



# Stability of ammonia oxidizer communities upon nitrogen fertilizer pulse disturbances is dependent on diversity

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## ABSTRACT

Diversity of the soil microbial community is an important factor affecting its stability against disturbance. However, the impact of the decline in soil microbial diversity on the stability of ammonia-oxidizing bacteria (AOB) and ammonia-oxidizing archaea (AOA) is not known, particularly considering the repeated soil nutrient disturbances occurring in modern agricultural systems. Here, we conducted a microcosm experiment and modified the soil microbial diversity using the dilution-to-extinction approach to determine the stability and population dynamics of AOB and AOA communities with repeated nitrogen (N) fertilizer application. Our results demonstrated that the AOB community became more abundant and stable against repeated disturbances by N in the treatments with the highest microbial diversity. In contrast, the abundance of AOA decreased following repeated N fertilizer application, regardless of the microbial diversity. Notably, during the initial application phase, AOA displayed a potential for increased abundance in treatments with high soil microbial diversity. These findings highlight that the soil microbial diversity controls the stability of ammonia oxidizers during short-interval repeated N disturbances.

## 1. Introduction

Biodiversity is important for the optimal functioning, productivity, and stability of ecosystems, particularly during stress and disturbance. In particular, the soil microbiome comprises thousands of species with a high degree of functional redundancy and is responsible for maintaining biogeochemical cycling in the soil (Delgado-Baquerizo et al., 2016; Locey and Lennon, 2016). A recent study revealed the importance of microbial diversity in key ecosystem functions, such as plant biomass production, plant diversity, litter decomposition, and soil carbon assimilation (Wagg et al., 2021). Additionally, a study on the specific functional microbial community driving the nitrogen (N) cycle in soil found a positive correlation between soil microbial diversity and functional redundancy (Luo et al., 2018). The loss of soil microbial diversity is related to the disruption of N cycle processes, such as nitrification and

denitrification (Philippot et al., 2013; Wertz et al., 2006). Therefore, understanding the relationship between microbial diversity and its functions is crucial to establish sustainable management practices for N cycling.

Ammonia oxidation is the first rate-limiting step of nitrification and is catalyzed by the enzyme ammonia monooxygenase (AMO). The key drivers of soil ammonia oxidation are ammonia-oxidizing bacteria (AOB), ammonia-oxidizing archaea (AOA), and complete ammonia-oxidizing (comammox) bacteria (Daims et al., 2015; Prosser and Nicol, 2008; Purkhold et al., 2000). Numerous studies have shown that AOB tend to be more dominant under high  $\text{NH}_4^+\text{-N}$  concentrations in soil, whereas AOA are favored under low  $\text{NH}_4^+\text{-N}$  concentrations (Farooq et al., 2022; Hink et al., 2018; Ying et al., 2017). Moreover, repeated short-interval N fertilizer application is known to have a more pronounced positive impact on the population size of AOB (Luchibia et al.,

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2020; Wakelin et al., 2013). Traditionally, this niche differentiation between AOB and AOA has been attributed to differences in their substrate affinities under varying  $\text{NH}_4^+\text{-N}$  concentrations. However, recent studies have challenged this perspective by demonstrating that AOA can grow under high  $\text{NH}_4^+\text{-N}$  concentrations (Zhao et al., 2020), and AOA isolates belonging to *Nitrososphaerales* exhibit similar substrate affinities to AOB (Jung et al., 2022). This indicates a greater variability in kinetic properties among the AOA community, which suggests that niche differentiation may be ascribed to differences in the competitive abilities of AOA and AOB for  $\text{NH}_4^+\text{-N}$ . Consequently, under primary N fertilizer application, AOB potentially become more dominant than AOA based on their competitive abilities.

Soil microbial diversity plays an important role in the population dynamics of ammonia oxidizers. A previous incubation study indicated that the loss of microbial diversity resulted in a significant loss of ammonia oxidizer abundance (Wertz et al., 2006). Additionally, a field experiment in a forest ecosystem showed that ammonia oxidizers became more abundant and phylogenetically diverse with increasing soil microbial diversity (Isobe et al., 2020). Moreover, in an agricultural field experiment, a positive correlation was observed between soil microbial diversity and the abundance and activity of ammonia oxidizers (Zheng et al., 2022). However, the effects of soil microbial diversity on the population dynamics of AOB and AOA during repeated N application remain unclear.

A recent study highlighted the importance of understanding the stability of functional microbial communities under multiple disturbances in natural ecosystems (Philippot et al., 2021). The stability of a soil microbial community is determined by its resilience and resistance to disturbance (Griffiths and Philippot, 2013). Resilience is the recovery of microbial composition and function after a disturbance. Resistance refers to the ability of a community or process to remain unchanged despite a specific disturbance. Within the agricultural environment, the application of chemical N fertilizer is recognized as a disturbance with a strong impact on soil microbial community composition (Allison and Martiny, 2008). Prokaryotic communities have been observed to exhibit resilience, undergoing cyclical and similar shifts in response to repeated disturbances, including N fertilizer application (Lourenço et al., 2018), dry-wet cycles (Zhou et al., 2016), and flooding events (Shabarova et al., 2021). This suggests that diverse prokaryotic communities can regenerate and revert to their original composition after repeated disturbances. Additionally, other studies have shown a negative association between the loss of soil microbial diversity and the stability of microbial communities under disturbance (Girvan et al., 2005; Tardy et al., 2014). However, the response of ammonia oxidizers or the influence of microbial diversity on their stability under repeated N application has scarcely been studied. Tao et al. (2021) discovered that AOB and AOA communities are likely to exhibit resilience or resistance to inorganic N fertilizer application, but this response can be influenced by variations in the soil microbial diversity.

Inoculation of sterilized soil with serially diluted soil suspensions can modify the diversity of the microbial community. Many studies have shown that this approach can successfully help reproduce soils with different levels of soil microbial diversity (Chiba et al., 2021; Trivedi et al., 2019; Wertz et al., 2006; Yan et al., 2017). Additionally, these studies have shown that the loss of soil microbial diversity significantly decreases the microbial function. In particular, AOB are known to be more sensitive to a decrease in soil microbial diversity than denitrifiers and heterotrophs because of their restricted phylogenetic distribution in *Betaproteobacteria* (Isobe et al., 2020; Wertz et al., 2006). However, few studies have focused on the stability of AOB and AOA communities under declining soil microbial diversity. Therefore, we investigated the relationship between the loss of soil microbial diversity, population dynamics, and stability of ammonia oxidizers under repeated disturbances by N fertilizer application. An incubation experiment was conducted with soil microcosms with different diversity levels using the serial dilution method. We hypothesized that the loss of soil microbial

diversity would negatively impact the (i) stability of the ammonia-oxidizing microbial community composition and (ii) growth of ammonia oxidizers under short intervals of repeated N fertilizer application.

## 2. Material and methods

### 2.1. Soil sampling

Soil was collected in November 2020 from agricultural fields in Obihiro, Hokkaido, Japan (43.22467° N, 143.29649° E). The initial soil chemical properties were characterized according to the Tokachi Federation of Agricultural Cooperatives (Table S1). The soil was classified as volcanic ash (Andosol), with 44.6, 21.5, and 33.9 % of sand, silt, and clay, respectively.

### 2.2. Experimental design and serial dilution inoculation setup

The sampled soil was sieved using a 2 mm diameter mesh. Subsequently, 17 g of the sieved fresh soil was packed in a 48 mL jam jar fitted with a screw lid, and Milli-Q water was added to establish water-filled pore space (WFPS) of 60 % with a bulk density of 0.74 g cm<sup>3</sup>. In total, 144 jars (four treatments × four replicates × nine sampling times) of soil were prepared to ensure sufficient soil for each sampling time. The soil samples within the jars were sterilized using 50 kGy of  $\gamma$ -irradiation from a <sup>60</sup>Co source (KOGA Isotope, Japan).

The same soil was used for both the sterilized and non-sterilized treatments. To establish a microbial inoculum with varying levels of microbial diversity, non-sterilized soils were subjected to serial dilution following established methodologies from previous studies (Wertz et al., 2006; Yan et al., 2015). In particular, 100 g (equivalent dry mass) of non-sterilized soil was thoroughly mixed with 200 mL of sterile Milli-Q water using a mixer for 2 min. The soil paste was serially (10-fold) diluted with sterile Milli-Q water. Subsequently, 2.3 mL (equivalent to 1 mL of inoculum applied to 5 g of dry soil) of diluted soil suspensions [ $10^{-1}$  dilution (D1),  $10^{-2}$  dilution (D2),  $10^{-3}$  dilution (D3), and  $10^{-4}$  dilution (D4)] were inoculated with sterilized soil samples in quadruplicate. To check for a decrease in prokaryotic diversity, we conducted a preliminary experiment using the following soil suspensions under the same conditions:  $10^{-1}$  dilution (D1),  $10^{-4}$  dilution (D4), and  $10^{-8}$  dilution (D8) (Fig. S1).

The samples were preincubated at 25 °C for ten weeks. This period was determined based on the findings from a preliminary experiment. The results demonstrated that microbial abundance recovered across all dilution treatments within eight weeks after microbial inoculation of the sterilized soils (Fig. S2). After preincubation, 200 kg N ha<sup>-1</sup> of ammonium sulfate ((NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>) was applied to all treatments. Subsequently, five weeks after the first N application, an additional 200 kg N ha<sup>-1</sup> of (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> was repeatedly applied to all treatments. The N fertilizer application rate was determined based on a previous study that examined the effect of short-interval repeated N application on ammonia oxidizer abundance (Wakelin et al., 2013). During the incubation period, the WFPS was maintained at 60 % by adding sterile Milli-Q water every week. Soil sampling for chemical and molecular analyses was conducted at 1, 2, 3, 5, 6, 7, 8, and 10 weeks after the first instance of N fertilizer application and stored at 4 °C prior to chemical analysis and at -30 °C for DNA extraction.

### 2.3. Measurement of soil pH and inorganic N

The soil pH was determined using a pH meter (AS800; AS ONE Co., Osaka, Japan) after shaking 3 g of fresh soil in 30 mL of deionized water for 1 h. To determine the inorganic N (NO<sub>3</sub><sup>-</sup>-N and NH<sub>4</sub><sup>+</sup>-N) concentration, 3 g of the fresh soil was shaken for 1 h with 30 mL of 2 M KCl and filtered with filter paper. The extracted solution was stored at -30 °C until the measurement was conducted using a colorimetric method by a

flow injection analyzer (AQLA-700; Aqualab, Tokyo, Japan). The net nitrification rate was calculated based on the difference in  $\text{NO}_3^-$ -N concentration between each time point.

#### 2.4. DNA extraction and quantitative polymerase chain reaction (PCR)

Soil DNA was extracted from 0.5 g of soil using NucleoSpin® Soil (Takara Bio, Japan) kits according to the manufacturer's protocol. To reduce DNA attachment to the organic matter and mineral components of the Andosol, skim milk (0.17 g) was added during extraction (Okada and Oba, 2008). To minimize the contamination, skim milk was sterilized by  $\gamma$ -irradiation prior to use. The concentration of the extracted DNA was determined using Quantus (Promega, Mannheim, Germany) and stored at  $-30^\circ\text{C}$  until further analysis.

The DNA was diluted 10-fold for the polymerase chain reaction (PCR) targeting the ammonia monooxygenase subunit A (*amoA*) gene. AOB and AOA *amoA* gene copies were quantified by a SYBR Green-based quantitative PCR (qPCR) technique using an MxPro300 (Agilent Technologies) with KAPA SYBR (Takara Bio). The primer pairs *amoA*-1Fmod (5'-CTGGGGTTTCTACTGGTGGTC-3') and GenAOBR (5'-GCAGTGATCATCCAGTTGCG-3') were used to quantify the AOB *amoA* gene (Meinhardt et al., 2015). The 25  $\mu\text{L}$  reaction volume contained 12.5  $\mu\text{L}$  of KAPA SYBR (Takara Bio), 0.5  $\mu\text{L}$  of 10  $\mu\text{M}$  AOB *amoA* gene primers, 2  $\mu\text{L}$  of 10-fold diluted DNA, and 9.5  $\mu\text{L}$  of nuclease-free water. Amplifications were conducted under the following cycling conditions:  $95^\circ\text{C}$  for 10 min, followed by 40 cycles at  $95^\circ\text{C}$  for 30 s,  $58^\circ\text{C}$  for 1 min, and  $72^\circ\text{C}$  for 1 min. The primer pair GenAOAF (5'-ATAGAGCCTCAAGTAGGAAAGTTCTA-3') and GenAOAR (5'-CCAAGCGGCATCCAGCTGTATGTCC-3') was used to quantify the AOA *amoA* gene. The mixture and cycling conditions were the same as those used for the AOB *amoA* gene, except for the annealing temperature ( $56^\circ\text{C}$ ). The efficiencies of standard curves were 88 % ( $R^2 = 0.99$ ) and 93 % ( $R^2 = 0.99$ ) for the *amoA* genes of AOB and AOA, respectively.

#### 2.5. DNA sequencing

The DNA was amplified by PCR. For the PCR of AOB *amoA*, we used the forward primer *amoA*-1Fmod labeled with the Ion Xpress Barcode Adapters Kit (Thermo Fisher Scientific K.K., Yokohama, Japan) and the reverse primer GenAOBR labeled with Ion P1. The mixture containing 2  $\mu\text{L}$  of 10-fold diluted DNA was mixed with 12.5  $\mu\text{L}$  of Amplitaq Gold Master mix (Applied Biosystems™, Foster City, U.S.A.), 0.5  $\mu\text{L}$  of 10  $\mu\text{M}$  forward and reverse primers, and 9.5  $\mu\text{L}$  of nuclease-free water; this mixture was amplified under the following cycle conditions:  $95^\circ\text{C}$  for 10 min, followed by 40 cycles at  $95^\circ\text{C}$  for 30 s,  $58^\circ\text{C}$  for 1 min, and  $72^\circ\text{C}$  for 1 min. For the PCR of the AOA *amoA*, the mixture and cycle conditions were the same as those for the AOB *amoA* gene, except for the primers (labeled forward and reversed primers GenAOAF and GenAOAR, respectively) and annealing temperature ( $56^\circ\text{C}$ ). All PCR products were purified using AMPure XP (Beckman Coulter, CA, USA) according to the manufacturer's protocol. The final length of the amplicons was determined using a Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA) and a Bioanalyzer High Sensitivity DNA Kit (Agilent Technologies, Santa Clara, CA, USA) according to the manufacturer's protocol. The libraries were diluted to 30 pM with nuclease-free water. We used an Ion Chef Instrument with the Ion PGM Hi-Q Chef kit (Thermo Fisher Scientific K.K., Tokyo, Japan) to load the library onto the Ion 318 chip (Thermo Fisher Scientific K.K., Tokyo, Japan). DNA sequencing was conducted using an Ion PGM Sequencer with Ion PGM Hi-Q View Sequence Solutions (Ion Torrent Life Technologies, Guilford, CT, USA). Sequence data were deposited in the Sequence Read Archive of the National Center for Biotechnology Information (NCBI) under the accession number PRJNA935527.

#### 2.6. Sequence data processing

The sequence FASTQ files were processed using DADA2 in R. In particular, the sequences were truncated by detecting the primer using the "cutadapt" command. Adapter trimming, quality filtering, denoising, and chimera removal were then conducted, followed by the generation of amplicon sequence variants (ASVs). In accordance with previous studies, the ASV approach was used to conduct subsequent statistical diversity analyses (Callahan et al., 2016; Cholet et al., 2022). Rarefaction was conducted to equalize the sample sizes to the smallest number of non-chimeric sequences among the samples, which were 19,396 and 8588 reads for AOB and AOA, respectively. The *amoA*-AOB and *amoA*-AOA databases in the FunGene pipeline (Fish et al., 2013) were used to assign the taxonomy after formatting the database into the dada2-compatible training FASTA format.

#### 2.7. Statistic analysis

To investigate the stability of the functional microbial community, we calculated the Bray–Curtis dissimilarity of the functional microbial community between the beginning of the experiment (week 0) and other time points (weeks 1, 3, 5, 6, 8, and 10) in the vegan package of R (Oksanen et al., 2007). Pairwise comparisons among treatments were conducted using the emmeans package (Searle et al., 1980), followed by an analysis of variance (ANOVA) test for the linear mixed model.

To further examine the community shift of AOB and AOA, permutational multivariate ANOVA (PERMANOVA) was conducted to test the significance of the effect of N fertilizer application on the microbial community structure using two factors: application and group. For the "application," the first application of N (weeks 1, 2, and 5) and the second application of N (weeks 6, 8, and 10) were used as the factors. For the "group," the treatment was grouped based on the passage of weeks after the closest N application: group 1 (weeks 1 and 6), group 2 (weeks 2 and 8), and group 3 (weeks 5 and 10). A non-metric multidimensional scaling analysis of community structure dissimilarity based on the Bray–Curtis index was conducted using the metaMDS function in the vegan package of R. We expected the group factor to be significant if the community underwent cyclical and similar shifts between the first and second phases of application.

The stability of the relative abundances of individual taxa over time, especially AOB and AOA, was assessed using the coefficient of variation (CV). The CV was calculated by dividing the standard deviation by the mean for the entire experimental period and multiplying the result by 100. This metric enabled the evaluation of variability in the relative abundance of each taxon over the course of the incubation period (Louca et al., 2016; Machado et al., 2021). Furthermore, to examine the impact of the dilution treatment on the variability of each taxon, we used the modified signed-likelihood ratio test (SLRT) for the equality of CVs (Krishnamoorthy and Lee, 2014). This statistical test was used to determine whether there was a significant treatment effect on the observed variability in each taxon. Furthermore, a Student's *t*-test was conducted to compare the relative abundance of each taxon between the first and repeated application phases at each time point.

For the concentrations of inorganic N, soil pH, and the absolute abundance of AOB and AOA, a two-way ANOVA was conducted to determine the effects of the treatments (D1 to D4), sampling time points (weeks 0 to 10), and their interactions, followed by post-hoc pairwise comparisons using Tukey's test. As soil sampling was conducted from different jam jars at each time point, the random block effect was not included in the analysis. Additionally, Pearson correlation analysis was conducted to examine the correlations between the  $\text{NO}_3^-$ -N concentration and relative abundance of each taxon. All statistical analyses were conducted using R (version 4.0.0).

### 3. Results

#### 3.1. Diversity and community structures of AOB and AOA

The dilution of the soil suspension and inoculation helped reproduce the soil microcosms with diverse communities of ammonia oxidizers (Fig. S3). A significant ( $P < 0.05$ ) difference in community richness was observed between D1 and D4 for AOB and between D2 and D4 for AOA. By decreasing the diversity of the microbial community, the dissimilarity in the community structure between the beginning of the incubation (week 0) and other time points (weeks 1, 3, 5, 6, 8, and 10) became higher in both AOB and AOA ( $P < 0.001$ ) (Fig. 1).

In D1 (samples characterized by the highest soil microbial diversity), we observed a shift in the AOB community similarity between the first and second phases of fertilizer application (Fig. 2). Thus, the dynamics of community composition exhibited a cyclic pattern as the incubation period progressed. The community was significantly ( $P < 0.001$ ) clustered with the factor of the group, namely, the number of weeks passed after the closest N application (Table S2). However, in D1, the community structure was not significantly affected by the factor of application, indicating no significant difference in the community between the first and second phases of application. In contrast, in D2 and D3, the AOB community clustered significantly among the application phases (first or second application phase,  $P < 0.05$ ), indicating that communities shifted after the second application of N. In contrast, the AOA community showed significant clustering among the application phases of N in D1 and D4 ( $P < 0.05$ ).

The relative abundances of each AOB and AOA species at each time point are visualized as a stacked bar diagram (Fig. 3). Furthermore, we compared the difference in the abundance of each taxon between the first and repeated N application phases for each dilution treatment (Figs. S4 and S5). In D1, no AOB species changed significantly in abundance when the first and repeated fertilizer applications were compared at each time point. In D2, uncultured *Nitrosospira* sp. was significantly less abundant and *Nitrosospira briensis* was significantly more abundant in the repeated application phase than in the first application phase at 3 and 5 weeks after the closest N fertilizer application, respectively. In D3, *Nitrosospira briensis* was significantly more abundant in the repeated application phase than in the first application phase at 3 weeks after N fertilizer application. The differences in the

abundance of these taxa between the application phases may have contributed to the community structural differences observed in D2 and D3.

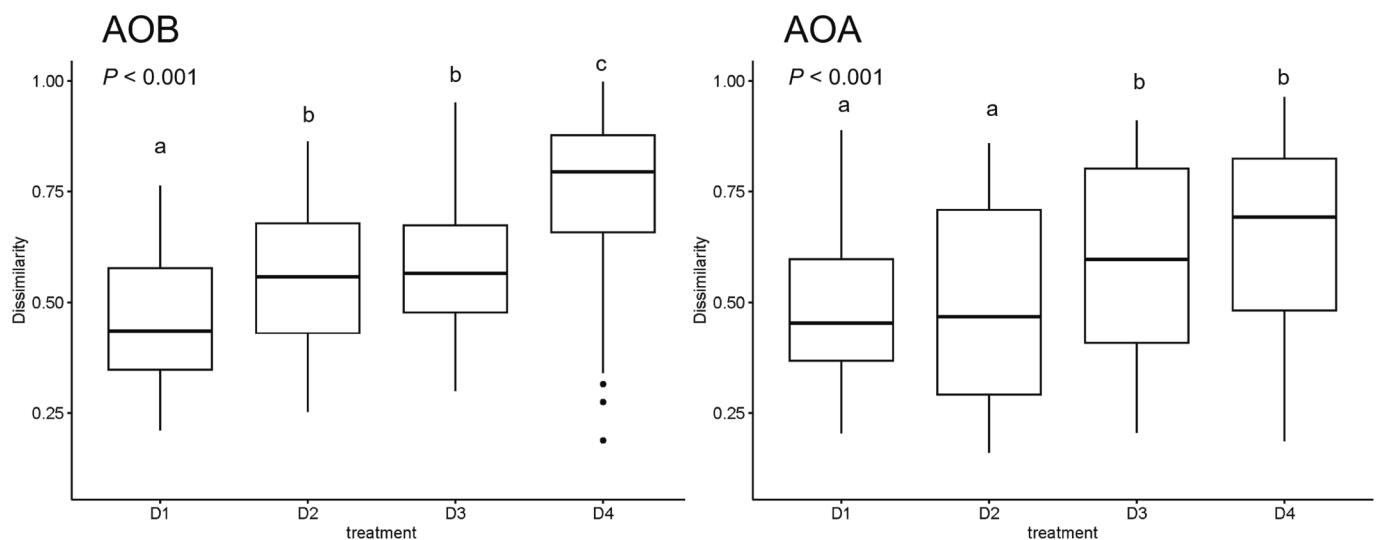
We observed a significant ( $P < 0.05$ ) effect of dilution treatments on the CV value of *Nitrosospira briensis* (Table 1). This indicated that the variability in the relative abundance of the dominant AOB *Nitrosospira briensis* was significantly affected by the dilution treatment. However, none of the AOA taxa were significantly affected by the dilution treatment.

We observed significant positive correlations between the relative abundance of *Nitrosospira briensis* and  $\text{NO}_3^-$ -N in D1 and D2 (Table 2). The relative abundance of uncultured *Nitrosospira* sp. was also correlated with  $\text{NO}_3^-$ -N in D3. No such correlation was observed in D4. Considering AOA, the relative abundance of *Candidatus Nitrosotalea devanattera* was positively correlated with  $\text{NO}_3^-$ -N concentration in D2, D3, and D4.

#### 3.2. AOB and AOA abundance and soil chemical properties

The dilution treatment significantly ( $P < 0.05$ ) decreased the initial (week 0) abundance of AOB. The abundance of AOB in D1 decreased compared with those in D3 and D4; similarly, the abundance of AOB in D2 decreased compared with that in D4. However, no significant difference was observed in the abundance of AOA among the treatments. ANOVA for the linear mixed model showed a significant interaction between the treatment and week for AOB abundance (Fig. 4). AOB became most abundant at week 2 in D1, week 7 in D2, and week 8 in D3. No significant increase was observed in the abundance of AOB in D4. For AOA, no interaction effect was observed between the treatment and week on abundance. AOA were significantly ( $P < 0.05$ ) more abundant in the initial application phase than in the repeated application phase. When the abundance was averaged by dilution treatment, it was significantly ( $P < 0.05$ ) higher in D1 than in D4. However, the abundance of AOA decreased after the second N fertilizer application, regardless of treatment.

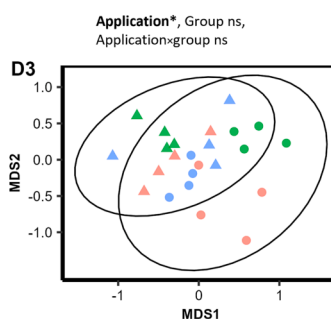
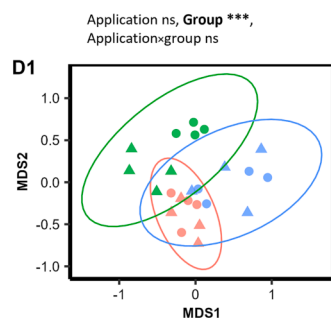
Following the first application of N, nitrification rates exhibited different response patterns across treatments. During the first application phase of N, the nitrification rate peaked during the first week in D1, whereas it peaked during the second week in D2 and D3. After the second N application phase, the nitrification rate reached a high level in



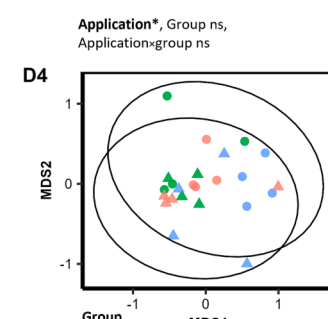
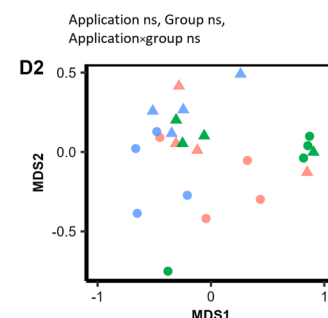
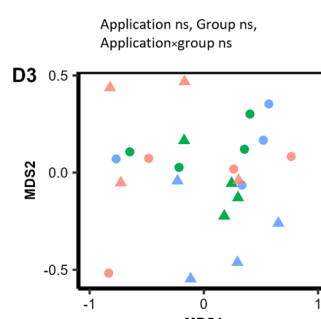
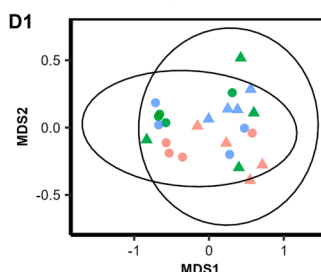
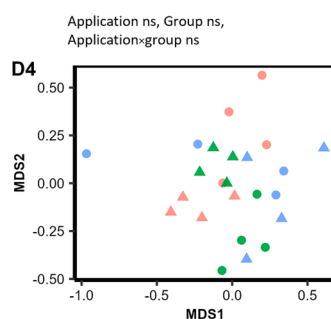
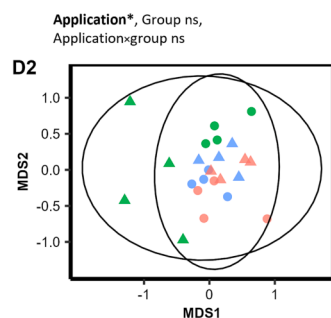
**Fig. 1.** Pairwise comparisons of Bray–Curtis dissimilarity of the ammonia-oxidizing bacteria (AOB) and ammonia-oxidizing archaea (AOA) communities among the dilution treatments. D1 to D4 represent diluted soil suspensions, namely,  $10^{-1}$  to  $10^{-4}$ , respectively. The  $P$  value indicates the significant difference in dissimilarity according to the dilution treatment calculated using ANOVA for a linear mixed model. The different letters indicate the significant difference ( $P < 0.05$ ) of the treatment calculated by Tukey's test. The dissimilarities between the beginning of the experiment (week 0) and at other time points (weeks 1, 3, 5, 6, 8, and 10) were included in the analysis.



## AOB



## AOA

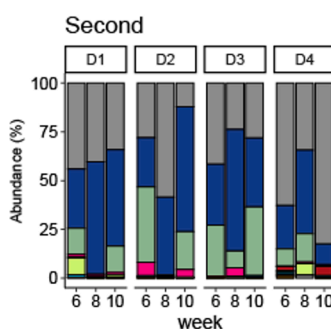
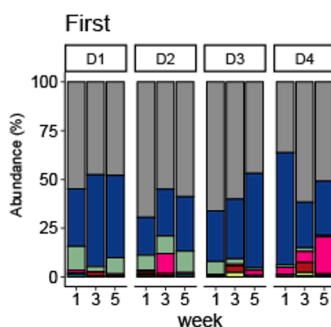


application ● First ▲ Second

group ● 1 ● 2 ● 3

**Fig. 2.** The non-metric multi-dimensional scaling (NMDS) plot based on Bray–Curtis dissimilarity for each dilution treatment. AOB and AOA are shown in two columns each. The group and color represent the three groups based on the passage of weeks after the latest N application: group 1 includes weeks 1 and 6; group 2 includes weeks 2 and 8; and group 3 includes weeks 5 and 10. The shape indicates the application period: the circle represents the first application, including weeks 1, 3, and 5; the triangle represents the second application, including weeks 6, 8, and 10. Asterisks in the upper left region indicate the significant ( $*P < 0.05$ ,  $**P < 0.01$ ,  $***P < 0.001$ ) PERMANOVA result for the group and application. Significant factors are surrounded by the 95 % confidence dispersion ellipses.

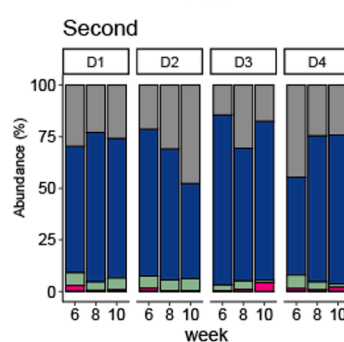
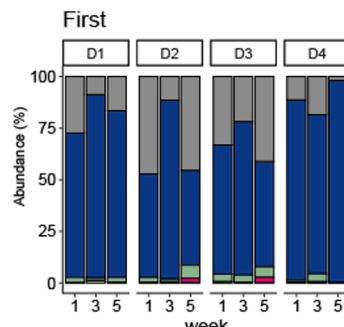
## AOB



## taxonomy

- unclassified bacteria
- Nitrosospora briensis*
- Nitrosospora* sp. (uncultured)
- Nitrosospora* sp. En13
- Nitrosospora* sp. Wyke8
- Nitrosospora multiformis* ATCC 25196
- Nitrosospora tenuis*
- Nitrosospora* sp. Wyke2
- Nitrosospora* sp. En1299
- Others

## AOA



## taxonomy

- unclassified archaea
- Candidatus Nitrosotalea devanattera*
- Candidatus Nitrososphaera gargensis* Ga9.2
- Nitrososphaera viennensis* EN76
- Others

**Fig. 3.** Average relative abundances (%) of AOB and AOA at the species level. The first and second rows represent the initial and second application phases of N fertilizer, respectively. Species with a relative abundance of less than 0.1% are classified as “Others.”

**Table 1**

The coefficient of variance (CV) was calculated using the relative abundance of each species across all sampling periods. Taxa with a relative abundance of less than 0.1% are classified as “Others.” The *P* values indicate the significant effects of the treatments, determined through the modified signed-likelihood ratio test values for the equality of CVs.

Taxa	D1	D2	D3	D4	<i>P</i> value
AOB					
<i>Nitrospira briensis</i>	40	74	60	96	0.018*
<i>Nitrospira</i> sp. (uncultured)	101	151	183	190	0.50
<i>Nitrospira</i> sp. En13	104	201	218	321	0.30
<i>Nitrospira</i> sp. Wyke8	185	134	211	167	0.77
<i>Nitrospira multiformis</i> ATCC 25196	366	185	321	270	0.78
<i>Nitrosobrio tenuis</i>	121	131	210	133	0.67
<i>Nitrospira</i> sp. Wyke2	380	238	282	270	0.88
<i>Nitrospira</i> sp. En1299	284	187	242	261	0.83
Others	490	406	339	481	0.99
AOA					
<i>Candidatus Nitrosotalea devanattera</i>	30	45	39	36	0.40
<i>Candidatus Nitrososphaera gargensis</i> Ga9.2	106	87	101	135	0.71
<i>Nitrososphaera viennensis</i> EN76	162	109	214	108	0.42
Others	253	252	305	225	0.88

**Table 2**

Correlation between the abundance of taxa and  $\text{NO}_3^-$ -N concentrations in soil. Taxa with relative abundances of < 0.1 % are classified as “Others.” Pearson’s *R* values are indicated by asterisks to show the *P* value (\**P* < 0.05, \*\**P* < 0.01, \*\*\**P* < 0.001).

Taxa	D1	D2	D3	D4
AOB				
<i>Nitrospira briensis</i>	0.55**	0.43*	0.00047	−0.044
<i>Nitrospira</i> sp. (uncultured)	−0.095	0.19	0.59***	−0.35
<i>Nitrospira</i> sp. En13	−0.1	−0.11	0.13	0.076
<i>Nitrospira</i> sp. Wyke8	−0.41*	0.01	−0.37	−0.24
<i>Nitrospira multiformis</i> ATCC 25196	−0.13	−0.012	−0.23	−0.2
<i>Nitrosobrio tenuis</i>	−0.25	−0.35	−0.014	0.021
<i>Nitrospira</i> sp. Wyke2	−0.13	−0.27	−0.2	0.37
<i>Nitrospira</i> sp. En1299	0.033	−0.38*	−0.23	−0.11
Others	−0.004	−0.3	0.11	−0.15
AOA				
<i>Candidatus Nitrosotalea devanattera</i>	0.24	0.47*	0.65***	0.52**
<i>Candidatus Nitrososphaera gargensis</i> Ga9.2	−0.053	−0.25	−0.6***	−0.43*
<i>Nitrososphaera viennensis</i> EN76	−0.065	−0.47*	−0.087	−0.22
Others	−0.059	−0.19	−0.3	−0.12

both D1 and D2 during the seventh week. However, in D3 during the repeated application phase and in D4 over the entire incubation period, the nitrification rate remained consistently low. At the end of the first or second phase of N fertilizer application, the nitrification rates were low (less than 10 mg N/day) or negative in all dilution treatments.

At the end of the first application phase, the  $\text{NH}_4^+$ -N concentration in the soil was 34.3 mg/kg in D1, which was significantly (*P* < 0.05) lower than those in D2 to D4, ranging from 173.3 to 270.4 mg/kg (Table 3). At the end of the second phase of N fertilizer application, the  $\text{NH}_4^+$ -N concentrations were significantly lower in D1 (64.0 mg/kg) and D2 (70.1 mg/kg) than those in D3 (251.3 mg/kg) and D4 (308.5 mg/kg).

#### 4. Discussion

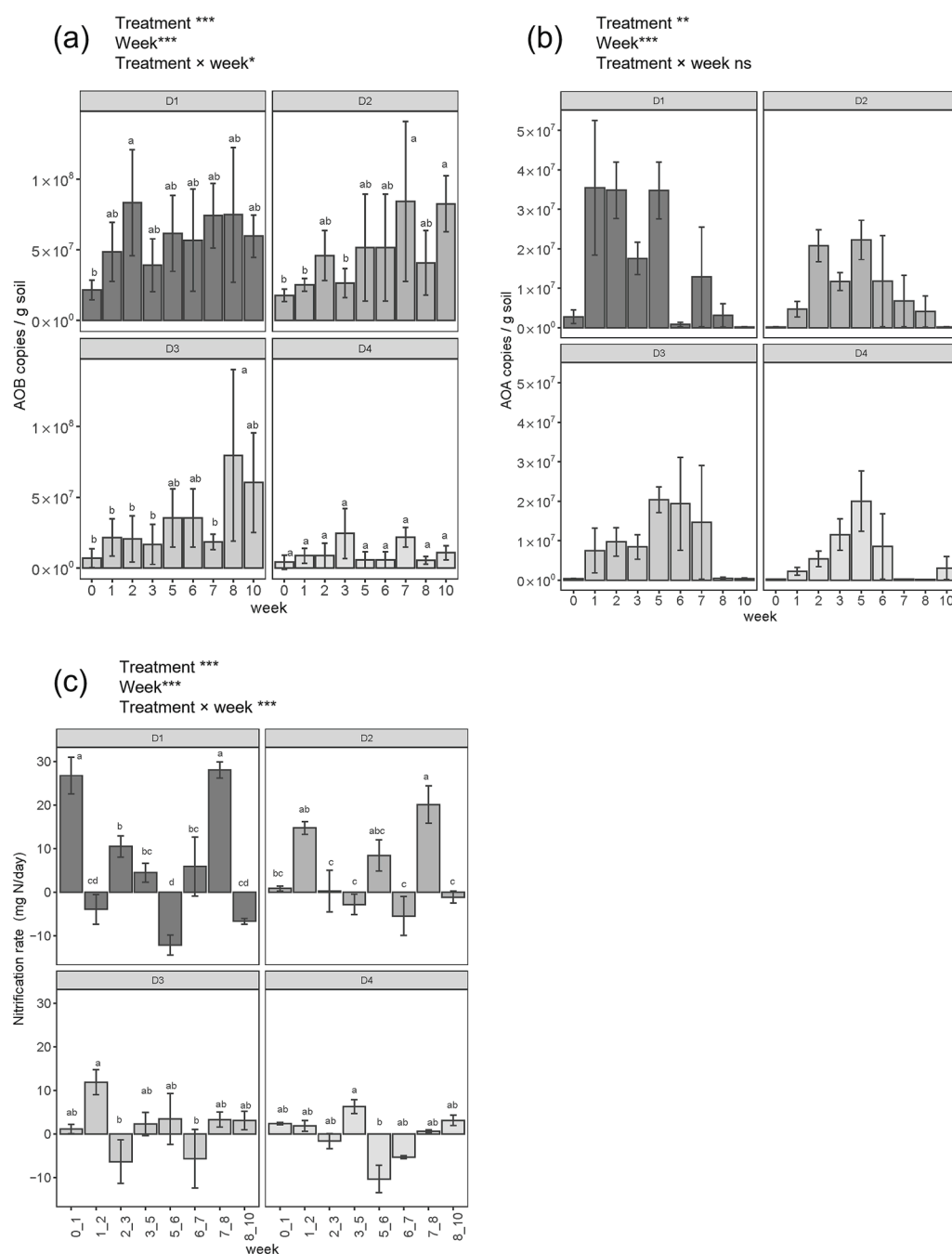
In this study, we observed a large dissimilarity in community composition with decreasing microbial diversity (Fig. 1). This suggests that the microbial community remained unchanged and stable in the high-diversity treatments. Furthermore, in the highest diversity treatment (D1), we observed the regeneration of similar AOB community

structures between the first and repeated application phases of N fertilizer (Fig. 2), indicating that changes in the community structures induced by N application are temporary and resilient. This finding is consistent with that of a previous study showing that the soil AOB community is resilient to chemical N fertilizer application (Tao et al., 2021). However, in the lower diversity treatments (D2 and D3), the composition of the AOB community differed significantly between the first and repeated application phases, suggesting that the community structure was not resilient to the repeated application of N fertilizer in the lower diversity treatments.

Our results also revealed that the stability of the relative abundance of *Nitrospira briensis*, namely, the most abundant AOB species in our experiment (occupying 43 % of the community), was significantly influenced by the dilution treatment. Higher diversity treatments resulted in less variation in the relative abundance of *Nitrospira briensis*, suggesting the importance of this microbe in maintaining the stability of AOB community structure. These results are consistent with those of Tao et al. (2021), who demonstrated the resilience of the AOB community in *Nitrospira*-dominated soils after N fertilizer application. *Nitrospira briensis* is known for its survival strategy under fluctuating  $\text{NH}_4^+$ -N availability; for instance, it can decrease its ammonia oxidation activity under conditions of N starvation but promptly recovers it upon N addition after a period of starvation (Bollmann et al., 2005). This supports our observation of a stable relative abundance of *Nitrospira briensis* under repeated application of N fertilizer in D1. In contrast, in D2 and D3, the relative abundance of *Nitrospira briensis* or uncultured *Nitrospira* sp. temporarily increased or decreased after repeated application of N fertilizer (Figs. 3 and S4). This suggests that the stability of the relative abundance of microbes is influenced by the soil microbial community diversity. These results support those of previous studies that proposed the co-dependence of AOB with diverse microorganisms through network-based analysis and activity measurements using culture media incubation (Liu et al., 2022; Yin et al., 2022). Our study is the first to demonstrate that the diversity gradient of the soil microbial community is associated with reformation of the community structure following repeated application of N fertilizer.

In addition to the structural shift in the community, the growth of AOB was restricted by dilution. In D1, the abundance of AOB significantly increased two weeks after the first application of N, whereas in D2 and D3, a significant increase in AOB was observed after the repeated application of N fertilizer (Fig. 4). This indicates that the dilution approach of microbial diversity could affect the timing of the onset of AOB growth after the soil receives N fertilizer. In a previous study conducted by Zou et al. (2022), the application of N fertilizer had a positive effect on both the abundance and diversity of AOB. Additionally, the nitrification ratio showed higher values in the higher diversity treatment, highlighting the importance of diverse microbial communities for the growth and activity of AOB under N fertilizer application. Although the dilution treatment partly influenced the initial abundance of AOB, the variation in the timing of initiation of their growth suggests a possible constraint on AOB growth associated with microbial diversity.

Similar to that of AOB, the community structure of AOA remained unchanged in the higher diversity treatments (D1 and D2). However, no significant differences were observed in the community compositional dynamics among the dilution treatments. Interestingly, D1 exhibited a higher abundance than other diversity treatments in the first N fertilizer application phase, indicating the potential importance of microbial diversity for AOA when the soil received an optimal amount of N. However, the abundance of AOA decreased after the repeated application of N fertilizer, consistent with the results of other studies showing that AOA was more favored than AOB in conditions of low  $\text{NH}_4^+$ -N availability (Farooq et al., 2022; Hink et al., 2018; Ying et al., 2017; Zhao et al., 2020). Notably, most studies primarily focused on the influence of available N concentration on the niche separation of AOB and AOA but have not extensively examined the impact of primary N fertilizer application. Therefore, further research is needed to investigate the



**Fig. 4.** The gene copy numbers of (a) AOB and (b) AOA, and the (c) nitrification ratio. The second application of N was conducted five weeks after the first application. Error bars represent standard errors of the mean. The results from two-way ANOVA for the linear mixed model are shown in the upper left (\* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ ). The different letters indicate the significant difference ( $P < 0.05$ ) in the gene copies or nitrification rates at different time points by Tukey's test.

niche differentiation of AOB and AOA under conditions of primary N fertilizer application.

Consistent with our first hypothesis, the AOB community structure became more stable with increasing soil microbial diversity in response to repeated N application. Within the AOB community, *Nitrosospira briensis* was identified as a key microbe that maintained consistent relative abundance under the high-diversity treatment. Additionally, with higher soil microbial diversity, AOB promptly became abundant during the initial N fertilizer application phase, whereas at lower diversity, the abundance increased during the repeated application phase. However, the stability of the AOA community did not appear to be clearly dependent on the soil microbial diversity, as AOA abundance

decreased in all treatments with repeated N fertilizer application. Our findings suggest that maintaining high microbial diversity can rapidly enhance AOB abundance after N fertilizer application and stabilize the community against repeated N fertilizer application. This enhanced stability of the AOB community could lead to a more efficient conversion of applied  $\text{NH}_4^+\text{-N}$  into  $\text{NO}_3^-\text{-N}$ , which might benefit nutrient cycling in agricultural systems.

#### Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence

Table 3

Average values for chemical properties at each time point and in each treatment. Pairwise comparisons by Tukey’s test among the treatments were conducted, followed by the ANOVA tests for the linear mixed model. The asterisk indicates the significant impact by each factor (\**P* < 0.05, \*\**P* < 0.01, \*\*\**P* < 0.001). The letters indicate the significant difference among the treatment at each time point (*P* < 0.05).

ANOVA test	NH <sub>4</sub> <sup>+</sup> -N		NO <sub>3</sub> <sup>-</sup> -N		pH					
Treatment	***		***		***					
Week	***		***		***					
Treatment × week	***		***		***					
Tukey’s test	Week									
	0	1	2	3	5	6	7	8	10	
NH <sub>4</sub> <sup>+</sup> -N (mg/kg soil)										
D1	12.3 ± 1.5 a	221.5 ± 23.9 a	191.8 ± 47.4 a	68.4 ± 23.4 b	34.3 ± 0.5 b	331.6 ± 44 b	162.8 ± 22.4 c	67.2 ± 8.3 c	64.0 ± 15.6 b	
D2	37.2 ± 7.5 a	311.8 ± 76.9 a	155.4 ± 4.9 a	149.1 ± 66.7 b	218.5 ± 41.9 a	297.7 ± 52.2 b	307.9 ± 74.2 bc	136.6 ± 35.1 c	70.1 ± 3.7 b	
D3	84.8 ± 23.7 a	306 ± 40.3 a	202.2 ± 40 a	216.6 ± 52.6 ab	270.4 ± 75.9 a	357.4 ± 62.6 b	424.2 ± 78.2 ab	356.7 ± 32.5 b	251.3 ± 48.6 a	
D4	89.7 ± 7.7 a	343.2 ± 10.3 a	246.5 ± 48.6 a	320.1 ± 37.9 a	173.3 ± 55.1 ab	526.7 ± 88.3 a	472.9 ± 32.6 a	577.7 ± 26 a	308.5 ± 38.9 a	
NO <sub>3</sub> <sup>-</sup> -N (mg/kg soil)										
D1	6.9 ± 1.7 a	194.1 ± 29.6 a	141.8 ± 45 ab	278.7 ± 31.7 a	394.6 ± 53 a	231 ± 32 ab	310.3 ± 91.2 a	689.4 ± 25.9 a	509.1 ± 17.2 a	
D2	21.5 ± 6.9a	32.7 ± 8 ab	230.8 ± 20.9 a	234.6 ± 63.7 ab	158.9 ± 64.4 b	272.8 ± 48.9 a	200.2 ± 59.9 ab	475.2 ± 59.8 b	443.9 ± 37.5 a	
D3	11.1 ± 7.3 a	26.7 ± 14.3b	191.3 ± 42.5 ab	108.7 ± 66.9 bc	171 ± 70.8 b	217.6 ± 77.2 ab	143 ± 91 bc	189 ± 23.6 c	272.5 ± 56.1 b	
D4	3.4 ± 1.1 a	40.5 ± 3.7 ab	64.6 ± 15.6b	43.9 ± 22.7 c	221.3 ± 42.5 b	83.6 ± 42.2 b	11.6 ± 4.7 c	20.7 ± 4.8 d	105.3 ± 32.7 c	
pH										
D1	6.6 ± 0.1 a	5.2 ± 0 a	4.7 ± 0.1 b	4.1 ± 0.1 c	4.6 ± 0.1 a	4.3 ± 0.1 ab	4.1 ± 0 b	3.6 ± 0.1 c	4.1 ± 0.1 b	
D2	6.2 ± 0.1 a	5.5 ± 0 a	4.5 ± 0 b	4.4 ± 0.1 bc	4.8 ± 0.2 a	4.2 ± 0.1 b	4.1 ± 0.1 b	3.9 ± 0.1 c	4.2 ± 0 b	
D3	6.4 ± 0.1 a	5.6 ± 0.1 a	4.8 ± 0.2 ab	4.6 ± 0.2 b	4.8 ± 0.2 a	4.6 ± 0.1 a	4.5 ± 0.2 ab	4.4 ± 0.1 b	4.7 ± 0.2 a	
D4	6.4 ± 0.1 a	5.5 ± 0 a	5.2 ± 0.1 a	5.5 ± 0.2 a	4.4 ± 0.1 a	4.7 ± 0.1 a	4.8 ± 0 a	5.1 ± 0.1 a	5 ± 0.1 a	

the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.geoderma.2023.116685>.

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