3-1-2012

Soil bacterial communities of a calcium-supplemented and a reference watershed at the Hubbard Brook Experimental Forest (HBEF), New Hampshire, USA

Ganapathi Sridevi
University of New Hampshire

Rakesh Minocha
Northern Research Station, Rakesh.Minocha@unh.edu

Swathi A. Turlapati
University of New Hampshire, Tony.Tagliaferro@unh.edu

Katherine C. Goldfarb
University of Colorado

Eoin L. Brodie
Lawrence Berkeley National Laboratory

RESEARCH ARTICLE

Soil bacterial communities of a calcium-supplemented and a reference watershed at the Hubbard Brook Experimental Forest (HBEF), New Hampshire, USA

Ganapathi Sridevi[1], Rakesh Minocha[2], Swathi A. Turlapati[1], Katherine C. Goldfarb[3], Eoin L. Brodie[3], Louis S. Tisa[4] & Subhash C. Minocha[1]

[1]Department of Biological Sciences, University of New Hampshire, Durham, NH, USA; [2]USDA Forest Service, Northern Research Station, Durham, NH, USA; [3]Ecology Department, Earth Sciences Division, Lawrence Berkley National Laboratory, Berkley, CA, USA; and [4]Department of Molecular, Cellular, and Biomedical Sciences, University of New Hampshire, Durham, NH, USA

Correspondence: Rakesh Minocha, USDA Forest Service, Northern Research Station, Durham, NH 03824, USA. Tel: +1 603 868 7622; fax: +1 603 868 7604; e-mail: rminocha@unh.edu or rminocha@fs.fed.us

Present addresses: Ganapathi Sridevi, Department of Plant Biotechnology, School of Biotechnology, Madurai Kamaraj University, Madurai, 625 021, Tamil Nadu, India;
Katherine C. Goldfarb, Department of Chemistry and Biochemistry, University of Colorado, Boulder, CO, 80309, USA.

Received 16 May 2011; revised 4 November 2011; accepted 7 November 2011.
Final version published online 15 December 2011.

DOI: 10.1111/j.1574-6941.2011.01258.x

Editor: Angela Sessitsch

Keywords
PhyloChip microarray; forest soil; bacterial diversity; Hubbard Brook; calcium amendment.

Abstract

Soil Ca depletion because of acidic deposition-related soil chemistry changes has led to the decline of forest productivity and carbon sequestration in the northeastern USA. In 1999, acidic watershed (WS) 1 at the Hubbard Brook Experimental Forest (HBEF), NH, USA was amended with Ca silicate to restore soil Ca pools. In 2006, soil samples were collected from the Ca-amended (WS1) and reference watershed (WS3) for comparison of bacterial community composition between the two watersheds. The sites were about 125 m apart and were known to have similar stream chemistry and tree populations before Ca amendment. Ca-amended soil had higher Ca and P, and lower Al and acidity as compared with the reference soils. Analysis of bacterial populations by PhyloChip revealed that the bacterial community structure in the Ca-amended and the reference soils was significantly different and that the differences were more pronounced in the mineral soils. Overall, the relative abundance of 300 taxa was significantly affected. Numbers of detectable taxa in families such as *Acidobacteriaceae*, *Comamonadaceae*, and *Pseudomonadaceae* were lower in the Ca-amended soils, while *Flavobacteriaceae* and *Geobacteraceae* were higher. The other functionally important groups, e.g. ammonia-oxidizing *Nitrosomonadaceae*, had lower numbers of taxa in the Ca-amended organic soil but higher in the mineral soil.

Introduction

Calcium (Ca) being an essential nutrient for living organisms plays a critical role in the health and productivity of forest ecosystems. A decrease in soil Ca has been correlated with a reduction in the biomass/carbon (C) sequestration of forest trees. Several factors influence Ca availability including acid deposition (Likens *et al.*, 1996, 1998), excess nitrogen (N) (Aber *et al.*, 1995), intensive forest harvesting (Federer *et al.*, 1989), and soil warming (Tomlinson, 1993). Acidic rain deposition has caused depletion of labile pools of nutrient cations particularly Ca^{2+} because of leaching that has resulted in decreasing amounts of net biomass in several Northeastern US forests, including the Hubbard Brook Experimental Forest (HBEF – www.hubbardbrook.org) in New Hampshire, USA – part of a network of intensive Long Term Ecological Research (LTER) sites (Bailey *et al.*, 1996; Likens *et al.*, 1996, 1998; Fernandez *et al.*, 2003). Over the past 48 years, HBEF has also faced a low input of the atmospheric deposition of base cations, especially Ca (Likens *et al.*, 1998).

In 1999, watershed (WS) 1 at HBEF was treated with Ca silicate (wollastonite) to restore soil Ca (www.hubbard

FEMS MICROBIOLOGY ECOLOGY

brook.org/research/longterm/calcium/w1_overview/index.html) in what was deemed to be Ca-depleted soil. Since then, extensive multidisciplinary studies at Ca-amended WS1 have revealed a multitude of changes in foliar physiology (of red spruce, sugar maple and other mixed hardwood species), stream water chemistry, soil N mineralization and cycling, mycorrhizae, and fine root biomass (Dasch *et al.*, 2006; Groffman *et al.*, 2006; Halman *et al.*, 2008; Cho *et al.*, 2010; Minocha *et al.*, 2010; Schaberg *et al.*, 2011). Comparisons of this watershed with either WS3 or WS6 as the reference watersheds used in the above studies revealed significant changes in soil chemistry as well as tree physiology in response to Ca amendment.

Groffman *et al.* (2006) reported that Ca addition had no significant effect on microbial (bacteria, fungi, and archaea) net nitrification and N mineralization rates during the period of 2000–2003. They also noted a lower microbial N biomass following Ca treatment. However, analyses of microbial population structure or its diversity have not been reported either in the reference or the Ca-amended watersheds at HBEF. This study in 2006 was undertaken to compare the bacterial population structure and diversity at a reference WS3 and the Ca-amended WS1. We hypothesized that the observed changes in soil chemistry as well as the various aboveground forest responses to Ca addition must be accompanied by shifts in soil microbial community structure. The diversity of microbes (specifically bacteria) in either the organic or the mineral soil horizons was not discernible in the earlier studies, even though gross differences in microbial biomass or N mineralization were reported. Our assumption was that Ca supplementation would lead to changes in bacterial taxa and/or their relative abundance either as a direct consequence of Ca addition or in relation to changes in soil chemistry and aboveground biological activity because of Ca treatment. We (Minocha *et al.*, 2010) have earlier reported significant physiological changes in the foliage of sugar maple at mid elevation as compared with low and high elevations of the Ca-amended WS1; thus, this study was limited to soils at mid elevations.

Some of the common and reliable methods for measuring microbial diversity involve culture-independent sequence comparisons of PCR amplified 16S rRNA genes (Janda & Abbott, 2007). The comparisons are made by either denaturing gradient gel electrophoresis (DGGE), sequencing of clone libraries, DNA microarrays, or more recently, pyrosequencing; all use PCR amplified 16S rRNA genes from the DNA isolated from various sources. In this study, G2 PhyloChip (a high-density 16S rRNA gene-based microarray) was used. It allows the detection of more than 8400 bacterial/archaeal taxa (= OTUs – operational taxonomic units) from samples taken from a variety of environments. This technique has been used for determining the impact of soil manipulations on complex microbial communities because of its higher sensitivity and reduced susceptibility to the effects of dominance in microbial population sampling (Brodie *et al.*, 2006, 2007; DeSantis *et al.*, 2007). Examples of its past use include Antarctic soils, mining-impacted soils, and soils along succession gradients (Yergeau *et al.*, 2009; Kuramae *et al.*, 2010; Rastogi *et al.*, 2010). We also used the complementary techniques of DGGE and sequencing of a limited number of 16S rRNA gene clones to detect taxa that may have been missed by the microarrays. The results show major differences in the microbial populations between the reference and the Ca-treated soils.

Materials and methods

Site description and soil sample collection

The Hubbard Brook Experimental Forest (HBEF) located within the White Mountain National Forest, West Thornton, NH, USA (43°56′N, 71°45′W) is a deciduous second-growth forest and part of a network of LTER sites (www.hubbardbrook.org). Climate, hydrology, topography, and vegetation of this site are described by Juice *et al.* (2006). American beech (*Fagus grandifolia* Ehrh.), sugar maple (*Acer saccharum* Marsh.), yellow birch (*Betula alleghaniensis* Britt.), and paper birch (*Betula papyrifera* Marsh.) are the dominant hardwood species at low and mid elevations; red spruce (*Picea rubens* Sarg.) and balsam fir (*Abies balsamea* (L.) Mill.) are dominant conifer species that mainly grow at the ridge top. Soils at HBEF were formed from glacial till and are moderately well-drained, acid Spodosols (Haplorthods) of sandy-loam to loamy-sand texture (Juice *et al.*, 2006).

Each WS spans a range of elevation and slope position, covering varying levels of soil development and growth conditions. This study only involved mid-elevation soils of a reference WS (WS3) and the nearby Ca-amended WS (WS1) at HBEF (Supporting Information, Fig. S1). Pretreatment Ca concentrations in stream flows from the Ca-amended and the reference watersheds were similar at 0.87 ± 0.01 and 1.0 ± 0.01 mg L^{-1}, respectively (data averaged from 1993 to 1999 for each WS). Following Ca treatment, stream flow Ca concentrations from WS1 rose to 1.47 ± 0.05 mg L^{-1}, while for reference WS, Ca concentration in the stream flow declined slightly to 0.88 ± 0.01 mg L^{-1} (data averaged from 1999 to 2005 for each WS) (Minocha *et al.*, 2010).

In October 1999, powdered and pelletized VANSIL-10, a dry commercial form of $CaSiO_3$, was applied uniformly (1.2 metric tons Ca per hectare) to WS1 by helicopter. The aim was to almost double the existing base saturation

(10%) of the soil and to increase the soil pH to the level that was estimated to exist about five decades prior to this treatment. More details on the treatment can be found at: http://www.hubbardbrook.org/research/longterm/calcium/w1_overview/index.html.

For this study, soil samples were collected in October 2006 from mid elevations (550–650 m) of the reference WS3 (Ref – GPS coordinates 43°58′N, 71°43′W) and the Ca-amended WS (Ca – GPS coordinates 43°57′N, 71°44′W). The two sample collection sites, although located in two different watersheds (WS3 and WS1), are only 125 m apart. Three replicate soil cores (7.0 cm dia., 15 cm depth) were sampled from each WS. The replicate samples were taken approximately 3 m apart from each other. After removing leaf litter from the surface, the top (5 cm) organic (Org) soil horizon samples designated as Ref-Org1, Ref-Org2, and Ref-Org3, and Ca-Org1, Ca-Org2, and Ca-Org3 were separated from the mineral soil horizon based on texture, color, and appearance. The bottom (10 cm) mineral horizon soil samples were designated as Ref-Min1, Ref-Min2, Ref-Min3, and Ca-Min1, Ca-Min2, and Ca-Min3. Each sample was mixed thoroughly, sieved first through 3.5 mm mesh and then through 2.0 mm mesh, and stored on ice during transport (2 h) to the laboratory. All samples were stored at −20 °C until further analyses.

Justification of study design

Binkley (2008) reported that replication and scale are important elements to consider in designing an experiment for ecological research. Whereas a WS-level study (although hard to replicate) provides a reasonably large scale needed to ask relevant questions under a specific set of conditions, a small well-replicated study design easily meets statistical requirements (Hurlbert, 1984; Oksanen, 2001; Cottenie & De Meester, 2003). This large WS scale study was set up in 1999 to answer several important questions related to the effects of Ca supplementation on forest functions. Even though a true evaluation of the effects of soil Ca addition on forest health would require the replication of this experiment at more than one location, replicating such a large-scale Ca fertilization within the same timeframe would not be feasible. Earlier work by several groups at this WS (which spans a range of topographic factors) showed a multitude of effects of Ca supplementation on soil chemistry, stream chemistry, forest growth and productivity, and foliar physiology of red spruce and sugar maple trees at Ca-amended vs. the reference watersheds (Juice *et al.*, 2006; Halman *et al.*, 2008; Cho *et al.*, 2010; Minocha *et al.*, 2010). This study complements previous reports and adds a new dimension to our knowledge about soil microbes.

Soil chemistry

Soil samples were air dried before chemical analyses at the Maine Soil Testing Service, Orono, ME. Total N and C were measured by combustion analysis at 1350 °C. Exchangeable base cations and exchangeable P and Al were extracted with 1 M NH_4Cl (Blume *et al.*, 1990) at a ratio of 2 g of organic soil or 5 g of mineral soil to 100 mL extraction solution. Samples were shaken for 1 h, the extracts vacuum-filtered through Whatman 42 filter paper (Whatman Inc., Clifton, NJ), and analyzed by flame emission (K and Na) or plasma emission spectroscopy (Ca, Mg, and exchangeable Al). Exchangeable acidity was measured by extraction with 1 M KCl (Blume *et al.*, 1990) and end point titration using phenolphthalein. Percentage soil organic matter was determined on oven-dried samples by loss-on-ignition (LOI) over 12 h at 550 °C using a muffle furnace. Effective cation-exchange capacity (ECEC) was calculated as the sum of the exchangeable base cations (Ca, Mg, K, and Na) plus exchangeable acidity.

Soil DNA extraction and PhyloChip microarray hybridization of 16S rRNA genes

Total DNA was extracted from 0.25 g of each soil sample using the Power Soil DNA isolation kit according to manufacturer's instructions (MO BIO Laboratories, Carlsbad, CA) and quantified by A_{260}; Pico Green assay was performed for equalizing DNA.

The PhyloChip (http://esd.lbl.gov/research/tech_transfer/r&d100/phylochip.html) used here is described in Brodie *et al.* (2006). It was designed with sequences representing a range of environments including marine and agricultural soils. Sample preparation for microarray hybridization was carried out following the procedure of Ivanov *et al.* (2009). This included the PCR amplification of 16S rRNA genes using bacterial targeted primers (27F and 1492R) across a gradient of eight different annealing temperatures in the range of 48–58 °C. The triplicate reactions for each sample were pooled, the products purified, and 500 ng of each sample used for further processing. The pooled PCR products were spiked with known concentrations of amplicons derived from known bacterial genes. This mix was fragmented to 50–200 bp size using DNase I, labeled with biotin, and hybridized to custom G2 PhyloChip (Affymetrix) at 48 °C and 60 r.p.m. for 16 h. PhyloChip washing and staining, background subtraction, data normalization, and probe-pair scoring were performed essentially as reported earlier (Ivanov *et al.*, 2009). Taxa were deemed present when the PosFrac value equaled or exceeded 0.9. Unlike cloning, but similar to DGGE, the three replicates of each treatment group were

hybridized separately (i.e. 3 replicate samples × 2 soil horizons × 2 treatments = 12 total arrays).

Statistical analyses of PhyloChip data

Following normalization, PhyloChip data were log transformed and a distance matrix was constructed using the Bray–Curtis distance. This distance matrix was represented in two dimensions using nonmetric multidimensional scaling (NMDS). The METAMDS function in the *vegan* package version 1.9-6 (http://cc.oulu.fi/~jarioksa/softhelp/softalist.html – Oksanen *et al.*, 2005) was used within the R programming environment version 2.1.1 by the R Development Core Team (2005 – www.R-project.org). To determine significant treatment effects on the individual bacterial taxa, we performed pair-wise ANOVA between the reference and the Ca-amended samples from the same horizon. Type II error was adjusted for by Benjamin–Hochberg correction as described previously (Ivanov *et al.*, 2009). To determine whether significant relationships existed between bacterial community composition (PhyloChip array data) and measured soil physical and chemical properties, we used the function *envfit* within the *vegan* package to overlay vectors and determine their correlations in NMDS ordination space. The *envfit* function was also used to display bacterial taxonomic groups responsible for the separation of treatment groups measured by 16S rRNA gene sequencing. Partitioning of variance was performed by permutational MANOVA (1000 permutations) using the *adonis* function (also in the *vegan* package) directly on the PhyloChip-derived inter-sample distance matrix to determine the relative power of soil horizon or Ca treatment in explaining the variance observed in bacterial community composition. Similarity of DGGE fingerprints was determined by Dice coefficient correlation (Röling *et al.*, 2001).

Denaturing gradient gel electrophoresis (DGGE)

For DGGE, the V3–V5 region of the 16S rRNA gene was amplified by PCR (polymerase chain reaction) using the primers 338F (5′-ACTCCTACGGGAGGCAGC-3′) with GC clamp and 907R (5′-CCGTCAATTCCTTTGAGTTT-3′) (Amann *et al.*, 1990; Lane, 1991) from each soil sample using 20 ng of soil DNA as template. Bio-Rad DCode™ Universal Mutation Detection System (Bio-Rad Inc., Hercules, CA) was used for DGGE according to the manufacturer's instructions. Samples were loaded on a 6% (w/v) polyacrylamide gel with a denaturing gradient from 40% to 60% (100% denaturant contains 7 M urea and 40% formamide). Gels were run in 1× TAE buffer for 16 h (60 V cm^{-1}, 60 °C), stained with ethidium bromide (0.5 mg L^{-1}), and photographed by UV transillumination using the NucleoVision gel documentation system (NucleoTech, San Mateo, CA). The replicate samples were loaded on the gel separately (3 replicates for each soil type × 2 soil horizons × 2 treatments = 12 samples). Amplified 16S rRNA genes from *Escherichia coli* were used as markers. The DGGE banding patterns were analyzed using the GELCOMPAR II version 6.1 software (Applied Maths, Austin, TX). A dendrogram was constructed using the unweighted pair-group method with arithmetic averages (UPGMA) algorithm. A similarity coefficient of < 0.70 indicates that the samples were significantly different (Röling *et al.*, 2001).

Cloning, sequencing, and computational analysis of rRNA gene libraries

Triplicate PCR reactions were performed for each of the three replicate soil samples (separately for the mineral and the organic soils) from each WS, and the amplified products were pooled to minimize PCR bias (Polz & Cavanaugh, 1998). The combined pools of nine amplification products for each soil horizon (3 replicate samples × 3 PCR reactions per sample) were used for library construction; the resulting four libraries (one library per soil horizon per WS) were designated as Ref-Org, Ref-Min, Ca-Org, and Ca-Min. The libraries were constructed using the TOPO TA Cloning® kit (Invitrogen Corp., Carlsbad, CA) with plasmid pCR2.1® and chemically competent *E. coli* (DH5α) according to the manufacturer's instructions. Transformed white colonies were selected on LB agar plates supplemented with 100 mg L^{-1} ampicillin and grown in 3 mL of LB broth containing 50 mg L^{-1} ampicillin for plasmid isolation (Zyppy™ Plasmid Miniprep kit; Zymo Research, Orange, CA). About 200 clones from each soil horizon/library, prescreened by PCR using the T7F and M13R primers to avoid the co-amplification of the *E. coli* (host) 16S rRNA gene, were sequenced (High-Throughput Genomics Unit, University of Washington, Seattle, WA) using the same set of primers.

All sequences belonging to a particular soil horizon were checked for quality (using Bioedit) and aligned using the NAST (Nearest Alignment Space Termination) tool (Greengenes – http://greengenes.lbl.gov/cgi-bin/nph-index.cgi); (DeSantis *et al.*, 2006a, b). The chimeric sequences identified by Bellerophon application (Greengenes) were discarded. The final NAST aligned dataset contained 302 sequences (151–HB-Ref-Org and 151–HB-Ref-Min) from the reference and 293 sequences (154 –Ca-Org and 139–Ca-Min) from the Ca-amended watersheds. The sequences were classified using the Greengenes tool with NCBI (http://blast.ncbi.nlm.nih.

gov) and RDPII databases (http://www.cme.msu.edu); (Cole *et al.*, 2005). The results of sequence analyses were also checked against the PhyloChip taxonomy outputs on the G2 server to compare the outcomes of cloning and PhyloChip data.

Nucleotide sequence accession numbers

The cloned library sequences for this study have been deposited in the GenBank database as separate files under accession numbers: HB_R_O (GU598579–GU598728), HB_R_M (GU598729–GU598817, GU598819–GU598878), HB_Ca_O (GU598879–GU598972, GU598974–GU598996, GU598998–GU599029), HB_Ca_M (GU599030–GU599 098, GU599100–GU599162).

Results and discussion

Calcium amendment-related changes in soil chemistry

In 2006, 7 years post-Ca amendment, both the Ca-Org and the Ca-Min soils from mid elevation of the Ca-amended WS1 had significantly higher Ca concentration as compared with the respective reference soils (Table 1). Concomitantly, there was significantly lower soluble Al in the Ca-amended organic horizon confirming the earlier reports of an inverse relationship seen between soil Ca and soil Al (Minocha *et al.*, 1997). Higher soil P along with lower acidity was also observed in the Ca-Org soil at WS1. Soil C and N concentrations were not different for the two watersheds (Table 1). The pH of the Ca-Org soil was not different from that of the Ref-Org soil, while the pH of the Ca-Min soil was significantly lower at WS1. This observation contradicts the earlier reports of Groffman *et al.* (2006) and Juice *et al.* (2006) who found significantly higher pH of both soil horizons at WS1 in the first few years after Ca addition. A study conducted on soil solution from WS1 during 2000–2003 (Cho *et al.*, 2010) also reported significantly higher Ca concentration, dissolved silica, pH, acid neutralizing capacity, and a lower inorganic monomeric Al (Al_i) at this site.

Data presented here indicate that the differences in soil pH of the two watersheds apparently diminished with time following Ca amendment and with soil depth. The small difference in pH in 2006 (Table 1) suggests that Ca supplementation had only a modest impact on ECEC 7 years after the application of Ca. The main reason for this could be that with time the positive effects of Ca amendment on ECEC were neutralized by a significant decrease in soil Al in the organic soil horizon. This decrease in Al, although not significant, was also observed for mineral soil as well where a significant decrease in pH of the Ca-Min soil was seen. Overall acidity was also lower in the mineral soil horizon of Ca-amended WS1. Changes in soil chemistry at this site as well as other sites have been shown to affect both the plant and the rhizosphere microbial ecosystem functions (Minocha *et al.*, 1997, 2010; Juice *et al.*, 2006).

Table 1. Chemical analysis of 2006 soil samples from the reference (WS3) and the Ca-amended (WS1) watersheds at HBEF

	Organic horizon		Mineral horizon	
Analyses (units)	Reference (WS3)	Ca-amended (WS1)	Reference (WS3)	Ca-amended (WS1)
Soil pH	4.26 ± 0.14	4.12 ± 0.17	4.66 ± 0.03*	4.09 ± 0.10†
Exch. Ca (mg kg^{-1})	248.50 ± 74.02	1271.68 ± 321.20†	56.38 ± 5.69	234.25 ± 61.45†
Exch. K (mg kg^{-1})	125.88 ± 28.98	163.22 ± 25.12	37.25 ± 11.37*	41.92 ± 13.04
Exch. Mg (mg kg^{-1})	55.07 ± 1.08	56.28 ± 10.73	16.55 ± 2.95*	17.91 ± 1.88
Exch. P (mg kg^{-1})	9.98 ± 1.92	21.15 ± 1.24†	3.74 ± 0.64*	3.13 ± 0.34
Exch. Al (mg kg^{-1})	1235.87 ± 321.78	182.50 ± 63.99†	667.86 ± 96.78	346.50 ± 107.30
Exch. Fe (mg kg^{-1})	82.05 ± 39.63	15.70 ± 5.67	40.89 ± 24.77	27.13 ± 8.28
Exch. Mn (mg kg^{-1})	6.30 ± 2.48	6.73 ± 0.81	1.19 ± 0.17	2.05 ± 0.85
Exch. Na (mg kg^{-1})	14.97 ± 0.88	12.57 ± 2.86	7.91 ± 1.42*	6.31 ± 0.08
Exch. Zn (mg kg^{-1})	6.23 ± 1.43	5.82 ± 2.92	1.51 ± 0.30*	1.45 ± 0.35
Acidity (meq 100 g^{-1})	16.48 ± 3.34	4.48 ± 1.39†	7.94 ± 1.26	4.61 ± 1.17
%LOI (organic matter)	42.93 ± 17.14	30.47 ± 6.78	17.50 ± 2.84	7.83 ± 0.30†
ECEC (meq)	18.56 ± 3.04	11.77 ± 2.47	8.48 ± 1.23*	6.06 ± 0.86
Total nitrogen (%)	1.194 ± 0.504	0.898 ± 0.177	0.423 ± 0.114	0.227 ± 0.012
Total carbon (%)	21.64 ± 8.74	16.56 ± 3.54	9.33 ± 1.91	4.37 ± 0.41

Data are compared separately between reference and Ca-amended soils for each of the two horizons. Data are mean ± SE of three replicate samples taken from different soil cores. ECEC was calculated by summation of milliequivalent levels of Ca, K, Mg, Na, and acidity.

*$P \leq 0.05$ for significant differences in the soil chemistry of reference organic and mineral soils.

†$P \leq 0.05$ for significant differences between Ca-amended and reference soils.

Calcium amendment-related changes in soil bacterial communities

Both the Ca-amended and the reference soils showed significant differences in the composition of bacterial communities at the phylum level as well as lower taxonomic levels using three different techniques. While DGGE profiling provided a quick snapshot of differences among the samples (Fig. S2), PhyloChip analyses, a more detailed community profiling technique, provided specific details on these differences. Limited sequencing analysis of clonal libraries allowed us to further evaluate the relative frequency distribution of the gene sequence of 16S rRNA generated from the reference and the Ca-amended soils. The sequence analysis also provided useful information in identifying some of the taxa that were not recognized by the PhyloChip because of the absence of probes representing these taxa.

More than 1000 taxa for each of the three replicate samples per soil type were identified in the organic as well as the mineral soil horizons by G2-PhyloChip used here. Separate comparisons between soils from the organic horizons of the two watersheds indicated significantly lower mean numbers of taxa (i.e. taxonomic richness) in the Ca-amended vs. the reference watershed ($P \leq 0.056$) (Table 2). Comparison of the mineral soil horizons showed no significant difference in taxa at the two watersheds. The total number of unique taxa (calculated by counting only one OTU for the 2–3 copies of same OTUs that may be present among the three replicates) within each soil type was higher than the mean value. This was perhaps because of micro site soil variability among replicates that lead to variability of taxa in terms of identity and the number of taxa (Table 2). From analysis on pooled data across all four combinations of treatments (plus and minus Ca) and horizons (mineral and organic), there were a total of 1756 different taxa detected. Pooled across the two watershed treatments, the organic horizons contained 1291 different taxa and the mineral horizons contained 1292 taxa. About 20% of the taxa present in the Ref-Org soil were absent in the Ca-Org soil samples, whereas 4% of the taxa that were present in the Ca-Org soil were absent in the Ref-Org soil (Table 2). Similarly, 12% of the total taxa present in the Ref-Min soil were absent in the Ca-Min soil, whereas another 12% that were present in the Ca-Min soil were absent in the Ref-Min soil samples (Table 2). The net change (percent of taxa moving into or of the PhyloChip window of detection) in microbial OTU composition of Ca-Org and Ca-Min soils was 23% and 22%, respectively (Table 2).

Altogether the 1756 taxa detected by the PhyloChip analysis spanned over 42 phyla, 53 classes, 127 orders, and 154 families from the soil samples analyzed. The total number of taxa detected in this study compared well with the numbers detected from Antarctic soils, mining-impacted soils, and soils along succession gradients using the same PhyloChip (Yergeau *et al.*, 2009; Kuramae *et al.*, 2010; Rastogi *et al.*, 2010).

Subjecting the PhyloChip microarray data to NMDS analysis further revealed that the bacterial community composition of the Ca-Min soil resembled that of the Ref-Org soil (Fig. 1). When Permutational MANOVA was used on the data to partition variance between the factors 'horizon' and the 'treatment', it became apparent that both were significant; however, more variance in bacterial

Table 2. Comparison of total numbers of OTUs (referred to as taxa in the table) of bacteria in reference and Ca-amended soils at the Hubbard Brook Experimental Forest

	Soil types			
Microarrays analyses	Ref-Org	Ca-Org	Ref-Min	Ca-Min
Taxa (OTUs)	1232 ± 79	1147 ± 22*	1202 ± 93	1381 ± 83
Number of unique† taxa within a soil type	1618	1343	1472	1466
Taxa that were absent in Ca-amended soils but were present in Ref soils	20% (327/1618)		12% (180/1472)	
Taxa that were present in Ca-amended soils but were absent in Ref soils		4% (52/1343)		12% (174/1466)
Total % change in Ca-amended soils = (absent + present taxa)/ (absent + present + common taxa)		23% (327 + 52)/ (327 + 52 + 1291)		22% (180 + 174)/ (180 + 174 + 1292)

Data are mean ± SE of three replicate samples each taken independently from a different soil core. The terms 'present' and 'absent' apply only to numbers of the total OTUs that could be detected at HBEF using G2 PhyloChip. The OTUs were designated using the G2 PhyloChip at PosFrac values ≥ 0.9.

*Significantly different numbers of OTU between Ca-treated and untreated soils ($P = 0.056$).

†Unique taxa per soil type were calculated by adding up all taxa from three replicate samples of each soil type and removing the duplicate taxa.

community composition could be explained by the Ca treatment (Fig. 1). To discern the relationship between soil variables that were measured and the bacterial community composition, environmental vectors were fit to NMDS ordinations of PhyloChip data; the analysis showed that Ca, P, and pH were more related to bacterial community structure ($P \leq 0.05$; Table 3) than other factors that were evaluated. The significance of changes in bacterial diversity and composition to the root physiology and overall health of young and mature trees at this site is currently not known.

Significant differences in bacterial populations at the family level were observed between soils from the two watersheds, presumably because of rearrangements in response to Ca treatment. This could be the combined result of direct and indirect effects of Ca through complex interactions with the soil and the rhizosphere, as well as through growth effects on plants. Overall, significant differences between the reference and the Ca-amended soil were observed in relative abundance of *c.* 300 taxa (Table 4). *Acidobacteria* have previously been shown to correlate strongly with soil pH, increasing in relative abundance as the pH declines (Fierer & Jackson, 2006; Lauber *et al.*, 2009; Rousk *et al.*, 2010). In this study, while some taxa within this group were lower in relative abundance in the Ca-Org as well as Ca-Min soils, some

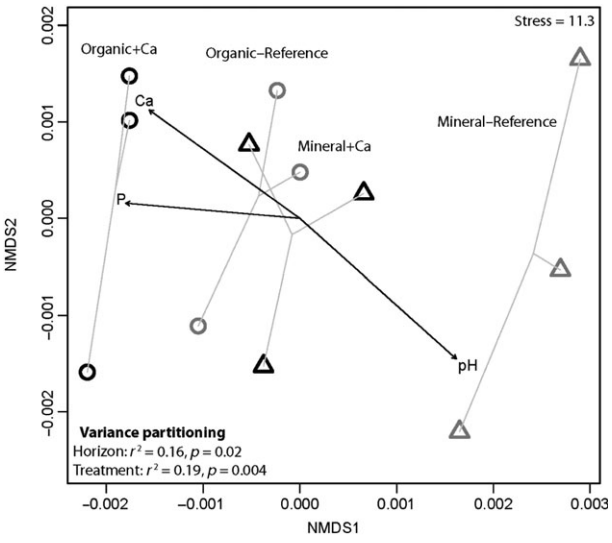

Fig. 1. NMDS ordination of bacterial community composition and structure analyzed by PhyloChip microarrays. Horizons are annotated by symbols: circles for the organic and triangles for the mineral horizons and treatments are annotated by color: black for the Ca-treated and gray for the reference watershed. Groups and their centroids are shown using spider plots. Significant ($P \leq 0.05$) environmental vectors for pH, Ca, and P are overlaid as black arrows. Variance partitioning shows that while both soil horizon and Ca treatment are significant, the treatment explains more variance in bacterial community composition than the soil horizon.

Table 3. Correlation coefficients between soil physical and chemical measurements and the NMDS ordination of bacterial community composition derived from PhyloChip G2 microarray data. These values were calculated using the function 'envfit' in the R *vegan* package as described in Materials and methods

	NMDS1	NMDS2	r^2	*P*
pH	0.744057	−0.668116	0.7274	0.0027**
Ca (mg kg^{-1})	−0.811314	0.58461	0.5671	0.0197*
P (mg kg^{-1})	−0.996205	0.087033	0.5033	0.045*
K (mg kg^{-1})	−0.923877	−0.38269	0.4314	0.0808
Mg (mg kg^{-1})	−0.956424	0.291982	0.3606	0.137
Mn (mg kg^{-1})	−0.877116	0.480279	0.2513	0.2747
Al (mg kg^{-1})	0.425442	−0.904986	0.2224	0.3274
Zn (mg kg^{-1})	−0.96443	0.264339	0.1383	0.5216
% N	−0.784476	−0.620159	0.1114	0.6007
Acidity (meq 100 g^{-1})	0.279803	−0.960058	0.1108	0.5963
Na (mg kg^{-1})	−0.831201	0.555973	0.0972	0.6307
% C	−0.734352	−0.678769	0.0959	0.6463
% LOI	−0.6691	−0.743172	0.0921	0.659
ECEC (meq 100 g^{-1})	−0.811531	−0.584309	0.0735	0.7101
Fe (mg kg^{-1})	0.999164	−0.040883	0.0165	0.9296

P values based on 10 000 permutations.
Significance codes: ** $\leq$ 0.01 and * $\leq$ 0.05.

unclassified *Acidobacteria* had a higher number of taxa in these soils vs. the reference soils (Table 4). *Actinobacteria* displayed differential responses to Ca addition by family and by soil horizon; for example, taxa within the *Acidimicrobiaceae* were higher in relative abundance in Ca-Org soil but lower in Ca-Min soil, whereas *Cellulomonadaceae* was relatively higher in abundance in the Ca-Min soil only. In the phylum *Bacteroidetes*, taxa belonging to *Flavobacteriaceae* were higher in both horizons of Ca-amended soil, while *Prevotellaceae* had lower number of taxa in both soil horizons. From these results, it can be argued that phylogenetically related organisms may inhabit distinct niches in these soils. In the phylum *Firmicutes*, taxa within many families declined in Ca-amended soil with the *Lactobacillaceae*, *Acidaminococcaceae*, and *Alicyclobacillaceae* showing lower relative abundance in both the organic and the mineral horizons. In contrast, the *Erysipelotrichaceae* showed higher relative abundance of taxa in the organic soils and lower in the mineral soils of Ca-amended watershed.

Relatively little is known about physiology of the common soil phylum *Gemmatimonadetes* because of their recalcitrance to growth in culture. This study shows that their relative abundance was greater in both horizons of the Ca-amended soil. Another poorly understood group, the *Planctomycetes*, had lower relative abundance of taxa in Ca-Org and higher in Ca-Min.

The phylum displaying the greatest differences in the number of families with altered relative abundance between treatments was the *Proteobacteria*. The families

Table 4. Phylochip data showing differences in relative abundance of bacterial families in Ca-amended organic and mineral soils in comparison with the respective reference soil samples. Relative abundance of each bacterial family is given as higher and lower in taxa (Columns) in Ca-amended soil in comparison with the respective reference soil ($P \leq 0.05$)

	Ca-amended organic soil		Ca-amended mineral soil	
Phylum	Increase	Decrease	Increase	Decrease
Acidobacteria	Unclassified	Acidobacteriaceae	Unclassified	Acidobacteriaceae Unclassified
Actinobacteria	Acidimicrobiaceae Actinomycetaceae Dermabacteraceae	Nocardiaceae Unclassified	Cellulomonadaceae Kineosporiaceae	Acidimicrobiaceae Corynebacteriaceae Mycobacteriaceae Rubrobacteraceae Unclassified
Bacteroidetes	Blattabacteraceae Flavobacteriaceae Crenotrichaceae	Prevotellaceae	Bacteroidaceae Cryomorphaceae Flavobacteriaceae Crenotrichaceae Flammovirgaceae Flexibacteraceae Sphingobacteriaceae	Prevotellaceae
Chlamydiae	–	–	–	Parachlamydiaceae
Chlorobi	–	Chlorobiaceae	–	–
Chloroflexi	Unclassified	–	–	Unclassified
Cyanobacteria	–	Unclassified	Unclassified	Unclassified
Dictyoglomi	–	–	Dictyoglomaceae	–
Deinococcus	–	–	–	Unclassified
Firmicutes	Lachnospiraceae Erysipelotrichaceae	Alicyclobacillaceae Bacillaceae Halobacillaceae Lactobacillaceae Acidaminococcaceae Unclassified	Streptococcaceae Mycoplasmataceae	Alicyclobacillaceae Enterococcaceae Lactobacillaceae Acidaminococcaceae Peptostreptococcaceae Erysipelotrichaceae Unclassified
Gemmatimonadetes	Unclassified	–	Unclassified	–
Marine group A	–	–	Unclassified	–
Planctomycetes	–	Planctomycetaceae	Planctomycetaceae	–
Proteobacteria	Caulobacteraceae Anaplasmatceae Rickettsiaceae Sphingomonadaceae Desulfobacteraceae Geobacteraceae Moraxellaceae Unclassified	Methylocystaceae Bradyrhizobiaceae Rhodobacteraceae Alcaligenaceae Comamonadaceae Nitrosomonadaceae Desulfobulbaceae Desulfovibrionaceae Shewanellaceae Methylococcaceae Pseudomonadaceae Unclassified	Caedibacteraceae Anaplasmataceae Burkholderiaceae Nitrosomonadaceae Desulfobacteraceae Geobacteraceae Myxococcaceae Polyangiaceae Ectothiorhodospiraceae Enterobacteriaceae Alcanivoraceae Oceanospirillaceae Moraxellaceae Francisellaceae Xanthomonadaceae Unclassified	Azospirillaceae Methylocystaceae Caulobacteraceae Rhodobacteraceae Alcaligenaceae Comamonadaceae Acidithiobacillaceae Alteromonadaceae Legionellaceae Pseudomonadaceae Unclassified
Spirochaetes	Spirochaetaceae	–		Spirochaetaceae
Termite group 1	–	–	Unclassified	–
Thermosulfobacteria	–	–	Thermosulfobacteriaceae	–
TM7	–	–	Unclassified	–
Verrucomicrobia	–	–	Verrucomicrobiaceae	Xiphinematobacteraceae

Desulfobacteraceae and *Geobacteraceae* (metal reducing genera) were higher in relative abundance in Ca-Org as well as Ca-Min soils, whereas methane oxidizers (*Methylocystaceae* and *Methylococcaceae*), *Pseudomonadaceae* and *Comamonadaceae* showed reduced relative abundance. To our knowledge, no studies on the effects of Ca fertilization on methane oxidizers in forest soils have been reported; thus, a direct comparison of these data cannot be made. In the park grass soils of Rothamsted, UK, the spatial distribution of methane assimilation by bacteria was controlled by concentrations of ammonia and pH in the local area which, in turn, depended on the quantity of N applied along with rate of its removal (Stiehl-Braun *et al.*, 2011). In another study conducted in a field of maize monoculture in Belgium, the methane oxidation function as well as the molecular and chemical composition of the methanotrophic community changed in response to NH_4NO_3 application (Seghers *et al.*, 2003a). In contrast, long-term addition of herbicides Atrazine® and a pesticide Metolachlor® were reported to alter the methanotroph communities without effecting their methane oxidation functions in agricultural soils (Seghers *et al.*, 2003b).

Groffman *et al.* (2006) reported no significant effect of Ca addition on net nitrification and mineralization by bacteria during the period 2000–2003 at HBEF. However, this study reveals a lower number of taxa of ammonia-oxidizing bacteria belonging to the family *Nitrosomonadaceae* in the Ref-Org soil but a higher number in the Ref-Min soil. In a study of a grassland soil, liming and NH_4NO_3 additions were found to be associated with a higher population of ammonia-oxidizing bacteria in the rhizosphere microcosm (Rooney *et al.*, 2010). Kennedy *et al.* (2004) had earlier suggested that the grassland rhizosphere bacterial communities are more likely to be impacted by liming treatments than by the plant species alone. Bäckman *et al.* (2003) reported an increase in some *Nitrospira* species in response to liming of an acidic spruce dominated forest. In our study, free-living N-fixing bacteria (*Bradyrhizobiaceae*, *Azospirillaceae*) were lower in relative abundance in both horizons of the Ca-amended vs. the reference soil; we did not, however, make a distinction between the rhizosphere bacteria and those in other parts of the soil. The results of this study strongly suggest that changes observed in the bacterial communities are most likely the consequence of Ca amendment. This, however, cannot be experimentally tested as zero-time data on bacterial populations at these watersheds were not collected because of the lack of any high-throughput technique for the study of bacteria in 1999.

Clone sequencing of 16S rRNA genes

While quite powerful in its ability to detect almost 8500 taxa, the G2-PhyloChip is limited in its capacity to detect novel sequences not represented by probes on the array. Furthermore, owing to differences in representation on the array and in hybridization efficiency between probe sets for different taxa, the PhyloChip does not provide accurate information on relative abundance of specific taxa within soil samples. To get a glimpse of this possibility, we performed a limited amount of sequencing of the representative 16S rRNA gene clone libraries from each of the soil samples. A total of 595 clones were sequenced for their entire length from 4 clone libraries, 302 from the two horizons of reference soil (151 from Ref-Org and 151 from Ref-Min), and 293 from the Ca-amended soil (154 from Ca-Org and 139 from Ca-Min). Phylogenetic analyses of the 16S rRNA gene clone library sequences revealed that the largest proportion (69–74%) of sequences belonged to the phylum *Acidobacteria*, which clustered (using RDP classifier in Greengenes) into subdivisions or groups 1, 2, 3, 5, 6, 10, 13, and 17 (Fig. S3). The dominance of *Acidobacteria* in terms of sequence abundance clearly supports an important role for this group of microbes in the forest soil ecosystem functioning (Lee *et al.*, 2008). *Acidobacteria*, being oligotrophs, remain viable in nutrient-limited environments and their abundance in turn indicates a limited availability of nutrients. Among the small number of OTUs that were identified by sequencing, most were found to be present on the PhyloChip with the exception of 2–13 new OTUs (depending on the soil sample) that were only seen in the rDNA clone libraries. These results indicate that the PhyloChip was quite powerful in covering the diversity of taxa found in these soils; and the sequencing of libraries complemented the PhyloChip results.

Relationship between soil chemistry and microbial community structure

Microorganisms inhabiting natural forest soils play vital roles in ecosystem functioning, including a major role in nutrient cycling. Many factors including pH, organic matter content, cation-exchange capacity, and microbial activities among others affect the bioavailability of soil nutrients (Ledin & Pedersen, 1996). Recent studies have shown that changes in soil microbial communities are often strongly correlated with changes in soil chemistry and vice versa (Frey *et al.*, 2004; Nilsson *et al.*, 2007; Lauber *et al.*, 2008; Jenkins *et al.*, 2009). This is particularly true for soil pH (Fierer & Jackson, 2006; Hartman *et al.*, 2008; Jenkins *et al.*, 2009; Lauber *et al.*, 2009; Rousk *et al.*, 2010), which was one obvious consequence of Ca addition at HBEF WS1 (Cho *et al.*, 2010). To date, little information is available regarding the response of forest soil microbial communities to cation amendment. At the beginning of the Ca supplementation study at

HBEF, it was envisioned that Ca addition would lead to changes in soil chemistry, and in turn, affect forest and animal growth and productivity; these predictions were indeed realized (Groffman *et al.*, 2006; Juice *et al.*, 2006; Vadeboncoeur *et al.*, 2007; Halman *et al.*, 2008; Cho *et al.*, 2010; Minocha *et al.*, 2010).

For this study, we hypothesized that changes in soil chemistry will be accompanied by long-term alterations in the soil bacterial communities. In a plot level study at HBEF and at another site in NY, fertilizer treatment (a mixture of N, P, K, Ca, and Mg) was shown to decrease fine root biomass, rhizosphere microbial biomass, and respiratory fluxes in hardwood forest soils (Phillips & Fahey, 2007). Addition of lime to pine and spruce forests in Sweden had led to increases in the proportions of culturable bacteria in the limed bulk soils as compared with the untreated soils (Bååth & Arnebrant, 1994). These authors suggested that observed alteration in the bacterial community composition was the result of a change in pH because of liming. Based on these observations, we had hypothesized that overall bacterial diversity would decrease with Ca application. Kennedy *et al.* (2004) have also reported that liming, which increased the pH of an acidic upland grassland soil, was accompanied by increased microbial activity and biomass and resulted in a decrease in bacterial taxa richness. Our results (Table 2) corroborate these findings of decline in richness of bacterial taxa in the Ca-Org soil horizon, with little or no effect on the mineral soil horizon. Whereas the pH of this soil had increased earlier between 2000 and 2004 (Groffman *et al.*, 2006), it was not significantly different from the reference for the organic horizon soil at the time of our sampling, i.e. in 2006. Thus, the intervening increase in soil Ca, along with changes in other soil parameters (e.g. pH and Al), may together have been responsible for the observed changes in microbial populations at WS1.

The NMDS ordination of PhyloChip data revealed that both the organic and the mineral soil bacterial communities diverged from their respective reference soils following Ca addition (Fig. 1). The data further show that both Ca treatment and soil horizon explain a significant amount of variance in the bacterial community composition, with Ca addition explaining more. Further analysis showed that among all the soil chemistry variables tested, only Ca, P, Al, and pH were significantly correlated with bacterial community structure (Table 3).

It must be kept in mind that observed shifts in the microbial community composition may have occurred over time because of the complex chemical reactions resulting from Ca addition in 1999. The exact chronology of changes in soil chemistry, plant and microbial communities, and the ecological and physiological mechanisms leading to changes in microbial diversity can only be speculated. It can be argued that most of these changes occur in an interactive manner, i.e. one change influencing the other in a reciprocal way. Such a reciprocal influences between plant and bacterial communities have been demonstrated for symbiotic associations (Brencic & Winans, 2005; Bright & Bulgheresi, 2010 and references therein). How the changes in the composition of bacterial population within specific families, as seen in this study, are linked with the aboveground ecosystem functions is yet to be explored. In fact, the functions of most bacterial taxa detected in this study are yet to be annotated. While all indications from soil chemistry and pretreatment stream chemistry data from the reference and the Ca-amended watersheds presented here indicate that soil microbial changes are probably related to Ca amendment, the possibility that the observed changes occurred because of preexisting geographic or historic factors cannot be ruled out.

Conclusions

Soil bacterial diversity in the two watersheds (the reference and the Ca-amended) at HBEF is typical of acidic forest systems with *Acidobacteria* being the most abundant group. Relative to the reference watershed, the Ca-amended watershed had significantly different soil chemical properties as well as the relative abundance of *c.* 300 bacterial taxa in the two soil horizons. Overall the study detected 1756 taxa spanning 42 phyla, 53 classes, 127 orders, and 154 families from the two watersheds. Calcium amendment led to a change in bacterial community composition of 23% in the organic and 22% in the mineral soil horizons. Families *Methylocystaceae* and *Methylococcaceae* (methane oxidizers) decreased in abundance in both horizons of the Ca-amended watershed. Whereas the relative abundance of ammonia-oxidizing bacteria decreased in the Ca-Org soil, it increased in the Ca-Min soil. Future experiments will focus on linking the functional importance of these microbes with ecosystem processes such as soil respiration, lignin degradation, biogeochemical cycling of nutrients, and changes in bacterial populations in response to short-term treatments with Ca. Zero time data will also be collected in these studies.

Acknowledgements

We thank Stephanie Long and Kenneth Dudzik for help in the collection of soil samples and for editing of the manuscript, and Lindsey Lemire and Matt Power (undergraduates) for help in plasmid isolations for bacterial library work. We also thank Dr Jo Handelsman, Dr Thomas Isenberger, and Dr Kevin Smith for their valuable suggestions during this study. A part of this work was

performed under the auspices of the US Department of Energy, at the University of California, Berkeley, CA under contract DE-AC02-05CH11231, and by the Laboratory Directed Research and Development Program of the Lawrence Berkeley National Laboratory. The Hubbard Brook Experimental Forest is owned and operated by the Northern Research Station, USDA Forest Service, and this study was part of the ongoing HBEF LTER study (www.hubbardbrook.org). The Northeastern States Research Cooperative (NSRC) provided partial funding for this research. Maine Soil Testing Service, University of ME is acknowledged for their help in soil analysis. This paper is scientific contribution Number 2365 from the New Hampshire Agricultural Experiment Station.

References

Aber JD, Magill A, McNulty SG, Boone RD, Nadelhoffer KJ, Downs M & Halle R (1995) Forest biogeochemistry and primary production altered by nitrogen saturation. *Water Air Soil Pollut* **85**: 1665–1670.

Amann R, Binder BJ, Chisholm SW, Olson RJ, R D & DA S (1990) Combination of 16S rRNA-targeted oligonucleotide probes with flow cytometry for analyzing mixed microbial populations. *Appl Environ Microbiol* **56**: 1919–1925.

Bååth E & Arnebrant K (1994) Growth rate and response of bacterial communities to pH in limed and ash treated forest soils. *Soil Biol Biochem* **26**: 995–1001.

Bäckman JSK, Hermansson A, Tebbe CC & Lindgren P-E (2003) Liming induces growth of a diverse flora of ammonia-oxidising bacteria in acid spruce forest soil as determined by SSCP and DGGE. *Soil Biol Biochem* **35**: 1337–1347.

Bailey SW, Hornbeck JW, Driscoll CT & Gaudette HE (1996) Calcium inputs and transport in a base-poor forest ecosystem as interpreted by Sr isotopes. *Water Resour Res* **32**: 707–719.

Binkley D (2008) Three key points in the design of forest experiments. *For Ecol Manage* **255**: 2022–2023.

Blume LJ, Schumacher PW, Schaffer KA, Cappo KA, Papp ML, Van Remortel R, Coffey D, Johnson M & Chaloud D (1990) *Handbook of Methods for Acid Deposition Studies. Laboratory Analyses for Soil Chemistry*. U.S. Environmental Protection Agency. EPA/600/4-90/023 Environmental Monitoring Systems Laboratory, Las Vegas, NV.

Brencic A & Winans SC (2005) Detection of and response to signals involved in host–microbe interactions by plant-associated bacteria. *Microbiol Mol Biol Rev* **69**: 155–194.

Bright M & Bulgheresi S (2010) A complex journey: transmission of microbial symbionts. *Nat Rev Microbiol* **8**: 218–230.

Brodie EL, DeSantis TZ, Joyner DC *et al.* (2006) Application of a high-density oligonucleotide microarray approach to study bacterial population dynamics during uranium reduction and reoxidation. *Appl Environ Microbiol* **72**: 6288–6298.

Brodie EL, DeSantis TZ, Moberg-Parker JP, Zubietta IX, Piceno YM & Andersen GL (2007) Urban aerosols harbor diverse and dynamic bacterial populations. *P Natl Acad Sci USA* **104**: 299–304.

Cho Y, Driscoll CT, Johnson CE & Siccama TG (2010) Chemical changes in soil and soil solution after calcium silicate addition to a northern hardwood forest. *Biogeochemistry* **100**: 3–20.

Cole JR, Chai B, Farris RJ, Wang Q, Kulam SA, McGarrell DM, Garrity GM & Tiedje JM (2005) The Ribosomal Database Project (RDP-II): sequences and tools for high-throughput rRNA analysis. *Nucleic Acids Res* **33**: D294–D296.

Cottenie K & De Meester L (2003) Comment to Oksanen (2001): reconciling Oksanen (2001) and Hurlbert (1984). *Oikos* **100**: 394–396.

Dasch AA, Blum JD, Eagar C, Fahey TJ, Driscoll CT & Siccama TG (2006) The relative uptake of Ca and Sr into tree foliage using a whole-watershed calcium addition. *Biogeochemistry* **80**: 21–41.

DeSantis TZ, Hugenholtz P, Keller K, Brodie EL, Larsen N, Piceno YM, Phan R & Andersen GL (2006a) NAST: a multiple sequence alignment server for comparative analysis of 16S rRNA genes. *Nucleic Acids Res* **34**: W394–W399.

DeSantis TZ, Hugenholtz P, Larsen N, Rojas M, Brodie EL, Keller K, Huber T, Dalevi D, Hu P & Andersen GL (2006b) Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB. *Appl Environ Microbiol* **72**: 5069–5072.

DeSantis TZ, Brodie EL, Moberg JP, Zubieta IX, Piceno YM & Andersen GL (2007) High-density universal 16S rRNA microarray analysis reveals broader diversity than typical clone library when sampling the environment. *Microb Ecol* **53**: 371–383.

Federer CA, Hornbeck JW, Tritton LM, Martin CW, Pierce RS & Smith CT (1989) Long-term depletion of calcium and other nutrients in eastern US forests. *Environ Manage* **13**: 593–601.

Fernandez IJ, Rustad LE, Norton SA, Kahl JS & Cosby BJ (2003) Experimental acidification causes soil base-cation depletion at the Bear Brook watershed in Maine. *Soil Sci Soc Am J* **67**: 1909–1919.

Fierer N & Jackson RB (2006) The diversity and biogeography of soil bacterial communities. *P Natl Acad Sci USA* **103**: 626–631.

Frey SD, Knorr M, Parrent JL & Simpson RT (2004) Chronic nitrogen enrichment affects the structure and function of the soil microbial community in temperate hardwood and pine forests. *For Ecol Manage* **196**: 159–171.

Groffman PM, Fisk MC, Driscoll CT, Likens GE, Fahey TJ, Eagar C & Pardo LH (2006) Calcium additions and microbial nitrogen cycle processes in a northern hardwood forest. *Ecosystems* **9**: 1289–1305.

Halman JM, Schaberg PG, Hawley GJ & Eagar C (2008) Calcium addition at the Hubbard Brook Experimental Forest increases sugar storage, antioxidant activity and cold

tolerance in native red spruce (*Picea rubens*). *Tree Physiol* **28**: 855–862.

Hartman WH, Richardson CJ, Vilgalys R & Bruland GL (2008) Environmental and anthropogenic controls of bacterial communities in wetland soils. *P Natl Acad Sci USA* **105**: 17842–17847.

Hurlbert SH (1984) Pseudoreplication and the design of ecological field experiments. *Ecol Monogr* **54**: 27–38.

Ivanov II, Atarashi K, Manel N *et al.* (2009) Induction of intestinal Th17 cells by segmented filamentous bacteria. *Cell* **139**: 485–498.

Janda JM & Abbott SL (2007) 16S rRNA gene sequencing for bacterial identification in the diagnostic laboratory: pluses, perils, and pitfalls. *J Clin Microbiol* **45**: 2761–2764.

Jenkins SN, Waite IS, Blackburn A, Husband R, Rushton SP, Manning DC & O'Donnell AG (2009) Actinobacterial community dynamics in long term managed grasslands. *Antonie Van Leeuwenhoek* **95**: 319–334.

Juice SM, Fahey TJ, Siccama TG, Driscoll CT, Denny EG, Eagar C, Cleavitt NL, Minocha R & Richardson AD (2006) Response of sugar maple to calcium addition to northern hardwood forest. *Ecology* **87**: 1267–1280.

Kennedy N, Brodie EL, Connolly J & Clipson N (2004) Impact of lime, nitrogen and plant species on bacterial community structure in grassland microcosms. *Environ Microbiol* **6**: 1070–1080.

Kuramae EE, Gamper HA, Yergeau E, Piceno YM, Brodie EL, DeSantis TZ, Andersen GL, van Veen JA & Kowalchuk GA (2010) Microbial secondary succession in a chronosequence of chalk grasslands. *ISME J* **4**: 711–715.

Lane DJ (1991) 16S/23S rRNA sequencing. *Nucleic Acid Techniques in Bacterial Systematics* (Stackebrandt E & Goodfellow M, eds.), pp. 115–175. John Wiley & Sons Ltd., Chichester, UK.

Lauber CL, Strickland MS, Bradford MA & Fierer N (2008) The influence of soil properties on the structure of bacterial and fungal communities across land-use types. *Soil Biol Biochem* **40**: 2407–2415.

Lauber CL, Hamady M, Knight R & Fierer N (2009) Pyrosequencing-based assessment of soil pH as a predictor of soil bacterial community structure at the continental scale. *Appl Environ Microbiol* **75**: 5111–5120.

Ledin M & Pedersen K (1996) The environmental impact of mine wastes – roles of microorganisms and their significance in treatment of mine wastes. *Earth Sci Rev* **41**: 67–108.

Lee S, Ka J & Cho J (2008) Members of the phylum Acidobacteria are dominant and metabolically active in rhizosphere soil. *FEMS Microbiol Lett* **285**: 263–269.

Likens GE, Driscoll CT & Buso DC (1996) Long-term effects of acid rain: response and recovery of a forest ecosystem. *Science* **272**: 244–246.

Likens GE, Driscoll CT, Buso DC *et al.* (1998) The biogeochemistry of calcium at Hubbard Brook. *Biogeochemistry* **41**: 89–173.

Minocha R, Shortle WC, Lawrence GB, David MB & Minocha SC (1997) A relationship among foliar chemistry, foliar polyamines, and soil chemistry in red spruce trees growing across the northeastern United States. *Plant Soil* **191**: 109–122.

Minocha R, Long S, Thangavel P, Minocha SC, Eagar C & Driscoll CT (2010) Elevation dependent sensitivity of northern hardwoods to Ca addition at Hubbard Brook Experimental Forest, NH USA. *For Ecol Manage* **260**: 2115–2125.

Nilsson LO, Bååth E, Falkengren-Grerup U & Wallander H (2007) Growth of ectomycorrhizal mycelia and composition of soil microbial communities in oak forest soils along a nitrogen deposition gradient. *Oecologia* **153**: 375–384.

Oksanen L (2001) Logic of experiments in ecology: is pseudoreplication a pseudoissue? *Oikos* **94**: 27–38.

Oksanen J, Kindt R & O'Hara B (2005) *Community Ecology Package. R Package Version 1.6-9. VEGAN Package*. (Oksanen J, Kindt R & O'Hara B, eds). http://cc.oulu.fi/~jarioksa/.

Phillips RP & Fahey TJ (2007) Fertilization effects on fineroot biomass, rhizosphere microbes and respiratory fluxes in hardwood forest soils. *New Phytol* **176**: 655–664.

Polz MF & Cavanaugh CM (1998) Bias in template-to-product ratios in multitemplate PCR. *Appl Environ Microbiol* **64**: 3724–3730.

Rastogi G, Osman S, Vaishampayan PA, Andersen GL, Stetler LD & Sani RK (2010) Microbial diversity in uranium mining-impacted soils as revealed by high-density 16S microarray and clone library. *Microb Ecol* **59**: 94–108.

Röling W, van Breukelen B, Braster M, Lin B & van Verseveld H (2001) Relationships between microbial community structure and hydrochemistry in a landfill leachate-polluted aquifier. *Appl Environ Microbiol* **67**: 4619–4629.

Rooney DC, Kennedy NM, Gleeson DB & Clipson NJW (2010) Responses of ammonia-oxidising bacterial communities to nitrogen, lime, and plant species in Upland grassland soil. *Appl Environ Soil Sci* 1–17.

Rousk J, Bååth E, Brookes PC, Lauber CL, Lozupone C, Caporaso JG, Knight R & Fierer N (2010) Soil bacterial and fungal communities across a pH gradient in an arable soil. *ISME J* **4**: 134–151.

Schaberg PG, Minocha R, Long SL, Halman JM, Hawley GJ & Eagar C (2011) Calcium addition at the Hubbard Brook Experimental Forest increases the capacity for stress tolerance and carbon capture in red spruce (*Picea rubens*) trees during the cold season. *Trees* **25**: 1053–1061.

Seghers D, Top EM, Reheul D, Bulcke R, Boeckx P, Verstraete W & Siciliano SD (2003a) Long-term effects of mineral versus organic fertilizers on activity and structure of the methanotrophic community in agricultural soils. *Environ Microbiol* **5**: 867–877.

Seghers D, Verthe K, Reheul D, Bulcke R, Siciliano SD, Verstraete W & Top EM (2003b) Effect of long-term herbicide applications on the bacterial community structure and function in an agricultural soil. *FEMS Microbiol Ecol* **46**: 139–146.

Stiehl-Braun PA, Powlson DS, Poulton PR & Niklaus PA (2011) Effects of N fertilizers and liming on the micro-scale distribution of soil methane assimilation in the long-term Park Grass experiment at Rothamsted. *Soil Biol Biochem* **43**: 1034–1041.

Tomlinson GH (1993) A possible mechanism relating increased soil temperature to forest decline. *Water Air Soil Pollut* **66**: 365–380.

Vadeboncoeur MA, Hamburg SP, Blum JD & Skeldon MA (2007) Terrestrial gastropod responses to an ecosystem-level calcium manipulation in a northern hardwood forest. *Can J Zool* **85**: 994–1007.

Yergeau E, Schoondermark-Stolk SA, Brodie EL, Déjean S, DeSantis TZ, Gonçalves O *et al.* (2009) Environmental microarray analyses of Antarctic soil microbial communities. *ISME J* **3**: 340–351.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Location of watersheds (WS) in Hubbard Brook Experimental Forest (HBEF).

Fig. S2. (a) Denaturing gradient gel electrophoresis of 16S rRNA genes amplified products (338F-GC clamp and 907R) from the reference and the Ca-amended sites at HBEF. Amplified 16S rRNA genes from *E. coli* were used as marker (lanes 2, 9 and 16). The legends Ref-Min1 to Ca-Min3 represent three different sub-plots sampled within each soil horizon at each site. (b) Cluster analysis of the DGGE banding patterns (Panel a) based on the position of the bands using UPGMA (unweighted). Dendrogram based on similarity values obtained with neighbor-joining method.

Fig. S3. Relative frequency distribution of 16S rRNA gene sequences generated from the reference and the Ca-amended sites at HBEF using G2 RDP database in Greengenes website.

Please note: Wiley-Blackwell is not responsible for the content or functionality of any supporting materials supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.