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# Liming Alters the Soil Microbial Community and Extracellular Enzymatic Activities in Temperate Coniferous Forests

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**Abstract:** Soil acidification caused by anthropogenic activities adversely affects forest ecosystems by altering soil pH, which is an important factor in soil quality and function. Liming is one suggested way to solve this problem. This study was performed to evaluate the effects of liming in acidic forest soils by determining soil microbial biomass, microbial community structure, and extracellular enzyme activities associated with carbon, nitrogen, and phosphorus cycling. Lime treatment increased soil pH by up to 40%, significantly increased organic matter (OM) content at some sites, and altered the enzyme activity of the soil. With liming, the microbial biomass appeared to be affected by the chemical properties of the soil, such as pH, $Ca^{2+}$, $Mg^{2+}$, $K^{+}$, and exchangeable aluminum (Ale) levels, although there were no significant differences at the site level. Enzymatic activity was found to be affected by pH, $Ca^{2+}$, $Mg^{2+}$, electrical conductivity (EC), and Ale; and acid phosphatase (AP) and phenol oxidase (POX) activity were significantly affected by lime treatment. AP activity decreased from 0.62 to 0.66, and POX activity increased from 1.75 to 3.00 in part of the sites. The bacterial community richness was influenced by pH as a direct effect of lime treatment. The fungal community richness was associated with changes in $K^{+}$ that were not due to lime treatment. The bacterial community structure was affected by soil OM, total nitrogen (TN), pH, and $Ca^{2+}$; and the fungal community structure was affected by pH, $Mg^{2+}$, and $K^{+}$. In conclusion, changes in soil environmental conditions by liming can affect soil microbial communities and functions through direct or indirect processes, further changing ecosystem processes.

**Keywords:** lime treatment; microbial biomass; enzyme activity; bacterial community; fungal community



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## 1. Introduction

Environmental pollution caused by anthropogenic activities is disturbing the ecosystem in various ways. Soil pH is an important factor that is affected by global changes through agricultural intensification, climate change, and other polluting events, such as acid rain [1–3]. Liming has been suggested as one method to solve this problem [2]. The application of lime to soils usually leads to significant increases in pH, resulting in changes in chemical and biochemical reactions and in microbiological processes. Traditional liming studies sought optimal application methods to increase tree health and growth, and they focused on soil chemistry responses to lime treatment [4,5]. As damage to the forest ecosystem by acidic precipitation has been reported in Korea [6], studies on liming have been conducted, but they focused only on the mitigation of soil acidification and plant growth responses [7–9].

Microbes are the principal components of soil and are considered the biogeochemical engines of ecosystems [10]. Soil microbial communities perform important functions in the carbon and nutrient dynamics of the soil through the decomposition of organic matter (OM) and the cycling of nutrients. Fungi and bacteria are the two groups that dominate

the microbial community, but as they represent eukaryotes and prokaryotes, respectively, they have potentially important differences in their properties, such as biomass elemental composition [11], nutrient demand [12–14], turnover rate [15], and metal tolerance [16]. Extracellular enzymes are major drivers of the decomposition of soil OM and nutrient acquisition [17,18]. Enzymes secreted by soil microbes catalyze the decomposition of soil OM and depolymerize carbon and nutrient sources [19,20]. Although not only microbes secrete extracellular enzymes, the extracellular enzyme activity is representative of microbial functions [21,22].

While various factors within the soil environment can affect the structure and composition of the soil microbial community, soil pH has been shown to be consistently related to microbial community structure. Soil pH strongly influences abiotic factors such as carbon availability [23,24], nutrient availability [24–26], and the solubility of metals [27,28]. In addition, soil pH may control biotic factors such as the community composition of fungi and bacteria in forest soils [29–31]. Previous studies showed that soil pH is correlated with bacterial community diversity to a predictable level across various spatial scales [31,32]. In addition, the rates of the extracellular enzyme-catalyzed reactions are dependent on the pH at which the reactions occur [33,34], and such changes can affect the biogeochemical cycle of the ecosystem [10,35].

We hypothesized that liming would have strong effects on both the microbial community and biological functions. To test these hypotheses, we sampled five lime-treated coniferous forest sites planted with *Pinus densiflora* Siebold & Zucc. (Korean red pine) and *Pinus koraiensis* Siebold & Zucc. (Korean pine) and investigated the effects of liming on soil microbial communities and extracellular enzyme activities.

## 2. Materials and Methods

### *2.1. Site Description*

This study used one Korean pine and four Korean red pine forests in the Republic of Korea. The Korean pine site is located in Chuncheon (Cc) in Gangwon-do, and the four Korean red pine sites are located in Gwangju (Gw) in Gyeonggi-do, Taean (Ta) in Chungcheongnam-do, Cheongju (Cj) in Chungcheongbuk-do, and Gyeongju (Gy) in Gyeonggibuk-do (Figure 1). The forests were under a temperate climate with hot, humid summers and cold, dry winters. The soil at the studied forests was typically a brown forest soil type that generally features acidic conditions.

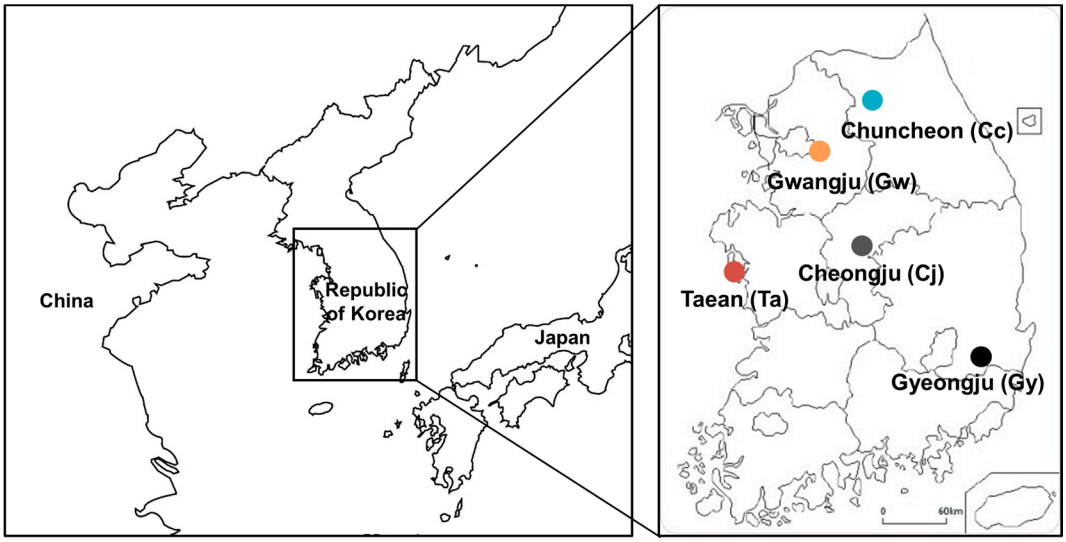


**Figure 1.** Study sites of soil conditioning for recovery from acidification.

To monitor the effect of conditioning on the acidified forest soil, one site per year was treated to restore the acidified forest soil. The amount of lime required per unit area was calculated according to the pH, OM, and texture of the site soil [36], and treatment was performed with granular dolomitic limestone. Soil conditioning with lime treatment was performed in Gwangju in 2011, Taean in 2012, Cheongju in 2013, Chuncheon in 2014, and Gyeongju in 2015. The target soil pH was 5.5–6.0, and 1.1–1.8 ton/ha of lime was applied over an area of about 50 ha at each site.

### 2.2. Soil Sampling

Soil samples were collected from depths of 0–10 cm at three randomly selected points in each five plot in June 2019. The three samples were combined and mixed to generate a single sample for analysis. After sieving (<2 mm) and removing visible plant material, soil samples were kept in a refrigerator at 4 °C until analysis of soil physicochemical properties, microbial biomass, and extracellular enzyme activity. In addition, a portion of the sieved soil was stored at −20 °C for microbial community structure and composition analyses. Soil microbial biomass and extracellular enzyme activity assays were performed within 1 week of soil sample collection.

### 2.3. Soil Chemical Properties

The soil samples were suspended in distilled water at a ratio of 1:5 for 1 h, and then, soil pH was measured using a pH meter (HM-30R, Toa Corp., Tokyo, Japan). Soil electrical conductivity (EC) was determined using a conductivity meter (CM-30R, Toa Corp., Tokyo, Japan) after suspending the soil in the same way as for pH measurement and filtering using Whatman No. 42 filter paper. The soil OM and total nitrogen (TN) were determined using an elemental analyzer (Vario MAX CN, Elementar Analysensysteme, Langenselbold, Germany). Available phosphorus ($P_2O_5$) was measured using an ultraviolet spectrophotometer (UV-2501PC, Shimadzu, Tokyo, Japan) with an extraction solution based on the ammonium molybdate reaction. The exchangeable potassium ($K^+$), sodium ($Na^+$), calcium ($Ca^{2+}$), and magnesium ($Mg^{2+}$) concentrations were analyzed by atomic absorption spectrophotometry (iCE 3000 Series, Thermo Fisher Scientific, Waltham, MA, USA) using 1 N ammonium acetate as the extraction solution. The exchangeable aluminum (Ale) concentration was measured by atomic absorption spectrophotometry after extraction in 1 M potassium chloride solution.

### 2.4. Soil Microbial Biomass and Extracellular Enzyme Activity

Soil microbial biomass was measured using the fumigation–extraction method [37]. Soil was extracted after 24 h of chloroform fumigation in 0.5 M potassium sulfate ($K_2SO_4$) solution at a ratio of 1:4 for 30 min and filtered. Soil that had not been fumigated with $CHCl_3$ was similarly extracted with 0.5 M $K_2SO_4$ and used as a control in this analysis. Microbial biomass C (MBC) was analyzed by the dichromate digestion method [37]. To convert the measured extractable C content into biomass C, we used the following formula [37]:

$$\text{Microbial biomass C} = 2.64 \times \text{EC (EC = fumigated} - \text{nonfumigated treatments).} \quad (1)$$

In addition, microbial biomass N (MBN) was evaluated by the ninhydrin method [38]. The measured ninhydrin-reactive N was converted to microbial biomass N using the following formula [38]:

$$\text{Microbial biomass N} = 5.0 \times \text{EN (EN = fumigated} - \text{nonfumigated treatments).} \quad (2)$$

Microbial biomass P (MBP) was evaluated by the ammonium molybdate-ascorbic acid method [39]. The measured extractable P was calculated to microbial biomass P using the following formula [39]:

$$\text{Microbial biomass P} = 2.5 \times \text{EP (EP = fumigated} - \text{nonfumigated treatments).} \quad (3)$$

A colorimetric method using *p*-nitrophenol-linked substrates was applied for determination of β-glucosidase (BG), N-acetylglucosaminidase (NAG), and acid phosphatase (AP) activities. These assay methods involve determination using an ultraviolet spectrophotometer (UV-2501PC, Shimadzu, Tokyo, Japan) of *p*-nitrophenol released when soil was incubated with toluene and the respective buffered substrate for 1 h at 37 °C [40–42]. In addition, a colorimetric method using L-3,4-dihydroxyphenylalanine (L-DOPA) was implemented to determine phenol oxidase (POX) activity [43]. For sample assays, blanks (soil suspension with 50 mM acetate buffer), negative controls (50 mM acetate buffer with L-DOPA solution), and sample controls (soil suspension with L-DOPA solution) were prepared. The microplates were incubated in the dark at 20 °C for up to 18 h. Activity was quantified by measuring the absorbance at 450 nm ($A_{450}$) using a microplate spectrophotometer (UVM 340, Biochrom, Cambridge, UK) and expressed in units of nmol $h^{-1}$ $g^{-1}$.

### 2.5. Soil Microbial Community Structure and Composition

Soil microbial genomic DNA was extracted from each sample using a DNeasyPowerSoil Kit (Qiagen, Hilden, Germany). The extracted DNA was quantified using Quant-IT PicoGreen (Invitrogen, Carlsbad, CA, USA). The primers used for amplification by polymerase chain reaction (PCR) were as follows: V3–V4 region of bacterial 16S rDNA, V3-F (5′-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG-3′) and V4-R (5′-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC-3′); fungal internal transcribed spacer (ITS) region, ITS3-F (5′-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGCATCGATGAAGAACGCAGC-3′) and ITS4-R (5′-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGTCCTCCGCTTATTGATATGC-3′). The PCR conditions consisted of an initial denaturation step at 95 °C for 3 min, followed by 25 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 30 s, with a final extension at 72 °C for 5 min. The first PCR product was purified with AMPure beads (Agencourt Bioscience, Beverly, MA, USA). Following purification, the first PCR product was amplified by PCR for final library construction containing the index using NexteraXT Indexed Primer. The cycle conditions for the second PCR were the same as for the first PCR except the reactions were repeated for 10 cycles, and the PCR product was purified with AMPure beads. Then, the final purified product was quantified according to the qPCR Quantification Protocol Guide (KAPA Library Quantification Kit for the Illumina Sequencing platform) and qualified using TapeStation D1000 ScreenTape (Agilent Technologies, Waldbronn, Germany). Paired-end (2 × 300 bp) sequencing was performed by Macrogen using the MiSeq™ platform (Illumina, San Diego, CA, USA).

### 2.6. Statistical Analysis

All data were tested for normality and homogeneity of variance (Shapiro–Wilk and Levene's statistics). Student's *t* test ($p < 0.05$) was applied to compare soil properties, microbial biomass, and extracellular enzyme activities between the lime-treated and control soils of each sites using SPSS software ver. 23.0 (IBM Corp., Armonk, NY, USA). We performed analysis of similarities (ANOSIM) using anosim functions in the Vegan library of R 3.6.1 (R De-velopment Core Team, Vienna, Austria) to verify the effect of lime treatment on the bacterial and fungal community, dividing the entire site into two groups: lime treatment and non-treatment. In addition, we used nonmetric multidimensional scaling (NMDS) to examine the relationships between the soil chemical properties and biological properties, including microbial biomass, extracellular enzyme activities, and microbial communities, using the meta-multidimensional scaling and envfit functions in the Vegan library of R. Stepwise multiple regression analysis was performed using SPSS software to investigate the relationships between soil microbial and enzymatic properties and selected soil chemical parameters based on the NMDS.

## 3. Results

### *3.1. Soil Chemical Property*

The effects of liming differed among the study sites, although the soil pH, $K^+$, $Ca^{2+}$, $Mg^{2+}$, and EC were higher, and Ale was lower, in the limed sites than in the control sites. In particular, changes in soil pH, the main effect of liming on soil properties, were significant only at Ta, Cj, and Gy, but not at Gw and Cc (Table 1). Soil pH was higher with lime treatments at Gy by 0.67 ($p < 0.001$) and at Cj by 2.37 ($p < 0.001$), and at Ta by 2.03 ($p < 0.001$) in comparison to the respective controls (Table 1). $Ca^{2+}$ content was higher with lime treatment than under the respective control conditions at Gy by 1.49 cmolc/kg ($p = 0.015$) and at Cj by 6.07 cmolc/kg ($p < 0.001$), at Ta by 5.64 cmolc/kg ($p = 0.003$), and at Gw by 0.21 cmolc/kg (($p < 0.001$) Table 1). $Mg^{2+}$ was higher with lime treatment at Gy by 2.41 cmolc/kg ($p = 0.027$) and at Cj by 5.48 cmolc/kg ($p < 0.001$) in comparison to the respective controls (Table 2). Conversely, Ale content was lower with lime treatment than under control conditions at Gy by 207.29 mg/kg ($p = 0.009$), at Cj by 268.410 mg/kg ($p < 0.001$), and at Ta by 257.43 mg/kg ($p = 0.002$) (Table 1). $K^+$ content and EC were higher with lime treatment than under control conditions at Cc by 0.22 cmolc/kg ($p = 0.012$) and at Cj by 0.16 cmolc/kg ($p = 0.003$) (Table 1)

**Table 1.** Soil chemical properties of study sites.

| | | pH | OM | TN | $P_2O_5$ | EC | Ale | $K^+$ | $Na^+$ | $Ca^{2+}$ | $Mg^{2+}$ |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | (1:5) | (%) | (%) | (mg $kg^{-1}$) | (1:5) | (mg $kg^{-1}$) | | (cmol$^+$ $kg^{-1}$) | | |
| Gw | Control | 4.20 | 13.24 | 0.18 | 8.64 | 0.30 | 294.88 | 0.10 | 0.08 | **0.13** | 0.23 |
| | | 0.13 | 2.97 | 0.05 | 1.44 | 0.04 | 92.71 | 0.02 | 0.02 | **0.03** | 0.04 |
| | Lime | 4.31 | 13.9 | 0.20 | 17.16 | 0.31 | 255.17 | 0.10 | 0.09 | **0.34** | 0.31 |
| | | 0.06 | 3.84 | 0.06 | 6.56 | 0.04 | 227.07 | 0.02 | 0.01 | **0.07** | 0.10 |
| Ta | Control | **4.61** | 6.7 | 0.13 | 12.89 | 0.32 | **264.16** | 0.15 | 0.12 | **1.11** | 0.96 |
| | | **0.28** | 1.27 | 0.03 | 8.82 | 0.12 | **84.32** | 0.06 | 0.03 | **0.47** | 0.35 |
| | Lime | **6.64** | 5.98 | 0.10 | 2.89 | 0.38 | **6.73** | 0.22 | 0.15 | **6.75** | 2.86 |
| | | **0.59** | 0.48 | 0.02 | 2.39 | 0.09 | **10.74** | 0.05 | 0.02 | **2.06** | 2.34 |
| Cj | Control | **4.9** | **7.39** | 0.09 | **24.25** | 0.21 | **271.02** | 0.21 | **0.07** | **0.96** | **0.74** |
| | | **0.10** | **0.27** | 0.01 | **1.05** | 0.04 | **22.95** | 0.06 | **0** | **0.18** | **0.06** |
| | Lime | **7.27** | **8.28** | 0.10 | **22.47** | 0.37 | **2.61** | 0.26 | **0.09** | **7.03** | **6.22** |
| | | **0.30** | **0.73** | 0.01 | **0.61** | 0.07 | **2.74** | 0.11 | **0.01** | **1.19** | **1.08** |
| Cc | Control | 4.85 | **8.4** | **0.12** | 9.19 | 0.18 | 546.93 | **0.12** | 0.07 | 0.83 | 0.44 |
| | | 0.12 | **0.72** | **0.02** | 4.54 | 0.03 | 88.23 | **0.04** | 0.01 | 0.43 | 0.22 |
| | Lime | 4.93 | **9.58** | **0.15** | 29.72 | 0.25 | 421.26 | **0.34** | 0.07 | 2.93 | 1.36 |
| | | 0.65 | **0.55** | **0.02** | 18.87 | 0.06 | 243.71 | **0.15** | 0.01 | 3.35 | 1.18 |
| Gy | Control | **4.55** | 8.88 | 0.14 | 25.59 | 0.28 | **465.25** | 0.32 | 0.09 | **0.92** | **0.88** |
| | | **0.11** | 1.95 | 0.05 | 8.33 | 0.07 | **134.88** | 0.18 | 0.01 | **0.55** | **0.38** |
| | Lime | **5.22** | 7.16 | 0.10 | 19.11 | 0.27 | **194.96** | 0.46 | 0.10 | **2.41** | **3.29** |
| | | **0.15** | 1.16 | 0.06 | 13.77 | 0.08 | **115.54** | 0.26 | 0.01 | **0.92** | **1.62** |

OM, organic matter; TN, total nitrogen; Ale, exchangeable aluminum; EC, electrical conductivity. The numbers in parentheses are the standard deviation. Bold letters specify significantly different means at each treatment based on the Student's *t*-test ($p < 0.05$).

**Table 2.** Soil microbial and enzymatic properties of study sites.

| | | MBC | MBN | MBP | BG | NAG | AP | POX |
|---|---|---|---|---|---|---|---|---|
| | | (mg kg$^{-1}$ soil) | | | (mg *p*−Nitrophenol kg$^{-1}$ soil h$^{-1}$) | | | (μmol h$^{-1}$ kg$^{-1}$) |
| Gw | Control | 273.25 | 70.33 | 92.41 | 424.19 | 174.14 | 993.31 | **130.64** |
| | | 56.97 | 21.54 | 34.84 | 102.46 | 73.97 | 144.88 | **68.50** |
| | Lime | 293.16 | 78.34 | 124.35 | 385.12 | 227.24 | 1061.05 | **228.37** |
| | | 91.75 | 22.34 | 71.25 | 113.78 | 65.48 | 260.33 | **59.86** |
| Ta | Control | 337.06 | 104.08 | 99.23 | 191.12 | 212.07 | **1196.20** | **150.0** |
| | | 146.91 | 37.66 | 53.68 | 45.49 | 57.07 | **106.82** | **51.09** |
| | Lime | 263.73 | 131.79 | 67.05 | 149.26 | 186.89 | **783.83** | **344.42** |
| | | 42.0 | 37.34 | 30.07 | 27.83 | 23.36 | **157.72** | **117.48** |
| Cj | Control | 249.09 | 76.39 | 66.07 | 193.73 | 175.22 | **1028.71** | **166.13** |
| | | 74.45 | 18.72 | 18.99 | 31.0 | 54.43 | **82.30** | **43.21** |
| | Lime | 334.92 | 109.80 | 72.95 | 214.52 | 193.54 | **634.51** | **314.08** |
| | | 180.78 | 30.21 | 23.60 | 49.81 | 34.55 | **100.45** | **90.86** |
| Cc | Control | 180.04 | 52.42 | 51.16 | 168.58 | 389.95 | 877.23 | 129.34 |
| | | 45.08 | 14.32 | 20.88 | 64.42 | 229.48 | 134.87 | 43.91 |
| | Lime | 295.31 | 69.83 | 54 | 164.49 | 285.84 | 935.87 | 127.03 |
| | | 127.03 | 23.18 | 10.70 | 52.39 | 137.86 | 153.71 | 45.05 |
| Gy | Control | 251.74 | 31.71 | 37.67 | 283.82 | 238.30 | 1108.50 | **70.34** |
| | | 55.47 | 19.01 | 27.41 | 39.93 | 68.70 | 64.68 | **39.09** |
| | Lime | 174.24 | 27.95 | 29.45 | 267.29 | 227.84 | 1097.16 | **210.87** |
| | | 79.37 | 21.37 | −12.22 | −140.09 | 75.95 | 108.44 | **111.12** |

MBC, microbial biomass C; MBN, microbial bioamss N; MBP, microbial bioamss P; BG, β-glucosidase; NAG, N-acetylglucosaminidase; AP, Acid phosphatase; POX, phenol oxidase. The numbers in parentheses are the standard deviation. Bold letters specify significantly different means at each treatment based on the Student's *t*-test ($p < 0.05$).

### *3.2. Microbial Biomass and Extracellular Enzyme Activity*

The microbial biomass showed no significant differences among the study sites (Table 2). Enzymatic activities showed differences according to lime treatment among the study sites (Table 2). There were no significant differences in BG or NAG activity between the lime treatments for all study sites, whereas significant differences in AP and POX activity were detected at some study sites (Table 2). AP activity was 0.38 ($p < 0.001$) and 0.34 ($p = 0.001$) times lower with lime treatment compared to control conditions at Cj and Ta, respectively (Table 3). POX activity was higher with lime treatment than in the control by 3.0 times at Gy ($p = 0.028$), by 1.89 times at Cj ($p = 0.011$), by 2.30 times at Ta ($p = 0.009$), and by 1.75 times at Gw ($p = 0.043$) (Table 2).

**Table 3.** Soil microbial community richness and diversity index of study sites.

| | | Bacteria | | | Fungi | | |
|---|---|---|---|---|---|---|---|
| | | OTU | Chao1 | Shannon | OTU | Chao1 | Shannon |
| Gw | Control | 545.4 | 655.55 | **6.11** | **151.0** | 155.20 | 4.27 |
| | | 67.29 | 88.06 | **0.51** | **16.72** | 17.33 | 0.82 |
| | Lime | 613.8 | 731.08 | **6.93** | **164.2** | 168.27 | 4.62 |
| | | 44.38 | 48.46 | **0.08** | **17.51** | 17.49 | 0.21 |
| Ta | Control | **625.60** | **728.67** | **6.98** | 170.0 | 178.40 | 4.55 |
| | | **70.13** | **66.42** | **0.20** | 37.22 | 37.26 | 0.43 |
| | Lime | **858.0** | **1032.06** | **7.44** | 153.2 | 160.80 | 4.96 |
| | | **71.58** | **93.45** | **0.34** | 20.84 | 29.07 | 0.38 |
| Cj | Control | **781.0** | **940.98** | 7.38 | 164.6 | 165.97 | 5.12 |
| | | **21.85** | **48.52** | 0.11 | 18.26 | 17.95 | 0.36 |
| | Lime | **897.2** | **1083.94** | 7.45 | 154.6 | 156.52 | 5.00 |
| | | **46.09** | **55.85** | 0.26 | 20.21 | 19.06 | 0.65 |

**Table 3.** *Cont.*

| | | Bacteria | | | Fungi | | |
|---|---|---|---|---|---|---|---|
| | | **OTU** | **Chao1** | **Shannon** | **OTU** | **Chao1** | **Shannon** |
| Cc | Control | 730.8 | 859.16 | 7.30 | **186.4** | **188.95** | **4.01** |
| | | 56.48 | 83.88 | 0.06 | **23.16** | **21.47** | **0.33** |
| | Lime | 742.8 | 882.91 | 7.41 | **262.8** | **271.41** | **4.91** |
| | | 114.95 | 152.32 | 0.22 | **39.88** | **49.90** | **0.35** |
| Gy | Control | **588.0** | **693.82** | **6.91** | 219.8 | 222.35 | 4.93 |
| | | **70.67** | **105.79** | **0.19** | 45.55 | 46.60 | 0.71 |
| | Lime | **728.4** | **866.76** | **7.33** | 221.8 | 223.26 | 4.79 |
| | | **75.21** | **100.07** | **0.26** | 45.38 | 45.92 | 0.76 |

OTU, operational taxonomic unit. The numbers in parentheses are the standard deviation. Bold letters specify significantly different means at each treatment based on the Student's *t*-test ($p < 0.05$).

### 3.3. Microbial Community Structure

The bacterial community was significantly affected by lime treatments (ANOSIM R = 0.111, $p = 0.005$), but the fungal community showed no effect of experimental manipulation (ANOSIM R = 0.014, $p = 0.219$). Bacterial richness was significantly increased at Gy ($p = 0.016$), Cj ($p = 0.001$), Ta ($p = 0.001$), and bacterial diversity was significantly increased at Gy ($p = 0.019$), Ta ($p = 0.031$), and Gw ($p = 0.021$) by lime treatment in comparison to the respective control conditions.

The bacterial community structure was dominated by four major bacterial phylogenic groups (Acidobacteria, Actinobacteria, Verrucomicrobia, and Proteobacteria) (Figure 2). These groups accounted for about 80% of the sequences among the study sites. Liming changed the 4th and 5th members of the bacterial community through decreases in *Firmicutes* ($p = 0.037$) and an increase in Bacteroidetes ($p = 0.02$). With liming, changes in Firmicutes were found at Cj ($p = 0.009$) and Ta ($p = 0.003$), and changes in Bacteroidetes were found at Cj ($p < 0.001$), Ta ($p = 0.001$), and Gw ($p = 0.007$). Fungal community structure was also dominated by four major fungal phylogenic groups (Ascomycota, Basidiomycota, Mortierellomycota, and Mucoromycota) (Figure 2). These four phyla accounted for >98% of the fungal community sequences. Although there was a change in the order of dominance between the treatment and control groups, there were no significant differences according to lime treatment.

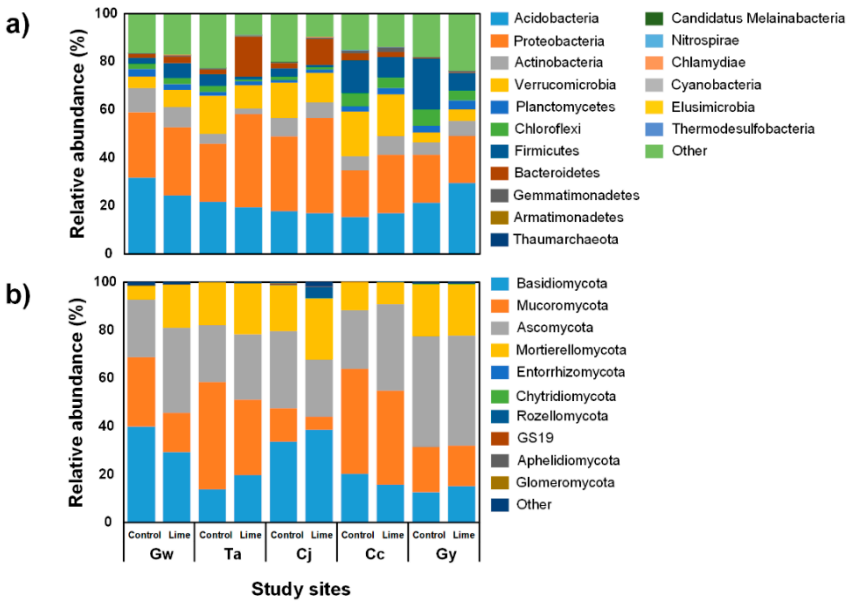


**Figure 2.** Relative abundance of soil bacterial phyla (**a**) and fungal phyla (**b**) at the study sites.

### *3.4. Factors Affecting Biological Property*

NMDS analysis indicated that lime treatment was associated with changes in soil microbial biomass, enzyme activity, and microbial communities (bacterial and fungal). NMDS analysis of the relationships between microbial biomass (MBC, MBN, and MBP) and the properties of the soil revealed significant correlations with pH, $Ca^{2+}$, $Mg^{2+}$, $K^{+}$, and Ale (Figure 3a). Stepwise linear regression analysis showed that MBN and MBP decreased with increasing Ale and $K^{+}$ concentrations (Table 4). MBC did not show any significant relationships with soil chemical properties. NMDS analysis showed that enzyme activity was significantly related to OM, total N, pH, $Ca^{2+}$, $Mg^{2+}$, EC, and Ale (Figure 3b). BG activity was strongly correlated with OM, and NAG was negatively related with the Ale concentration (Table 4).

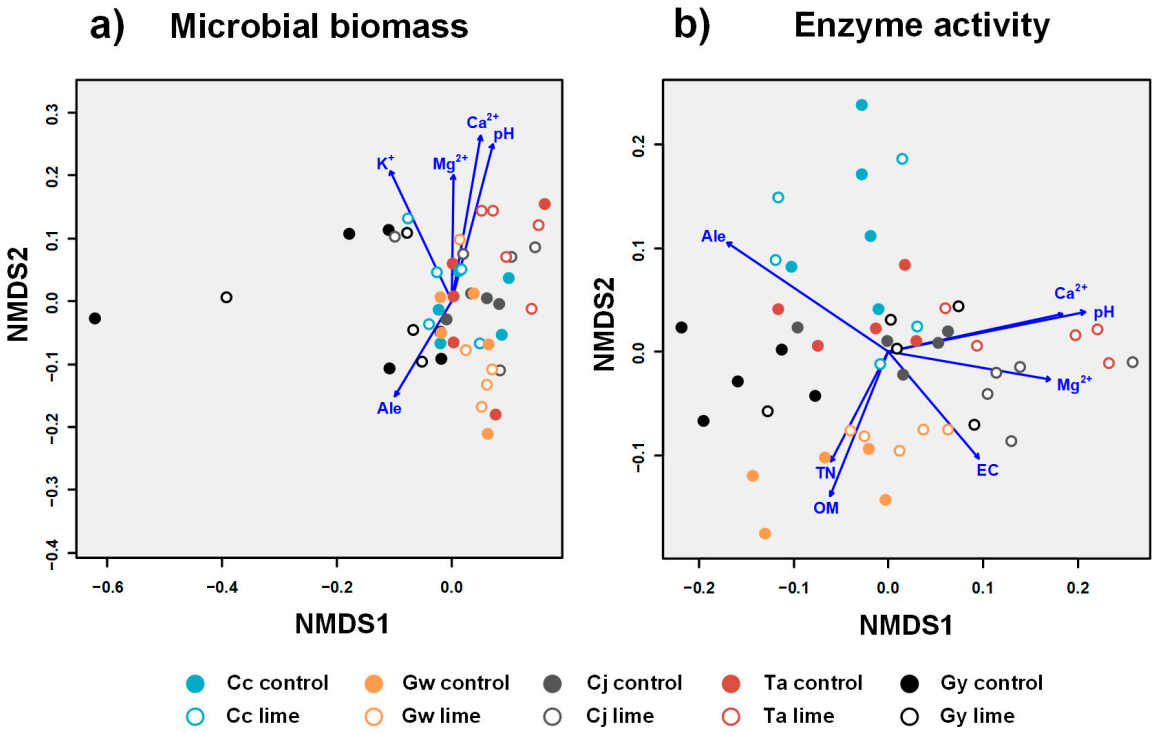


**Figure 3.** Nonmetric multidimensional scaling ordination between the soil chemical properties and (**a**) microbial biomass and (**b**) extracellular enzyme activities. Arrow directions indicate the correlation slopes, and arrow lengths indicate the degrees of influence of the various factors.

The bacterial community structure was related to the OM, TN, pH, and $Ca^{2+}$, and the fungal community structure was related to the pH, $Mg^{2+}$, and $K^{+}$ (Figure 4a). The richness of the bacterial community showed positive relations with pH and Ale, and diversity showed a negative relation with TN (Table 4). NMDS analysis of the relationships between fungal community structure and the properties of the soil revealed significant correlations with pH, $P_2O_5$, EC, $Mg^{2+}$, and $K^{+}$ (Figure 4b). The richness of fungal communities showed positive relations with $K^{+}$ and Ale (Table 4).

**Table 4.** Stepwise multiple regression analysis of soil microbial biomass, extracellular enzyme activity, microbial community index, and selected soil parameters from nonmetric multidimensional scaling (NMDS).

| Dependent Variable | Regression Equation | $R^2$ | *F*-Value |
|---|---|---|---|
| Microbial biomass | | | |
| MBN | = 110.2 + (−0.128 × Ale) | 0.472 | 7.14 ($p$ = 0.028) |
| | = 144.2 + (−0.140 × Ale) + (−136.0 × $K^+$) | 0.709 | 8.53 ($p$ = 0.013) |
| MBP | =111.687 + (−185.75 × $K^+$) | 0.585 | 11.28 ($p$ = 0.010) |
| Enzyme activity | | | |
| BG | = −25.75 + (30.16 × OM) | 0.707 | 19.32 ($p$ = 0.002) |
| NAG | = 160.11 + (0.261 × Ale) | 0.504 | 8.14 ($p$ = 0.021) |
| | = −127.95 + (0.454 × Ale) + (45.72 × pH) | 0.726 | 9.23 ($p$ = 0.011) |
| AP | = 1678.68 + (−137.4 × pH) | 0.678 | 16.86 ($p$ = 0.03) |
| POX | = −177.11 + (70.78 × pH) | 0.670 | 16.23 ($p$ = 0.004) |
| Bacterial community | | | |
| OTU | = 182.94 + (102.64 × pH) | 0.792 | 30.51 ($p$ = 0.001) |
| | = 257.81 + (130.98 × Ale) + (−771.08 × EC) | 0.918 | 39.08 ($p$ < 0.001) |
| Shannon | = 8.14 + (−7.76 × TN) | 0.471 | 7.08 ($p$ = 0.029) |
| Fungal community | | | |
| OTU | = 135.99 + (214.77 × $K^+$) | 0.467 | 7.02 ($p$ = 0.029) |
| | = 94.14 + (239.32 × $K^+$) + (0.133 × Ale) | 0.918 | 20.88 ($p$ = 0.001) |

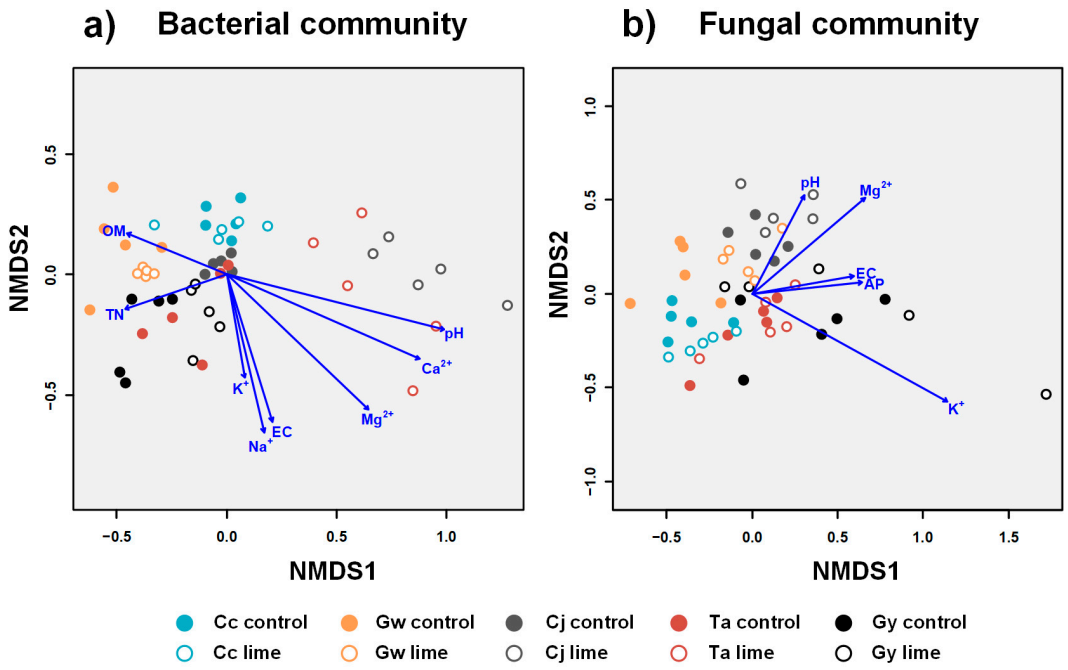


**Figure 4.** Nonmetric multidimensional scaling ordination between the species composition and relative abundances of (**a**) the bacterial community and (**b**) fungal phyla and soil chemical properties. Arrow directions indicate the correlation slopes, and arrow lengths indicate the degrees of influence of the various factors.

## 4. Discussion

### *4.1. Effects of Liming on Microbial Biomass and Extracellular Enzyme Activity*

The results of this study indicate inconsistent effects of lime treatment on microbial biomass at the site level. MBC, MBN, and MBP did not show any significant differences between the control and lime treatment groups at any sites, but the effects of lime treatment were confirmed based on the relationships between the changes in soil chemical properties and microbial biomass. NMDS analysis showed that the microbial biomass was influenced

by soil pH, $K^+$, $Ca^{2+}$, $Mg^{2+}$, and Ale. The supply of $Ca^{2+}$ and $Mg^{2+}$ by the liming material increased the pH of the soil, and the increase in pH decreased the amount of Ale. Changes in pH, $Ca^{2+}$, $Mg^{2+}$, and Ale, which were the direct effects of lime treatment, were found to have particularly affected MBN (Table 4). Al toxicity is one of the most important factors limiting the growth of plants in acidic soils [44] and may affect the circulation of soil microbial and microbial-mediated effects. Kraal et al. [45] reported that Al inhibited soil respiration, nitrification, and microbial absorption of $NH_4^+$. In the present study, the significant negative correlation between MBN and Ale was due to the suppression of N absorption by Al toxicity in the acidic soil of the control and suppression of Al toxicity due to Ale with lime treatment.

BG and NAG activities were unaffected by liming at any of the study sites (Table 2). On the other hand, AP activity was significantly decreased at Cj and Ta by lime treatment, and POX activity was significantly increased at all sites except Cc (Table 2). On NMDS analysis, extracellular enzyme activity was found to be affected by soil OM, TN, pH, EC, $Ca^{2+}$, $Mg^{2+}$, and Ale. BG activity did not show any differences among treatment sites but showed a significant linear relation with soil OM (Figure 3, Table 4). BG is the most abundant cellulose degradation enzyme in soil and is essential for the carbon cycle [46]. The BG enzyme activity is influenced by the substrate concentration [47]. BG activity increased with the amount of OM, which is a substrate, but the OM was not significantly affected by lime treatment, so there was no difference between the experimental plots. Lime treatment showed effects on NAG, AP, and POX. NAG showed a negative relation with EC and a positive relation with Ale. AP showed significant negative relations with soil pH, $Ca^{2+}$, and $Mg^{2+}$. As the optimum pH of AP is between 4.0 and 5.0 [48], the increase in soil pH resulting from lime treatment may markedly reduce the activity of AP. The activity of AP was different at Cj and Ta, where the soil pH increased significantly due to the influence of liming. POX activity was affected by pH, $Ca^{2+}$, $Mg^{2+}$, EC, and Ale. POX activity has a positive relation with soil pH; as pH increases, phenols deprotonate, which reduces their redox potential and increases their solubility, both of which may enhance their reaction potential [49]. In addition, various microorganisms that vary according to soil pH can widely produce POXs with little conserved homology, thereby changing the soil enzyme pool and potentially altering its range of action [49]. Such relations support the importance of the influence of the soil on enzyme activity, implying that soil chemical changes caused by liming can affect the biogeochemical cycle of ecosystems by altering the enzyme activity in soil.

### *4.2. Effects of Liming on Microbial Community Structure*

The results of this study show that treatment with lime changes the soil microbial community, and the main factor underlying these changes is soil pH. The richness and diversity of bacterial communities in Gw, Ta, and Gy soils and fungal communities in Cc soils were increased by lime treatment. NMDS analysis showed that the changes in the chemical properties of soil by lime treatment affected the microbial community structure at the species level. The bacterial community structure was related to the OM, TN, pH, and $Ca^{2+}$ of the soil, and the fungal community structure was related to the pH, $Mg^{2+}$, and $K^+$ of the soil. Bacterial richness showed significant relations with TN, pH, $Ca^{2+}$, and $Mg^{2+}$, and species diversity showed significant relations with OM and TN. The fungal richness of soil was significantly related to $K^+$, and the species diversity did not show significant relations with the chemical properties of the soil. Soil pH is an important indicator of microbial diversity [49]. Various factors affect the microbial community structure, but the factors that lead to complex and diverse structures are those that change due to the direct effects of lime treatment, such as pH, $Ca^{2+}$, and $Mg^{2+}$. In this study, changes in soil pH caused by lime treatment had strong effects on the diversity and composition of bacterial communities across the entire gradient. On the other hand, the fungal community structure was unaffected by soil pH (Figure 4). This was because most bacterial taxa have a relatively narrow growth-tolerance range [50]. Bacterial activity decreases by 50% between

environments where the pH differs by 1.5 units [51]. The decrease in activity of bacteria according to changes in pH maintains their homeostasis, and bacteria have systems to maintain the cytoplasm at an appropriate pH [52]. Bacteria that show reductions in activity due to the operation of the homeostasis maintenance system can be rapidly overtaken by other bacteria that are unaffected by changes in pH, leading to changes in the community. This is because fungi have a relatively wide tolerance range of 5–9 pH units [53,54], unlike bacteria, and therefore, they are not significantly affected by changes in soil pH values in the range pH 4–7.

The shifts in the relative abundances of specific taxonomic groups of bacteria at the phylum level across pH gradients were similar to the changes observed in species richness in response to pH. Six of the 16 phyla that appeared at the site (Armatimonadetes, Bacteroidetes, Chlamydiae, Nitrospirae, Proteobacteria, and Thermodesulfobacteria) were affected by soil pH, and six of these showed a tendency to increase in relative abundance with pH. Major changes affecting diversity were observed in the specific dominant phyla. In particular, the dominant phyla, Proteobacteria and Bacteroidetes, showed strong positive correlations in both relative abundance and species number with changes in soil pH, leading to changes in bacterial communities with lime treatment. The correlation between soil pH and these key bacterial species was consistent with the results of previous studies [32,55,56]. OM, a major factor influencing species diversity, affected the relative abundance of four phyla (Chloroflexi, Firmicutes, Planctomycetes, Proteobacteria), and negatively affected that of one phylum (Proteobacteria). Previous studies indicated that Proteobacteria is a copiotrophic phylum that grows well in environments rich in sources of C [57–63]. However, in the present study, contrary to previous reports, Proteobacteria tended to decrease as OM increased. Although some studies showed that Proteobacteria is an oligotrophic phylum [64], our results are thought to be the result of artificial changes in the soil environment as a result of lime treatment. In this study, changes in soil pH due to lime treatment showed a negative correlation with soil OM. This was because the effect of lime treatment was reduced by the buffering action of OM against changes in soil pH and the obstruction of the thickly accumulated OM layer on the forest floor. Although OM influences the abundance of Proteobacteria [65], soil pH had a stronger influence on their relative abundance.

We propose Ale as one factor inducing the bacterial community shift with changes in pH. In this study, high substitutional aluminum decreased MBN and increased the activity of NAG. Aluminum toxicity inhibits the absorption and nitrification of $NH_4^+$ by microorganisms, thus hindering nitrogen fixation by microorganisms, resulting in lower MBN. Microorganisms with increased demand for N due to the disturbance of absorption of inorganic nitrogen can increase the expression of N-acquiring enzymes to increase activity [66]. Inorganic nitrogen is one of the factors influencing microbial community composition [67,68], and fungi have lower N demands at the cell wall than do bacteria [68]. In this study, Al showed a negative correlation with bacterial operational taxonomic unit (OTU), demonstrating its relationship to the bacterial community

The relative abundances of specific taxonomic groups of fungi at the phylum level, as well as species richness and diversity, showed no significant effects of lime treatment. Among the four major fungal phyla present at the study sites, only Ascomycota and Mortierellomycota showed significant relations with soil chemical properties. Significant positive relations were observed between Ascomycota and $K^+$ and between Mortierellomycota and $Mg^{2+}$. The significant relationship between Mortierellomycota and $Mg^{2+}$ indicates that the fungal community structure was affected by the changes in soil chemical properties resulting from lime treatment. However, unlike $Ca^{2+}$ and $Mg^{2+}$, $K^+$ is not a nutrient that is increased by lime treatment. In contrast, the positive relation between $K^+$ and Ascomycota was thought to be a case in which soil fungi influenced the chemical properties of the soil. The ability of the fungi to release nutrients from minerals (i.e., weathering) is due to the action of fungal extracellular polysaccharides (EPSs) in cooperation with organic acids synthesized by the fungi, resulting in the precipitation and dissolution of soil minerals through

the acidification and chelation of different ions [69–71]. Acidic metabolites adsorbed on the EPS surface, high-molecular-weight sugar-based polymers synthesized and secreted by microorganisms, bind with $SiO_2$ ions of silicate minerals and contribute to the release of $K^+$ from soil solutions [72].

### *4.3. Ecological Implications of Liming*

Our observations indicate that extracellular enzyme activity and the microbial community are significantly responsive to lime treatment of the soil (Table 2). Extracellular enzymes are key components that regulate C storage and nutrient cycling in an ecosystem, and extracellular enzyme activities can indicate C storage and nutrient availability [73]. According to economic principles, microorganisms do not synthesize or secrete extracellular enzymes when available energy and nutrients are sufficient [74]; the release of extracellular enzymes is not controlled by the host but is affected by environmental factors [49,75,76]. Lime treatment altered the activities of AP and POX at the experimental sites. These observations reveal changes in activity depending on the optimum pH of the enzyme, although it has been reported that the optimum pH of extracellular enzymes adapts and changes if a change in soil pH is maintained for a long time in an acidic environment as a result of lime treatment [77]. The evidence indicates that with liming, the activity of AP, which is optimal in an acidic environment, decreases, and that of POX, which is optimal in a neutral environment, increases [33,78]. These changes in extracellular enzyme activity can affect biogeochemical cycling.

Lime treatment can affect soil microbial communities through direct or indirect processes. The increased soil pH resulting from lime treatment acted as a powerful force to alter the composition and proportion of bacterial communities, increasing their diversity (Figure 4 and Table 3; Table 4). These results are consistent with previous observations showing that bacterial communities are sensitive to pH and stimulated at neutral pH [31,32,50,79,80]. In addition, we proposed that Ale affects the bacterial community. It has been reported that the microbial community composition is important for several soil functions associated with specific microbial-mediated processes, such as nitrification, denitrification, and specific enzyme activity [81–84]. Changes in soil chemical properties caused by lime treatment not only affect soil function through changes in the bacterial community but also alter the nutrient cycling process and microbial uptake as a result of the aluminum toxicity of the soil, which is likely to affect the soil N pool.

## 5. Conclusions

This study demonstrated that lime treatment to condition an acidic soil environment greatly impacts the biological properties of the soil. Although some biological properties, including MBC, MBN and MBP, did not show significant differences at the site level, changes in soil chemical properties resulting from liming were correlated with microbial biomass, enzymatic activity, and microbial community structure. Both direct and indirect effects of lime treatment were evident. The change in pH resulting from liming directly altered the activities of enzymes such as AP and POX, and the bacterial community richness. By decreasing toxicity due to leaching of Ale, liming indirectly increased microbial biomass N and N-acetyl glucosaminidase activity. In this study, we interpreted various phenomena of various biological properties of soil induced by changes in the soil environment, and our findings will be helpful in elucidating the shifts in soil microbes and associated ecosystem functions. However, further studies are needed to evaluate the direct relationships between changes in enzymatic activities and microbial communities due to soil conditioning. Nevertheless, our findings can help elucidate the shifts in soil microbes and associated ecosystem functioning resulting from lime treatment in Republic of Korea.

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