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1 Surprising Relationships Between Soil pH and Microbial Biomass and Activity in a 2 **Northern Hardwood Forest** 3 4 RENATA ONTMAN¹ PETER M. GROFFMAN^{1,2,3,*} 5 6 CHARLES T. DRISCOLL⁴ ZHONGOI CHENG^{1,2} 7 8 ¹Brooklyn College of the City University of New York, Department of Earth and Environmental 9 Sciences, Brooklyn, NY 11210 10 11 ²City University of New York Advanced Science Research Center at the Graduate Center, New York, NY 10031 12 13 14 ³Cary Institute of Ecosystem Studies, New York, NY 12545 15 16 ⁴Syracuse University, Department of Civil and Environmental Engineering, Syracuse, NY 13244 17 18 *Corresponding author 19 20 21 **Abstract** 22 Soil microbes mediate major biogeochemical processes in forest ecosystems. Soil pH is 23 considered a "master variable" with a strong positive effect on many biogeochemical processes. 24 To better understand how soil pH influences microbial activity and nitrogen dynamics in forests, 25 we utilized a set of long-term measurements of surface soil pH, nitrogen availability, and 26 microbial biomass and respiration from Hubbard Brook Experimental Forest (HBEF), a northern 27 hardwood forest in New Hampshire, USA. Further, we compared the strengths of these 28 relationships in an unmanipulated watershed, where naturally acidic soils have been further 29 acidified by anthropogenic acid deposition, to those in a nearby watershed, where soils were 30 treated with calcium silicate to ameliorate the effects of acid deposition. While we expected to 31 observe strong positive relationships between soil pH and microbial biomass and activity, we

instead found weak and/or curvilinear relationships. Further, microbial biomass and activity

peaked at unexpectedly low pH values (~4.5), and decreased at higher pH values, especially in the calcium-treated soils. It is likely that complexities in plant-microbial interactions inhibit and/or mask microbial response to changes in pH in these acidic soils. These results raise questions about pH as a controller of microbial processes and how ecosystems recover in response to decreases in acid deposition.

- **Key words:** microbial respiration, nitrogen cycle, mineralization, nitrification, denitrification,
- 40 pH, forest soil, northern hardwood forest

Introduction

Soil microorganisms play a major role in nutrient cycling, plant production, soil formation, and other critical ecosystem processes. However, much remains unknown about how microbes mediate these processes, and how they respond to environmental change and disturbances. Such changes include but are not limited to atmospheric deposition and climate change, which in turn impact abiotic factors such as soil pH (Paul 2014). Soil has considerable capacity to buffer acid deposition; however, when hydrogen ion inputs exceed the soil buffering capacity, soil acidification occurs (van Breemen et al. 1983). Acidification alters mineral solubility and nutrient availability, and thus impacts the availability and toxicity of nutrients and elements such as free Fe ³⁺ and Al ³⁺ in the soil (Driscoll et al. 2001). In forests, acid addition has been found to negatively impact Mg ²⁺, Ca ²⁺, soil respiration, fine root biomass, microbial biomass C, microbial biomass N, and bacterial biomass, while positively affecting soil iron, NH₄ ⁺-N, available phosphorus (P), and fungal biomass (Meng et al. 2019).

Many studies have found pH to be a strong regulator of microbial community

composition (Bahram et al. 2018; Fierer et al. 2009; Lauber et al. 2009) and function (Meng et al. 2019). Fierer et al. (2009) found that bacterial community composition and fungal: bacterial gene ratios were strongly driven by soil pH and soil C:N ratios. Lauber et al. (2009) found significant correlations between soil pH and the abundance of acidobacteria, actinobacteria, and bacterioidetes. Hartman et al. (2008) found that soil pH predicted the diversity of bacterial 371phyla and "species" across wetland sites. This regulation is based on the limited pH tolerance of prokaryotic taxa as well as the effects of pH regulation on nutrient availability (Zhalnina et al. 2015). The ability of specific soil microbes to adapt to pH changes is an important regulator of ecosystem response to global phenomena such as acid rain and calcium additions (Bardgett & van der Putten 2014).

The Hubbard Brook Experimental Forest (HBEF) Long Term Ecological Research site in the White Mountains of New Hampshire (Figure 1) presents unique opportunities for study of patterns in soil microbial biomass and activity in responses to changes in pH. The HBEF is dominated by northern hardwood forest and has been impacted by acid deposition for over 50 years (Likens et al. 2021). In October 1999, a watershed at HBEF (Watershed 1) was experimentally treated with wollastonite (CaSiO₃) to replace calcium estimated to have been leached from the ecosystem by decades of acid deposition (Cho et al. 2010; Cho et al. 2012). This treatment allows for evaluation of pre- and post-treatment effects of the calcium addition as well as comparisons to an untreated, reference watershed (Watershed 6) at HBEF.

Long-term measurements of microbial biomass and activity were initiated in a reference area (adjacent to the untreated reference Watershed 6) at the HBEF in 1994 and in the calciumtreated watershed in 1998 (Groffman 2019). Early analysis (Bohlen et al. 2001) focused on relationships among microbial biomass, N transformation rates (net N mineralization, net

nitrification, denitrification potential), and soil, plant communities, and stream chemistry. This analysis established that microbial biomass is a useful indicator of broad-scale variations in N cycling processes within ecosystems and that plant-soil-microbial relationships regulate spatial and temporal patterns of N cycling processes.

The extensive body of long-term monitoring and research on the calcium-treated and reference watersheds provided an excellent opportunity for analysis of the importance of pH as a controller of microbial and ecosystem processes. Previous research on these watersheds has highlighted the importance of plant:microbial interactions as a regulator of this control (Groffman & Fisk 2011a). We hypothesized that there would be strong positive relationships between pH and C and N cycle processes in soils at Hubbard Brook and that these relationships would emerge in comparisons between the calcium-treated and reference watersheds and in long-term temporal patterns where pH was increasing (reference watershed) or decreasing (calcium-treated watershed) over time. The analysis provides basic scientific information on an important controller of microbial biomass and activity variables that are central to forest ecosystem nutrient cycling processes. More practically, the results are relevant to a widespread environmental problem, acid deposition, which is ongoing in many parts of the world while in other areas, such as the northeast U.S., recovery or deacidification is underway (Jandl et al. 2012; Likens et al. 1996; Oulehle et al. 2017; Oulehle et al. 2011)

Materials and Methods

The HBEF was established in 1955 in the White Mountains of New Hampshire (43 °56'N, 71°45' W) with 3,160 ha (~7,800 acres) of forest. The climate is continental with short cool summers and long cold winters featuring constant snowpack averaging 1.5 m. The average air

temperature is 19 °C in July and -9 °C in January and annual precipitation averages 140 cm (Campbell et al. 2021). Soils are dominated by Spodosols (Haplorthods) of various drainage classes formed from glacial till, with pH values ranging from 3.4 to 3.8 in the surface organic horizons and from 4.1 to 4.7 in the mineral horizons (Johnson et al. 2000). The forest floor organic horizons are typically 5-9 cm in thickness and are comprised of the undecomposed litter layer (Oi), a partially decomposed fibrous layer with a noticeable root mat (Oe), and a highly decomposed humic layer (Oa/A). The mineral soils are fairly shallow with high amounts of coarse fragments and loamy sand to sandy loam texture (Johnson et al. 2000). Sugar maple (*Acer saccharum* Marsh.), American beech (*Fagus grandifolia* Ehrh.), and yellow birch (*Betula alleghaniensis* Michx.) dominate the overstory vegetation, with some red spruce (*Picea rubens* Sarg.) and balsam fir (*Abies balsamea* L.) at higher elevations (Bohlen et al. 2001).

Sample collection has taken place every July since 1994 at the HBEF for analysis of soil NO₃⁻ and NH₄⁺ concentrations, microbial biomass C and N content, microbial respiration, potential net N mineralization and nitrification rates, and denitrification potential in a reference area located just west of the Watershed 6, the biogeochemical reference watershed at HBEF. Sampling plots (2 x 3 m) were located at 4 elevations (525, 585, 685, and 775 m above sea level) on each watershed, with 5 replicate plots at each elevation for a total of 40 sampling plots in each year. Two to eight samples were taken from each plot and composited by horizon (Oie, Oa, and the top 10 cm of the mineral soil) (Groffman et al. 2006). Sampling was conducted at least a week following rainfall events to avoid the effects of re-wetting dry soils on microbial activity. In 1994 and 1995, only the first two forest floor soil horizons (Oie and Oa/A) were sampled. Beginning in 1996 the Oie, Oa/A horizons and the upper 10 cm of mineral soil were sampled. Samples were divided by horizon in the field and each horizon was placed into a sample bag.

Soil samples were placed in a cooler and transported to the Cary Institute of Ecosystem Studies in Millbrook, New York, USA and stored at 4 °C until analyses, which were completed within 10 days of sampling. Analysis of soil pH of these samples began in 2000.

In mid-October 1999, 55 tons of pelletized calcium in the form of wollastonite (CaSiO₃) were added to the 11.8 ha Watershed 1 to replenish calcium estimated to have been leached from the ecosystem by several decades of acid deposition. In 1998, the sampling routine for microbial biomass and activity was extended to four matching elevations with five replicate plots per elevation on this watershed. Composite soil samples were mixed by hand, and roots and stones over 2 mm were removed. Soil moisture content was determined by drying at 60° C for 48 h (McInnes et al. 1994). Amounts of inorganic N (NO₃- and NH₄+) in soil were determined by extraction with 2 M KCl followed by colorimetric analysis using a Perstorp Flow Solutions 3000 or Lachat 8000 series flow injection analyzer. Soil pH was measured with a glass electrode in a 1:2 (Oa/A, mineral soil) or 1:4 (Oie) soil:water slurry.

Field-moist soils were used for microbial biomass, respiration, N mineralization, and denitrification potential analyses. The chloroform-fumigation-incubation method (Jenkinson & Powlson 1976) was used to determine soil microbial biomass. A 20-g soil sample was fumigated for 24 hours with distilled chloroform in a humid vacuum desiccator, and then it was inoculated with 0.5 g of fresh soil and placed in 1-L air-tight glass jars and incubated for 10 days at room temperature. In separate jars, controls composed of 20.5 g of soil were incubated simultaneously alongside the fumigated samples. Gas samples collected at the end of the incubations were analyzed for CO₂ content by thermal conductivity gas chromatography using a Shimadzu GC-2014. Inorganic N (NO₃- and NH₄+) was extracted from fresh samples before the incubations and from jars at the end of the incubation as described above.

Microbial biomass C was calculated as $B_c=F_c/0.41$, where B_c is microbial biomass C, F_c represents the C CO₂-C produced over the 10-day incubation of flush from fumigated soils, and 0.41 is a correction factor that accounts for the extraction efficiency of the method for microbial biomass C (Voroney & Paul 1984). Estimates of microbial biomass N were not corrected and are determined as the inorganic N produced over the 10-day incubation of fumigated soils. The production of CO_2 in the unfumigated incubation serves as an estimate of microbial respiration, and the accumulation of inorganic N in these incubations is a measure of potential net N mineralization. The accumulation of NO_3 in the unfumigated incubation is a measure of potential net nitrification.

Denitrification potential was measured using a denitrification enzyme activity (DEA) assay that involves quantifying the production of nitrous oxide (N₂O) from soils treated with NO₃⁻ (100 mg N/kg), glucose (500 mg/kg), chloramphenicol (125 mg/kg) and acetylene (C₂H₂, 10 kPa) and incubated under anaerobic conditions for 90 minutes (Groffman et al. 1999; Smith & Tiedje 1979). Gas samples taken at 30 and 90 minutes were analyzed for nitrous oxide by electron capture gas chromatography using a Shimadzu GC-2014. The denitrification enzyme activity was then calculated as the accumulation of nitrous oxide between 30 and 90 minutes.

Data were analyzed using R v. 1.1.3.1093 (R Core Team 2021) and all R codes are available on Github (https://figshare.com/s/e9dc374a99c9ea02d39a). Data analysis began with graphical analysis of relationships between soil microbial biomass and activity and pH within and between different watersheds and soil horizons. GGplot was utilized to plot microbial biomass/activity parameters versus pH (Wickham 2016). The packages dplyr, plyr, and tidyr were used for data manipulation (Wickham et al. 2022). These analyses revealed that 1) the strong positive relationships between these variables that we expected were not present, 2) in

many cases there appeared to be a peak in biomass/activity at an intermediate pH value, and 3) relationships were weak, with lots of visible scatter. These findings led us to fit both linear and polynomial regressions to confirm these results.

The graphical analysis facilitated treatment of possible outlier values. All values were left in the data set unless it was evident that there was a contamination or laboratory procedure problem (Groffman 2019). In some cases, data points were 5 - 10 times higher than the next highest value. However, if this pattern was evident in multiple parameters we determined that it was not due to laboratory error, and must represent a "hot spot" of activity in the soil. Thus, these data were left in the dataset.

Linear and polynomial regression analyses with soil pH as the driver/x-variable were run for each watershed (calcium-treated and reference) and soil horizon (Oie/Oe, Oa/A, and Mineral Soil). Analyses with data from the two watersheds combined were also run, to expand the range of pH values for the analysis. Changes in pH with time were explored for each watershed and soil horizon separately. Linear and polynomial regression models were run using the lm function in R (R Core Team 2021) for each separate soil horizon with the combined watershed data points for a total of 176 individual models. The summary feature allowed us to extract the r-squared value and p-value for both the linear and polynomial models. To account for the multiple (nine) variables being regressed against soil pH, a Bonferroni correction significance value of 0.006 (0.05/9) was used as a criterion for statistically significant relationships (Haynes 2013).

Results

Soil pH significantly increased over time in the Oie, Oa/A and mineral soil horizons in the reference watershed (Figure 2a) and in the mineral soil horizon in the calcium-treated watershed (Figure 2b). Soil pH significantly decreased over time in the Oi/Oe and Oa/A horizons in the calcium-treated Watershed 1 (Figure 2b). However, graphical analysis showed that the strong positive, linear relationships between microbial biomass and activity and soil pH that that we expected were not present and that in many cases there was an apparent peak in biomass/activity at an intermediate pH value, with peak values at ~4.5 and lower values at lower and higher pH (Table 1, Figures 3, 4, and 5). Peak values for the majority of curvilinear relationships occurred in the 2008-2011 time frame.

There were no statistically significant relationships between soil microbial biomass C and pH in any horizon, in the analysis across both the reference and calcium-treated watersheds (Figure 3a). The only statistically significant relationship between soil microbial biomass C and pH was in the Oa/A horizon of the reference watershed (Table 1).

Microbial respiration showed a statistically significant curvilinear relationship with pH in the Oa/A horizon (reference watershed only) with peak values at pH ~4.5, and lower values at higher and lower pH (Figure 3b). The curvilinear peak in the reference watershed occurred in 2005-2006. There were no significant relationships between microbial respiration and pH in the Oie or mineral soil horizons, except in the Oa horizon of the reference watershed (Table 1).

Microbial biomass N exhibited a significant curvilinear relationship with pH in the Oie horizon, with peak values at pH \sim 4 (Figure 4a). In the reference watershed, peak values occurred in 2009 - 2010 and in the calcium-treated watershed, peak values occurred in 2009-2010. There was a significant negative linear relationship between microbial biomass N and pH in the Oa/A horizon. There were no relationships between microbial biomass N and pH in the mineral soil horizons.

There was a significant curvilinear relationship between potential net N mineralization

and pH in the Oie horizon with peak values at pH ~4.25, and significant linear relationships with pH in the Oa/A and mineral soil horizons (Figure 4b). The curvilinear peak for potential net mineralization occurred in 2009-2010 for the reference watershed and in 2010-2011 in the calcium-treated watershed.

There were no significant relationships between potential net nitrification (Figure 4c) or denitrification potential (Figure 4d) and pH in any horizon. There were significant negative linear relationships between ammonium and soil pH in all three soil horizons (Figure 5a) and significant curvilinear relationships between nitrate and pH in the Oa/A and mineral soil horizons (Figure 5b). The mineral horizon had peak nitrate values at pH ~4 .25, the Oa/A horizon had peak values at pH ~3.8, and the Oie horizon had peak values at pH ~4.25 (Figure 5b). In the reference watershed, peak values occurred in 2009-2010. In the calcium-treated watershed, peak values occurred in 2006-2007 in the Oi/Oe horizon, 2008-2009 in the Oa/A horizon, and 2009 in the mineral soil horizon.

Discussion

Our results are surprising given previous studies showing strong positive relationships between soil pH and microbial community composition and activity and thus raise questions about relationships between soil pH and biological processes. While we expected strong positive relationships between soil pH and microbial biomass and activity, we instead observed significant curvilinear relationships with peaks at unexpectedly low values, between pH ~3.5 and ~4.5. These relationships are based on 20 years of data that span reference and calcium-treated watersheds and organic and mineral soil horizons.

Microbial biomass C is a key functional pool for C and N cycling in organic detritus

since it represents the active biological component of soil organic matter. Microbial respiration is a more dynamic, short-term indicator of soil biological activity. The fact that there were no significant relationships between these variables and pH in the Oie horizon, which is the most biologically active zone of the soil profile (Coleman et al. 2017), suggests that effects of soil pH on biological activity at this northern forest site are complex. The weak and/or curvilinear relationships observed may suggest that the soil communities are adapted to the inherently low pH at the site. Addition of calcium to Watershed 1 experimentally increased the pH but clearly did not shift this adaptation to a higher pH. The curvilinear relationships contrast with our expectations and other results from the calcium-treated watershed (Sridevi et al. 2012) showing that decreased soil calcium was correlated with reduced microbial biomass and C sequestration of forest trees.

Our results also show potential adaptation of N cycling processes to low pH in Hubbard Brook soils with weak and/or curvilinear relationships between potential net N mineralization and potential net nitrification and soil pH. Relationships between nitrification and pH have long been mysterious, with laboratory studies showing marked declines in growth at pH below 7 contrasting with field studies showing active nitrification activity at much lower pH values (Nicol et al. 2008; Yao et al. 2011). The N cycle processes interacted to create significant relationships between pH and ammonium (linear, negative) and nitrate (curvilinear) in all three soil horizons.

An alternative explanation to microbial adaptation to low pH for the curvilinear relationships observed centers on plant: microbial interactions. Increases in pH could stimulate microbial biomass and activity to a certain level at which plants begin to respond, e.g., by establishing mycorrhizal associations inhibited by low pH (Juice et al. 2006). This plant response

can then limit the ability of heterotrophic microbes to respond further. Similar dynamics have been observed in other studies (discussed below) at HBEF. Thus, the complexities of plant: microbial interactions may give the appearance that microbes are adapted to the low values while in reality, their ability to respond to increases in pH is being inhibited by plants. This complexity also likely contributes to the generally low r-squared values that we observed in the relationships between microbial biomass and activity and pH.

Complex effects of soil pH at a naturally acidic watershed

Forest soils at HBEF are naturally acidic, with low base cation supply due to low rates of mineral weathering and low exchangeable base cation concentrations in the O horizon (Lawrence et al. 1995; Likens et al. 1996). The low levels of exchangeable calcium in mineral soils are due to low rates of mineral weathering at the site. They are also associated with elevated concentrations of Al in the O horizon that inhibit root growth and the establishment of mycorrhizal associations (Juice et al. 2006; Likens et al. 1998; Palmer et al. 2004).

The addition of calcium silicate to Watershed 1 in 1999 rapidly (by 2000) increased exchangeable Ca, soil pH, cation exchange capacity, and base saturation and decreased exchangeable acidity in organic soil horizons (Cho et al. 2010; Cho et al. 2012; Johnson et al. 2014). In the mineral soil layer, short-term increases of exchangeable Ca, soil pH, cation exchange capacity, and base saturation were much smaller and exchangeable acidity exhibited small decreases to no change.

Over the last 20 years, pH has declined in the Oa/A and Oie horizons of the calcium-treated watershed, consistent with processing and loss of the added calcium (Johnson et al. 2014). Soil pH has increased in the mineral and Oa/A horizons of the reference watershed,

consistent with deacidification and reduction of acid deposition (Likens et al. 2021). Our sampling sites, which range across elevation gradients in both reference and treated watersheds, thus encompass a wide range of pH conditions for the northern hardwood forests represented by the HBEF.

Several studies at HBEF have found that soil pH controls on soil biological processes are more complex that might be expected from consideration of the chemical dynamics of base cations, acidity and aluminum. Fiorentino et al. (2003) investigated the effects of the calcium addition to Watershed 1 on P pools and cycling and N cycle processes. While there were no significant differences in soil solution or fine root P concentration, microbial biomass P, microbial C to P ratios, available organic and inorganic P fractions were lower in the Oe horizon of the calcium-treated watershed than the reference watershed. They concluded that the soil pH changes associated with the calcium addition had increased the rates of P cycling in the forest floor horizons, with enhanced flow of P to plants at the expense of microbes (Fiorentino et al. 2003).

Several studies of the Watershed 1 experiment have reported complex effects of calcium additions on N cycling processes (Battles et al. 2014; Juice et al. 2006; Rosi-Marshall et al. 2016). Groffman et al. (Groffman et al. 2006) observed declines in the N content of the microbial biomass, potential net and gross N mineralization rates, and soil inorganic N pools in the Oie horizon of the treated watershed. These results clearly suggested that the calcium addition did not stimulate microbial N cycling, and the authors suggested that plants had responded more dynamically to the calcium addition than microbes, inhibiting the ability of the microbes to respond to the increases in calcium and soil pH. This idea was supported by soil mesocosm studies without plants where calcium additions resulted in marked increases in soil microbial N

cycling process rates (Groffman & Fisk 2011a; Groffman & Fisk 2011b). It is also supported by increases in plant growth (but not changes in community composition) in the calcium-treated watershed (Battles et al. 2014).

These results for P and N response to the calcium addition to Watershed 1 lead to the idea that plant:microbial interactions underlie the surprising curvilinear relationships between microbial biomass and activity and soil pH observed in this study. It is logical that increases in pH caused either by natural variation or calcium addition should stimulate microbial biomass and activity. However, if the increases in pH reach a certain level where plants begin to respond, e.g., by forming mycorrhizal associations, the flow of C, N, and P to non-mycorrhizal microbes could be reduced by plant uptake(Gadgil & Gadgil 1971), resulting in apparent declines in microbial biomass and activity at higher pH. Thus, the complexities of plant: microbial interactions may make it appear that the microbes are adapted to/optimized for low pH while in reality their ability to respond to increases in pH is inhibited by plants.

The previous research at the HBEF highlight that there are important temporal trends in microbial biomass and activity that are not addressed in our analysis of pH controls here. Key trends include marked declines in acid and N deposition, which combined with a lengthening growing season and increases in atmosphere CO₂ concentrations have led to a marked reduction in N availability (oligotrophication) at the site (Groffman et al. 2018). These temporal trends are being monitored and analyzed in other studies.

The importance of microbial community composition

Much of the effects of soil pH and acid deposition on ecosystem processes are mediated via effects on microbial community composition. Despite advances in technology and

understanding, soil biota have proven to be very difficult to study and characterize. A metaanalysis of over 1300 published datapoints, found that variation in microbial biomass is
predictable across biomes with microbial biomass C representing 0.6-1.1% of soil organic C and
1-20% of total plant biomass C (Fierer et al. 2009). A study that utilized marker gene sequencing
to characterize soil bacterial communities found that 53% of microbes had predictable habitat
preferences and strong links between microbial community composition and plant productivity
(Delgado-Baquerizo et al. 2018). These studies suggest that there are predictable patterns in soil
microbial communities that should be useful for assessing and predicting the effects of
environmental changes on these communities.

Consistent with the global studies cited above, previous studies (Sridevi et al. 2012) have found significant differences in bacterial populations in the reference and calcium-treated watersheds at HBEF. *Acidobacteriaceae*, *Comamonadaceae*, and *Pseudomonadaceae* were lower in the Ca-amended soils, while *Flavobacteriaceae* and *Geobacteraceae* showed an opposite pattern. Ammonia-oxidizing *Nitrosomonadaceae* were lower in organic horizon soils of the Ca-amended watershed and an opposite pattern was observed in the mineral soil. The decreases in *Acidobacteriacease* and *Nitrosomonadaceae* support our assertion here that microbes do not always respond to increases in pH as expected. The presence of *Acidobacteria* in HBEF soils is important because these bacterial taxa play a major role in the function of the forest soil ecosystem and are viable in acidic, nutrient-limited environments such as forests at HBEF. The presence of specific bacteria can be indicative of how pH impacts the bioavailability of soil nutrients and can potentially be a limiting agent in nutrient pathways.

A metagenomics survey in the reference watershed at the Hubbard Brook focused on the genes involved in denitrification, a process that responds to N enrichment, found evidence that

acidity affected the communities that carry out this process (Roco et al. 2019). It has long been recognized that low soil pH inhibits expression of the genes responsible for the last step of the denitrification process (Payne 1981; Zumft 1997). The more recent study showed a series of adaptations to acidity, including removal of toxic intermediates in the denitrification process (nitric oxide) that suggest significant capacity for adaptation to highly acid conditions in HBEF soils (Roco et al. 2019). Further evidence for adaptive capacity was found under experimental soil warming and increased soil freeze/thaw cycles which suppressed bacterial taxa with the genetic potential for the final steps of denitrification (Garcia et al. 2020). This analysis suggested that a key to adaptation to new conditions in HBEF soils is the emergence of taxa that trade-off growth for stress tolerance traits. Another study showing adaptive capacity found that winter snowpack decline associated with global warming increased bacterial richness and phylogenetic diversity (Sorensen et al. 2016). However, any effects of changing soil climate conditions may have been mediated by effects on root growth, which is impaired by soil freezing associated with the snowpack decline (Sorensen et al. 2019).

Much of the microbial community response to changing environmental conditions may be focused in "microzones" or "microbial hotspots" of higher pH in the generally acidic soil matrix at the HBEF (Kuzyakov & Blagodatskaya 2015). These microzones could be associated with Ca-containing mineral particles, or plant roots and could serve as refugia for pH sensitive microbes. Detailed analysis of hotspot characteristics would be required to evaluate this idea.

Regulation of microbial biomass and activity in forest soils

It is undeniable that pH has a strong effect on soil microbial biomass, activity and community composition. However, our results in the context of other findings suggest that these effects may

be mediated by plants and that evaluations of responses of the soil microbial community to environmental change should consider plant: microbial interactions. These interactions greatly complicate assessment and prediction of how ecosystem C and N cycling will respond to environmental change, which will depend on the varying resistance or resilience of taxa within groups or "functional guilds" of microbes that cycle C and N. At the same time, plant responses to environmental change can be more dynamic than microbial responses, as they have access to both aboveground and belowground resources and the ability to shift allocation of these resources in response to environmental stress (Drake et al. 2011; Groffman & Fisk 2011a). Thus, our initial hypothesis that it is useful to view soil pH as a "master variable" controlling belowground biogeochemical processes may be only partially correct. Rather, the effects of pH, and other environmental variables must be evaluated in the context of whole-ecosystem, multicomponent interactions and dynamics.

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Table 1. Regression statistics (coefficient of determination (r^2) , p-value) for linear and polynomial relationships between microbial biomass and activity variables and soil pH over all samples as well as stratified by reference versus calcium-treated watersheds and by soil horizon. Significant relationships, adjusted using the Bonferroni correction, are shown in bold, p < 0.006.

	Linear	Polynomial	Linear	Polynomial	Linear	Polynomial	Linear	Polynomial
	Microbial biomass C		Microbial biomass N		Microbial Respiration		Nitrate	
Reference	$r^2 = 0.001,$	$r^2 = 0.003,$	$r^2 = 0.020,$	$r^2 = 0.027,$	$r^2 = 0.002,$	$r^2 = .0001,$	$r^2 = 0.001,$	$r^2 = 0.018,$
	p = 0.75	p = 0.77	p < 0.001	p = 0.005	p = 0.19	p = 0.74	p = 0.82	p < 0.001
Treated	$r^2 = 0.001,$	$r^2 = 0.001,$	$r^2 = 0.002,$	$r^2 = 0.001,$	$r^2 = 0.001,$	$r^2 = 0.009,$	$r^2 = 0.024,$	$r^2 = 0.025,$
	p = 0.84	p = 0.29	p = 0.10	p = 0.56	p = 0.62	p = 0.38	p < 0.001	p = 0.14
Oi/Oe	$r^2 = 0.001,$	$r^2 = 0.005,$	$r^2 = 0.015,$	$r^2 = 0.040,$	$r^2 = 0.001,$	$r^2 = 0.003,$	$r^2 = 0.001,$	$r^2 = 0.011,$
	p = 0.49	p = 0.028	p < 0.001	p < 0.001	p = 0.55	p = 0.05	p = 0.35	p = 0.01
Reference	$r^2 = 0.010,$	$r^2 = 0.011,$	$r^2 = 0.018,$	$r^2 = 0.020, p$	$r^2 = 0.009,$	$r^2 = 0.015,$	$r^2 = 0.010,$	$r^2 = 0.030,$
	p = 0.031	p = 0.26	p = 0.007	=0.17	p = 0.039	p = 0.08	p = 0.036	p = 0.004
Treated	$r^2 = 0.010,$	$r^2 = 0.011,$	$r^2 = 0.036,$	$r^2 = 0.054$, p	$r^2 = 0.007,$	$r^2 = 0.008,$	$r^2 = 0.038,$	$r^2 = 0.043,$
	p = 0.033	p = 0.28	p < 0.001	= 0.007	p = 0.059	p = 0.31	p < 0.001	p = 0.09
Oa/A	$r^2 = 0.001,$	$r^2 = 0.002,$	$r^2 = 0.029,$	$r^2 = 0.028$, p	$r^2 = 0.022,$	$r^2 = 0.001,$	$r^2 = 0.001, p$	$r^2 = 0.027,$
	p = 0.45	p = 0.73	p < 0.001	=0.54	p = 0.11	p = 0.60	= 0.54	p = < 0.001
Reference	$r^2 = 0.001,$	$r^2 = 0.044,$	$r^2 = 0.067,$	$r^2 = 0.064$, p	$r^2 = 0.055,$	$r^2 = 0.087,$	$r^2 = 0.008$, p	$r^2 = 0.025,$
	p = 0.40	p = < 0.001	p = < 0.001	= 0.68	p < 0.001	p < 0.001	= 0.054	p = 0.008
Treated	$r^2 = 0.001,$ p = 0.38	$r^2 = 0.001,$ p = 0.330	$r^2 = 0.003,$ p = 0.92	$r^2 = 0.003$, p = 0.084	$r^2 = 0.002,$ p = 0.59	$r^2 = 0.005,$ p = 0.702	$r^2 = 0.029$, p < 0.001	$r^2 = 0.029,$ p = 0.319
Mineral	$r^2 = 0.001, p = 0.89$	$r^2 = 0.001,$ p = 0.44	$r^2 = 0.001,$ p = 0.71	$r^2 = 0.004$, p = 0.039	$r^2 = 0.001,$ p = 0.30	$r^2 = 0.001,$ p = 0.33	$r^2 = 0.002, p$ = 0.11	$r^2 = 0.018,$ p < 0.001
Reference	$r^2 = 0.001,$	$r^2 = 0.002,$	$r^2 = 0.003,$	$r^2 = 0.001$, p	$r^2 = 0.007,$	$r^2 = 0.013,$	$r^2 = 0.008$, p	$r^2 = 0.009,$
	p = 0.25	p = 0.48	p = 0.91	= 0.15	p = 0.063	p = 0.07	= 0.055	p = 0.26
Treated	$r^2 = 0.002,$	$r^2 = 0.002,$	$r^2 = 0.002,$	$r^2 = 0.001, p$	$r^2 = 0.001,$	$r^2 = 0.001,$	$r^2 = 0.0025$, p	$r^2 = 0.012,$
	p = 0.22	p = 0.26	p = 0.19	= 0.47	p = 0.22	p = 0.34	= 0.48	p = 0.017

Table 1 continued

	Linear	Polynomial	Linear		Polynomial	Linear	Polynomial	Linear	Polynomial			
	p-value											
	Denitrificati	Ammonium			Mineralization		Nitrification					
Reference	$r^2 = 0.027,$ p < 0.001	$r^2 = 0.029,$ p = 0.09	$r^2 = 0.020,$ < 0.001	p	$r^2 = 0.023,$ p = 0.028	$r^2 = 0.005,$ p = 0.013	$r^2 = 0.030,$ p < 0.001	$r^2 = 0.011,$ p < 0.001	$r^2 = 0.023, p$ < 0.001			
Treated	$r^2 = 0.009,$ p = 0.79	$r^2 = 0.002,$ p = 0.63	$r^2 = 0.004, = 0.026$	р	$r^2 = 0.004,$ p = 0.38	$r^2 = 0.003,$ p = 0.039	$r^2 = 0.003,$ p = 0.51	$r^2 = 0.002,$ p = 0.11	$r^2 = 0.008, p$ = 0.007			
Oi/Oe	$r^2 = 0.001,$ p = 0.81	$r^2 = 0.003,$ p = 0.039	$r^2 = 0.039,$ < 0.001	p	$r^2 = 0.039,$ p = 0.30	$r^2 = 0.001,$ p = 0.16	$r^2 = 0.021,$ p < 0.001	$r^2 = 0.009,$ p = 0.007	$r^2 = 0.008, p$ = 0.71			
Reference	$r^2 = 0.003,$ p = 0.81	$r^2 = 0.005,$ p = 0.06	$r^2 = 0.001,$ < 0.001	p	$r^2 = 0.002,$ p = 0.20	$r^2 = 0.008,$ p = 0.050	$r^2 = 0.042,$ p < 0.001	$r^2 = 0.002,$ p = 0.203	$r^2 = 0.008, p$ = 0.08			
Treated	$r^2 = 0.002,$ p = 0.52	$r^2 = 0.001,$ p = 0.18	$r^2 = 0.032,$ < 0.001	p	$r^2 = 0.029,$ p = 0.81	$r^2 = 0.003,$ p = 0.17	$r^2 = 0.006,$ p = 0.14	$r^2 = 0.022,$ p = 0.003	$r^2 = 0.030, p$ = 0.05			
Oa/A	$r^2 = 0.001$, $p = 0.88$	$r^2 = 0.002,$ p = 0.44	$r^2 = 0.052,$ < 0.001	p	$r^2 = 0.061,$ p = 0.006	$r^2 = 0.024,$ p < 0.001	$r^2 = 0.027,$ p = 0.08	$r^2 = 0.001,$ p = 0.83	$r^2 = 0.011,$ p = 0.002			
Reference	$r^2 = 0.270,$ p = 0.003	$r^2 = 0.024,$ p = 0.68	$r^2 = 0.140,$ < 0.001	p	$r^2 = 0.151,$ p = 0.010	$r^2 = 0.038,$ p < 0.001	$r^2 = 0.048,$ p = 0.033	$r^2 = 0.003,$ p = 0.99	$r^2 = 0.008$, p = 0.027			
Treated	$r^2 = 0.006,$ p = 0.09	$r^2 = 0.004,$ p = 0.46	$r^2 = 0.003,$ < 0.001	p	$r^2 = 0.005,$ p = 0.74	$r^2 = 0.005,$ p = 0.11	$r^2 = 0.004,$ p = 0.45	$r^2 = 0.010,$ p = 0.037	$r^2 = 0.007, p$ = 0.86			
Mineral soil	$r^2 = 0.002,$ p = 0.96	$r^2 = 0.0014,$ p = 0.09	$r^2 = 0.023,$ < 0.001	p	$r^2 = 0.027,$ p = 0.06	$r^2 = 0.017,$ p < 0.001	$r^2 = 0.019,$ p = 0.12	$r^2 = 0.003,$ p = 0.09	$r^2 = 0.009, p$ = 0.023			
Reference	$r^2 = 0.001,$ p = 0.37	$r^2 = 0.002,$ p = 0.17	$r^2 = 0.023,$ = 0.003	р	$r^2 = 0.042,$ p < 0.001	$r^2 = 0.022,$ p = 0.003	$r^2 = 0.025,$ p = 0.16	$r^2 = 0.002,$ p = 0.18	$r^2 = 0.006, p$ = 0.14			
Treated	$r^2 = 0.003,$ p = 0.76	$r^2 = 0.007,$ p = 0.039	$r^2 = 0.031,$ < 0.001	p	$r^2 = 0.030,$ p = 0.52	$r^2 = 0.006,$ p = 0.083	$r^2 = 0.003,$ p = 0.73	$r^2 = 0.007,$ p = 0.070	$r^2 = 0.004$, p = 0.64			

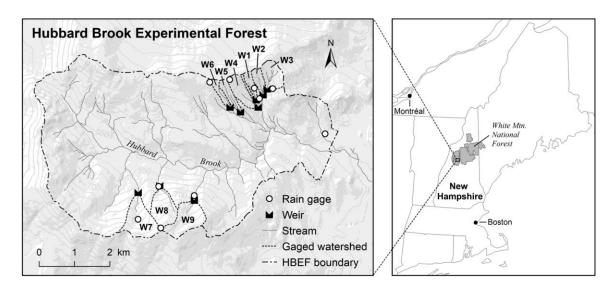


Figure 1: A map of watershed study locations at the Hubbard Brook Experimental Forest. From Campbell et al. (2021)

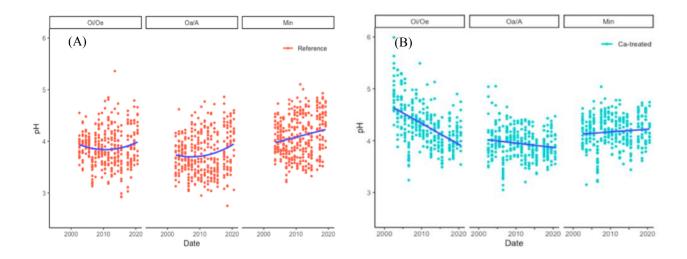


Figure 2: pH in reference (A) and calcium-treated (B) watersheds in Oie, Oa/A, and mineral soil horizons at the Hubbard Brook Experimental Forest in samples collected annually between 1994 and 2018. Lines indicate statistically significant regression relationships, with linear or polynomial relationships shown depending on which was most statistically significant.

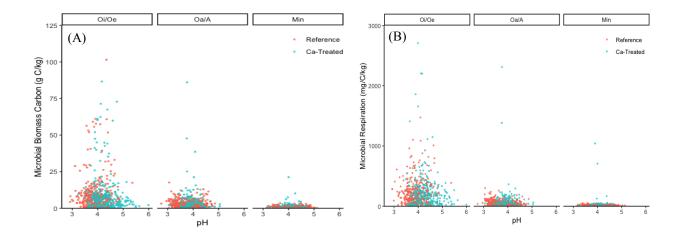


Figure 3: Microbial biomass carbon (A) and respiration (B) with variations in pH in Oie, Oa/A, and mineral soil horizons in reference (red) and calcium-treated (blue) watersheds at the Hubbard Brook Experimental Forest in samples collected annually between 1994 and 2018. One outlier value for both variables was excluded for visual clarity. There were no statistically significant regression relationships with pH in an analysis across the reference and calcium-treated watersheds, with linear or polynomial relationships shown depending on which was most statistically significant.

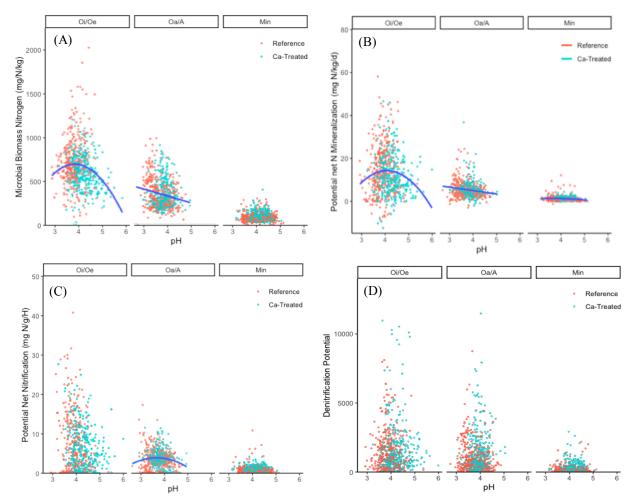


Figure 4: Microbial biomass nitrogen (A), potential net N mineralization (B), potential net nitrification (C), and denitrification potential (D) with variation in pH in Oie, Oa/A, and mineral soil horizons in reference (red) and calcium-treated (blue) watersheds at the Hubbard Brook Experimental Forest in samples taken annually between 1994 and 2018. One outlier value was removed from the microbial biomass nitrogen, potential net N mineralization, and potential net nitrification graphs, and four outlier values were removed from the denitrification potential graph for visual clarity. Lines indicate statistically significant regression relationships with pH across the reference and calcium-treated watersheds, with linear or polynomial relationships shown depending on which was most statistically significant.

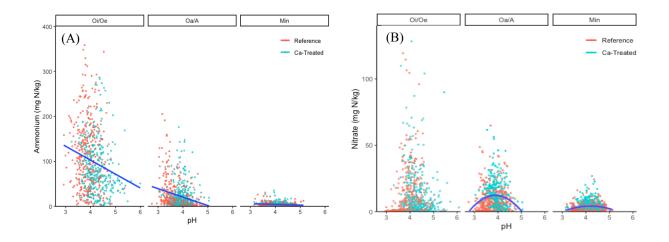


Figure 5: Ammonium (A) and nitrate (B) with variation in pH in Oie, Oa/A, and mineral soil horizons in reference (red) and calcium-treated (blue) watersheds at the Hubbard Brook Experimental Forest in samples taken annually between 1994 and 2018. One outlier value was removed from each graph for visual clarity. Lines indicate statistically significant regression relationships with pH across the reference and calcium-treated watersheds, with linear or polynomial relationships shown depending on which was most statistically significant.