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Abstract

Soil pH has been shown to structure microbial community composition, yet few studies have manipulated soil pH to understand its impact on microbial community composition and function. In this study, we examine how watershed liming, which increases soil pH, has changed organic matter decomposition, and how the fungal and bacterial community structure has been altered 25 years after lime application in two organic forest soil horizons. We studied the watersheds of Woods Lake in the Adirondack region of New York, where watershed liming has doubled organic matter stocks in the forest floor. We hypothesized liming would reduce the potential activities of extracellular enzymes and increase microbial diversity. We used the Illumina miseq platform to sequence soil bacterial and fungal DNA, and measured microbial biomass, carbon mineralization, and extracellular decomposition enzyme activities (α-glucosidase, β-xylosidase, β-glucosidase, cellobiohydrolase, N-acetyl glucosaminidase, leucine aminopeptidase, acid phosphatase, polyphenol oxidase, and peroxidase). Liming increased soil pH by one unit and significantly reduced the activities of five decomposition enzymes in the Oa horizon, where the largest accumulation of organic matter was observed. As hypothesized, soil pH was significantly positively correlated with bacterial and fungal diversity, and liming significantly altered bacterial and fungal communities in the Oa horizon. Overall, more bacterial OTUs responded positively to liming, with 80% of the responding OTUs in the Oe horizon and 75% of the responding OTUs in the Oa horizon showing a higher relative abundance in the limed soils. In contrast, liming did not significantly impact fungal communities in the Oe horizon, but more commonly reduced abundances of fungal OTUs with 78% of them decreasing in abundance. Fungal OTU richness was also positively correlated with several enzyme activities measured indicating that the negative impact on fungal communities has also impaired decomposition in the Oa horizon. Our results indicate that bacterial and fungal microbial communities may be driven by different factors (pH vs. C:N), and that differences in these edaphic factors in organic soil may drive decomposition of organic matter. This study demonstrates that liming can have a long-term impact on soil pH, and is associated with structural shifts in the bacterial and fungal communities with functional consequences for nutrient cycling and carbon storage in soils at Woods Lake.
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First reported in 1960, acid deposition in northeastern U.S. has been a critical environmental stress on forest and aquatic ecosystems for the last half century. Acid deposition depletes base cations (like calcium and magnesium) in soils of these regions and lowers their pH. Recent research has revealed that soil pH is a fundamental driver of the structure of soil microbial communities, which are responsible for important ecosystem functions like decomposition of organic matter. Through forest management practices like liming (the addition of calcium carbonate), it is possible to raise soil and stream water pH back to pre-deposition levels. However, the impacts of liming on soil microbial community structure and their ecosystem functions are largely unknown. To study this, we used a unique watershed liming experiment initiated in 1989 at Woods Lake in the Adirondack region of New York. Previous work at this site found that twenty years after lime addition, the organic matter stocks in the forest floor soil horizons had nearly doubled, indicating a large-scale alteration of the ecosystem carbon balance. This previous study suggested that liming had altered how the microbial community decomposes organic matter, although the mechanism for this remained unclear.

We sequenced soil DNA to uncover how bacterial and fungal microbial communities had changed 24 years after lime application and measured microbial functions like production of decomposition enzymes and mineralization of organic carbon. We sampled soils from the Oe and Oa forest floor horizons, which contain partially and highly decomposed organic matter respectively. As expected, increasing the soil pH via liming lead to increases in bacterial and fungal diversity. Bacterial communities were significantly different between the limed and control soils in both soil horizons. Of the significantly responding bacterial OTUs (unique DNA markers), 75-80% increased in relative abundance due to liming. In contrast, liming only significantly impacted fungal community composition in the Oa horizon, which contains highly decomposed organic matter. In this horizon, 78% of significant fungal OTU responders declined in relative abundance. These results indicate that bacterial and fungal communities in the soils respond differently to liming and that their response may also depend on the quality of organic matter substrates present in each soil horizon. Liming significantly inhibited the activities of five decomposition enzymes in the Oa horizon, which supports the idea that organic matter is accumulating in these limed forest floor soils due to the limited ability of the microbial community to decompose it. Liming, in addition to increasing soil pH, also increases the availability of Ca ions, which can bind to organic matter, making it less available for decomposition. Finally, liming may benefit bacterial communities and relatively disadvantage fungal communities that decompose organic matter. These competing effects seem to be at play in the forest floor of Woods Lake.
This study demonstrates that liming can have a long-term impact on soil pH, is associated with structural shifts in the bacterial and fungal communities, and change how they cycle nutrients and store carbon in soils. This study also highlights the importance of studying microbes as drivers of soil carbon cycling and storage. Further research is required to understand how calcium may physically or chemically stabilize carbon in organic soils if forest liming is to be used to aid in the recovery of acid-impacted forests.
1 Introduction

An important question in ecosystem and microbial ecology is whether and to what extent microbial community structure is linked to ecosystem function. Microbes are considered the biogeochemical engines of ecosystems (Falkowski et al. 2008) and soil microbial communities perform critical functions like decomposition of organic matter and the cycling of nutrients. However, we are only beginning to understand these important functions in the context of the microbial community’s structure, given recent advances in next generation sequencing that allow us unprecedented access to the identities of soil microbes. While a multitude of factors may determine the structure and composition of the soil microbial community in a given space, soil pH has emerged as a soil chemical factor that consistently correlates with microbial community structure. Studies show that soil pH correlates with bacterial community diversity in a predictable fashion (Rousk et al. 2010a) across various spatial scales: at the continental scales (Fierer and Jackson 2006; Lauber et al. 2009), across land-use types at a given location (Lauber et al. 2008; Jenkins et al. 2009), and across sub-meter plots (Philippot et al. 2009). However, very few studies have directly manipulated soil pH and reported changes in both microbial community structure and ecosystem function, and none have used high throughput next generation sequencing techniques to answer this question (Bier et al. 2015). From 454-pyrosequencing studies, which have limited sample sizes, it is understood that soil pH has a strong influence on bacterial diversity (Fierer and Jackson 2006; Lauber et al. 2009) which increases with pH, while fungal diversity appears to be less influenced by soil pH due to their wider optimal pH range (Rousk et al. 2009). There is recent evidence that raising soil pH may reduce the activity of certain extracellular enzymes involved in decomposition of organic matter, while concurrently changing the mycorrhizal fungal growth and diversity in soils (Carrino-Kyker et al. 2016). Does soil pH influence decomposition of organic matter in forest soils by mediating the structure of soil microbial communities?

The Adirondack region provides a useful system to answer this question. Subject to years of acid deposition, the soils are recovering from loss of base cations. An experimental watershed liming experiment was initiated in 1989 by Driscoll et al. (1996) to increase lake water pH. Eighteen years later Melvin et al. (2013) explored how N and C cycling had changed in the limed watersheds of Woods Lake. They found that organic matter mass in the forest floor horizons of the limed watersheds had nearly doubled (175 vs. 94 Mg OM/ha). Preliminary work suggested that organic matter decomposition had been inhibited in the limed soils, however, the mechanisms driving these changes in organic matter stocks were unclear and warranted further exploration (Melvin et al. 2013).
This study seeks to examine how liming, which increases soil pH, has changed organic matter decomposition, an important ecosystem function, and how the microbial community structure has been altered 25 years after lime application at Woods Lake. We used high throughput next generation sequencing techniques to understand how both bacterial and fungal community structure had changed in the limed soils compared to soils from nearby reference watersheds and also how microbial decomposition processes had changed in different organic soil horizons. The hypothesis is that decomposition enzyme activities would be lower in the limed forest floor, explaining the accumulation of organic matter found in the previous study, but that liming would increase bacterial and fungal diversity. Alternatively, if pH induced shifts in microbial community structure are accompanied by no change in decomposition enzyme activity, this may suggest that microbial communities have physiologically adapted to their new high pH environment and are able to decompose organic matter similarly despite being structurally different.
2 Methods

2.1 Field Site and Experimental Design

Woods Lake is the site of the Experimental Watershed Liming Study, which was initiated in October 1989 by Driscoll et al. (1996). It is located within the Adirondack Park, in Herkimer County NY (43°52’ N, 74°57’ W) and has mean annual temperature of 5.28°C and mean annual precipitation of 1230 mm (Yavitt et al. 1995). In 1989, 6.89 tons of CaCO$_3$ ha$^{-1}$ (2.76 t Ca ha$^{-1}$) were applied by helicopter to two ~50 ha watersheds in a single application. Two additional watersheds of Woods Lake were maintained as controls. Lime was applied in pellet form with 82% CaCO$_3$, 8% MgCO$_3$, and 4% organic binder. The watersheds contain mixed hardwood forest species with American beech ($Fagus$ grandifolia), red maple ($Acer$ rubrum), and yellow birch ($Betula$ alleghaniensis) dominating the canopy and lesser amount of red spruce ($Picea$ rubens), sugar maple ($Acer$ saccharum), and striped maple ($Acer$ pensylvanicum) (Smallidge and Leopold 1994).

The bedrock is a hornblende granitic gneiss, which is covered by a glacial sandy till comprised of quartz and feldspar with some hornblende, ilmenite, and magnetite (April and Newton 1985). The soils are Orthod Spodsols (Smallidge and Leopold 1994) with mean mineral soil depth of 30 to 35 cm (Brocksen et al. 1988). The forest floor had a mean soil pH of 3.7 before lime was added (Simmons et al. 1996) and Ca was the dominant soil cation (Blette and Newton 1996). Within one to two years of liming the forest floor soil, pH rose to 4.9 in the Oe horizon and 4.0 in the Oa horizon. Ca availability also rose from 8.5 to 35 cmol, Kg$^{-1}$ in the Oe and 6 to 10 cmol, Kg$^{-1}$ in the Oa (Blette and Newton 1996). Following these studies, the site was not studied again until in 2010, when aboveground biomass, litter chemistry, soil carbon and nitrogen stocks, nitrification and nitrogen mineralization rates, and soil basal respiration were measured by Melvin et al. 2013.

2.2 Soil Field Measurements

Soil collection was conducted in August 2014, almost 25 years after the lime application. In each of the four 50 ha watersheds, five plots that were used in the 2013 study by Melvin et al. were reestablished. Three soil cores were taken from the four corners of each plot and separated into two organic soil horizons. This yielded 80 samples of each limed and control soils. Soils were collected in ziplock bags and transported on ice back to the Goodale Lab at Cornell University where they were held at 4°C. Following a 4mm sieving to remove roots and stones, a subsample was taken from each sample and stored at -80°C for enzyme and microbial community analyses.
2.3 Lab Analyses

2.3.1 Soil physicochemical analyses

Soil gravimetric moisture was assessed for each sample by calculating the difference in weight between 1g of fresh soil and soil dried at 105°C for 24 hours. All following biomass and activity variables are reported as per gram dry weight for each sample. To prepare for the carbon mineralization assay, the 100% water holding capacity of the organic soils was ascertained by comparing the difference in soil weights when they were saturated with DI water and when they were dried at 105°C for 24 hours. This, along with gravimetric soil moisture was used to arrive at 60% water holding capacity for each sample. Soil pH was measured on a 1:1 soil/deionized water slurry following 10 min of equilibration using an Accumet basic AB15 pH meter with a flushable junction soil probe. Total carbon and nitrogen were analyzed by combustion with a Vario EL III elemental analyzer.

2.3.2 Carbon mineralization

The size of the fast cycling pool of C, as per Bradford et al. (2008), was ascertained using a 60 day incubation of limed and control soils, held at 60% water holding capacity and at 22°C for the entire duration of the experiment. Three grams dry weight soil were measured into 50 ml centrifuge tubes with caps modified to have airtight septum lids. The headspace of the centrifuge tubes was flushed with CO₂–free air and incubated for 15 hours at ambient atmospheric pressure and at 22°C. The concentration of headspace CO₂ was determined after the incubation period by injecting 2 ml from the headspace into an infrared gas analyzer (LI-6200, LI-COR, Lincoln, Nebraska, USA). CO₂ flux was then calculated per gram dry weight soil per hour for each sample. This procedure was done on day 1 of the incubation and repeated on days 4, 10, 20, 30, 45, and 60. CO₂ flux was interpolated between these days and cumulated over the entire 60 day assay to arrive at the cumulative C respired for each sample.

2.3.3 Microbial biomass C

An estimate of the active microbial biomass pool as per Fierer et al. (2003) was ascertained using the substrate induced respiration technique. Three grams of pre-incubated (at 22°C) dry weight soil were measured into 50 ml centrifuge tubes with caps modified to have airtight septum lids. Four milliliters of autolysed yeast extract solution were added to tubes and shaken on a desktop shaker for 2 hours to allow the microbiological media substrate to penetrate the soil matrix and become biologically available. Following this, the headspace of the centrifuge tubes was flushed with CO₂-free air and incubated at
atmospheric pressure and room temperature for 4 hours. Finally, maximum CO$_2$ flux was calculated by measuring the CO$_2$ headspace concentration using an infrared gas analyzer (LI-6200, LI-COR, Lincoln, Nebraska, USA). Maximum CO$_2$ flux was converted into microbial biomass C as per the formula put forth by Anderson and Domsch (1978).

### 2.3.4 Extracellular enzyme assays

Fresh soil samples were sieved and stored at -80°C until analysis. The potential activities in soil for seven hydrolytic and two oxidative enzymes were measured to assess functional changes in decomposition. These enzymes included α-glucosidase (AG), β-xylosidase (BX), β-glucosidase (BG), cellobiohydrolase (CB), N-acetyl glucosaminidase (NAG), leucine aminopeptidase (LAP), acid phosphatase (AP), polyphenol oxidase (POX) and peroxidase (PER) (Table 1). The hydrolytic enzyme activities were measured using fluorescently labeled substrates 4-methylumbelliferone (MUB) and 7-amino-4-methylcoumarin (AMC) at 200 µM. The oxidative enzyme activities POX and PER were measured spectrophotometrically using L-3,4-dihydroxyphenylalanine (25 mM) as the substrate (Saiya-Cork et al. 2002; German et al. 2011). Soil slurries were prepared by blending 2 g fresh soil with 100 ml pH 5.0 sodium acetate buffer (50mM) for 1 min. We used white 96-well microplates for the fluorimetric assays and transparent-bottom 96 well plates for the absorbance based assays. Standards were prepared by adding 200 µl of soil slurry and 50 µl of MUB or AMC standards (0, 2.5, 5, 10, 15, 25, 50, and 100 µM) into wells of standard plate. Substrate plates were prepared by adding 200 µl of soil slurry and 50 µl of appropriate substrates into wells. Plates were incubated in the dark at 23°C for three hours and 10 µl of 1.0M NaOH was added to hydrolytic plates to stop the reaction. Fluorescence was measured on a microplate reader (BioTeK) with excitation wavelength at 365 nm and emission wavelength at 450 nm. Oxidative enzyme plates consisted of a blank (250 µl buffer), a L-3,4-blank (200 µl buffer + 50 µl L-3,4-dihydroxyphenylalanine), sample blank (200 µl soil slurry + 50 µl buffer), and the sample wells (200 µl soil slurry + 50 µl L-3,4-dihydroxyphenylalanine). Peroxidase plates received 10 µl 0.3% H$_2$O$_2$ solution. Absorbance was measured at 460 nm with the BioTek microplate reader after a four-hour incubation. We calculated activity based on equations from Saiya-Cork et al. 2002.
Table 1. Extracellular enzymes assayed in soil, their enzyme commission number (EC), corresponding substrate, and the abbreviation used in this study

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>EC</th>
<th>Substrate</th>
<th>Abbreviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-1,4-glucosidase</td>
<td>3.2.1.20</td>
<td>4-methylumbelliferyl-α-d-glucoside</td>
<td>AG</td>
</tr>
<tr>
<td>β-1,4-glucosidase</td>
<td>3.2.1.21</td>
<td>4-methylumbelliferyl-β-d-glucoside</td>
<td>BG</td>
</tr>
<tr>
<td>β-1,4-xylosidase</td>
<td>3.2.1.37</td>
<td>4-methylumbelliferyl-β-d-xyloside</td>
<td>BX</td>
</tr>
<tr>
<td>Cellulohydrolase</td>
<td>3.2.1.91</td>
<td>4-methylumbelliferyl-β-d-cellobioside</td>
<td>CB</td>
</tr>
<tr>
<td>L-leucine aminopeptidase</td>
<td>3.4.11.1</td>
<td>L-Leucine-7-amino-4-methylcoumarin</td>
<td>LAP</td>
</tr>
<tr>
<td>β-1,4-N-acetylglucosaminidase</td>
<td>3.1.6.1</td>
<td>4-methylumbelliferyl-N-acetyl-β-d-glucosaminide</td>
<td>NAG</td>
</tr>
<tr>
<td>Acid phosphatase</td>
<td>3.1.3.2</td>
<td>4-methylumbelliferyl-phosphate</td>
<td>AP</td>
</tr>
<tr>
<td>Peroxidase</td>
<td>1.11.1.7</td>
<td>L-3,4-dihydroxyphenylalanine</td>
<td>PER</td>
</tr>
<tr>
<td>Phenol Oxidase</td>
<td>1.10.3.2</td>
<td>L-3,4-dihydroxyphenylalanine</td>
<td>POX</td>
</tr>
</tbody>
</table>

2.3.5 Fungal and bacterial sequencing

DNA was extracted from each sample using the 96-well PowerMag Microbiome RNA/DNA Isolation Kit (MO BIO Laboratories, Inc., Carlsbad, CA, USA). The recommended protocol was modified for use with highly organic soils with approximately 0.3 g fresh soil used for DNA extraction. Quantification was performed with the standard DNA quantification protocol for Picogreen (Thermo Fisher Scientific, Inc., Waltham, MA, USA). All pipetting for DNA extraction and normalization was conducted with a Hamilton Vantage Liquid handling system robot (Hamilton Robotics, Reno, USA). We amplified 16 S rRNA gene and the internal transcribed spacer (ITS) regions in triplicate from the extracted DNA. Dual-indexed custom barcoded libraries were generated as described by Kozich et al. (2013). PCR primers targeted the bacterial/archaeal 16S rRNA gene variable region 4 (515 F/806 R), and the fungal ITS1-F and ITS2 primers were used to amplify the internal transcribed spacer (ITS1) region of the rRNA operon from fungal genomes. We pooled triplicate amplified samples and normalized them using SequalPrep Normalization Plates (Applied Biosystems, Norwalk, CT). We combined equal concentrations of all barcoded samples and then diluted the pooled, barcoded amplicons for submission to the Cornell Life Sciences Sequencing Core for multiplexed paired-end sequencing on the Illumina MiSeq platform. Raw sequence data were demultiplexed using an in-house (Cornell University) Python script and then processed following the USEARCH pipeline (Edgar RC 2010). Demultiplexed reads were used to construct a de novo database by removing sequences with the maximum expected error rate per sequence, removing replicated and unique sequences, and clustering the remaining sequences into operational taxonomic units (OTUs) at the 97% similarity threshold. We assigned taxonomy to our OTUs using the SILVA database and removed unassigned sequences.
2.4 Statistical Analyses

We used the R statistical package (Rproject.org) to analyze microbial biomass C, soil pH, cumulative carbon respired, and enzyme activity data using ANOVA with a linear mixed effects model with watershed and plot as random effects. All data sets were tested for normality using the Shapiro–Wilk W-test and log transformed when necessary. Statistical significances of these comparisons are from the application of a post hoc Tukey test using lime effect and soil horizon as the dependent variables. For the microbial community analyses, we used the physolseq package in R (McMurdie and Holmes 2013) to produce Bray-Curtis distance matrices. PERMANOVA was performed to identify significantly different clusters using the vegan package in R (Oksanen 2016). Differential enrichment of OTUs was modeled using the DESeq2 pipeline (Love 2014). DESeq2 enables robust estimates of standard error in addition to reliable ranking of logarithmic fold change (LFC) in OTU relative abundance even with low count OTUs (Pepe-Ranney et al. 2016). Correlations between enzyme activities and microbial richness were assessed using Pearson’s product moment correlation in R.
3 Results

3.1 Soil Physicochemical Analyses

Soil pH was significantly higher in limed soils ($F = 58.8, p < 0.001$) with the limed soil pH around 1 unit higher compared to the control soils in both soil horizons. Soil horizon was also a significant factor for pH ($F = 13.7, p < 0.001$) with the Oe horizon (5.1) having a higher pH compared to the Oa horizon (4.9) (Figure 1, Table 2). Soil moisture was not significantly different between limed and control soils, but was higher in the Oe horizon (Table 2).

3.2 Extracellular enzyme activity

Extracellular enzymes showed a consistent response to liming (Figure 2a-i). Overall, soil horizon explained a large part of the variance in enzyme activity rates ($p < 0.001$) with generally higher activity in the Oe horizon. However, there was a significant interaction between soil horizon and lime effect ($p < 0.05$) for almost all extracellular enzymes measured. Liming significantly reduced the activities of AG ($p = 0.04$), BX ($p = 0.008$), and LAP ($p = 0.009$) in the Oa horizon (Table 3). Additionally, AP, which is associated with the mineralization of organic phosphorus, also showed diminished activity in the limed soils in the Oa horizon ($p = 0.02$). Similarly, PER activity was suppressed in the limed soils of the Oa horizon ($p = 0.02$). In contrast, in the Oe horizon, liming tended to slightly increase extracellular activity (Figure 2), although none of the comparisons to control soils in that horizon were statistically significant. Enzymes associated with cellulose and chitin degradation (BX, CB and NAG), and phenol oxidase activity did not respond to liming in either soil horizon.

3.3 Soil microbial Analyses

Limed soils had 1.04 times the log microbial biomass C than control soils, however this result was not significant ($F = 3.5, p = 0.07$). The difference between the soil horizons however, was significant, with the Oe horizon having 1.3 times the log microbial biomass C of the Oa horizon ($F = 281.17, p < 0.001$) (Table 2). There was no significant difference between the cumulative amount of carbon respired from the limed and control soils during the 60-day incubation. The difference between the soil horizons was significant, with the Oe horizon having 1.2 times the log cumulative C respired of the Oa horizon ($F = 690.71, p < 0.001$) (Table 2)
Figure 1. Soil pH as measured in the Oe and Oa soil horizons

Table 2. Soil microbial biomass and respiration measured at Woods Lake

<table>
<thead>
<tr>
<th>Soil Property</th>
<th>Control Oe</th>
<th>Limed Oe</th>
<th>Control Oa</th>
<th>Limed Oa</th>
</tr>
</thead>
<tbody>
<tr>
<td>Microbial biomass C</td>
<td>438.6 ± 53.1</td>
<td>718.1 ± 96.1</td>
<td>104.9 ± 3.5</td>
<td>117.5 ± 7.8</td>
</tr>
<tr>
<td>Cumulative C respired</td>
<td>1907.8 ± 87.8</td>
<td>1887.2 ± 92.3</td>
<td>540 ± 32.5</td>
<td>518.5 ± 37.6</td>
</tr>
<tr>
<td>Soil moisture (g)</td>
<td>3.5bc ± 0.12</td>
<td>3.58c ± 0.12</td>
<td>2.32a ± 0.19</td>
<td>2.75bc ± 0.16</td>
</tr>
<tr>
<td>Soil pH</td>
<td>4.6a ± 0.05</td>
<td>5.6b ± 0.08</td>
<td>4.4c ± 0.04</td>
<td>5.4d ± 0.09</td>
</tr>
</tbody>
</table>

Means that are significantly different (p < 0.05) across rows have different letters. Errors are standard error of the mean and n = 40. Microbial biomass C and cumulative C respired are in μg C per gram soil.
Figure 2. Extracellular enzyme activity rates in μmol hr$^{-1}$ g$^{-1}$ for (a) α-glucosidase (AG), (b) β-glucosidase (BG), (c) β-xylosidase (BX), (d) cellobiohydrolase (CB), (e) leucine aminopeptidase (LAP), (f) N-acetyl β-glucosaminidase (NAG), (g) acid phosphatase (AP)
Figure 3. Correlations between soil pH and (a) bacterial diversity and (b) fungal diversity.

Triangles represent samples from the Oe horizon and circles represent samples from the Oa horizon. Limed soils are in blue and control soils are in black.

Table 3. Abbreviation of extracellular enzymes assayed in soil, their function, and a summary of statistically significant results using the following significance level codes ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Function</th>
<th>Soil horizon effect</th>
<th>Interaction</th>
<th>Lime effect in Oa</th>
</tr>
</thead>
<tbody>
<tr>
<td>AG</td>
<td>Starch degradation</td>
<td>***</td>
<td>***</td>
<td>*</td>
</tr>
<tr>
<td>BG</td>
<td>Cellulose degradation</td>
<td>***</td>
<td>***</td>
<td></td>
</tr>
<tr>
<td>BX</td>
<td>Hemicellulose degradation</td>
<td>***</td>
<td>*</td>
<td>**</td>
</tr>
<tr>
<td>CB</td>
<td>Cellulose degradation</td>
<td>***</td>
<td>***</td>
<td></td>
</tr>
<tr>
<td>NAG</td>
<td>Chitin degradation</td>
<td>***</td>
<td>**</td>
<td></td>
</tr>
<tr>
<td>LAP</td>
<td>Peptide degradation</td>
<td>***</td>
<td>***</td>
<td>**</td>
</tr>
<tr>
<td>AP</td>
<td>Mineralizes organic P into phosphate</td>
<td>***</td>
<td>***</td>
<td>*</td>
</tr>
<tr>
<td>POX</td>
<td>Degrades lignin, aromatic polymers</td>
<td>***</td>
<td>.</td>
<td></td>
</tr>
<tr>
<td>PER</td>
<td>Catalyzes oxidation reactions</td>
<td>**</td>
<td>**</td>
<td></td>
</tr>
</tbody>
</table>
Table 4. Soil bacterial richness and diversity metrics (means ± SE) across limed and control watersheds in two soil horizons at Woods Lake

<table>
<thead>
<tr>
<th>Diversity Index</th>
<th>Control</th>
<th>Limed</th>
<th>Control</th>
<th>Limed</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Oe</td>
<td>Oa</td>
<td>Oe</td>
<td>Oa</td>
</tr>
<tr>
<td>Chao1 Estimator</td>
<td>173.5 ± 16.8</td>
<td>272.5 ± 32.2</td>
<td>205.5 ± 19.7</td>
<td>238.7 ± 30.1</td>
</tr>
<tr>
<td>Inverse Simpson</td>
<td>66.8b ± 5.0</td>
<td>96.6ab ± 9.4</td>
<td>51.8a ± 4.8</td>
<td>67.1a ± 6.5</td>
</tr>
<tr>
<td>Shannon’s H (log 10)</td>
<td>4.5a ± 0.1</td>
<td>4.9a ± 0.1</td>
<td>4.5a ± 0.1</td>
<td>4.6a ± 0.1</td>
</tr>
</tbody>
</table>

Means that are significantly different (p < 0.05) across rows have different letters. Errors are standard error of the mean and n = 40.

Table 5. Soil fungal richness and diversity metrics (means ± SE) across limed and control soil

<table>
<thead>
<tr>
<th>Diversity Index</th>
<th>Control</th>
<th>Limed</th>
<th>Control</th>
<th>Limed</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Oe</td>
<td>Oa</td>
<td>Oe</td>
<td>Oa</td>
</tr>
<tr>
<td>Chao1 Estimator</td>
<td>415.7 ± 16.1</td>
<td>484.6 ± 17.9</td>
<td>343.1b ± 17.5</td>
<td>321.4b ± 18.7</td>
</tr>
<tr>
<td>Inverse Simpson</td>
<td>15.4b ± 1.1</td>
<td>14.1ab ± 1.0</td>
<td>10.8a ± 0.9</td>
<td>11.4ab ± 0.8</td>
</tr>
<tr>
<td>Shannon’s H (log 10)</td>
<td>3.4b ± 0.1</td>
<td>3.6c ± 0.1</td>
<td>3.1ac ± 0.1</td>
<td>3.1ab ± 0.1</td>
</tr>
</tbody>
</table>

Means that are significantly different (p < 0.05) across rows have different alphabets. Errors are standard error of the mean and n = 40.

3.4 Bacterial community structure

Across all 160 samples, 2,782,994 total sequences were obtained after quality filtering and removal of chimeric sequences. Clustering by 97% similarity yielded 7,190 OTUs. Members of the Proteobacteria (~45%), Actinobacteria (~29%), Planctomyces (~10%), and Acidobacteria (~8%) phyla dominated the bacterial community. Soil pH explained a significant amount of variation in the bacterial Shannon diversity index (H) and was positively correlated with it ($R^2$ marginal = 0.11; $p = 0.03$) (Figure 3a). Overall, bacterial diversity, measured by Shannon and Inverse Simpson indices, was higher in the limed soils across both soil horizons ($p < 0.05$) (Table 4). Similarly, the Chao1 estimator showed that limed soils had significantly higher bacterial OTU richness compared to control soils ($p = 0.04$) (Table 4). The NMDS ordination plots revealed distinct bacterial communities in the Oe and Oa soil horizons ($p < 0.05$) (Figure 4c). Within each soil horizon, bacterial communities were also distinct between limed and control soils ($p < 0.05$) (Figure 4a 4b). We also found 1,394 OTUs spanning 15 phyla that had significantly higher or lower abundance in the Oe relative to the Oa horizon.
Within the Oe horizon, we found 1,158 OTUs spanning 14 phyla that responded significantly to liming (Figure 6) with 929 OTUs that responded positively and 229 OTUs that were relatively more abundant in the control soils. Liming also significantly changed the relative abundances of 779 OTUs in the Oa horizon (Figure 7). In the Oa horizon, 584 OTUs increased in relative abundance in response to liming while 195 decreased in relative abundance.

3.5 Fungal community structure

Overall, 2,746 fungal OTUs belonging to 9 phyla and 475 genera were detected. The fungal community was dominated by members of the Basidiomycota (36%) and Ascomycota (52%) phyla. Once again, soil pH exerted a strong positive influence on fungal Shannon diversity ($R^2$ marginal = 0.25; $p < 0.001$) (Figure 3b). Fungal diversity, as measured by Shannon and Inverse Simpson Indices, was significantly higher in the Oe horizon ($p < 0.001$) but was not significantly different with liming (Table 5). Similarly, fungal Chao1 richness was significantly higher in the Oe horizon ($p < 0.001$) (Table 5). NMDS ordinations revealed that fungal communities were distinct in each soil horizon ($p < 0.05$) (Figure 8c). Fungal communities significantly clustered by liming in the Oa horizon ($p < 0.05$) (Figure 8b), but not in the Oe horizon (Figure 8a). We found 116 fungal OTUs that were significantly different between the two soil horizons spanning 32 genera (Figure 9). Within the Oe horizon, we found 5 OTUs that responded positively to liming and 5 that responded negatively ($p < 0.01$) (Figure 10). In the Oa horizon, 68 OTUs responded to liming with significant changes in relative abundance ($p < 0.01$) (Figure 11). Over 60% of the OTUs that responded to liming in the Oa horizon were from the phylum Ascomycota.
Figure 4. Nonmetric multidimensional scaling (NMDS) plot showing clustering of bacterial community by soil horizon (c) and by lime effect within the Oe (a) and Oa (b) horizon.

Triangles represent samples from the Oe horizon and circles represent samples from the Oa horizon. Limed soils are in blue and control soils are in black. Ordinations are based on Bray-Curtis distance metric.
Figure 5. Log fold change in relative abundance of bacterial OTUs that show significantly different changes across phyla.

Positive values indicate OTUs that are relatively more abundant in the Oe horizon and negative values indicate OTUs relatively more abundant in the Oa horizon.

Figure 6. Log fold change in relative abundance of bacterial OTUs in the Oe horizon that show significantly different changes across phyla.

Positive values indicate OTUs that are relatively more abundant in the limed soils and negative values indicate OTUs relatively more abundant in control soils.
Figure 7. Log fold change in relative abundance of bacterial OTUs in the Oa horizon that show significantly different changes across phyla.

Positive values indicate OTUs that are relatively more abundant in the limed soils and negative values indicate OTUs relatively more abundant in control soils.
Figure 8. Nonmetric multidimensional scaling (NMDS) plot showing clustering of the general fungal community by soil horizon (c) and by lime effect within the Oe horizon (a) and within the Oa horizon (b).

Triangles represent samples from the Oe horizon and circles represent samples from the Oa horizon. Limed soils are in blue and control soils are in black. Ordinations are based on Bray-Curtis distance metric.
Figure 9. Log fold change in relative abundance of fungal OTUs that show significantly different changes across phyla.

Positive values indicate OTUs that are relatively more abundant in the Oe horizon and negative values indicate OTUs relatively more abundant in the Oa horizon.

Figure 10. Log fold change in relative abundance of fungal OTUs in the Oe horizon that show significantly different changes across phyla.

Positive values indicate OTUs that are relatively more abundant in the limed soils and negative values indicate OTUs relatively more abundant in control soils.
Figure 11. Log fold change in relative abundance of fungal OTUs in the Oa horizon that show significantly different changes across phyla.

Positive values indicate OTUs that are relatively more abundant in the limed soils and negative values indicate OTUs relatively more abundant in control soils.
Figure 12. Significant correlations between fungal richness and enzyme activity rates in μmol hr\(^{-1}\) g\(^{-1}\) for (a) α-glucosidase (AG), (b) β-glucosidase (BG), (c) cellobiohydrolase (CB), (d) β-xylosidase (BX), (e) N-acetyl β-glucosaminidase (NAG), (f) leucine aminopeptidase (LAP), (g) acid phosphatase (AP), and (h) phenol oxidase (POX).

Triangles represent samples from the Oe horizon and circles represent samples from the Oa horizon. Limed soils are in blue and control soils are in black.
3.6 Microbial community structure and function

We found some positive relationships between microbial richness and enzyme activities measured as potential predictors of decomposition. Bacterial richness did not show significant correlations with enzyme activities. Fungal richness was significantly and positively correlated with several enzyme activities including AG (r = 0.33, p < 0.001), BG (r = 0.41, p < 0.001), CB (r = 0.35, p < 0.001), BX (r = 0.36, p < 0.001), AP (r = 0.45, p < 0.001), LAP (r = 0.43, p < 0.001), NAG (r = 0.38, p < 0.001), and POX (r = 0.31, p < 0.01) (Figure 12).
4 Discussion

Forest liming is a commonly used tool to neutralize acidic deposition in Canada, Sweden, Norway, and the US (Driscoll et al. 1996). This study demonstrates that liming can have a long-term impact on soil pH, and is associated with structural shifts in the bacterial and fungal communities with functional consequences for nutrient cycling and carbon storage at Woods Lake. Our results also show that the two organic soil horizons studied have distinct microbial communities that process nutrients differently, and that bacterial and fungal communities respond differently to liming. Aboveground, tree biomass and litter production were not affected by liming when measured in 2009 (Melvin et al. 2013) indicating that inputs of organic matter had not changed. However, a reduction in soil basal respiration and nitrogen mineralization in the same study pointed to an inhibition of organic matter decomposition. We found further evidence that liming may be inhibiting decomposition particularly in the Oa horizon, where five out of nine extracellular enzyme activities were significantly lower in the limed soils. The exact mechanisms by which liming has altered the decomposer community and its function warrant further investigation, as liming may have changed the physical or chemical recalcitrance of organic matter or led to transient or unobserved changes in organic matter inputs to the soil.

4.1 Liming effect on bacterial community

Our study supports the hypothesis that liming, by increasing soil pH, increases bacterial diversity. Several studies have reported that soil pH is highly positively correlated with bacterial diversity (Fierer and Jackson 2006; Lauber et al. 2009). Using this watershed-scale pH manipulation study, it is apparent this relationship could be causal even when pH changes by only one unit. Liming also induced compositional shifts in the bacterial community in both forest floor soil horizons. These results may reflect that bacteria have narrow optimal pH ranges for growth (Rousk et al. 2009), or that soil pH imposes a direct stress on bacterial cells selecting for some bacterial taxa over others (Lauber et al. 2008; Kowalchuk et al 1997). Our method allowed us to detect changes in ~2,000 OTUs, which is an order of magnitude greater than the detection in PhyloChip (Sridevi et al. 2011) and Denaturing Gradient Gel Electrophoresis (DGGE) (Clivot et al. 2012) studies that have been used previously to assess the effects of lime or calcium amendments on forest soils. We found that within the dominant phyla (Actinobacteria, Proteobacteria, Acidobacteria, and Planctomycetes), bacterial OTUs responded to liming by both increasing and decreasing in relative abundance. Some of these responses are consistent with previous studies that have shown that liming can increase the recovery of Actinomycetes 100-fold in acidic agricultural soils (Tsao et al. 1960; El-Tarabily et al. 1996) and decrease the abundance of Acidobacteria in northern hardwood forest soils (Sridevi et al. 2011). Acidobacteria subgroups 1, 2, and 3 have been
shown to negatively correlated with soil pH, while subgroups 5 and 7 are positively correlated (Rousk et al. 2010b; Kielak et al. 2016). We found 10 OTUs within Nitrospirae that were significantly more abundant in the limed soils, a pattern that was also observed in a limed spruce forest (Bäckman and Klemedtsson 2003). In the Oa horizon, OTUs belonging to Firmicutes and Thermatogae only decreased in response to liming, while OTUs belonging Fibrobacteres and Armatimonadetes solely increased in abundance. Overall, more bacterial OTUs responded positively to liming, with 80% of the responding OTUs in the Oe horizon and 75% of the responding OTUs in the Oa horizon showing a higher relative abundance in the limed soils. There was no correlation between bacterial richness and extracellular enzyme activities, indicating that increasing bacterial diversity by liming may not increase their potential to decompose organic matter. Bacterial richness and hydrolytic extracellular enzyme activities (BG, AP, and NAG) were positively correlated in freshly decomposing leaf litter (Purahong et al. 2016), but this relationship may not hold in the moderately and heavily decomposed organic horizons of this study. Soils at Woods Lake had a relatively low abundance of Acidobacteria, just 8%, compared to 70% in northern hardwood soils (Sridevi et al. 2011) and 46% in spruce-fir forest soils (Clivot et al. 2012). Acidobacteria dominance has been negatively correlated with C mineralization rate and organic carbon availability (Fierer et al. 2007; Jones et al. 2009), which may explain the low relative abundance at Woods Lake.

4.2 Liming effects on fungal communities

Liming changes the soil fungal community in complex ways. In our study, fungal diversity showed a significant positive correlation with soil pH, and showed a positive, but non-significant response to liming. Lime additions at Woods Lake raised soil pH and also changed the availability of calcium and nitrogen in soil organic matter (Melvin et al. 2013). Increasing soil pH increased fungal diversity, but liming may have mitigated these positive impacts on fungal diversity by changing nutrient availability. Unlike bacteria, where diversity tends to follow soil pH closely, fungal communities have been shown to have a wider range of optimal pH growth (Rousk et al. 2009) and tend to be most closely associated with changes in soil nutrient status (Lauber et al. 2008). While there was no significant impact of liming on fungal richness, Suz et al. (2014) found that ectomycorrhizal fungal richness was positively correlated with soil organic horizon pH and soil C:N.

The study demonstrated that different fungal communities operate in the two organic soil horizons, potentially tuned to mineralizing different carbon substrates that dominate these horizons. We also found that fungal communities were not significantly different in the limed and control soils in the Oe horizon, but were significantly different in the Oa horizon. The effect of liming on the soil fungal
community appears to be closely tied to the way liming affected the C:N ratio of the two forest floor horizons. Melvin et al. (2013) reported that soil C:N ratio significantly increased with liming in the Oa horizon, but not the Oe horizon. This idea is supported by Lauber et al. (2008) who found that soil C:N was significantly correlated with phylogenetic distances between fungal communities across land use types. Shifts in substrate quality (high vs low soil C:N) and have previously been linked to shifts in fungal abundance and community composition (Bossuyt et al. 2001; Siles and Margesin 2016).

Ascomycota and basidiomycota represented most of the fungal sequences in this study. Similar numbers of fungal OTUs increased and decreased in abundance in response to liming in the Oe horizon, whereas 22% of significant responders in the Oa horizon increased in abundance and 78% decreased in abundance. Fungal OTUs that showed significant negative responses to liming in the Oa horizon were predominantly from the ascomycota (56%) and basidiomycota (19%) phyla, and many of them have ectomycorrhizal trophic strategies (Birkebak et al. 2013). Of the basidiomycota, five OTUs within the genus *Russula* and one OTU from the genus *Lactaria* were significantly less abundant in the limed Oa soils. Ectomycorrhizal members of these two genera have been shown to be associated with low soil pH (Suz et al. 2014). Of the ascomycota, the genus *Cenecoccum*, which tends to be commonly found in temperate forest soils and associated with low pH organic soils (Suz et al. 2014), showed significant declines in abundance when limed.

Fungi, particularly some ascomycetes and bacidiomycetes, are important sources of extracellular enzymes like peroxidases, phenol oxidases, and laccases in soils (Sinsabaugh et al. 2012). Other members of these phyla produce hydrolytic enzymes like proteases and chitinases for the purpose of mining nitrogen from humus, rather than for carbon acquisition (Sinsabaugh et al. 2008; Suz et al. 2014). We found that fungal richness across the Oe and Oa horizons was significantly positively correlated with all hydrolytic and oxidative enzyme activities measured except for peroxidase. Fungal richness in freshly decomposing litter is negatively correlated with laccase and peroxidase (Purahong et al. 2016), but may not be the case in the Oe and Oa horizon.

### 4.3 Liming effect on extracellular enzyme activities

Differential rates of extracellular enzyme activity between limed and control soils suggest that there may be functional consequences of the microbial community shifts described above. Liming decreased the potential activities of five extracellular enzymes in the Oa horizon soils. Limed soils in the Oa horizon had a diminished potential for C decomposing enzymes including ones that degrade starch, hemicellulose, and phenols, as inferred by AG, BX, and PER activity respectively. We did not find
Evidence for cellulose degradation, as measured by BG and CB activity, being suppressed by liming in our study. Lime additions have been shown to have no effect on BG and CB in a deciduous forest soil (DeForest et al. 2011; Carrino-Kyker et al. 2016), a negative effect on ectomycorrhizal root tip CB production in a beech forest soil (Rineau et al. 2009), a negative effect on BG in Arctic tundra soils (Stark et al. 2014), but a positive effect on AG and BG in agricultural soils (Acosta-Martinez and Tabatabai 2000; Tabatabai et al. 2010). Very few studies have described the effects of lime or calcium additions on bulk soil C degrading enzyme activities in forest soils. The responses of C degrading enzymes in our study may have to do with the specific change in soil pH achieved by liming and the optimum pH at which these enzymes operate. Soil pH can affect the efficiency of enzymes by changing conformation of the enzyme active site (Frankenberger and Johanson 1982). For example, PER has an optimum pH of 4.5 (Wang et al. 2012), and increasing the soil pH from 4.4 to 5.4 in the Oa horizon may have reduced its potential activity. Rineau and Garbaye (2009) found that members of Cenecococcus, an ectomycorrhizal genus, were the main contributors to total enzyme activities measured in root tips in beech forest soils. The study found that members of Cenecococcus were up to 32 times less abundant in limed Oa horizon soils suggesting that the changes in fungal community composition might partially explain the reduced C degrading potential in the limed Oa horizon.

Liming also altered the dynamics of N and P acquisition in the Oa forest soils. Here, liming reduced the potential activity of NAG, the enzyme that catalyzes the hydrolysis of N-acetyl-b-D-glucosamine from chitobiose and other chito-oligosaccharides, which are a large source of organic N in soils (Sinsabaugh et al. 2012). This reduction in N-acquiring enzyme activity along with lower rates of N mineralization (Melvin et al. 2013) implies that soil microbes may be N limited in the Oa horizon. Liming also reduced the potential of plant and microbial mineralization of organic P in the Oa horizon, as measured by AP activity. This effect has been observed in a limed deciduous forest soil (Carrino-Kyker et al. 2016) and in pot experiments with forest soils (Illmer and Schinner 1991). The effect of liming on extracellular enzyme activities was significantly different in the Oe horizon, where there was no detectable difference between limed and control soils.

4.4 Liming effects on forest floor C cycling and decomposition

Liming marginally increased Oe horizon microbial biomass C, but no difference was detected in the Oa horizon. Similarly, we did not detect any effect of liming on cumulative C mineralized. Most liming studies show a stimulation of microbial activity, C mineralization, and decreases in organic matter stocks (Baath et al. 1980; Lohm et al. 1984; Persson et al. 1989; Smolander et al. 1996; Ingvar Nilsson et al. 2001). Liming studies have reported increases (Kemmitt et al. 2006; Aciego Pietri and Brookes
in microbial biomass C and no response in C mineralization (Minick et al. 2011). Previous *in situ* measurements of soil respiration at Woods Lake within the first year after liming showed no significant difference of CO₂ efflux between control and limed soil (Yavitt et al. 1995), but Melvin et al. (2013) showed soil basal respiration had decline by up to 40% in the Oa horizon, suggesting a suppression of decomposition. This discrepancy may be due to differences in methods used to measure respiration in the two studies. In comparison to the previous study, we doubled the duration and the number of field replicates, which increased the variance we observed. Yet, evidence from enzyme activities measured support the idea that liming reduced decomposition in the Oa horizon. Because enzymes persist in soils (Burns 1982), they reflect an integrated measure of decomposition capacity of the soil in the long term (Spalding 1980), whereas the response of C mineralization and biomass measurements may be dominated by seasonal C cycle dynamics in the forest floor. Relative to the Oa horizon, microbial activity was consistently higher in the Oe horizon, evidenced by significantly higher microbial biomass C and rates of carbon mineralization. This likely reflects differences in substrate quality between the horizons typical in northern hardwood forests (Minick et al. 2011; Morse et al. 2014), but the result highlights the need for a finer scale of horizon resolution when studying microbial community structure and function than is typically employed.

### 4.5 Competing effects of liming

We found support for our hypotheses that liming would suppress decomposition enzyme activities and alter the microbial community structure, which could explain the accumulation of OM stocks observed in the Oa horizon of Woods Lake’s limed watersheds. Liming and soil pH can interact, leading to complex effects on the soil microbial community structure and function through the addition of nutrient cations like calcium. Whittinghill and Hobbie (2011) demonstrated this interaction on tundra soils, where high pH increased microbial activity relative to low pH soils, but adding calcium diminished this response. Calcium may also reduce the solubility of OM in the forest floor (Balaria et al. 2014). We found a marked difference in the effect of liming between the moderately decomposed litter in the Oe horizon and the well decomposed humic material in the Oa horizon. This result points to the idea that liming may initially augment microbial activity enhancing C loss, following which, the remaining C pool becomes relatively enriched in more recalcitrant compounds (Chan and Heenan 1999).
5 Conclusions

Liming in the Woods Lake watersheds resulted in a decline in enzyme activities and a profound shift in fungal and bacterial community structure within the forest floor. The results of this study are in concert with some liming studies, but also show some dramatic differences, suggesting that liming can have differential effects on microbial community structure and function driven by a suite of physicochemical and biological processes. This work highlights the importance of studying microbes as mechanistic drivers of soil C cycling and demonstrates their potential to influence soil C storage, which has direct implications for climate change. As a land management strategy, ecosystem scale forest liming endeavors should monitor changes in microbial communities and be aware of long term impacts of altering the relationship between decomposers and soil organic matter. This enhances our understanding of the effects of liming on New York forests and provides additional insights into how alterations in pH and Ca affect C cycling and storage.
6 References


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