Does Soil Microbial Community Respond to Moderately Elevated Nitrogen Deposition? A Correlation Analysis in a Cool Temperate Forest Surrounded by Pasture Grasslands in Northern Japan

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Abstract

We analyzed relationships between nitrogen deposition (deposition of nitrate and ammonium ions) and soil microbial properties, which were spatially varied in a cool temperate forest surrounded by normally fertilized pasture grasslands in northern Japan. The aim of the present study was to gain the primary information on soil microbial response to moderately elevated nitrogen deposition (< 10 kg N ha$^{-1}$ y$^{-1}$). We established three experimental plots in the forest edge adjacent to the grasslands and other three plots in the forest interior at least 700 m away from the grasslands. During May to November 2018, nitrogen deposition in each plot was measured. In August 2018, litter and soil (0–5 cm depth) samples were collected from all plots to measure net nitrogen mineralization and nitrification rates as indicators of microbial activity, and microbial biomass carbon and nitrogen and various gene abundances (i.e. bacterial 16S rRNA, fungal ITS, bacterial amoA, and archaeal amoA genes) as indicators of microbial abundance. Nitrogen deposition in the forest edge was 1.4-fold greater than that in the forest interior, even while the maximum deposition was 3.7 kg N ha$^{-1}$. Nitrogen deposition was significantly correlated to the net nitrogen mineralization and nitrification rates and the 16S rRNA and bacterial amoA gene abundances. Microbial community structures in litter and soil samples were also analyzed using a high throughput DNA sequencer for the bacterial 16S rRNA and fungal ITS gene amplicons. Microbial community structures were different between litter and soil samples but were similar between the forest edge and interior. Significant correlations of nitrogen deposition to the soil carbon-to-nitrogen ratio, and the nitrate and ammonium contents were also observed. Thus, our results show that moderately elevated nitrogen deposition in nitrogen-limited forest edges likely stimulate microbial activities and abundances in soils.

Introduction

Nitrogen deposition (deposition of nitrate (NO$_3^-$) and ammonium (NH$_4^+$) ions) is expected to increase until the end of 21st century in global scale (Decina et al. 2019; Dentener et al. 2006; Galloway et al. 2004; Kanakido et al. 2016; Reay et al. 2008) and thus to cause various effects on forest ecosystem structures and functions such as biodiversity, productivity, biogeochemical cycles, and energy dynamics (Chiwa et al. 2018; Groffman et al. 2018; Janssens et al. 2010; Reay et al. 2008.; Zhang et al. 2018). The effects of increased nitrogen deposition in the ecosystem structures and functions have been investigated mostly in the environments manipulated with more than 20 kg N ha$^{-1}$ y$^{-1}$ of nitrogen addition (Janssens et al. 2010; Zhang et al. 2018). A large extent of forest ecosystems over the world is, however, receiving less than 10 kg N ha$^{-1}$ y$^{-1}$ of nitrogen deposition because of the heterogeneous localization of extensively elevated nitrogen deposition around urbanized areas (Reay et al. 2008). Moreover, nitrogen limitation in these forest ecosystems continues to occur due to the increased nitrogen demand of forest vegetations under the increasing atmospheric CO$_2$ concentrations (Groffman et al. 2018; McLauchlan et al. 2017). Therefore, we need to know more about the effects of moderately elevated nitrogen deposition (less than 10 kg N ha$^{-1}$ y$^{-1}$) on nitrogen-limited forest ecosystems, in order to obtain reliable responses of forest
ecosystems against increased nitrogen deposition and their feedbacks to climate change through the changing biogeochemical and energy dynamics.

Soil microbial community is one of the fundamental components sensitive to changes in nitrogen deposition and availability (Janssens et al. 2010; Niu et al. 2016; Tian et al. 2017; Waldrop et al. 2004; Zhang et al. 2018). In soils amended with extensively elevated nitrogen deposition, CO₂ release resulting from microbial decomposition of soil organic matter has generally been reduced (Janssens et al. 2010; Zhang et al. 2018). Microbial transformations of inorganic nitrogen compounds, such as nitrification and denitrification, are generally enhanced by nitrogen addition (Niu et al. 2016), increasing the risks of nitrogen leaching to surrounding water body and emission of nitrous oxide (a greenhouse gas 300-fold effective than CO₂; IPCC 2013) to the atmosphere (Butterbach-Bahl and Willibald 2002; Niu et al. 2016). There is little publication focusing on the effect of moderately-elevated nitrogen deposition on soil microbial community under field conditions, except for Allison et al. (2009). According to Aliison et al. (2009), fungal species isolated from boreal forest soils responded nonlinearly but parabolically to nitrogen addition from 0 to 200 µg N which are equivalent to only 0.1% or less of the amount of organic substrates in the soils. Thus, soil microbial community can be activated rather than restricted by moderately elevated nitrogen deposition.

In the present study, we focus on the relationship between spatially varied nitrogen deposition and soil microbial properties within a cool temperate forest in eastern area of Hokkaido, Japan. The eastern Hokkaido including the investigated forest is receiving relatively low nitrogen deposition from the atmosphere (Chiwa et al. 2015; EANET, https://monitoring.eanet.asia/document/public/index), while the boundary area of the forest (i.e. forest edge) is possibly receiving more nitrogen deposition than the interior area of the forest (forest interior) owing to nitrogen fertilization in the surrounding pasture grasslands (Reinmann and Hutyra 2017; Remy et al. 2016, 2017, 2018a, 2018b). These grasslands have been fertilized with nitrogen in normal extent as a normal agricultural management practice since the land reclamation from forest to grassland in 1950s. Therefore, investigating the relationship between nitrogen deposition and soil microbial properties in this forest ecosystem would provide the primary information on soil microbial responses to moderately elevated nitrogen deposition for over 60 years, rather than those responses to manipulationally and extremely elevated nitrogen deposition in short-term.

**Methods/experimental**

**Site description**

This study was conducted in a natural, deciduous, broad-leaved forest in the Shibecha branch of the Hokkaido Forest Research Station, Field Science Education and Research Center, Kyoto University (N43° 24.2', E144° 38.5', 115 m above sea level) in eastern Hokkaido, northern Japan. This station is registered as an associate site of JaLTER (Shibecha/Shiranuka forest, http://www.jalter.org/en/researchsites/) and used in a diverse range of ecological researches (e.g. Hosokawa et al 2017; Isobe et al. 2018; Nakayama et al. 2019; Nakayama and Tateno 2018; Tateno et al. 2019; Urakawa et al 2014 and 2016). Briefly, the
mean annual air temperature and precipitation for 1981–2010 were 6.2 °C and 1169.7 mm, respectively. The growing season is usually from June to October, and the season with a persistent snowpack is generally from December to April. Mean annual maximum snow depth for 1981–2010 was 64 cm. More detailed features of this forest ecosystem are given in Christopher et al (2008).

**Establishment of experimental plots**

In May 2018, we established six experimental plots in the forest (Fig. 1a). Three of the plots were located in the forest edge (Edge 1 to 3), a boundary between the forest and adjacent pasture grasslands, while the other three were located in the forest interior (Interior 1 to 3) at least 700 m away from the grasslands (Fig. 1a). All experimental plots were 10 m × 40 m in size. Soils in this forested area have been classified as Andosols, using the classification of the Food and Agriculture Organization (IUSS Working Group WRB 2015). Dominant vegetations of the plots were natural, deciduous broadleaved trees, mainly Japanese oak (Quercus crispula), with dense understory vegetations of Sasa nipponica. The mean diameter at breast height of standing trees was 17.3 cm throughout the plots. Mean stand density was 829 trees ha⁻¹. The maximum height of Sasa vegetation was 80–100 cm. There was no remarkable difference in vegetation status (i.e. species composition, standing trees density and canopy structure) between forest edges and interiors.

**Nitrogen deposition observation**

The amounts of nitrogen deposition in the six experimental plots were measured during the period of May 9th to November 20th, 2018, by continuously collecting throughfall water from the atmosphere to ground through the canopy vegetation. Seven of shaded plastic buckets equipped with collecting tubes and funnels (21 cm in diameter) were randomly put on each of the experimental plots. Throughfall water was collected by the buckets at an almost bi-weekly interval and then filtrated using a 0.45 µm pore sized membrane filter (ADVANTEC 25CS045AN, Toyo Roshi Kaisya LTD., Tokyo, Japan). Then, the concentrations of NO₃⁻ and NH₄⁺ were analyzed using an ion chromatography (Dionex-Integrion, Thermo Fisher Scientific, MA, USA). The amount of nitrogen deposition for the individual collection interval was quantified by multiplying the ion concentrations in the collected water sample and the amount of throughfall. Then, the total amount of nitrogen deposition during the six-month observation period was quantified by summing up the nitrogen deposition for all collection intervals.

**Litter and soil sampling**

Litter and surface mineral soil (0–5 cm depth) samples were collected in August 10th, 2018. Three sets of litter and soil samples were collected from each of the experimental plots to obtain the representative mean and the interspatial variation of soil microbial properties within a plot. Here, we determined the locations of litter and soil sampling avoiding the area directly below trees to reduce the possibility of specific effects from roots and rhizospheres on collected samples. Litter samples were collected by gloved hands from an area of 30 cm × 30 cm randomly selected within each of the plots, and soil samples were then collected using a shovel. Collected litter and soil samples were cooled and transferred
to the laboratory within a day. Soil samples were gently passed through a 4-mm sieve to remove gravel and plant tissues, while litter samples were pieced into a smaller size (ca. less than 2 mm × 2 mm) to obtain a homogenized sample. Both the litter and soil samples were immediately used for further analysis of soil microbial properties. Portions of the samples were air-dried and analyzed for total carbon and nitrogen contents (Koarashi et al. 2018) and pH (H₂O), as presented in Table 1. Ammonium and nitrates contents in fresh litter and soil samples are also measured (Urakawa et al. 2014, 2016) and presented in Table 1. All data of soil properties in this study are presented with the unit per area after the conversion with measured bulk density in Table S1.
Table 1
Chemical properties of litter and soil (0–5 cm) samples a) and significance of their correlations to nitrogen deposition b).

| Sample type | Property                           | Experimental plot | p value for correlation to N deposition |
|-------------|------------------------------------|-------------------|-----------------------------------------|
|             |                                    | Interior 1        | Interior 2                              | Interior 3                              | Edge 1         | Edge 2 | Edge 3 |                                    |
| Litter      | Total carbon [Mg ha\(^{-1}\)]      | 5.84 ± 0.26       | 5.85 ± 0.29                             | 5.70 ± 0.63                             | 4.19 ± 0.87   | 3.94 ± 0.38 | 5.87 ± 0.12 | 0.14                     |
|             | Total nitrogen [Mg ha\(^{-1}\)]   | 0.30 ± 0.03       | 0.30 ± 0.01                             | 0.28 ± 0.03                             | 0.24 ± 0.03   | 0.22 ± 0.04 | 0.34 ± 0.01 | 0.95                     |
|             | Carbon-to-nitrogen ratio           | 19.74 ± 0.88      | 19.63 ± 0.53                            | 20.00 ± 0.28                            | 17.09 ± 1.48  | 18.51 ± 1.68 | 17.13 ± 0.05 | <0.05,↓ |
| Soil        | Total carbon [Mg ha\(^{-1}\)]      | 25.84 ± 3.95      | 24.23 ± 2.84                            | 25.10 ± 0.45                            | 21.64 ± 5.39  | 26.83 ± 0.93 | 27.96 ± 2.89 | 0.52                     |
|             | Total nitrogen [Mg ha\(^{-1}\)]   | 2.01 ± 0.28       | 1.82 ± 0.18                             | 1.86 ± 0.10                             | 1.77 ± 0.45   | 2.03 ± 0.07  | 2.26 ± 0.27  | 0.17                     |
|             | Carbon-to-nitrogen ratio           | 12.87 ± 0.42      | 13.32 ± 0.27                            | 13.54 ± 0.84                            | 12.20 ± 0.38  | 13.23 ± 0.52 | 12.40 ± 0.18 | <0.05,↓ |
|             | NO\(_3^-\) [kg N ha\(^{-1}\)]    | 0.03 ± 0.01       | 0.06 ± 0.07                             | 0.04 ± 0.02                             | 0.08 ± 0.02   | 0.10 ± 0.05  | 0.06 ± 0.02  | 0.20                     |
|             | NH\(_4^+\) [kg N ha\(^{-1}\)]    | 1.55 ± 0.29       | 0.90 ± 0.52                             | 0.96 ± 0.41                             | 1.20 ± 0.54   | 0.72 ± 0.22  | 1.70 ± 1.07  | 0.46                     |
|             | NO\(_3^-\) [kg N ha\(^{-1}\)]    | 0.23 ± 0.02       | 0.19 ± 0.12                             | 0.27 ± 0.17                             | 0.37 ± 0.15   | 0.51 ± 0.08  | 0.44 ± 0.30  | <0.05,↑ |
|             | NH\(_4^+\) [kg N ha\(^{-1}\)]    | 1.62 ± 0.28       | 1.33 ± 0.42                             | 1.65 ± 0.43                             | 0.73 ± 0.27   | 1.20 ± 0.68  | 1.00 ± 0.19  | <0.05,↓ |

a) Mean ± Standard deviation for 3 replicates.

b) p < 0.05 is defined as the probability level suggesting the statistically significant level. Upward and downward arrows indicate significantly positive and negative correlations to nitrogen deposition, respectively.
### Sample type

| Property | Experimental plot | p value for correlation to N deposition |
|----------|-------------------|----------------------------------------|
|          | Interior 1        | Interior 2                               | Interior 3 | Edge 1 | Edge 2 | Edge 3 |                 |
| pH (H₂O) | 5.0 ± 0.1         | 4.9 ± 0.3                               | 5.3 ± 0.1  | 4.9 ± 0.1 | 4.9 ± 0.2 | 4.9 ± 0.1 | 0.10 |

a) Mean ± Standard deviation for 3 replicates.

b) $p < 0.05$ is defined as the probability level suggesting the statistically significant level. Upward and downward arrows indicate significantly positive and negative correlations to nitrogen deposition, respectively.

### Analysis of soil microbial property

The litter and soil samples were analyzed for net nitrogen mineralization and nitrification rates as indicators of microbial activity, and for microbial biomass carbon and nitrogen and various gene abundances, such as bacterial 16S rRNA, fungal ITS, bacterial amoA, and archaeal amoA genes, as indicators of microbial abundance and structure. The net nitrogen mineralization and nitrification rates were determined, respectively, as the changes in the concentrations of total inorganic nitrogen ($\text{NO}_3^- + \text{NH}_4^+$) and $\text{NO}_3^-$ only after aerobic incubation of soils at 25 °C for 4 weeks (Urakawa et al. 2014, 2016). In the investigated forest, these net mineralization and nitrification rates can be indicative metrics for gross mineralization and nitrification rates, respectively (Urakawa et al. 2016). Microbial biomass carbon and nitrogen were measured with the chloroform fumigation extraction methods (Vance et al. 1987). Total DNA were extracted from the soil samples using DNeasy Power Soil Kit (Qiagen, Hilden, Germany). Abundances of microbial genes were then quantified with an Illumina's Eco Real-Time PCR System (Illumina, CA, USA) and commercial regent kits or primer sets targeting the specific gene regions. Femto Bacterial and Fungal DNA Quantification Kits (Zymo research, CA, USA) were used for bacterial 16S rRNA and fungal ITS genes. For bacterial and archaeal amoA genes, the primer sets of amoA1f/amoA2r (Rotthauwe et al. 1997) and CrenamoA23f/Cremamo616r (Tourna et al. 2008) were used, respectively, with FastStart Essential DNA Green Master (Roche, Basel, Switzerland) as a PCR reaction mixture. The PCR conditions were shown in Table S2.

Microbial community structures were also evaluated using a high throughput DNA sequencer (MiSeq, Illumina) for the bacterial 16S rRNA and fungal ITS gene amplicons in litter and soil samples. DNA samples extracted from Edge 2 and Interior 1 plots were used in this evaluation to capture the difference in the microbial community structure between these two contrasting plots. The amplicon libraries of bacterial 16S rRNA and fungal ITS genes were prepared with the 16S (V3–V4) Metagenomic Library Construction Kit for NGS (TaKaRa Bio) and the prime set of ITS3-F/ITS4-R (Waud et al. 2014), respectively, in the coupling with the Nextera XT Index Kit (Illumina). Total 0.16 million of 2 × 250 bp pair-end reads of bacterial 16S rRNA genes and total 1.26 million of 2 × 150 bp pair-end reads of fungal ITS genes were obtained from 12 total genomic DNA samples (2 depths × 2 sites × 3 replications). These sequences were then binned into operational taxonomic units (OTUs) of 1592 for bacterial 16S rRNA
genes and 2625 for fungal ITS genes by using CD-HIT-OUT (Li et al. 2012) configured with a clustering threshold value of 0.97 and per-base PCR error value of 0.01.

**Statistical analysis**

Statistical analysis in the present study was performed with R software ver. 3.6 (R Core Team 2019). The two-way ANCOVA using glm function in base package (R Core Team 2019) was applied to examination of significant correlation between nitrogen deposition and s and significant interactive effect of different soil layers (i.e. litter vs. 0–5 cm soil) on those correlations. The correlation between nitrogen deposition and possibly confounding environmental factors, i.e. mean soil water content and temperature for the observation period was also examined. Soil water content and temperature can vary between forest interior and edge locations (Reinmann and Hutyra 2017; Remy et al. 2016, 2017, 2018a, 2018b). The correlations between nitrogen deposition and soil chemical properties, i.e. carbon and nitrogen contents, pH (H₂O) and NO₃⁻ and NH₄⁺ contents, were also examined to capture their possible changes associating with moderately elevated nitrogen deposition and microbial properties. The bacterial and fungal community structures were compared among different soil layers and sites with the permutational multivariate analysis of variance (perMANOVA, 9999 random permutations) by adonis function in vegan package (Oksanen et al. 2018). The probability levels suggesting statistically significance (i.e. p values) are defined as 0.05 in the present study.

**Results**

**Summary of nitrogen deposition**

Cumulative nitrogen deposition via throughfall for the six-month period (from May to November 2018) was ranged from 2.2 to 3.7 kg N ha⁻¹ in the six experimental plots (Fig. 1). The maximum and minimum amounts of nitrogen deposition were observed in the most northern plot of the forest edge (Edge 3; Fig. 1a) and in the most northern plot of the forest interior (Interior 3), respectively. In summary, mean nitrogen deposition for three plots of the forest edge was 3.5 ± 0.9 kg N ha⁻¹, which was 1.4-fold higher than that of the forest interior (2.5 ± 0.7 kg N ha⁻¹). A large proportion (> 76%) of the nitrogen deposition was in the form of NH₄⁺ form in the study area.

**Soil microbial properties vs. nitrogen deposition**

Both of the net mineralization and nitrification rates showed positive correlations to nitrogen deposition (Fig. 2). These positive correlations were statistically significant without any significant interactive effects from combinations of soil layer and nitrogen deposition (p > 0.05).

The abundances of 16S rRNA and bacterial amoA genes showed positive correlations to nitrogen deposition (Fig. 3). The positive correlation between 16S rRNA gene abundance and nitrogen deposition was statistically significant without significant interactive effects from the combinations of soil layer and nitrogen deposition (p > 0.05). The positive correlation between bacterial amoA gene abundance and
nitrogen deposition was also statistically significant, while there was significant interactive effect from the combinations of soil layer and nitrogen deposition. The slope value for the relationship between nitrogen deposition and amoA gene abundance in the surface mineral soil was 3.5-fold greater than that in the litter layer. There was no significant correlation between other microbial properties and nitrogen deposition (p > 0.05). There was also no significant difference in the microbial species composition between Edge 2 and Interior 1, while the microbial composition was significantly different between the litter and soil layers (Fig. 4).

Environmental factors vs. nitrogen deposition

There was no apparent relationship between nitrogen deposition and environment factors (i.e. temperature and soil water content) (Fig. 5). Comparing mean values of these environmental factors between the forest edge and interior, the differences were only 0.2 °C in temperature and 1% in soil water content.

Soil chemical properties vs. nitrogen deposition

In contrast to the environmental factors, some soil chemical properties were found to be significantly correlated with nitrogen deposition (Table 1). Carbon-to-nitrogen ratios of the litter and soil samples showed negative correlations to nitrogen deposition. In soil samples, NO$_3^-$ content showed a positive correlation to nitrogen deposition, while NH$_4^+$ content showed a negative correlation to nitrogen deposition. This should include the findings of the study including, if appropriate, results of statistical analysis which must be included either in the text or as tables and figures.

Discussion

Soil microbial activity vs. nitrogen deposition

Nitrogen disposition was greater in the forest edge than in the forest interior (Fig. 1). A significant contribution of nitrogen fertilizer applied to surrounding pasture grasslands to the forest was suggested because more than 76% of the deposited nitrogen was observed to be in the form of NH$_4^+$. However, the amount of nitrogen deposition in this forest was less than half of threshold amount to cause adverse effects on temperate and boreal forest ecosystems (i.e. 10–15 kg N ha$^{-1}$ y$^{-1}$; Bobbink et al. 2010; Nordin et al. 2005). Thus, the bioavailability of nitrogen in the investigated soils in both the interior and edge plots was likely limited.

The moderately elevated nitrogen deposition likely enhanced soil microbial activity in the forest edge (Fig. 2). This microbial response is different from observations of the reduction in soil microbial CO$_2$ release in forests under extensively elevated nitrogen deposition (Janssens et al. 2010; Zhang et al. 2018). In our forest, enhancement of soil organic matter decomposition may be possible due to the enhancement of microbial activity at the edge sites; this is partly supported by the observations of the
negative correlations between soil carbon-to-nitrogen ratio and nitrogen deposition (Table 5). The relative abundance of carbon to nitrogen in organic matter generally decreases with the progress of microbial decomposition where organic carbon is mineralized and released as CO$_2$ while nitrogen is retained and reutilized by soil microbial community (Koarashi et al. 2014; Kramer et al. 2017). Correlations between nitrogen deposition and individual content of soil inorganic nitrogen species (Table 1) were probably resulted from the enhanced consumption of NH$_4^+$ and production of NO$_3^-$ through nitrification under the moderately elevated nitrogen deposition (Fig. 2).

Such an enhancement of soil microbial activity under moderately elevated nitrogen deposition (Fig. 2) can contribute to increasing CO$_2$ production through decomposition of soil organic matter, and thus to increasing atmospheric CO$_2$ concentration. Moreover, taking into account for the previously-known sensitive responses of microbial processes to elevated nitrogen deposition and addition (Allison et al. 2009; Butterbach-Bahl and Willibald 2002; Jassal et al. 2011; Niu et al. 2016; Smith et al. 2000), microbially driven nutrition dynamics may also be altered even by moderately elevated nitrogen deposition particularly at forest edge sites.

**Soil microbial abundances vs. nitrogen deposition**

There was a remarkable difference between bacterial and fungal abundances in the terms of their correlations to nitrogen deposition (Fig. 3). This difference between bacteria and fungi is considered to reflect the different nitrogen demand between these two different types of microbes (Strickland and Rousk 2010). In general, bacterial biomass is relatively enriched in nitrogen compared with fungal biomass, suggesting a higher nitrogen demand of bacterial body (Strickland and Rousk 2010). Therefore, the observed linkage between the bacterial abundance and nitrogen deposition can be reliable when a high sensitivity of bacteria to changing nitrogen availability is assumed. The different responses to nitrogen deposition between bacteria and fungi in our forest can also be inferred from the lower ratio of microbial biomass carbon to nitrogen and the lower ratio of fungal to bacterial gene abundances at the edge sites than at the interior sites (Fig. 3), while the differences in these ratios between the edge and interior sites were not statistically significant (p > 0.05).

The bacterial amoA gene abundance appeared to respond to nitrogen deposition, but the archaeal amoA gene abundance was not (Fig. 3). In the investigated forest, Isobe et al. (2018) also found the synchronous temporal change in gross nitrification rate and bacterial amoA gene abundance during the wintertime. This was somewhat different from previous suggestion that archaeal ammonia oxidizer plays an important role in soil nitrification process in temperate forest and agricultural upland soils in Europe (Leininger et al. 2006). One of the possible interpretations of this discrepancy between European and Japanese forest soils is that soil conditions of our forest is preferable for bacterial ammonia oxidizers which have larger-sized cell body and higher cell-specific-unit activity compared with archaea (Jia and Conrad et al. 2009). Then, the specific dependence of bacterial ammonia oxidizer on autotrophic growth while contrary dependence of archaeal oxidizers on autotrophic growth and heterotrophic growth as well...
(Jia and Conrad et al. 2009) might result in the dominant contribution of bacterial community to nitrification in our forest soils.

Moreover, this specific sensitivity of bacterial ammonia oxidizers might be associated with the changes in species compositions of those bacteria (Isobe et al. 2020). While the overall compositions of bacterial and fungal communities were less sensitive to moderately elevated nitrogen deposition in the investigated forest (Fig. 5), Isobe et al. (2020) found the significant changes in species compositions of bacterial ammonia oxidizers along with forest slope gradients. Accordingly, Isobe et al. (2020) pointed out the importance of specific microbial community compositions on elucidating the soil nitrogen dynamics under changing environmental conditions.

**Environmental factors vs. nitrogen deposition**

In our forest, there was a low possibility that environment factors other than nitrogen deposition had caused the pseudo-correlation between nitrogen deposition and soil microbial properties, because the environmental factors observed in the present study were all similar between the forest edge and interior (Fig. 5). In Andosols, Urakawa et al. (2016) suggested the amounts of substrates for nitrogen mineralization were sufficient for soil microbial activities and soil chemical properties such as soil acidity would be the next significant factor. However, the moderately elevated nitrogen deposition in our forest may not be large enough to create the gradient of environmental factors directly affecting microbial properties. This situation was strictly different from the situations in previous studies in forests in Europe (Remy et al. 2016, 2017, 2018a, 2018b) and USA (Reinmann and Hutyra 2017), where not only nitrogen deposition but also other environmental factors changes gradually from edge to interior.

**Implication for future work**

Finally, we offer to promote further studies investigating the response of plant productivity, together with the response of soil microbial property, to nitrogen deposition, because plant productivity is functioning as the major offset process against the enhanced microbial CO$_2$ production (Reay et al. 2008). In the investigated forest, effects of nitrogen deposition on plant production are still under investigation and will be reported elsewhere in near future. Understanding both the responses of plant productivity and soil microbial community against the moderately elevated nitrogen deposition are essential for precisely capturing changes in the terrestrial carbon cycle under changing Earth's environment. This is not only because of the significant coverage of nitrogen limited forest ecosystems over the world (Groffman et al. 2018; McLauchlan et al. 2017; Reay et al. 2008) but also because of the significant increase in fragmented forest ecosystems (Haddad et al. 2015; Smith et al. 2018) where the spatial gradient of nitrogen deposition from edge to interior zones is apparently increased (Reinmann and Hutyra 2017; Remy et al. 2016).

**Conclusions**

In a Japanese forest surrounded by pasture grasslands, we found that soil microbial activities and their abundances were increased along with spatial gradients of nitrogen deposition between forest interior
and boundary edge area, nevertheless elevated level of nitrogen deposition in forest edges was not extreme, but moderately (< 10 kg N ha\(^{-1}\) year\(^{-1}\)). Our finding was different from the most of previous studies which mainly focused on the effects of > 20 kg N ha\(^{-1}\) year\(^{-1}\) nitrogen deposition reporting the reduction of soil CO\(_2\) release (Janssens et al. 2010; Zhang et al. 2018). Because of the significant coverages of nitrogen limited forest ecosystems (Groffman et al. 2018; McLauchlan et al. 2017; Reay et al. 2008) and the significant increase in fragmented forest ecosystems over the world (Haddad et al. 2015; Smith et al. 2018), understanding the responses of soil microbial community against the moderately elevated nitrogen deposition are essential, in order to capture the reliable insights of carbon cycles under changing environments. Through this study, we provided the primary information on soil microbial response to moderately elevated nitrogen deposition.

**Declarations**

**Availability of data and material**

The Illumina datasets obtained in this study are available at NCBI (National Center for Biotechnology Information) Sequence Read Archive (SRA) under accession number PRJNA612411 (from SRX7906297 to SRX7906320 as the SRA experiment accession numbers). Other data that support the findings of this study are available from the corresponding author upon reasonable request. The codes that process the data of this study are also available from the corresponding author upon reasonable request.

**Competing interests**

The authors declare that they have no competing interest.

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**Authors' contributions**

HN, GK, KF, TY, MW, and JK conceived and designed the study. HN, MN, GK, KF, TY, MW, JK, RT carried out all of the field works. KF, GK, and TK analyzed throughfall water samples. HN, JK, and MA analyzed soil physicochemical properties. MN and KF measure net nitrification and nitrogen mineralization rate of soil sample. HN, MN, and TK analyzed soil microbial abundances and community structures. HN conducted the statistical analysis of data and wrote first version of the manuscript. All authors advised on the content and revised the manuscript. All authors read and approved the final manuscript.
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References

1. Allison SD, LeBauer DS, Ofrecio MR et al (2009) Low levels of nitrogen addition stimulate decomposition by boreal forest fungi. Soil Biol Biochem 41:293–302. https://doi.org/10.1016/j.soilbio.2008.10.032

2. Bobbink R, Hicks K, Galloway J et al (2010) Global assessment of nitrogen deposition effects on terrestrial plant diversity: a synthesis. (Special Issue: Perspectives on the modern nitrogen cycle.). Ecol Appl 20:30–59

3. Butterbach-Bahl K, Willibald G, Papen H (2002) Soil core method for direct simultaneous determination of N2 and N2O emissions from forest soils. Plant Soil 240:105–116. https://doi.org/10.1023/A:1015870518723

4. Chiwa M, Inoue S, Tashiro N et al (2015) Assessing the role of forests in mitigating eutrophication downstream of pasture during spring snowmelt. Hydrol Process 29:615–623. https://doi.org/10.1002/hyp.10189

5. Chiwa M, Tateno R, Hishi T, Shibata H (2018) Nitrate leaching from Japanese temperate forest ecosystems in response to elevated atmospheric N deposition. J For Res 00:1–15. https://doi.org/10.1080/13416979.2018.1530082

6. Christopher SF, Shibata H, Ozawa M et al (2008) The effect of soil freezing on N cycling: Comparison of two headwater subcatchments with different vegetation and snowpack conditions in the northern Hokkaido Island of Japan. Biogeochemistry 88:15–30. https://doi.org/10.1007/s10533-008-9189-4
7. Decina SM, Hutyra LR, Templer PH (2019) Hotspots of nitrogen deposition in the world’s urban areas: a global data synthesis. Front Ecol Environ fee.2143. https://doi.org/10.1002/fee.2143
8. Dentener F, Drevet J, Lamarque JF et al (2006) Nitrogen and sulfur deposition on regional and global scales: A multimodel evaluation. Global Biogeochem Cycles 20:. https://doi.org/10.1029/2005GB002672
9. Galloway JN, Dentener FJ, Capone DG et al (2004) Nitrogen cycles: Past, present, and future. Biogeochemistry 70:153–226. https://doi.org/10.1007/s10533-004-0370-0
10. Groffman PM, Driscoll CT, Durán J et al (2018) Nitrogen oligotrophication in northern hardwood forests. Biogeochemistry 141:523–539. https://doi.org/10.1007/s10533-018-0445-y
11. Haddad NM, Brudvig LA, Clobert J et al (2015) Habitat fragmentation and its lasting impact on Earth’s ecosystems. Sci Adv 1:e1500052. https://doi.org/10.1126/sciadv.1500052
12. Hosokawa N, Isobe K, Urakawa R et al (2017) Soil freeze–thaw with root litter alters N transformations during the dormant season in soils under two temperate forests in northern Japan. Soil Biol Biochem 114:270–278. https://doi.org/10.1016/j.soilbio.2017.07.025
13. IPCC (Intergovernmental Panel on Climate Change) (2013) Climate Change 2013: The Physical Science Basis. Contribution of Working Group I to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change. Cambridge University Press, Cambridge
14. Isobe K, Ise Y, Kato H et al (2020) Consequences of microbial diversity in forest nitrogen cycling: diverse ammonifiers and specialized ammonia oxidizers. ISME J 14:12–25. https://doi.org/10.1038/s41396-019-0500-2
15. Isobe K, Oka H, Watanabe T et al (2018) High soil microbial activity in the winter season enhances nitrogen cycling in a cool-temperate deciduous forest. Soil Biol Biochem 124:90–100. https://doi.org/10.1016/j.soilbio.2018.05.028
16. IUSS Working Group WRB (2015) World Reference Base for Soil Resources 2014, update 2015 International soil classification system for naming soils and creating legends for soil maps. World Soil. FAO, Rome
17. Janssens IA, Dieleman W, Luyssaert S et al (2010) Reduction of forest soil respiration in response to nitrogen deposition. Nat Geosci 3:315–322. https://doi.org/10.1038/ngeo844
18. Jassal RS, Black TA, Roy R, Ethier G (2011) Effect of nitrogen fertilization on soil CH4 and N2O fluxes, and soil and bole respiration. Geoderma 162:182–186. https://doi.org/10.1016/j.geoderma.2011.02.002
19. Jia Z, Conrad R (2009) Bacteria rather than Archaea dominate microbial ammonia oxidation in an agricultural soil. Environ Microbiol 11:1658–1671. https://doi.org/10.1111/j.1462-2920.2009.01891.x
20. Kanakidou M, Myriokefalitakis S, Daskalakis N et al (2016) Past, present, and future atmospheric nitrogen deposition. J Atmos Sci 73:2039–2047. https://doi.org/10.1175/JAS-D-15-0278.1
21. Koarashi J, Atarashi-Andoh M, Takeuchi E, Nishimura S (2015) Topographic heterogeneity effect on the accumulation of Fukushima-derived radiocesium on forest floor driven by biologically mediated
processes. Sci Rep 4:6853. https://doi.org/10.1038/srep06853

22. Koarashi J, Nishimura S, Atarashi-Andoh M et al (2018) Radiocesium distribution in aggregate-size fractions of cropland and forest soils affected by the Fukushima nuclear accident. Chemosphere 205:147–155. https://doi.org/https://doi.org/10.1016/j.chemosphere.2018.04.092

23. Kramer MG, Lajtha K, Aufdenkampe AK (2017) Depth trends of soil organic matter C:N and 15N natural abundance controlled by association with minerals. Biogeochemistry 136:237–248. https://doi.org/10.1007/s10533-017-0378-x

24. Leininger S, Urich T, Schloter M et al (2006) Archaea predominate among ammonia-oxidizing prokaryotes in soils. Nature 442:806–809. https://doi.org/10.1038/nature04983

25. Li W, Fu L, Niu B et al (2012) Ultrafast clustering algorithms for metagenomic sequence analysis. Brief Bioinform 13:656–668. https://doi.org/10.1093/bib/bbs035

26. McLauchlan KK, Gerhart LM, Battles JJ et al (2017) Centennial-scale reductions in nitrogen availability in temperate forests of the United States. Sci Rep 7:1–7. https://doi.org/10.1038/s41598-017-08170-z

27. Nakayama M, Imamura S, Taniguchi T, Tateno R (2019) Does conversion from natural forest to plantation affect fungal and bacterial biodiversity, community structure, and co-occurrence networks in the organic horizon and mineral soil? For Ecol Manage. https://doi.org/10.1016/j.foreco.2019.05.042

28. Nakayama M, Tateno R (2018) Solar radiation strongly influences the quantity of forest tree root exudates. Trees - Struct Funct 32:871–879. https://doi.org/10.1007/s00468-018-1685-0

29. Niu S, Classen AT, Dukes JS et al (2016) Global patterns and substrate-based mechanisms of the terrestrial nitrogen cycle. Ecol Lett 19:697–709. https://doi.org/10.1111/ele.12591

30. Nordin A, Strengbom J, Witzell J et al (2005) Nitrogen deposition and the biodiversity of boreal forests: Implications for the nitrogen critical load. Ambio 34:20–24. https://doi.org/10.1579/0044-7447-34.1.20

31. Oksanen JFG, Guillaume Blanchet F, Friendly M et al (2018) Vegan: community ecology package. http://cran.r-project.org/package=vegan%0Ahttp://cran.rproject.org/package vegan

32. R Core Team (2017) R: A Language and Environment for Statistical Computing

33. Reay DS, Dentener F, Smith P et al (2008) Global nitrogen deposition and carbon sinks. Nat Geosci 1:430–437. https://doi.org/10.1038/ngeo230

34. Reinmann AB, Hutyra LR (2017) Edge effects enhance carbon uptake and its vulnerability to climate change in temperate broadleaf forests. Proc Natl Acad Sci 114:107–112. https://doi.org/10.1073/pnas.1612369114

35. Remy E, Gasche R, Kiese R et al (2017) Edge effects on N 2 O, NO and CH 4 fluxes in two temperate forests. Sci Total Environ 575:1150–1155. https://doi.org/10.1016/j.scitotenv.2016.09.196

36. Remy E, Wuyts K, Boeckx P et al (2016) Strong gradients in nitrogen and carbon stocks at temperate forest edges. For Ecol Manage 376:45–58. https://doi.org/10.1016/j.foreco.2016.05.040
37. Remy E, Wuyts K, Van Nevel L et al (2018) Driving Factors Behind Litter Decomposition and Nutrient Release at Temperate Forest Edges. Ecosystems 21:755–771. https://doi.org/10.1007/s10021-017-0182-4

38. Remy E, Wuyts K, Verheyen K et al (2018) Altered microbial communities and nitrogen availability in temperate forest edges. Soil Biol Biochem 116:179–188. https://doi.org/10.1016/j.soilbio.2017.10.016

39. Rotthauwe JH, Witzel KP, Liesack W (1997) The ammonia monooxygenase structural gene amoa as a functional marker: Molecular fine-scale analysis of natural ammonia-oxidizing populations. Appl Environ Microbiol 63:4704–4712

40. Smith IA, Hutyra LR, Reinmann AB et al (2018) Piecing together the fragments: elucidating edge effects on forest carbon dynamics. Front Ecol Environ 16:213–221. https://doi.org/10.1002/fee.1793

41. Smith KA, Dobbie KE, Ball BC et al (2000) Oxidation of atmospheric methane in Northern European soils, comparison with other ecosystems, and uncertainties in the global terrestrial sink. Glob Chang Biol 6:791–803. https://doi.org/10.1046/j.1365-2486.2000.00356.x

42. Strickland MS, Rousk J (2010) Considering fungal: Bacterial dominance in soils - Methods, controls, and ecosystem implications. Soil Biol Biochem 42:1385–1395. https://doi.org/10.1016/j.soilbio.2010.05.007

43. Tian D, Jiang L, Ma S et al (2017) Effects of nitrogen deposition on soil microbial communities in temperate and subtropical forests in China. Sci Total Environ 607–608:1367–1375. https://doi.org/10.1016/j.scitotenv.2017.06.057

44. Tourna M, Freitag TE, Nicol GW, Prosser JI (2008) Growth, activity and temperature responses of ammonia-oxidizing archaea and bacteria in soil microcosms. Environ Microbiol 10:1357–1364. https://doi.org/10.1111/j.1462-2920.2007.01563.x

45. Urakawa R, Ohte N, Shibata H et al (2016) Factors contributing to soil nitrogen mineralization and nitrification rates of forest soils in the Japanese archipelago. For Ecol Manage 361:382–396. https://doi.org/10.1016/j.foreco.2015.11.033

46. Urakawa R, Ohte N, Shibata H et al (2014) Biogeochemical nitrogen properties of forest soils in the Japanese archipelago. Ecol Res 30:1–2. https://doi.org/10.1007/s11284-014-1212-8

47. Vance ED, Brookes PC, Jenkinson DS (1987) An extraction method for measuring soil microbial biomass C. Soil Biol Biochem 19:703–707. https://doi.org/10.1016/0038-0717(87)90052-6

48. Waldrop MP, Zak DR, Sinsabaugh RL (2004) Microbial community response to nitrogen deposition in northern forest ecosystems. Soil Biol Biochem 36:1443–1451. https://doi.org/10.1016/j.soilbio.2004.04.023

49. Waud M, Busschaert P, Ruyters S et al (2014) Impact of primer choice on characterization of orchid mycorrhizal communities using 454 pyrosequencing. Mol Ecol Resour 14:679–699. https://doi.org/10.1111/1755-0998.12229
50. Zhang T, Chen HYH, Ruan H (2018) Global negative effects of nitrogen deposition on soil microbes. ISME J 12:1817–1825. https://doi.org/10.1038/s41396-018-0096-y

Figures
Figure 1

(a) Map showing the distribution of forest and pasture grassland with different nitrogen (N) levels and marked sampling points (Interior 1, 2, 3, Edge 1, 2, 3).

(b) Bar graph showing throughfall nitrogen deposition (kg N ha⁻¹ 6 months⁻¹) for Interior 1, 2, 3, Edge 1, 2, 3 with separate bars for nitrate (NO₃⁻) and ammonium (NH₄⁺).
Figure 1

Locations of six experimental plots in a Japanese cool temperate forest (a), and nitrogen deposition via throughfall for 6 months (May 9th to November 20th, 2018) in each experimental plot (b). Purple arrows in the top panel represent major wind flow which transport fertilizer from pasture grasslands to our forest. Error bars in bottom panel represent standard deviations (n = 7).
Figure 2

![Graph showing nitrogen mineralization and nitrification as a function of throughfall nitrogen deposition.](image_url)

- **Nitrogen mineralization** ([kg N ha⁻¹ day⁻¹]):
  - Litter sample: N: ρ < 0.01
  - Soil sample: L × N: ρ = 0.06

- **Nitrification** ([kg N ha⁻¹ day⁻¹]):
  - L: N: ρ < 0.01
  - L × N: ρ = 0.08
Figure 2

Correlations of net nitrogen mineralization and nitrification rates to nitrogen deposition. The probability level (p value) for statistically significance examined by two-way ANCOVA was presented above panels. The two-way ANCOVA was applied to the correlation and the difference in correlations between litter and soil samples (see text for details). Arrows represent correlations with p < 0.05.
Figure 3

![Graphs showing relationships between throughfall nitrogen deposition and microbial carbon, nitrogen, carbon/nitrogen ratio, ITS, 16S rRNA, AOB amoA, AOA amoA, and AOA amoA/AOB amoA ratios.](image)

- **Graph 1:** Microbial carbon [kg C ha⁻¹] vs. throughfall nitrogen deposition [kg N ha⁻¹ 6 months⁻¹]
  - N: ρ = 0.06
  - L × N: ρ = 0.15

- **Graph 2:** Microbial nitrogen [kg N ha⁻¹] vs. throughfall nitrogen deposition [kg N ha⁻¹ 6 months⁻¹]
  - N: ρ = 0.05
  - L × N: ρ = 0.18

- **Graph 3:** Microbial carbon/nitrogen ratio vs. throughfall nitrogen deposition [kg N ha⁻¹ 6 months⁻¹]
  - N: ρ = 0.71
  - L × N: ρ = 0.38

- **Graph 4:** ITS [×10¹⁸ copy ha⁻¹] vs. throughfall nitrogen deposition [kg N ha⁻¹ 6 months⁻¹]
  - N: ρ = 0.27
  - L × N: ρ = 0.68

- **Graph 5:** 16S rRNA [×10¹⁸ copy ha⁻¹] vs. throughfall nitrogen deposition [kg N ha⁻¹ 6 months⁻¹]
  - N: ρ < 0.01
  - L × N: ρ = 0.11

- **Graph 6:** ITS/16S rRNA vs. throughfall nitrogen deposition [kg N ha⁻¹ 6 months⁻¹]
  - N: ρ = 0.97
  - L × N: ρ = 0.29

- **Graph 7:** AOB amoA [×10¹⁷ copy ha⁻¹] vs. throughfall nitrogen deposition [kg N ha⁻¹ 6 months⁻¹]
  - N: ρ < 0.01
  - L × N: ρ < 0.01

- **Graph 8:** AOA amoA [×10¹⁷ copy ha⁻¹] vs. throughfall nitrogen deposition [kg N ha⁻¹ 6 months⁻¹]
  - N: ρ = 0.10
  - L × N: ρ = 0.26

- **Graph 9:** AOA amoA/AOB amoA vs. throughfall nitrogen deposition [kg N ha⁻¹ 6 months⁻¹]
  - N: ρ = 0.99
  - L × N: ρ = 0.99
Figure 3

Correlations of microbial biomass carbon and nitrogen and various gene contents, such as bacterial 16S rRNA, fungal ITS, bacterial amoA, and archaeal amoA genes, to nitrogen deposition. Statistical analysis of correlation was conducted in the same manner as Figure 2 (see text for details).
Figure 4

**perMANOVA:**

Site, $\rho = 0.10$; Depth, $\rho < 0.01$; Site $\times$ Depth, $\rho = 0.24$

Proportional composition of fungal phylum [%]

|       | Litter | Soil in 0-5 cm |
|-------|--------|----------------|
| Edge 2|        |                |
| Interior 1 |    |                |

- Ascomycota
- Basidiomycota
- Chytridiomycota
- Glomeromycota
- Mortierellomycota
- Mucoromycota
- Olpidiomyctota
- Rozellomyctota
- unidentified
- Other

**perMANOVA:**

Site, $\rho = 0.08$; Depth, $\rho < 0.01$; Site $\times$ Depth, $\rho = 0.46$

Proportional composition of bacterial phylum [%]

|       | Litter | Soil in 0-5 cm |
|-------|--------|----------------|
| Edge 2|        |                |
| Interior 1 |    |                |

- Acidobacteria
- Actinobacteria
- AOA
- Armatimonadetes
- Bacteroidetes
- Chlamydiae
- Chlorobi
- Chloroflexi
- Crenarchaeota
- Cyanobacteria
- Elusimicrobia
- FCPS426
- Firmicutes
- GAL15
- Gemmatimonadetes
- Nitrospirae
- OD
- Planctomycetes
- Proteobacteria
- TM6
- TM7
- Verrucomicrobia
- WP5-2
- Other
Figure 4

Correlations of microbial biomass carbon and nitrogen and various gene contents, such as bacterial 16S rRNA, fungal ITS, bacterial amoA, and archaeal amoA genes, to nitrogen deposition. Statistical analysis of correlation was conducted in the same manner as Figure 2 (see text for details).
Figure 5

![Graph showing the relationship between throughfall nitrogen deposition and soil water content and temperature. The graph includes error bars for each data point.]

- Water content;
- Temperature

Throughfall nitrogen deposition [kg N ha\(^{-1}\) 6 months\(^{-1}\)]

Mean soil water content [% v/v for 6 months]

Mean soil temperature [°C for 6 months]
Figure 5

Correlations of soil water content and temperature to nitrogen deposition. The presented soil water content and temperature are seasonal means for the observation period from May to November 2018. No significant correlation was observed between nitrogen deposition and the environmental factors.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Graphicalabstracts200719.pdf
- TableS2200719.docx
- TableS1200719.docx