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ORIGINAL PAPER

Effects of repeated fertilization and liming on soil microbial biomass in *Betula maximowicziana* Regel and *Abies sachalinensis* Fr. Schmidt stands in Japan

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Abstract

Few long-term fertilization experiments have been performed in forests, even though the effects of nitrogen (N) addition on soil microbial biomass are a cause for concern. Our objective was to examine the effects of repeated fertilization for 36 years on soil microbial biomass in two forest stands. We measured soil chemical properties and microbial biomass carbon (C) and N in soils in fertilized and non-fertilized plots in a birch stand (*Betula maximowicziana* Regel) and a fir stand (*Abies sachalinensis* Fr. Schmidt). We also performed lime amendments and a 21-day laboratory incubation, and measured microbial biomass to clarify the effects of acidification due to fertilization. Soil pH was significantly lower in fertilized plots in both stands, and soil microbial biomass C and N were lower (significantly so in the fir stand) in the fertilized plots after 36 years of repeated fertilization. In the laboratory incubation, lime amendment did not significantly affect the microbial biomass C, N, or C:N ratio, despite an increase of about 1 unit in soil pH. Our results therefore indicate that factors other than soil pH also have important effects on soil microbial biomass in repeatedly fertilized forest stands.

Keywords Available carbon · Microbial biomass carbon:nitrogen ratio · Nitrogen addition · Soil pH

Abbreviations

Al	Aluminum
C	Carbon
C:N	The carbon to nitrogen ratio
DBH	Diameter at breast height
Fe	Iron
K	Potassium
N	Nitrogen
P	Phosphorus
WEOC	Water-extractable organic carbon

Introduction

Increasing deposition of atmospheric nitrogen (N) has altered N dynamics in forest ecosystems throughout the USA (Fenn et al. 2005), Europe (Corre et al. 2007; Neirynck et al. 2008; Tietema et al. 1997), and Japan (Ohrui and Mitchell 1997; Yoh et al. 2004). High levels of N addition elevate nitrate (NO_3) concentrations in stream water during the growing season, which can indicate N saturation in forests. Most atmospheric N is immobilized by soil microbes or is taken up by plants and passed through the forest N cycle before being leached into streams (Tabayashi and Yamamuro 2012). Soil microbes play important roles in regulating this internal N cycling, including in decomposition processes and in N transformation and retention. Soil microbial biomass is correlated with soil enzyme activity (Zaman et al. 1999) and N mineralization rates (Hassink 1994; Zaman et al. 1999). Moreover, the soil microbiota immobilizes N and functions as an important N pool. Thus, the effect of high levels of N addition on the soil microbial biomass is a subject of concern.

The effects of N addition on soil microbial biomass have been studied with fertilization experiments. N fertilization in forests can increase the soil microbial biomass (Zhang

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and Zak 1998) or decrease it (Insam and Palojarvi 1995; Thirukkumaran and Parkinson 2000) shortly after fertilization. Over longer time spans (more than several years), fertilization often decreases the soil microbial biomass (Corre et al. 2003; Nohrstedt et al. 1989; Smolander et al. 1994; Wallenstein et al. 2006), and although the reasons for this decrease have been discussed, they are not well understood. Soil microbial biomass is positively correlated with soil pH within the pH range from 3 to 7 (Anderson and Joergensen 1997; Baath and Anderson 2003); the decreased biomass that has sometimes been observed might therefore be a result of the lower soil pH that occurs after long-term N addition. Wallenstein et al. (2006) suggested that long-term excess N deposition can also lead to loss of base cations and to related changes in soil pH, which can also decrease microbial biomass. A decrease in available substrates for microbial growth could also occur under N addition (Corre et al. 2003). For example, N addition as ammonium decreased lignin degradation by decreasing the activity of ligninolytic enzymes (Keyser et al. 1978), and high concentrations of N may decrease the decomposition rate both during late stages of litterdecomposition (Berg and MacClaugherty 2003) and in soil organic matter in general (Berg 1986). Reduced rates of decomposition may, in turn, decrease the availability of carbon (C) substrates to microorganisms (Corre et al. 2003).

Increasing the pH of acidic forest soils can create a more favorable environment for microorganisms, improve soil nutrient status, and increase the availability of soluble C sources (Foster et al. 1980; Salonius 1972; Smolander and Malkonen 1994; Soderstrom et al. 1983). Therefore, liming is a popular management practice for decreasing acidification of forest soils and is expected to suppress the negative effects of N addition on soil microbial biomass. Soil microbial biomass C increased in forests that were fertilized several times and limed once or twice over a 10-year period relative to the levels in forests that were fertilized but not limed (Corre et al. 2003; Smolander et al. 1994). However, the effects of liming on microbial biomass are not consistent in forests with acidic soils that receive lime amendment but not fertilizer. Soil microbial biomass C increased in the organic (O) horizon 11 years after liming (Priha and Smolander 1994) and 20 or 30 years after liming (Smolander and Malkonen 1994). In other studies, soil microbial biomass C in the O and A horizons did not change 5–6 years after liming (Baath et al. 1980), or decreased after 7 years (Lorenz et al. 2001), despite the increased pH. Zelles et al. (1990) found that bacterial populations increased but fungal populations decreased in the O and A horizons up to 18 years after liming.

The effects of lime amendment on soil microbial biomass in acidic soils were also inconsistent in laboratory experiments in which incubation periods varied from weeks to months. Carter (1986) found that soil microbial biomass C increased with lime amendment over several weeks; Neale et al. (1997) observed an initial increase followed by a decrease or constant biomass and finally a higher biomass; in contrast, others reported that microbial biomass did not change (Badalucco et al. 1992; Illmer and Schinner 1991). Badalucco et al. (1992) suggested that the increase in soil microbial biomass under lime amendment is probably a result of increased C availability. In addition, populations of microorganisms that are adapted to higher soil pH could increase under lime amendment after an initial decline in populations that thrive under more acidic conditions (Badalucco et al. 1992; Neale et al. 1997). These alkalinity-tolerant microorganisms can use substrates that are not readily metabolized by the microorganisms that are active in acidic soils (Neale et al. 1997), and their biomass may not change or may decrease with depletion of these substrates (Badalucco et al. 1992; Wachendorf 2015). Therefore, pH is not the only factor that affects microorganisms in acidic soils: C availability is also important, and lime amendment experiments can provide information about the status of the C sources available as substrates for soil microbes. There have been few long-term fertilization experiments in forests; therefore, experiments that combine fertilization and lime amendment will increase our understanding of the dominant factors that affect soil microbial biomass dynamics in forests that receive continuous N addition.

The objective of the present work was to examine the effects of long-term fertilization on soil microbial biomass. We used experimental forest stands in Hokkaido, Japan, where yearly fertilization has been performed since 1978. The continuous fertilization has led to a decrease in soil pH and in exchangeable cations in the surface soil (Aizawa et al. 2012). Continuous experiments such as this one are rare; they therefore represent a unique opportunity to examine the long-term effects of fertilization on the properties of soil microbes. We examined the effects of fertilization on soil microbial biomass in the stands, and we assessed the effects of lime amendment on microbial biomass in soils from these forests in a laboratory experiment.

Materials and methods

Study site

The long-term fertilization study was performed in a *Betula maximowicziana* Regel (monarch birch) stand and an *Abies sachalinensis* Fr. Schmidt (Sakhalin fir) stand in the experimental forest of the Hokkaido Research Center, Forestry and Forest Products Research Institute (FFPRI), Sapporo, Japan (42°59′N, 141°23′E). The mean annual temperature and total precipitation in the study area are 7.4 °C (2000–2013) and 1121 mm (2007–2012), respectively (Mizoguchi et al.

2014; Y. Mizoguchi, unpublished data). Mean monthly temperatures range from − 5.1 °C in January to 20.1 °C in August (Mizoguchi et al. 2014). The birch and fir stands were planted in 1974 and 1973, respectively, and three treatment plots [non-fertilized, N–phosphorus (P)–potassium (K)-fertilized, and NP-fertilized plots) were established in each stand in 1978. Each plot was 16 m × 18 m (288 m^2) in the birch stand and 20 m × 24 m (480 m^2) in the fir stand. Details of the fertilization treatments are shown in Table 1. N, P, and K have been applied once per year to the NPK-fertilized plots as a commercial fertilizer [N: phosphorus pentoxide (P_2O_5): potassium oxide (K_2O), 24:16:11 from 1978 to 2008 or 2009, 20:10:10 thereafter). N and P have been applied annually in the NP-fertilized plots, as ammonium sulfate ($[NH_4]_2SO_4$) and lime superphosphate ($Ca[H_2PO_4]_2$+$CaSO_4$).

Table 2 summarizes the stand characteristics (tree density, diameter at breast height, and height) in the stands in 2010 drawing on data from Aizawa et al. (2012) and S. Aizawa, FFPRI (unpublished data). The bamboo species *Sasa cernua* Makino covers the forest floor in the birch stand; understory vegetation is sparse in the fir stand. The soils in both stands are classified as black soils and light-colored black soils derived from volcanic ejecta (Aizawa et al. 2012). The pH of the surface mineral soil (0–10 cm) ranged from 5.8 to 6.1 in 1981, 3 years after fertilization began in the stands, but had decreased to between 4.2 and 4.9 in the fertilized plots by 2007 (Aizawa et al. 2012).

Table 1 Annual and total quantities of fertilizer added to the experimental forest stands from 1978 to 2014

	NPK-fertilized plot			NP-fertilized plot	
	N	P	K	N	P
Betula maximowicziana (monarch birch) stand					
Annual quantity (kg ha^{-1} $year^{-1}$)					
1978	214	62	82	214	62
1979	236	69	90	236	69
1980	259	75	99	259	75
1981–1985	285	83	109	285	83
1986–2009	110	32	42	152	57
2010–2014	110	24	46	152	57
Total (kg ha^{-1})	5322	1508	2046	6529	2265
Average (kg ha^{-1} $year^{-1}$)	144	41	55	176	61
Abies sachalinensis (Sakhalin fir) stand					
Annual quantity (kg ha^{-1} $year^{-1}$)					
1978	94	27	36	94	27
1979	103	30	39	103	30
1980	113	33	43	113	33
1981–1985	125	36	47	125	36
1986–1990	125	36	47	125	36
1991–2007	125	36	48	124	34
2008	125	36	48	91	34
2009–2014	130	28	54	124	34
Total (kg ha^{-1})	4587	1278	1773	4511	1272
Average (kg ha^{-1} $year^{-1}$)	124	35	48	122	34

N Nitrogen, *P* phosphorus, *K* potassium

Table 2 Tree density, diameter at breast height (*DBH*), and height in the birch and fir stands in 2010

	Density (trees ha^{-1})	DBH (cm)	Height (m)
Birch stand			
Non-fertilized plot	1984	15.9 (6.3)	14.7 (3.8)
NPK-fertilized plot	1235	20.7 (5.9)	18.1 (3.6)
NP-fertilized plot	1190	22.7 (5.7)	19.1 (3.7)
Fir stand			
Non-fertilized plot	2232	17.0 (4.3)	16.0 (1.6)
NPK-fertilized plot	2018	20.1 (4.7)	16.9 (1.3)
NP-fertilized plot	2246	19.2 (4.3)	17.7 (1.2)

Values are means (SD) (n = 40, 28, and 21 trees per plot for the birch non-fertilized, NPK-fertilized, and NP-fertilized plots, respectively; 60, 62, and 69 trees per plot for the fir non-fertilized, NPK-fertilized, and NP-fertilized plots, respectively). Unpublished data for tree density from S. Aizawa (Forestry and Forest Products Research Institute, Japan); DBH and height data from Aizawa et al. (2012). For other abbreviations, see Table 1

Soil sampling

We collected soil samples from the mineral layer (0–5 cm) in each plot in both stands in August 2014, thirty-six years after fertilization started. Five subplots were established in each plot, and a composite sample consisting of four soil cores (50-mm diameter × 50-mm depth) was obtained from each subplot; thus, we collected 30 composite samples in total. The soil samples were sieved to pass through a 2-mm mesh and stored at 4 °C until use. A subsample of each sample was air-dried and ground to measure the soil total C and N contents, as described in the next section. Wealso collected and weighed the humus layer (the O layer) in a 0.25-m × 0.25-m quadrat in each plot in both stands in August 2015 and determined the moisture content to allow calculation of the oven dry weight (24 h, 105 °C).

We collected soil samples to analyze the water-extractable organic C (WEOC) content in the mineral layer (0–5 cm) in each plot in both stands in October 2017. A soil core (50-mm diameter × 50-mm depth) was obtained from each subplot. Roots and litter were removed manually from the sample. The samples were stored in a cooler and analyzed as soon as possible on return to the lab. Fertilization treatments have been continued at 2014 levels since 2015.

Soil chemical properties and soil microbial biomass

Soil chemical properties and microbial biomass were measured before and after lime amendment and incubation. Soil pH and electrical conductivity were measured in aqueous suspensions (soil-to-water ratio = 1:2.5 w/w). Soil total C and N contents were measured with an NC analyzer (Sumigraph NC900; Sumika Chemical Analysis, Tokyo). Exchangeable cations were extracted from 4 g of air-dried soil using 40 mL of 1 N ammonium acetate at pH 7 and determined by inductively coupled plasma optical-emission spectrometry (Optima 4300DV; PerkinElmer, Yokohama, Japan). Extractable P was measured using the procedure described by the Editorial Committee of the Standard Method of Soil Analysis (1986): air-dried soil (2 g) was extracted with 40 mL of Bray-2 extractant (0.03 M ammonium fluoride + 0.1 M hydrochloric acid), the extract was filtered immediately through no. 6 Advantec filter paper, and P in the extract was determined spectrophotometrically. WEOC was determined according to the method of Gong et al. (2009): in summary, a moist 10-g subsample of each soil sample was shaken in 50 mL of distilled water for 0.5 h, the suspension was centrifuged at 15,000 r.p.m. for 10 min, the supernatant was filtered through a 0.45-µm membrane filter, and total organic C in the extract was measured with a total organic carbon analyzer (TOC-5000; Shimadzu, Kyoto, Japan).

Soil microbial biomass C and N were determined by the chloroform-fumigation method (Vance et al. 1987), according to the protocols described by Voroney et al. (2008), within 7–10 days after sampling. Briefly, two moist soil subsamples (each equivalent to 5 g of oven-dried soil) were prepared. One subsample was fumigated with chloroform for 24 h and then extracted in 20 mL of 0.5 M potassium sulfate (K_2SO_4). The other subsample was extracted immediately in 20 mL of 0.5 M K_2SO_4 and used as an unfumigated control. The extracts were stored in a freezer until analysis of the C and N concentrations, which were determined with a total organic carbon analyzer (TOC-5000) and a flow injection analyzer (FI-N50; Mitsubishi Chemical Analytech, Tokyo), respectively.

Microbial biomass C and N (mg kg^{-1} dry soil) were calculated as follows:

$$\text{Biomass C} = E_C/K_C, \quad (1)$$

where E_C = 0.5 M K_2SO_4–extractable C from each chloroform-fumigated sample minus that from the unfumigated control, and K_C is a conversion factor [0.45 (Wu et al. 1990)], and

$$\text{Biomass N} = E_N/K_N, \quad (2)$$

where E_N = 0.5 M K_2SO_4–extractable N from each chloroform-fumigated sample minus that from the unfumigated control, and K_N is a conversion factor [0.50 (Voroney et al. 2008)].

The 0.5 M K_2SO_4–extractable N in the unfumigated control was also used to calculate the extractable N content of the soil (mg kg^{-1} dry soil).

Lime amendment and incubation

Lime amendment and incubation were conducted according to Marumoto et al. (1990), within 3 weeks of storage of the soil samples at 4 °C. Two subsamples (moist soil equivalent to 20 g of oven-dried soil) were prepared in plastic vials. To one of them, 0.15 g of powdered lime (calcium carbonate; $CaCO_3$), equivalent to 1747 kg $CaCO_3$ ha^{-1}, was added and mixed. The other subsample was an unlimed control. Both subsamples were incubated for 21 days at 25 °C and 60% of water-holding capacity. Marumoto et al. (1990) and other studies (e.g., Neale et al. 1997) demonstrated that changes in the microbial species composition and biomass increases could occur within the first few days after lime addition and incubation, and that both parameters could then become constant within 10 days, thus we believe that 21 days is sufficient for the incubation. Soil pH and microbial biomass C and N were evaluated at the end of the incubation as described above.

Statistical analysis

All statistical tests were performed with version 6.0 of the JMP software (SAS Institute, Cary, NC). The Steel–Dwass test for multiple comparisons was used to assess the effects of the fertilizer treatment on soil chemical and microbiological properties, and on the weight of the O layer, after repeated fertilization for 36 years. For the lime amendment and incubation experiment, homogeneity of variance of the soil microbial biomass C and N data was examined using Bartlett's test. All variances were homogeneous, thus two-way ANOVA was performed to detect the effects of the lime application and the fertilizer treatment on soil microbial biomass C and N.

Results

Soil chemical properties and soil microbial biomass after repeated fertilization for 36 years

After repeated fertilization for 36 years, soil pH was significantly lower in the fertilized plots than in the non-fertilized plots of both species (Table 3), and was lowest in the NP-fertilized plot of both species (but not significantly lower than in the NPK-fertilized plot). Fertilizer treatment increased soil electrical conductivity, although the increase

Table 3 Chemical properties of the soils in the birch and fir stands after repeated fertilization for 36 years

	pH	Electrical conductivity (μS cm^{-1})	Total C (g kg^{-1} soil)	Total N (g kg^{-1} soil)	C:N ratio
Birch stand					
Non-fertilized plot	6.0 (0.1) a	47.1 (15.9) a	112.4 (24.5) a	7.0 (1.5) a	15.9 (0.4) a
NPK-fertilized plot	4.4 (0.2) b	87.5 (34.1) a	110.1 (14.4) a	7.3 (0.9) a	15.1 (0.5) a
NP-fertilized plot	3.9 (0.2) b	105.9 (56.6) a	112.9 (16.5) a	7.4 (1.0) a	15.3 (0.5) a
Fir stand					
Non-fertilized plot	5.5 (0.2) a	48.6 (6.4) a	78.7 (8.8) a	5.3 (0.8) a	15.1 (1.2) a
NPK-fertilized plot	4.2 (0.1) b	74.3 (12.9) b	93.4 (8.7) a	6.3 (0.5) a	14.8 (0.5) a
NP-fertilized plot	4.0 (0.1) b	139.3 (35.2) c	116.8 (20.6) a	7.8 (1.5) a	15.0 (0.4) a

Values are means (SD). Values of a parameter labeled with *different letters* differ significantly between fertilizer plots within a species (Steel–Dwass test, $P < 0.05$)

C Carbon; for other abbreviations, see Table 1

was significant only in the fir stand. There were no significant effects of fertilization on the soil total C and N contents or on the C:N ratio of either species (Table 3).

Exchangeable cation concentrations were lower in both fertilized plots than in the non-fertilized plot, but only some of the differences were significant (Table 4). In the birch stand, exchangeable calcium (Ca) and magnesium (Mg) were significantly lower in both fertilized plots than in the non-fertilized plot; in the fir stand, exchangeable Ca was significantly lower in the NPK-fertilized plot than in the non-fertilized plot, and exchangeable Mg was significantly lower in both fertilized plots than in the non-fertilized plot. In the birch stand, exchangeable K and Na were significantly lower in the NP-fertilized plot than in the non-fertilized plot. WEOC did not differ significantly between fertilizer treatments in the birch stand, but in the fir stand, it was significantly higher in the NP-fertilized plot than in the non-fertilized plot (Table 5). The 0.5 M K_2SO_4–extractable N was only significantly higher in the NP-fertilized plot than in the non-fertilized plot in the fir stand (Table 5). In both species, the Bray 2-extractable P increased significantly in the fertilized plots; in the birch stand, it was also significantly higher in the NP-fertilized plot than in the NPK-fertilized plot (Table 5). Fertilizer treatment significantly increased the oven-dry mass of the O layer in both species, but this mass did not differsignificantly between the two fertilizer treatments (Table 6).

Soil microbial biomass C and N were lower in the fertilized plots than in the non-fertilized plot, and most of these differences were significant in the fir stand (Table 7), but none were significant in the birch stand. The C:N ratio of microbial biomass was greater in the fertilized plots than in the non-fertilized plot in both stands, and these differences were significant in the NP-fertilized plot in the birch stand and in both fertilized plots in the fir stand. The ratios of the microbial biomass C to soil total C and of the microbial biomass N to soil total N were lower in the fertilized plots than in the non-fertilized plot in both species, but the differences were significant only in the fir stand (Table 7).

Lime amendment and incubation

At the end of the 21-day incubation of soil from all plots of both stands, the pH of unlimed soil had decreased by

Table 4 Exchangeable potassium (*K*), sodium (*Na*), calcium (*Ca*), and magnesium (*Mg*) in the soils of the birch and fir stands after repeated fertilization for 36 years

	K ($cmol_c$/kg)	Na ($cmol_c$/kg)	Ca ($cmol_c$/kg)	Mg ($cmol_c$/kg)
Birch stand				
Non-fertilized plot	0.67 (0.38) a	0.10 (0.02) a	15.19 (4.91) a	3.13 (0.92) a
NPK-fertilized plot	0.28 (0.12) ab	0.06 (0.01) ab	2.81 (1.11) b	0.49 (0.23) b
NP-fertilized plot	0.16 (0.06) b	0.06 (0.01) b	2.64 (0.78) b	0.34 (0.08) b
Fir stand				
Non-fertilized plot	0.17 (0.09) a	0.14 (0.03) a	9.56 (2.60) a	1.40 (0.36) a
NPK-fertilized plot	0.14 (0.03) a	0.09 (0.02) a	2.41 (0.89) b	0.27 (0.09) b
NP-fertilized plot	0.14 (0.02) a	0.09 (0.02) a	3.91 (1.30) ab	0.36 (0.11) b

Values are means (SD). Values of a parameter labeled with *different letters* differ significantly between fertilizer plots within a species (Steel–Dwass test, $P < 0.05$). For other abbreviations, see Table 1

Table 5 Water-extractable organic C (*WEOC*), 0.5 M potassium sulfate (K_2SO_4)–extractable N, and Bray 2–extractable P in soils of the birch and fir stands after repeated fertilization for 39 years (WEOC) or 36 years (extractable N and P)

	WEOC (mg kg^{-1} soil)	0.5 M K_2SO_4–extractable N (mg kg^{-1} soil)	Bray 2–extractable P (mg kg^{-1} soil)
Birch stand			
Non-fertilized plot	110.3 (33.2) a	25.2 (7.3) a	27.6 (10.7) a
NPK-fertilized plot	118.4 (29.8) a	32.5 (6.8) a	1069.5 (364.7) b
NP-fertilized plot	109.8 (37.1) a	31.7 (5.4) a	2314.2 (618.6) c
Fir stand			
Non-fertilized plot	83.8 (11.6) a	28.5 (5.2) a	31.1 (11.5) a
NPK-fertilized plot	118.0 (45.7) ab	39.8 (6.2) ab	691.7 (284.2) b
NP-fertilized plot	126.4 (17.8) b	52.9 (21.1) b	1391.2 (373.7) b

Values are means (SD). Values of a parameter labeled with *different letters* differ significantly between fertilizer plots within a species (Steel–Dwass test, $P < 0.05$). For other abbreviations, see Table 1

Table 6 Oven-dry mass of the O layer in the birch and fir stands after repeated fertilization for 36 years

	Mass of O layer (kg/m^2)
Birch stand	
Non-fertilized plot	0.79 (0.16) a
NPK-fertilized plot	4.60 (2.87) b
NP-fertilized plot	9.39 (1.51) b
Fir stand	
Non-fertilized plot	3.05(0.73) a
NPK-fertilized plot	7.33 (1.22) b
NP-fertilized plot	7.42 (1.11) b

Values are means (SD). Values of a parameter labeled with *different letters* differ significantly between fertilizer plots within a species (Steel–Dwass test, $P < 0.05$). For abbreviations, see Table 1

0.1–0.3 units and the pH of limed soil had increased by about 1 unit (Table 8). In the birch stand, lime amendment had not affected soil microbial biomass C and N at the end of the incubation, but the fertilizer treatment had significantly affected these parameters (Table 9). Microbial biomass C and N in soil from both fertilized plots tended to be lower than that from the non-fertilized plot (Fig. 1). Results were similar in the fir stand, where lime amendment had not significantly affected soil microbial biomass C or N at the end of the incubation, whereas fertilizer treatment significantly affected these values (Table 9). At the end of the incubation, microbial biomass C and N tended to be lower in soil from both fertilized plots in the fir stand compared to that in the non-fertilized plot (Fig. 2). There were no significant interactive effects of lime amendment and fertilizer treatment on microbial biomass C or N in soil from either stand (Table 9).

Discussion

The lower soil pH values in both fertilized plots after repeated fertilization for 36 years are consistent with previous observations of soil pH from 1981 to 2007 in the same plots (Aizawa et al. 2012). The lower values of most exchangeable cations in the soil in the fertilized plots are also consistent with previous research (Aizawa et al. 2012; Takahashi et al. 1999). The N added in the fertilizer transforms into NO_3 and then into HNO_3 in the soil, thereby

Table 7 Soil microbial biomass C and N, microbial biomass C:N ratio, ratio of microbial biomass C to soil total C, and ratio of microbial biomass N to soil total N in soils of the birch and fir stands after repeated fertilization for 36 years

	Soil microbial biomass				
	C (mg kg^{-1})	N (mg kg^{-1})	C:N ratio	Microbial C/total C (%)	Microbial N/total N (%)
Birch stand					
Non-fertilized plot	897 (138) a	134 (28) a	6.7 (0.4) a	0.8 (0.1) a	1.9 (0.3) a
NPK-fertilized plot	761 (216) a	111 (39) a	7.0 (0.7) a	0.7 (0.1) a	1.5 (0.4) a
NP-fertilized plot	763 (135) a	94 (16) a	7.9 (0.2) b	0.7 (0.1) a	1.3 (0.3) a
Fir stand					
Non-fertilized plot	732 (117) a	118 (18) a	6.2 (0.4) a	0.9 (0.1) a	2.2 (0.1) a
NPK-fertilized plot	442 (52) b	52 (10) b	8.6 (1.2) b	0.5 (0.0) b	0.8 (0.2) b
NP-fertilized plot	599 (115) ab	73 (16) b	8.2 (1.0) b	0.5 (0.0) b	0.9 (0.1) b

Values are means (SD). Values of a parameter labeled with *different letters* differ significantly between fertilizer plots within a species (Steel–Dwass test, $P < 0.05$). For abbreviations, see Table 1

Table 8 Changes in soil pH during the 21-day incubation experiment using soils sampled from the birch and fir stands

	pH before incubation	pH at the end of the 21-day incubation	
		Unlimed	Limed
Birch stand			
Non-fertilized plot	6.0 (0.1) a	5.8 (0.2) a	6.5 (0.2) a
NPK-fertilized plot	4.4 (0.2) b	4.1 (0.1) b	5.3 (0.2) b
NP-fertilized plot	3.9 (0.2) b	3.8 (0.1) b	5.0 (0.2) b
Fir stand			
Non-fertilized plot	5.5 (0.2) a	5.3 (0.2) a	6.5 (0.2) a
NPK-fertilized plot	4.2 (0.1) b	4.1 (0.1) b	5.4 (0.1) b
NP-fertilized plot	4.0 (0.1) b	4.0 (0.1) b	5.1 (0.2) c

Values are means (SD). Values of a parameter labeled with *different letters* differ significantly between fertilizer plots within a species (Steel–Dwass test, $P < 0.05$). For abbreviations, see Table 1

Table 9 Results of two-way ANOVA for the effects of lime amendment and fertilizer treatment on soil microbial biomass in soils from the birch and fir stands at the end of the 21-day incubation

	Biomass C		Biomass N	
	F-value	*P*-value	*F*-value	*P*-value
Birch stand				
Liming	0.02	0.90	0.07	0.80
Fertilizing	10.43	< 0.01	20.79	< 0.0001
Liming × fertilizing	0.16	0.85	0.86	0.43
Fir stand				
Liming	1.85	0.19	3.12	0.09
Fertilizing	15.96	< 0.0001	62.93	< 0.0001
Liming × fertilizing	0.01	0.99	3.18	0.06

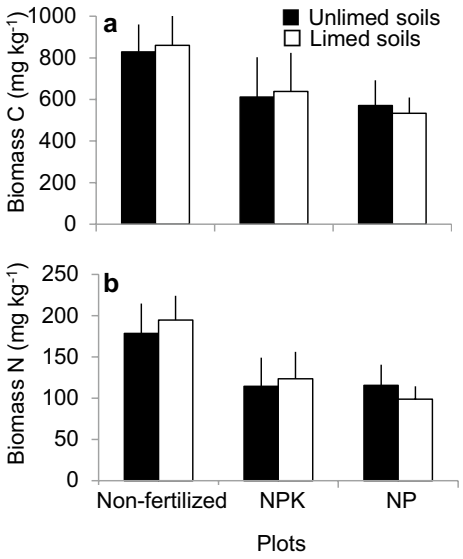

Fig. 1 Microbial biomass **a** carbon (*C*) and **b** nitrogen (*N*) in soils from plots in the birch stand at the end of the 21-day incubation. Values are means ± SD. Results are on a dry-weight basis. *P* Phosphorus, *K* potassium

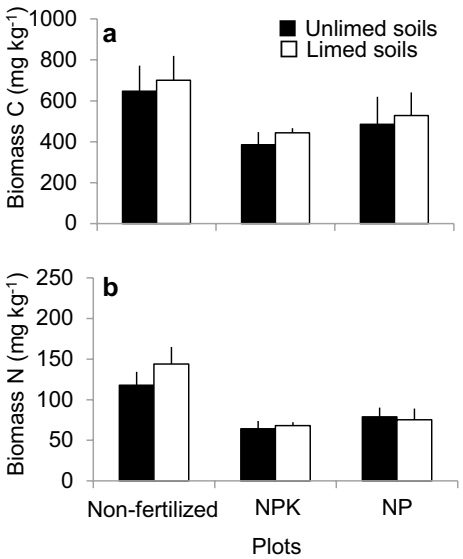

Fig. 2 Microbial biomass **a** C and **b** N in soils from plots in the fir stand at the end of the 21-day incubation. Values are means ± SD. Results are on a dry-weight basis. For abbreviations, see Fig. 1

lowering the soil pH. The significantly higher extractable N in the NP-fertilized plot in the fir stand suggests that there is much NO_3 in this soil, although this extractable N may also contain other forms of inorganic and organic N. The higher electrical conductivity in the fertilized plots may also result from increased NO_3. However, other forms of extractable N, various forms of extractable P, and the release of aluminum (Al) would also increase the soil's electrical conductivity. This is because primary and secondary minerals in the soil could dissolve in reaction with the acid and release Al into the soil solution under acidic conditions, as would be the case at pH < 5 in both fertilized plots in both stands (Sumner and Noble 2003).

The total soil N content did not differ significantly between the treatments. This suggests that the N from the fertilizer has not accumulated, and that instead it was leached from the soil, taken up in plant tissues, or both. Any surplus NO_3 leaches with cations (Fog 1988; Takahashi et al. 1999), and this may explain the lower exchangeable cation contents in the soil of the fertilized plots (Table 4); in addition, organic acids produced in the thick O layer of the fertilized plots (Table 6) may also accelerate leaching of cations (Takahashi et al. 1999). Takahashi et al. (1999) also suggested that, as the trees grow, they increase their uptake of cations, thereby decreasing both the level of exchangeable

cations and the soil pH. These changes would be especially noticeable in the fertilized plots, where the oven-dry weight of the O layer increased greatly and significantly and where tree growth was faster than in the control plot (Table 2).

N fertilizer often increases the mass or C content of the O horizon, and this increase is generally associated with-increased aboveground primary productivity, leading to increased inputs as litterfall (Nohrstedt et al. 1989; Rifai et al. 2010). Another reason for the increased mass of the O horizon is that N fertilization decreases soil microbial activity (Fog 1988; Soderstrom et al. 1983) and thus decreases decomposition (Nohrstedt et al. 1989). At our study site, the quantity of litterfall did not differ between plots in the birch stand (Suetsugu et al. 2012), but was greater in the fertilized plots than in the non-fertilized plot in the fir stand (S. Aizawa, unpublished data). Thus, the increased mass of the O layer could be explained by a combination of suppression of microbial activity in both stands with increased litterfall in the fir stand.

Fertilization noticeably decreased soil microbial biomass C and N in both stands after repeated fertilization for 36 years, but the difference was significant only in the fir stand, except for C in the NP-fertilized plot (Table 7). The ratios of microbial biomass C to soil organic C or to total C have been used as sensitive indicators of changes in soil organic matter (Sparling 1992) and as predictors of C assimilation by microorganisms (Xu et al. 2014). The lower ratios of microbial biomass C to total C in the fertilized plots of both species (with significant decreases in the fir stand) also suggest that microbial growth (i.e., C assimilation by microorganisms) was inhibited. Decreasing microbial biomass C and N after fertilization is consistent with previous studies of fertilizer effects on soil microbial biomass more than 11 years after fertilization (Corre et al. 2003; Nohrstedt et al. 1989; Smolander et al. 1994; Wallenstein et al. 2006). These previous studies provide some plausible mechanisms for a negative long-term effect of fertilization on microbial biomass, although the mechanisms are not well understood.

One possible mechanism is that the decreases in soil pH caused by long-term excess N deposition can decrease microbial biomass (Wallenstein et al. 2006). Acidic conditions stress microbial cells, as the pH in cells must be maintained at or near neutrality, and cell-surface and membrane-associated mechanisms must be upregulated to maintain the optimal internal pH under acidic external conditions (Tate 2000). Moreover, soil acidity indirectly affects soil microbes owing to changes in soil nutrient concentrations and levels of toxins such as Al (Tate 2000). The solubility of elements is also controlled by pH. For example, iron (Fe) is more soluble at low pH (Tate 2000), and Alalso dissolves more readily in acidic soil solutions (Sumner and Noble 2003). Dissolved Fe and Al can bind with phosphate, leading to its strong fixation in acidic soil (Haynes and Swift 1988). However, the available P would not have limited microbial biomass in the fertilized plots in our study because the extractable P content was much higher in fertilized plots than in the control plot. Increased leaching of cations caused by acidic soil conditions may also decrease microbial biomass (Wallenstein et al. 2006). This decrease might result from the decreased availability of cations, which would have a potential effect on soil microbes (Wolters and Schaefer 1994). Moreover, Hattori et al. (1972) observed that the growth of bacteria absorbed on an anion-exchange resin was faster than in bacteria growing freely in a liquid medium. From this observation, Oyanagi et al. (2001) assumed that the base cations are important microsites on which soil microorganisms grow, and assumed that increasing exchangeable cations could promote an increase of microbial biomass. The multiple influences of decreased pH, loss of base cations, and increase of Al concentrations could have adversely affected the microbial biomass C and N in our study.

The microbial biomass C:N ratio was higher in the fertilized plots in both stands (significantly so in the NP-fertilized plot in both stands and in the NPK plot in the fir stand), which suggests that fungi, which have a higher C:N ratio than bacteria, might be the dominant microorganisms in the fertilized plots (Anderson and Domsch 1980). The relative importance of fungi and bacteria, which comprise most of the microbial biomass, can be affected by pH: fungi tend to dominate at low pH, as in the fertilized plots, whereas bacteria are favored by more neutral pH (Lavelle and Spain 2001; Zelles et al. 1990). Soil acidification due to fertilization for 36 years in our stands may therefore have led to increased dominance of fungi.

A second possibility relates to a decrease in C substrates that support microbial growth under excess N addition (Corre et al. 2003). Many reports suggested that easily available C as a substrate for microbes plays an important role in determining microbial biomass (Demoling et al. 2007, 2008; Joergensen and Scheu 1999). N addition decreases the decomposition rate both during late stages of litter decomposition and in soil organic matter (Berg 1986; Berg and MacClaugherty 2003; Fog 1988). Reduced rates of decomposition may, in turn, decrease the availability of C substrates to microorganisms (Corre et al. 2003). In our study, the available C did not seem to limit soil microbial biomass at our site according to the WEOC results, which showed no significant difference between treatments in the birch stand and showed increases (significant in the NP-fertilized plot) in the fir stand. Moreover, limitation of microbial growth by N has been reported (Demoling et al. 2007). However, significantly higher levels of 0.5 M K_2SO_4–extractable N, which serves as an indication of soluble N, were observed in the fertilized plots in the fir stand (Table 5), so N was not a limiting factor in the fertilized plot. Because our results are inconsistent with some of these previous reports, further

research will be necessary to clarify why C availability was not restricted in our study. This research should determine the composition of the WEOC, since it is possible that it may not be a good indicator of the availability of C to microbes.

A third possibility is that microbial growth can be suppressed by the presence of tannins and humic acids or other organic acids in acidic soils (Harrison 1971; Tate 2000), as these substances are produced or released during decomposition in the thick O layer of the fertilized plots. A negative feedback loop between accumulation of the O layer and microbial biomass may exist if suppression of microbial activity by acidification decreases the decomposition rate of organic matter (Wolters and Schaefer 1994), and organic acids from the accumulated O layer, in turn, decrease microbial biomass and activity.

The effects of fertilization on soil microbial biomass C, N, and the ratio of microbial biomass C to total C were not significant in the birch stand before the lime amendment and incubation (Table 7), suggesting that there was no significant difference between these plots under field conditions. In contrast, most decreases were significant in the fir stand. Both species also showed significant negative effects of fertilization on soil microbial biomass C and N in the lime amendment and incubation experiment (Fig. 1; Table 9). Murugan et al. (2014) indicated that inputs of forms of organic matter that are consumed immediately by soil microorganisms, such as root exudates, strongly influence the composition and biomass of the soil microbial community. The birch plots also had *S. cernua* in the understory, whereas the fir stand mostly lacked understory vegetation. The addition of root exudates from *S. cernua* might stimulate microbial activity and offset the negative effect of fertilization on soil microbial biomass. However, the effects of understory plants and belowground processes have not yet been studied at our site, so further experimentation will be required to test these hypotheses. In addition, the role of root exudates and their relationship with both WEOC and easily available C should be explored in future research.

No significant effect of lime on soil microbial biomass C or N was observed in either stand (Table 9; Figs. 1, 2), despite an increase of about 1 unit in soil pH in all plots as a result of liming (Table 8). This suggests that microbial biomass does not increase solely in response to increased pH. The lack of a liming effect on soil microbial biomass may be the result of an insufficient increase in the soil pH. However, Corre et al. (2003) showed in an N-fertilized beech forest that liming increased soil pH from 3.8 to 4.6 (i.e., only 0.8 units), but microbial biomass C nonetheless increased. Priha and Smolander (1994) also showed that liming increased pH by up to 1 unit from the non-fertilized plot value of 4.5 and that soil microbial biomass C increased. Therefore, the magnitude of the increase in pH that we observed could have affected the microbial biomass; the fact that microbial biomass C and N did not increase therefore requires some explanation. Neale et al. (1997) suggested that microbial species that are relatively inactive under acidic conditions would proliferate under the increased pH that resulted from liming, and these alkalinity-tolerant microorganisms can increase their biomass by using substrates that arenot readily metabolized by microorganisms that are active in acidic soils. The change of microbial species composition and increase of biomass could occur within the first few days after lime addition and incubation (Marumoto et al. 1990; Neale et al. 1997), then could become constant within about 10 days (Marumoto et al. 1990). However, the microbial biomass may not change or may decrease in response to depletion of these substrates (Badalucco et al. 1992; Wachendorf 2015). Alternatively, Wachendorf (2015) suggested that microbial biomass C probably decreased because the Ca in lime increases the linkage of organic matter to minerals, resulting in increased stability of the organic matter (Oades 1988). The lack of any significant effect of lime on soil microbial biomass C or N in our study suggests that the soil in the fertilized plot may have no potential to increase the available C for soil microbes even if pH increased, or that the available C did not increase because it was stabilized (made less available) by Ca. On the other hand, the effects of fertilization were significant in the incubation experiment, suggesting that the decrease of exchangeable cations in the fertilized plots (Table 4) and the presence of tannins, humic acids, or both may have affected the soil microbial biomass C and N in the fertilized plots during the laboratory incubation.

If we extrapolate the laboratory results to field conditions, this suggests that microbial growth would be inhibited to some extent by the indirect effects of decreased pH, such as the decrease in concentrations of exchangeable cations, possibly combined with the release of tannins, humic acids, and other organic acids from the O layer, rather than by the direct effect of decreased pH. However, it is not clear whether the results of a 21-day trial can adequately reflect the ability of liming to compensate for changes in the soil caused by decades of fertilizer application.

Although our ability to draw conclusions from our findings is limited by the lack of replication of the treatments in each stand, we nonetheless observed a significant decrease in soil pH and in microbial biomass after 36 years of fertilization in both the birch and fir stands, but no change in soil microbial biomass in response to liming. Our study thus demonstrates that long-term fertilization has negative effects on soil microbial biomass that were not counteracted by a short-term lime-induced increase in soil pH. These findings suggest that continuous N addition over a period of several decades might adversely affect the soil microbiota and decrease their ability to immobilize N in an important N pool.

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