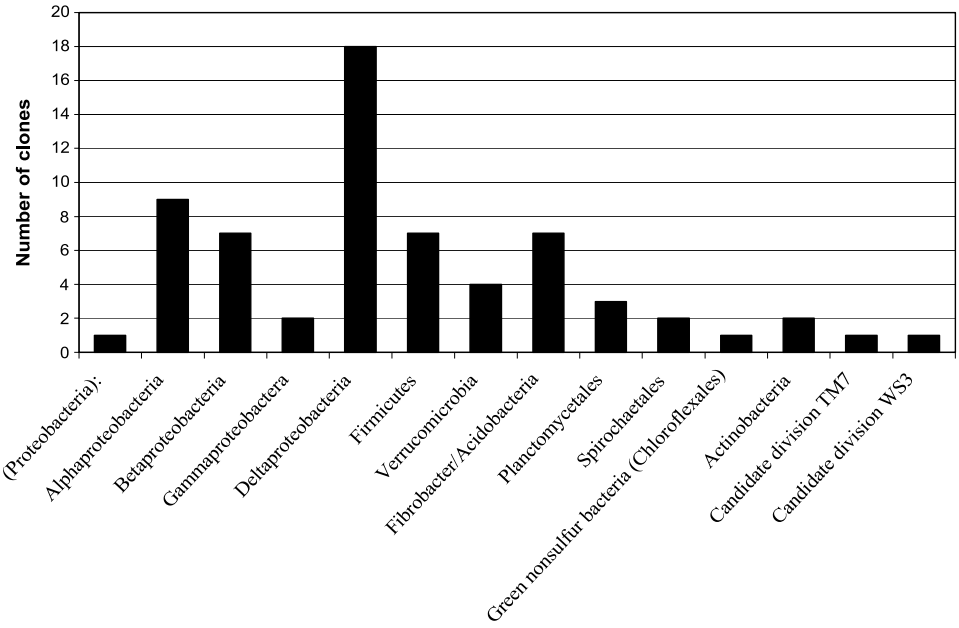

Figure 3. Phylogenetic representation within the 16S clone library. Phylogenetic placement was assigned based on neighbor-joining phylogenetic trees and BLASTN against the Ribosomal Database Project (RDP) rRNA database.

Table 5. T-RFLP and 16S rRNA clone library comparison

		Predicted TRFs			Observed HaeIII TRF		Observed HhaI TRF		Observed MspI TRF	
Clone	*Taxonomic assignment*	*HaeIII*	*HhaI*	*MspI*	*Size*	*Abundance*[a]	*Size*	*Abundance*[a]	*Size*	*Abundance*[a]
63	Acidobacteria, Acidobacteriales, Acidobacteriaceae, Acidobacterium	216	282	267	216.45 213.75	14.15 15.28	281.6 279.55	3.05 3.76	268.46 266.33	14.56 18.74
21	Acidobacteria, Acidobacteriales, Acidobacteriaceae, Acidobacterium	214	93	265	216.45 213.75	14.15 15.28	93.53 91.4	3.57 7.42	266.33	18.74
34	Deltaproteobacteria, Syntrophobacterales, Syntrophobacteraceae, Syntrophobacter	175	97	166	178.09	6.4	97.83	5.84	167.9	4.48
92	Verrucomicrobia, Verrucomicrobiae, Verrucomicrobiales, Verrucomicrobiaceae, Verrucomicrobium	175	97	166	178.09	6.4	97.83	5.84	167.9	4.48
31	Acidobacteria, Acidobacteria, Acidobacteriales, Acidobacteriaceae, Holophaga	203	361	162	203.1 201.49	2.58 5.52	364.23	2.18	163.16	2.33
51	Candidate division WS3	237	214	66	237.45	3.2	213.17	2.09	63.93	2.48
23	Alphaproteobacteria, Rhizobiales, Methylocystaceae, Methylosinus	203	61	148	203.1 201.49	2.58 5.52	58.45	7.74	147.09	7.43
7	Deltaproteobacteria, Syntrophobacterales, Syntrophobacteraceae, Syntrophobacter	263	100	169	263.24	1.52	97.83	5.84	167.9	4.48

Predicted TRFs from a clone library were compared against observed TRFs from the same site.
[a]Percentage of total fluorescence.

Figure 4. Rarefaction curve generated from the 16S rRNA clone library. The number of different clones was determined by alignment based 90% sequence identity. The number of OTU's is plotted versus the number of clones.

clones were identified as possible members of separate candidate divisions (TM7 and WS3). Other sequences were identified as being part of deep-rooted lineages such as Actinobacteria [12] and Planctomycetes [15, 36, 38].

A comparison of the clone library to the ribotypes taken from the same site yielded eight matches dominated by Acidobacteria (Table 5). The phylum Acidobacteria has commonly been identified from environmental DNA using rRNA targets [4, 25, 33]. The ecological roles of members of that phylum however remain mostly undescribed because of the lack of cultivated representatives.

Rarefaction curves were constructed based on operational taxonomic unit (OTU) groups formed at 90% sequence similarity (Fig. 4). Another rarefaction curve was generated by grouping clones based on their top BLAST match accession number. Clones sharing the highest similarity to the same organism were grouped together under the same OTU. The curve generated from this data set correlated to the 90% similarity rarefaction curve (Fig. 4). The rarefaction curve generated did not flatten toward an asymptote, suggesting an incomplete coverage of the richness within the community.

Discussion

We used rapid community fingerprinting to compare bacterial community structure in surface and subsurface microhabitats of *Sphagnum* bogs throughout northern New England. We first compared bacterial communities among 24 *Sphagnum* bogs; we tested the hypothesis that *Sphagnum* bogs represented a sufficiently homogeneous collection of environments that bacterial community structure would be relatively similar among several individual sites. We also compared communities between

surface and subsurface samples; we expected to find distinct communities in these two microhabitats because of anoxic conditions in the subsurface samples.

Although bogs are oligotrophic and acidic, overall diversity was high, with 817 ribotypes detected. There were more unique ribotypes in surface *vs* subsurface samples (199 *vs* 64), but total bacterial counts were higher for the subsurface samples (Fig. 1 and Table 2). Although no data are available to explain this increase in abundance with depth, an increase in bacterial numbers in anoxic zones could correlate with a decreased amount of competition or predation by organisms present in the aerated zones. Alternatively, as counts were normalized based on cells/mL of sample, higher counts might have been an effect of solids content.

Canonical correspondence analysis of the ribotype data showed a clear separation of samples based on sampling depth but relatively poor separation of bogs sampled at the same depth (Fig. 2). At this level of discriminating power, all of the truly ombrotrophic bogs sampled were fairly similar in their bacterial community composition. Chickering bog, which has been classified elsewhere as a poor fen, was identified as an outlier based on T-RFLP profiles. This was possibly correlated to increased calcium levels, a mineral nutrient distinguishing fens from bogs [30]. The identification of a second bog (Colchester) with increased Ca^{2+} levels, but which was not an outlier in the CCA analysis, implies that Ca^{2+} levels are not solely responsible for the unique composition of Chickering Bog. However, analyses to identify other environmental parameters driving the community structure did not reveal correlation with any of the other tested parameters.

Analysis of individual ribotypes recognized Deltaproteobacteria as a key group in bog ecology, having the highest number of representatives among the most frequently assigned or "core" ribotypes (Table 3) and the clone library. It was not possible to conclude relative dominance of individual taxa, however; diversity indices require species abundance data not obtained by our use of T-RFLP [9] or limited clone library analyzed.

Because these Northeastern U.S. *Sphagnum* bogs contained similar bacterial communities (total variance of 4%), the sequence library derived from a single surface sample might yield information regarding *Sphagnum* bog bacterial community composition across the region, and perhaps over an even wider geographic range. Thirteen library sequences gave BLAST hits with sequences obtained from wetlands. Ten of the matching sequences, including a TM7 Candidate Division match, came from a forested wetland in Georgia with a pH range of 2–4 [3], whereas the remaining three were from a *Sphagnum* Bog in Siberia [35]. Clones were derived from Proteobacteria, Verrucomicrobia, Firmicutes, Acidobacteria, Chloroflexales, Spirochaetales, Actinobacteria, Planctomycetes as well as two Candidate Divisions. The clones included a high proportion of Proteobacteria, particularly those of the Delta subclass. Most inferences about physiological capability based solely on rDNA sequence are tenuous, but the metabolic diversity of the Deltaproteobacteria leaves many potential ecological roles open; members of that subclass are capable of dissimilatory sulfate reduction, iron reduction, fermentative metabolisms, even predatory behavior and developmental life cycles [6, 8, 29, 32]. The identified Deltaproteobacteria sequences, however, were most similar to Syntrophobacterales or Desulfovibrionales sequences, two Orders with representatives from the sulfate-reducing bacteria [5, 20, 28]. Published data for Molly bog [31] indicates a direct correlation between pitcher plant density and increased sulfate concentration. Thus we may have biased the sampling to favor sulfate-dependent or -responsive taxa.

The taxonomic diversity suggested from this study indicates that *Sphagnum* bog environments represent a rich source of potentially novel organisms. We have established the homogeneity between similar wetlands and our capacity to separate distinct ones based on their bacterial community. The communities described were vertically stratified, possibly indicating that unique aerobic and anaerobic taxa are present [24]. A core bog community was supplemented in each site with unique ribotypes observed in only that or a limited group of sites. Further testing of community fingerprinting of bog ecosystems may enable the use of microbial communities as indicators of environmental changes. Isolation of bacteria from this environment by culturing would also allow for correlation with nucleic acid techniques and more detailed examination of potential ecological functions.

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