**Supplementary methods**

**Title:** Consequences of microbial diversity in forest nitrogen cycling: Diverse ammonifiers and specialized ammonia oxidizers

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**1. Study site**

The study was conducted in a planted coniferous forest in the Fukuroyamasawa Experimental Watershed located in the University Forest in Chiba, the University of Tokyo, Japan (35° 12′ N, 140° 06′ E). The forest is located in a temperate climatic region and a mountainous area at an elevation of 124–227 MASL. The mean annual precipitation and air temperature (1994–2003) were 2,230 mm and 14°C, respectively (Oda *et al.* 2019).

We established a 10 × 100-m2 study plot in September 2013 on a steep slope (ca. 30°) from the ridge to the valley in a small-scaled (1.09 ha) watershed (**Fig. S1**). The uppermost part of the slope is A1 of Oda et al. (2019), while the lowest part of the slope is A2. The evergreen coniferous tree species *Cryptomeria japonica* was planted from 1928 to 1930 and thus larger trees can be caused by a higher growth rate (Oda et al., 2019). The tree heights were measured with an ultrasonic rangefinder (Vertex IV; Haglöf Sweden) as described by Larjavaara & Muller-Landau (2013). The height was 19.2 ± 4.2 m (n = 44) around the upper end of the slope and 26.9 ± 5.0 m (n = 129) around the lower end of the slope (100 m from the ridge). The diameter at breast height was 30.8 ± 10.5 cm around the upper end and 37.7 ± 13.0 cm around the lower end (Oda *et al.* 2019). The volumetric soil water content at a 10-cm depth at the ridge and valley was monitored over time during the actively growing season of trees (Nishizono *et al.* 2018) from May 11, 2014, to August 4, 2014 using time domain reflectometry (TDR) as described by Noborio (2001) with the probe (Campbell Sci.\* CS 615; Campbell Scientific Ltd., Shepshed, U.K.) connected to a datalogger (CR10X; Campbell Scientific Ltd.). The probes were inserted directly into the soil for the *in situ* measurement.TDR is an indirect measure of soil water content. It measures the transit time of a high frequency electromagnetic pulse through the soil that is used to calculate the permittivity of the soil. Then we can calculate the soil water content because the permittivity correlates with the water content (Noborio 2001).

**2. Soil chemical analysis**

The soil water content was measured by drying 20 g of soil at 105°C for 24 h in a ventilated oven. The soil pH was measured using a pH meter (D-54; Horiba, Tokyo, Japan) after extracting 5 g of soil in 25 mL of water. Soil inorganic N (NH4+ and NO3‒), organic N, and organic C were extracted with 2 M KCl solution (7 g of soil with 35 mL of 2 M KCl, shaking for 1 h). The extracts were then centrifuged at 3500 rpm for 10 min and filtered by glass fiber filters (GF/F; Whatman Int. Ltd., Maidstone, UK). The NH4+ concentration of the extracts was measured by the indophenol method and the NO3‒ concentration in the extracts was measured by the sulfanilamide-naphthylethylenediamine method after reduction by a Cd-Cu column. Both colorimetric methods used a flow injection system (FIU-300; Jasco Corp., Tokyo, Japan). The total dissolved N (TDN) and dissolved organic carbon (DOC) concentrations in the extracts were measured using a TOC/TN analyzer (TOC-V; Shimadzu, Kyoto, Japan).The dissolved organic nitrogen (DON) concentration was calculated by subtracting the inorganic N from the TDN in the extracts.

**3. Measurement of the gross and net ammonification and nitrification rates**

The gross ammonification and nitrification rates in soils were determined in the laboratory using the isotope dilution method (Hart *et al.* 1994). Two subsamples (7 g each, equivalent to approximately 3.5 g of dry soil) from each soil sample were used for the analysis of ammonification or nitrification during 24 h of incubation. Briefly, 7 g of soils supplemented with 1 mL of 1 mM 15NH4Cl (99.7 15N atom %) or Na15NO3 (99.8 15N atom %) was incubated at 20°C. We extracted both NH4+ and NO3− from the soils with 35 mL of 2 M KCl solution as described above after incubation for 2 and 26 h. The NH4+ concentration in the extracts was measured colorimetrically as described above. The denitrifier method was used to measure the 15N atom% of NH4+ and NO3−, and concentration of NO3− in the extracts was measured using gas chromatography with a mass spectrometer (GCMS, GCMS-QP2010 Plus; Shimadzu Corp., Kyoto, Japan). The detailed method is described elsewhere (Isobe et al., 2011). Briefly, we converted NO3− in the extract to N2O by using denitrifying bacteria lacking N2O reducing ability. We also captured the NH4+ in the extract as NH3 by using the diffusion method, converted NH4+ to NO3− by persulfate oxidation, and converted NO3− to N2O by using the denitrifier method. Then, we measured 15N atom% and the concentration of N2O with GCMS. The gross soil ammonification (NH4+ production) and nitrification (NO3−­ production) rates were calculated according to the equations of Hart *et al.* (1994).

The net ammonification and nitrification rates in soils were also determined in the laboratory. Two subsamples (50 g each, equivalent to approximately 25 g of dry soil) from each soil sample were used for the analysis of ammonification or nitrification during 28 days of incubation. Briefly, 50 g of soil was dispensed into glass vessels covered with aluminum foil to minimize water evaporation and incubated for 28 days at 20°C. No moisture adjustment was performed during the incubation; soil was incubated under the initial moisture content at field sampling. The moisture loss during the incubation was lower than 2% in terms of the gravimetric water content. Initial and incubated soil extraction was conducted. The methods for extraction, filtration, and concentration analysis were the same as described for the gross rate measurement. The net ammonification and nitrification rates were calculated as the concentration changes in soil NH4+ and NO3−, respectively, during 28-day incubation.

**4. Quantification of 16S rRNA genes, bacterial and archaeal ammonia monooxygenase genes (*amoA*), and bacterial copper-containing reductase genes (*nirK*)**

Microbial DNA was extracted from the soils and purified using an ISOIL kit for bead beating (Nippon Gene, Tokyo, Japan), as described previously (Isobe *et al.* 2012).

Bacterial 16S rRNA gene, bacterial and archaeal *amoA*, and bacterial *nirK* abundances were quantified by quantitative PCR (qPCR) to estimate the abundances of total bacteria, bacterial and archaeal nitrifiers, and bacterial denitrifiers, respectively. Bacterial 16S rRNA gene was determined using the primers 515f-806r (Caporaso *et al.* 2011). The bacterial and archaeal *amoA* were determined using the primers amoA1f-amoA2r (Rotthauwe *et al.* 1997) and CrenamoA23f-CrenamoA616r (Nicol *et al.* 2008), respectively. Bacterial *nirK* was determined using the primers nirKC1F-nirKC1R (Wei et al. 2015). Denitrifying bacteria in soil have *nirK* grouped into four or cytochrome cd1-containing reductase gene (*nirS*) grouped into two (Wei *et al.* 2015), but we previously found that *nirK* amplified by the primers nirK1F-nirK2R was the most abundant (Wei et al. 2015).

qPCR was conducted using a StepOne real-time PCR system (Applied Biosystems, Warrington, UK). All PCR procedures were performed in 0.2 mL tubes with 20 μL reaction volumes, using the hot start technique to reduce non-specific amplification. The reaction mixture contained the reaction buffer (1 × SYBR Premix Ex Taq II; Takara Bio, Shiga, Japan), 10 ng of DNA for the 16S rRNA gene or 20 ng of DNA for bacterial and archaeal *amoA* and bacterial *nirK*, 0.2 μM of each primer, 0.5 μg μL–1 of bovine serum albumin, and 1 × ROX Reference Dye (Applied Biosystems). The reactions were performed under the following conditions: initial annealing at 95 °C for 10 min, followed by 40 cycles of 95 °C for 30 s, 55 °C for 30 s for bacterial 16S rRNA gene, bacterial and archaeal *amoA*, or 58 °C for 30 s for bacterial *nirK*, and 72 °C for 30 s for bacterial 16S rRNA gene and *nirK* or 72 °C for 35 s for bacterial and archaeal *amoA*. The amplification efficiencies of all genes for standard curve generation were >90%, and the standard curves had high correlation coefficients (R2 > 0.95).

**5. Sequence analysis of 16S rRNA gene**

Bacterial 16S rRNA genes in soils were PCR-amplified using the primers 515f-806r. These primers were identical to those used in the qPCR assay except for the addition of appropriate Illumina adapters and 12-bp barcodes for multiplex sequencing on the Illumina platform (Caporaso *et al.* 2011). The PCR mixture (30 μl) contained 1× Ex Taq buffer (Takara Bio, Shiga, Japan), 0.2 mM of each dNTP, 0.2 μM of each primer, 2 U/100 μl of TaKaRa Ex TaqHS (Takara Bio), and 10 ng of DNA template. The amplification was performed under the following conditions: initial annealing at 94 °C for 3 min, followed by 21 cycles at 94 °C for 30 s, 50 °C for 45 s, and 72 °C for 90 s, and final extension at 72 °C for 5 min. The PCR amplicons were cleaned by electrophoresis and with a Wizard SV Gel and PCR Clean-Up System (Promega, USA), quantified fluorescently with a Qubit dsDNA HS kit (Life Technologies Inc., USA), and pooled at equimolar concentration. Libraries were checked for quality in terms of concentration and amplicon size using an Agilent 2100 Bioanalyzer (Agilent Technologies, USA) and sequenced on an Illumina MiSeq platform (Illumina, Inc., USA).

Sequence data were processed using a combination of the UPARSE (Edgar 2013) and QIIME (Caporaso *et al.* 2011) pipelines. The UPARSE pipeline was used to merge the de-multiplexed sequences, conduct quality filtering, and cluster sequences into operational taxonomic units (OTUs). Briefly, we set a minimum overlap of 30 bp for merging paired-end reads, and the minimum length of merged reads was set as 200 bp. A maximum number of expected errors (E\_max) of 0.5 was used to quality-filter sequences, and singletons were removed to reduce sequencing noise. OTU clustering was performed at >97% sequence similarity, and chimeric sequences were removed. The taxonomy of representative sequences of each OTU was assigned in the QIIME pipeline using the RDP classifier at an 80% confidence threshold trained on the Greengenes database (version 13\_08). Chloroplast and mitochondrial OTUs (i.e., plant-derived sequences) and unidentified OTUs at the kingdom level were excluded. A total of 1,588,970 (17,878–94,756) and 1,011,690 (13,572-142,157) sequences were obtained from 55 and 30 samples for the field survey and water-manipulation experiment samples, respectively. The number of sequences per sample was rarefied to the minimum depth within the samples (17,800 for the field survey samples or 13,000 for manipulation experiment samples) by random sampling within the QIIME pipeline. The OTUs’ sequences were aligned with PyNAST and a phylogenetic tree was constructed with FastTree after the aligned sequences were filtered with the default lanemask file within QIIME.

PCR primers for quantifying microbial genes responsible for litter degradation and NH4+ production have not been developed, unlike *amoA* in nitrification and *nirK* and *nirS* in denitrification. Thus, to estimate the abundance of these microbial genes, we used PICRUSt (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States) (Langille *et al.* 2013), which was designed to computationally infer metagenome functional contents from 16S rRNA gene sequences. To perform PICRUSt, the taxonomy of the representative sequences of each OTU in the 17,800 rarefied sequences was reassigned in the QIIME pipeline using BLASTn trained on the Greengenes database (version 13\_05), and OTU names in the OTU table were replaced with the taxonomy ID of the Greengenes database. The resulting OTU table was fed into PICRUSt (version 1.1.1) and functional predictions were made according to the metagenome inference workflow described by the developers (http://picrust.github.com/picrust/tutorials/). We predicted the occurrence of the genes necessary for enzymes for NH4+ production, N-acetylglucosaminidase (EC 3.2.1.52), arginase (EC 3.5.3.1), and urease (EC 3.5.1.5). The activity of these enzymes in forest soils is frequently measured in N biogeochemical studies, and N-acetylglucosaminidase genes are known to be phylogenetically conserved (Zimmerman *et al.* 2013). N-acetylglucosaminidase hydrolyzes dimer or trimer N-acetylglucosamine, which forms the cell walls of bacteria. Although a number of intercellular metabolic pathways can be used for NH4+ production, we chose the urea cycle as a representative pathway. Arginase converts arginine, which is an amino acid generated in the urea cycle, into ornithine and urea. Urease hydrolyzes urea into NH3 and CO2.

**6. Clone library analysis of bacterial and archaeal *amoA***

We used microbial DNA extracted from one soil sample each from the ridge and valley. The bacterial and archaeal *amoA* sequences were amplified with the primers used in the qPCR assay. The PCR amplicons were cleaned with a Wizard SV Gel and PCR Clean-Up System (Promega, USA). The purified PCR products were cloned into pGEM-T Easy vector (Promega) and transformed into *E. coli* JM109 high-efficiency competent cells (Promega), in accordance with the manufacturer’s instructions. The plasmids with the insert were extracted using a Qiagen Plasmid Mini Kit (Qiagen, Valencia, CA, USA), and used as a template for sequencing reactions. Occasionally, cloned insert DNA was amplified by PCR with vector primers M13 vector primers M3 (5′-GTA AAA CGA CGG CCA GT-3′) and RV-P (5′-GGA AAC AGC TAT GAC CAT G-3′), purified as mentioned above, sent to Takara Bio (Shiga, Japan), and directly sequenced.

The Phred and Phrap pipelines (Ewing *et al.* 2005) were used to conduct quality filtering and merge the paired-end reads. We obtained 37 and 40 bacterial *amoA* clones and 48 and 47 archaeal *amoA* clones from the ridge and valley, respectively. We removed the primer sequences and clustered the sequences into OTUs at the >97% sequence similarity using the MOTHUR program (Schloss *et al.* 2009). Then, we obtained 3 and 4 bacterial *amoA* OTUs (>97% sequence similarity) and 6 and 11 archaeal *amoA* OTUs from the ridge and valley, respectively. Reference sequences were retrieved from NCBI GenBank. The OTU sequences and reference sequences were aligned and the phylogenetic trees were constructed using the maximum-likelihood method with bootstrap analysis (n = 1,000) by using MEGA X software (Kumar *et al.* 2018).

**7. Statistical analysis**

All statistical analyses were performed using R software. The relationships of the slope position with the soil N and C concentrations, net and gross N transformation rates, and gene abundances were tested via correlation analysis.

A structural equation model (SEM, Eisenhauer *et al.* (2015)) was used to test the hypothesis that the soil environmental gradient affects the gross ammonification and nitrification rates by affecting the abundances of ammonifiers and nitrifiers. SEM was computed using the “sem” function in the *lavaan* package. We tested the simple hypothetical relationship (**Fig. 2**) showing that the gross ammonification and nitrification rates are determined by the abundance of ammonifiers and nitrifiers, and the substrate for the processes, and that the abundance of ammonifiers and nitrifiers is determined by soil environmental properties (i.e., soil pH and water content) and the substrate. In the analysis of the ammonification rate, we used the soil DON and DOC contents as the substrate for ammonifiers and the abundance of bacterial 16S rRNA gene as the abundance of ammonifiers. In the nitrification rate analysis, we used the NH4+ content and gross NH4+ production rate as the substrate supply and the abundance of the bacterial and archaeal *amoA* as the abundance of nitrifiers. The gene abundances were log10-transformed. Reduced models were created by eliminating the paths with the highest probability values in a stepwise manner until all paths between two variables were significant (p < 0.05), as described in Petersen *et al.* (2012). For model goodness-of-fit, we used chi-square (χ2) statistics and comparative fit index (CFI) (Eisenhauer *et al.* 2015). If the χ2 was statistically non-significant (p > 0.05) and CFI value is greater than 0.9, the model was a good fit to the dataset. The final hypothetical relationship between variables were adequate fits to the dataset for both ammonification (χ2 = 3.901, p = 0.564, CFI = 1) and nitrification (χ2 = 3.294, p = 0.193, CFI = 0.980).

Spatial change in the community composition was visualized via a nonmetric multidimensional scaling ordination with Bray–Curtis similarities using the *phyloseq* package (McMurdie & Holmes 2013). Then, the community composition was statistically compared among the slope positions via a permutational multivariate analysis of variance (PERMANOVA) test using the “adonis” function in the *vegan* package (Oksanen *et al.* 2017). The Bray–Curtis similarity between two communities was statistically compared with the geographic distance between the communities via a Mantel test with 999 permutations using the “mantel” function in the *vegan* package. The soil environmental variables that strongly correlated with the compositional similarity were explored using the “envfit” function with 999 permutations in the *vegan* package.

**References**

Caporaso, J.G., Lauber, C.L., Walters, W.A., Berg-Lyons, D., Lozupone, C.A., Turnbaugh, P.J., *et al.* (2011). Global patterns of 16S rRNA diversity at a depth of millions of sequences per sample. *Proc. Natl. Acad. Sci.*, 108, 4516–4522.

Edgar, R.C. (2013). UPARSE: highly accurate OTU sequences from microbial amplicon reads. *Nat. Methods*, 10, 996–8.

Eisenhauer, N., Bowker, M.A., Grace, J.B. & Powell, J.R. (2015). From patterns to causal understanding: Structural equation modeling (SEM) in soil ecology. *Pedobiologia (Jena).*, 58, 65–72.

Ewing, B., Ewing, B., Hillier, L., Hillier, L., Wendl, M.C., Wendl, M.C., *et al.* (2005). Base-Calling of Automated Sequencer Traces Using. *Genome Res.*, 175–185.

Hart, S.C., Stark, J.M., Davidson, E.A. & Firestone, M.K. (1994). Nitrogen mineralization, immobilization, and nitrification. In: *Methods of Soil Analysis: Part 2—Microbiological and Biochemical Properties*. Soil Science Society of America, pp. 985–1018.

Isobe, K., Koba, K., Suwa, Y., Ikutani, J., Fang, Y., Yoh, M., *et al.* (2012). High abundance of ammonia-oxidizing archaea in acidified subtropical forest soils in southern China after long-term N deposition. *FEMS Microbiol. Ecol.*, 80, 193–203.

Isobe, K., Suwa, Y., Ikutani, J., Kuroiwa, M., Makita, T., Takebayashi, Y., *et al.* (2011). Analytical Techniques for Quantifying 15N/14N of Nitrate, Nitrite, Total Dissolved Nitrogen and Ammonium in Environmental Samples Using a Gas Chromatograph Equipped with a Quadrupole Mass Spectrometer. *Microbes Environ.*, 26, 46–53.

Kumar, S., Stecher, G., Li, M., Knyaz, C. & Tamura, K. (2018). MEGA X: Molecular evolutionary genetics analysis across computing platforms. *Mol. Biol. Evol.*, 35, 1547–1549.

Langille, M., Zaneveld, J., Caporaso, J.G., McDonald, D., Knights, D., Reyes, J., *et al.* (2013). Predictive functional profiling of microbial communities using 16S rRNA marker gene sequences. *Nat. Biotechnol.*, 31, 814–21.

Larjavaara, M. & Muller-Landau, H.C. (2013). Measuring tree height: A quantitative comparison of two common field methods in a moist tropical forest. *Methods Ecol. Evol.*, 4, 793–801.

McMurdie, P.J. & Holmes, S. (2013). phyloseq: An R Package for Reproducible Interactive Analysis and Graphics of Microbiome Census Data. *PLoS One*, 8, e61217.

Nicol, G.W., Leininger, S., Schleper, C. & Prosser, J.I. (2008). The influence of soil pH on the diversity, abundance and transcriptional activity of ammonia oxidizing archaea and bacteria. *Environ. Microbiol.*, 10, 2966–2978.

Nishizono, T., Zushi, K., Hiroshima, T., Toyama, K., Kitahara, F., Terada, F., *et al.* (2018). Latitudinal variation in radial growth phenology of Cryptomeria japonica D. Don trees in Japan. *Forestry*, 91, 206–216.

Noborio, K. (2001). Measurement of soil water content and electrical conductivity by time domain reflectometry: A review. *Comput. Electron. Agric.*, 31, 213–237.

Oda, T., Imamura, N., Egusa, T. & Ohte, N. (2019). The effects of canopy alteration–induced atmospheric deposition changes on stream chemistry in Japanese cedar forest. *For. Ecol. Manage.*, 448, 85–93.

Oksanen, J., Blanchet, F.G., Kindt, R., Legendre, P., Minchin, P.R., Hara, R.B.O., *et al.* (2017). vegan: Community Ecology Package. *Available at http://CRAN.R-project.org/package=vegan.*

Petersen, D.G., Blazewicz, S.J., Firestone, M., Herman, D.J., Turetsky, M. & Waldrop, M. (2012). Abundance of microbial genes associated with nitrogen cycling as indices of biogeochemical process rates across a vegetation gradient in Alaska. *Environ. Microbiol.*, 14, 993–1008.

Rotthauwe, J., Witzel, K. & Liesack, W. (1997). The Ammonia Monooxygenase Structural Gene amoA as a Functional Marker : Molecular Fine-Scale Analysis of Natural Ammonia-Oxidizing Populations, 63, 4704–4712.

Schloss, P.D., Westcott, S.L., Ryabin, T., Hall, J.R., Hartmann, M., Hollister, E.B., *et al.* (2009). Introducing mothur: Open-source, platform-independent, community-supported software for describing and comparing microbial communities. *Appl. Environ. Microbiol.*, 75, 7537–7541.

Wei, W., Isobe, K., Nishizawa, T., Zhu, L., Shiratori, Y., Ohte, N., *et al.* (2015). Higher diversity and abundance of denitrifying microorganisms in environments than considered previously. *ISME J.*, 9.

Zimmerman, A.E., Martiny, A.C. & Allison, S.D. (2013). Microdiversity of extracellular enzyme genes among sequenced prokaryotic genomes. *ISME J.*, 7, 1187–1199.