

Cropping system modulates the effect of spring drought on ammonia-oxidizing communities

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ABSTRACT

The severity of drought is predicted to increase across Europe due to climate change. Droughts can substantially impact terrestrial nitrogen (N) cycling and the corresponding microbial communities. Here, we investigated how ammonia-oxidizing bacteria (AOB), archaea (AOA), and complete ammonia oxidizers (comammox) as well as inorganic N pools and N₂O fluxes respond to simulated drought under different cropping systems. A rain-out shelter experiment was conducted as part of a long-term field experiment comparing cropping systems that differed mainly in fertilization strategy (organic, mineral, or mixed mineral and organic) and plant protection management (biodynamic versus conventional pesticide use). We found that the effect of drought varied depending on the specific ammonia-oxidizing (AO) groups and the type of cropping system. Drought had the greatest impact on the structure of the AOA community compared to the other AO groups. The abundance of ammonia oxidizers was also affected by drought, with comammox clade B exhibiting the highest sensitivity. Additionally, drought had, overall, a stronger impact on the AO community structure in the biodynamic cropping system than in the mixed and mineral-fertilized conventional systems. The responses of ammonia-oxidizing communities to drought were comparable between bulk soil and rhizosphere. We observed a significant increase in NH₄⁺ and NO₃⁻ pools during the drought period, which then decreased after rewetting, indicating a strong resilience. We further found that drought altered the complex relationships between AO communities and mineral N pools, as well as N₂O fluxes. These results highlight the importance of agricultural management practices in influencing the response of nitrogen cycling guilds and their processes to drought.

1. Introduction

Future climate projections indicate increasing drought frequency and intensity across Europe by the end of the 21st century (Hari et al., 2020; Suarez-Gutierrez et al., 2023). Large areas of Europe are already experiencing prolonged drought events as a result of climate change, which is primarily caused by anthropogenic activities (Hari et al., 2020; Min et al., 2011). Severe drought had been reported in 2018–2019, and more recently in 2022, significantly affecting around 30 % of the European continent (Barker et al., 2024; Blauhut et al., 2022; van der Woude et al., 2023). Drought, one of the most prominent environmental stresses in terrestrial ecosystems, shapes the soil microbiome because water content controls cell viability, activity, and functions (Schimel,

2018). Recent studies suggest that drought can also indirectly affect microbes via plants and that these indirect effects can outweigh the direct effects in the rhizosphere (de Vries et al., 2020). The consequences of severe drought on soil microbial communities may be detrimental because of its cascading effects on ecosystem functions. Among soil microbial processes, nitrogen (N) cycling is fundamental in agroecosystems as N is the most limiting essential nutrient for plant growth and crop production (Gruber and Galloway, 2008). Drought can decrease microbial biomass, lower N transformation rates (Homyak et al., 2017), and reduce plant N uptake (Flynn et al., 2023), which potentially affects yield quality and quantity. As droughts are expected to become more frequent and severe, a better understanding of their impact on N-cycling and the corresponding soil microbial communities

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is needed to better predict its potential impacts on soil functions and ecosystem services.

It is widely reported that changes in soil properties due to agricultural practices can directly or indirectly affect soil microbial communities including those involved in N-cycling (Hallin et al., 2009; Philippot et al., 2024; Z.-B. Zhao et al., 2020). Furthermore, soil physico-chemical properties can also influence the resistance (the ability to withstand a disturbance) and resilience (recovery towards no or pre-disturbance state) of soil microbial communities when exposed to disturbances, including drought (Griffiths and Philippot, 2013). This suggests that the effect of drought on N-cycling communities may be determined by fertilization regimes and soil management practices. Previous studies demonstrated that long-term organic farming can enhance soil organic matter (Krause et al., 2022; Kundel et al., 2020; Ullah et al., 2020), which can improve the soil water-holding capacity and therefore potentially mitigate the effect of drought on the soil microbial communities. Distinct microbial communities were observed in organic and conventional systems (M. Hartmann et al., 2015), which may also lead to differences in the response of N-cycling communities to drought. For example, organic amendments have been reported to increase the diversity of microbial communities (Sun et al., 2022), making them more likely to contain members capable of resisting to disturbances, as suggested by the insurance hypothesis (Philippot et al., 2021; Yachi and Loreau, 1999). Therefore, taking agricultural practices into account when analyzing the impact of drought on soil microbial communities involved in N-cycling is crucial for developing climate-resilient cropping systems.

Within the N-cycle, nitrification consists of the oxidation of ammonia (NH_4^+) to nitrite (NO_2^-) followed by the oxidation of NO_2^- to nitrate (NO_3^-) (Kuypers et al., 2018). It has a major role in the global N-cycle because it links organic matter decomposition, NH_4^+ release, and denitrification, making it being a key process in controlling N-availability for plants (Kuypers et al., 2018; Prosser, 2014). Nitrification can lead to N loss through NO_3^- leaching and emission of the potent greenhouse gas nitrous oxide (N_2O) (Hansen et al., 2019; Prosser et al., 2020). Ammonia oxidation, the rate-limiting step of nitrification, is mediated by ammonia-oxidizing bacteria (AOB), archaea (AOA), as well as complete ammonia oxidizers (comammox *Nitrospira*) (Daims et al., 2015; Leininger et al., 2006; van Kessel et al., 2015). There is evidence for physiological differences between ammonia-oxidizers with AOB having a lower affinity for ammonium compared to AOA, making ammonium availability an important factor in the niche differentiation of ammonia oxidizers (Kits et al., 2017; Straka et al., 2019). Based on such niche differentiation and their susceptibility to environmental changes in soil ecosystem at varying scales, ammonia oxidizers have been proposed as proper and relevant bioindicators for monitoring soil status (Karpouzias et al., 2022; Wessén and Hallin, 2011). Thus, it has been reported that the nitrification process is sensitive to drought with reduced nitrification activity and limited substrate availability to nitrifiers due to lower substrate diffusion (Séneca et al., 2020; Stark and Firestone, 1995). However, studies investigating the resistance and resilience of AO communities to drought are scarce and often inconsistent. For example, some studies showed that AOA and comammox clade B were more sensitive to drought than AOB (Bello et al., 2019; Séneca et al., 2020), while Krüger et al. (2021) found that AOB was more responsive to drought. Moreover, Fuchslueger et al. (2014) showed that the effect of drought on AO communities was modulated by land management, with decreased AOA abundance in managed meadows, while the AO abundances in abandoned grassland sites remained unaffected. On the other hand, Kaurin et al. (2018) showed the AO communities were resistant to drought regardless of management practices in agricultural fields.

Here, we determine the extent to which different cropping systems modulate the response of ammonia-oxidizing communities to drought in bulk and rhizosphere soil. For this purpose, we monitored the abundance and structure of AO communities, mineral N pools, as well as N_2O emissions over 5 months during and after simulated drought using

rainout-shelters in the DOK (bio-Dynamic, bio-Organic, and “Konventionell”) long-term experiment, which compares organic and conventional cropping systems since 1978. We hypothesized that (i) the effect of drought on AO communities will depend on the cropping system, with organically fertilized system enhancing resistance, (ii) the effect of drought will be group specific considering the physiological differences among AO groups, and (iii) the response of AO will differ between rhizosphere and bulk soil.

2. Materials and methods

2.1. Experimental design

The rainout-shelter experiment was conducted from mid-November 2021 to September 2022 in the DOK long-term experimental field in Therwil, Switzerland. The experiment has been established in 1978 in a strip-split-plot design under five cropping systems that received system-specific fertilization and pesticide management (Mäder et al., 2002). For this study, three cropping systems were chosen: a biodynamic system receiving composted manure and crop protection measures according to biodynamic regulations (BIODYN), a mixed-conventional system receiving both stacked manure and mineral fertilizer as well as pesticides according to Swiss thresholds for conventional farming (CON-FYM), and a purely mineral fertilized conventional system and the same crop protection treatments as the mixed conventional system (CONMIN). The three cropping systems were selected because they exhibited distinct microbial communities and contrasting physico-chemical properties (Hartmann et al., 2015; Mäder et al., 2002). The experimental design included three types of cropping systems as the main plots and two levels of water regime (control, drought) were installed at the sub-plot level (=6 treatment combinations). The agricultural practices and their timeline (e.g. fertilization, pesticide application, and weed management) were performed according to the assigned cropping system (Kost et al., 2024). Drought treatment was established by installing rainout-shelters ($L \times W \times H = 6 \times 4 \times 2.4$ m) in each plot to exclude rainfall. The control plots had no rainout-shelters installed and were established next to the corresponding drought plots. The study was performed in four replications for each treatment combination with a total of 24 plots. The field was planted with a commercial variety of winter wheat (*Triticum aestivum* L. cv. “Wiwa”) in mid-October 2021 before installing the rainout-shelters. The rain-out shelters were installed in mid-November 2021 when the crops were at the emergence stage. Irrigation was performed in the sheltered plots during winter until March 31st 2022 with a total of 55 mm of water. The drought period then started on April 1st 2022 when plants were at the tillering stage, until July 14th 2022 when plants were at the ripening stage. The rainout-shelters were removed on July 6–7th 2022, followed by wheat harvesting on July 13th 2022. A rewetting event were performed on July 14th 2022 by watering the plots with 36 mm of water.

2.2. Soil sampling and analyses

Soil samples were collected at five timepoints i.e. three timepoints during the drought period and at two timepoints after rewetting. The first sampling was performed at stem elongation on April 28th 2022 (stage 3, the first node of stem visible; $n = 24$ bulk soil, $n = 24$ rhizosphere). The second sampling was performed at flowering on June 1st (stage 6; $n = 24$ bulk soil, $n = 24$ rhizosphere). The third sampling was performed at wheat ripening on July 5th (stage 8; $n = 24$ bulk soil, $n = 24$ rhizosphere) before the rainout-shelters were removed (July 6–7th) and plots were rewetted (July 14th). The fourth ($n = 24$) and fifth ($n = 24$) sampling campaigns were conducted on July 20th (one week after rewetting) and September 13th (eleven weeks after rewetting), respectively, by collecting bulk soil only (plant were already harvested). A total of 120 bulk soil and 72 of rhizosphere soil samples were collected. Bulk soils were sampled between plant rows using a 5 cm soil core

sampler at 15 cm of depth and sieved through 5 mm sieve to remove plant debris and to achieve more homogenous soil particles. Root-attached rhizosphere soils were collected from within plant rows directly at the root stalk using a root auger set (Royal Eijkelamp, NL) to a depth of 15 cm. The rhizosphere soils were then separated from the roots by washing with a buffer solution (6.75 g KH₂PO₄ and 8.75 g K₂HPO₄ in 1000 mL deionized water with added 200 µL Tween 20). Soil samples were stored at -20 °C for further analyses. The measured soil parameters included gravimetric water content (GWC), pH, mineral nitrogen content (NO₃⁻, NH₄⁺) as well as N₂O fluxes (Kost et al., 2024). Briefly, N₂O fluxes were measured using the non-steady-state, static chamber method (Hutchinson and Mosier, 1981) with chambers of 30 cm diameter and 30 cm height as described earlier (Barthel et al., 2022). Chambers were installed in the field early January. For measuring the fluxes, chambers were closed for 1 h, and four air samples were collected at 20-min intervals. Nitrous oxide (N₂O) concentrations in samples were measured by gas chromatography (456-GC; Scion Instruments, Goes, The Netherlands) using standards covering the expected range of the concentrations.

2.3. Amplicon library preparation and sequencing of *amoA* genes

Soil DNA was extracted from a total of 192 samples using DNeasy® PowerSoil Pro Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol from 0.25 g homogenized rhizosphere and bulk soil. The quality and quantity of the DNA were assessed via UV/VIS spectrophotometry with the QIAxpert (Qiagen) and normalized to 10 ng µL⁻¹. The analysis of ammonia-oxidizing communities was conducted by sequencing the *amoA* genes of AOB, AOA, and comammox. The sequencing libraries were performed using two-step polymerase chain reaction (PCR) amplification approach. The first-step PCR amplification of *amoA* genes of AOB and AOA were conducted using primers *amoA*-1F (5'-GGGGTTTCTACTGGTGGT-3') and *amoA*-2R (5'-CCCCTCKGSAAAGCCTTCTTC-3') (Rotthauwe et al., 1997); and CrenamoA23f (5'-ATGGTCTGGCTWAGACG-3') and CrenamoA616r (5'-GCCATCCATCTGTATGTCCA-3') (Tourna et al., 2008), respectively. The resulting amplicon sizes were 491 bp for AOB and 628 bp for AOA. The PCR conditions used to amplify the *amoA* genes of AOB and AOA were as follows: 3 min at 94 °C; 25 cycles consisting of 30 s at 94 °C, 30 s at 55 °C, and 30 s at 72 °C; and a final cycle of 10 min at 72 °C. Amplifications were performed in 15 µL total mixtures in a 96-well PCR plate containing 1x Phusion High-Fidelity (HF) Master Mix (Thermo Scientific™, Waltham, MA, USA), 250 ng T4 Gene 32 Protein (T4gp32) (QIAGEN, Hilden, Germany), 0.5 µM of each primer, and 6 ng of template DNA. The first-step PCR was performed twice, and the products from the first and second run were pooled for the second-step PCR template. The second-step PCR (barcoding) was performed to construct amplicon libraries by introducing multiplex index-sequences (barcode) to the overhang adapters using multiplex primer pairs specific for each sample.

Comammox *amoA* genes were amplified using primers comamoA-F (5'-AGNGAYTGGGAYTCTGG-3') and comamoA-R (5'-CGGACA-WABRTGAABCCCAT-3') (Z. Zhao et al., 2019), yielding an amplicon size of 436 bp. The PCR amplifications were set up in duplicates following the conditions: 3 min at 94 °C; 40 cycles consisting of 30 s at 94 °C, 30 s at 52 °C, and 30 s at 72 °C; and a final cycle of 10 min at 72 °C. The PCR reaction solutions were prepared in a total volume of 15 µL in a 96-well 0.2 mL PCR plate containing 1x Phusion Green Hot Start II High-Fidelity Master Mix (Thermo Scientific™, Waltham, MA, USA), 250 ng T4gp32, 0.5 µM of each primer, and 6 ng of template DNA. For comammox, the first-step PCR products were cleaned up using the SequalPrep™ Normalization Plate (96) Kit (Invitrogen™, Waltham, MA, USA) before being used as a template for the second-step PCR.

Final PCR products of AOB, AOA, and comammox were purified and normalized according to the manufacturer's protocol of the Sequal-Prep™ Normalization Plate (96) Kit. Barcoded, purified, and normalized

amoA gene amplicons of AOB, AOA, and comammox were sequenced at the GenoScreen sequencing facility in Lille, France, using Illumina MiSeq platform with reagent kit v2 and paired-end reads sequencing format (2 x 250 bp).

2.4. *amoA* gene amplicon sequence analysis

The raw *amoA* gene sequence data of AOB, AOA, and comammox were analyzed using the AMOA-SEQ sequence pipeline (<https://github.com/miasungeunlee/AMOA-SEQ/tree/main>) by Lee (2024). The AMOA-SEQ pipeline implements the DADA2 tool (Callahan et al., 2016) to perform filtering and correcting sequence errors to generate Amplicon Sequence Variant (ASVs). The demultiplexed sequences were processed by removing primers and ambiguous bases, followed by quality filtering using the DADA2 standard filtering parameters (maxN = 0, truncQ = 2, rm.phix = TRUE, and maxEE = 2). To ensure the quality of the data, we discarded any reads that did not meet the minimum length requirements (200 bp for AOB and AOA, and 204 bp for comammox) and truncated the reads to a specific length (200 bp for AOB and AOA, and 210 bp for comammox). Dereplication was performed to identify unique sequences. Full denoised sequences were then generated by either merging the forward and reverse reads for comammox (-c FALSE) or simply concatenating the non-overlapping forward and reverse reads for AOB and AOA (-c TRUE). Furthermore, an ASV table was constructed, and any chimeric sequences were eliminated from the table. The next step in the AMOA-SEQ pipeline was selecting the DADA2-generated ASV sequences that match the expected quality-filtered amplicon size (410 bp for AOB and AOA, and 396 bp for comammox) using SeqKit (Shen et al., 2016) to generate correct ASV sequences. Taxonomic annotation of these ASV sequences against the reference data sets of the AMOA sequence database was performed using DIAMOND BLASTx with e-value of 0.00001 to generate the best-hit annotation (Buchfink et al., 2021; Lee, 2024). The AMOA database incorporated in this AMOA-SEQ pipeline was constructed by Lee (2024) by curating *amoA* gene sequences from different resources, such as NCBI and IMG-JGI databases, and also from previous studies (Aigle et al., 2019; Alves et al., 2018; Palomo et al., 2022).

2.5. Quantification of total bacterial and ammonia-oxidizing communities

Real-time quantitative PCR (qPCR) assays of 16S rRNA and *amoA* genes were performed to quantify the abundances of total bacterial and ammonia-oxidizing communities (AOB, AOA, comammox clade A and B), respectively. Total bacterial communities were quantified using 341F and 534R primer pair (Muyzer et al., 1993), which amplifies the V3 region of the 16S rRNA gene, according to the previous studies (López-Gutiérrez et al., 2004). Ammonia-oxidizing bacterial and archaeal abundances were determined using the *amoA* gene-targeted primers (*amoA*-1F and *amoA*-2R for AOB, and CrenamoA 23F and CrenamoA 616r for AOA) as described previously (Bru et al., 2011; Leininger et al., 2006; Tourna et al., 2008). The abundances of comammox *amoA* genes were assessed using two primer sets targeting comammox *Nitrospira* clade A (comaA-244F and comaA-659R) and B (comaB-244F and comaB-659R) (Pjevac et al., 2017). Two independent qPCR runs were performed for each gene. The fluorescent SYBR Green dye-based qPCR was performed in a 15 µL reaction mix containing the Takyon™ low ROX SYBR 2X MasterMix blue dTTP (Eurogentec, Seraing, Belgium), 250 ng T4gp32, 1 µM of each primer, and 3 ng of template DNA. Tenfold serial dilutions of linearized plasmids (pGEM-T) containing cloned target genes were used as template to determine standard curves. In addition, negative controls containing RNase-free water as template were included for measurement. The PCR efficiencies were 86–88% for AOB, 88–89% for AOA, 72–75% and 82–83% for comammox A and B, respectively. Prior to qPCR, the presence of PCR inhibitors in the DNA samples was tested by adding known copies of standard

plasmid DNA (pGEM®-T Easy Vector Systems) (Promega, Madison, WI, USA) into the diluted DNA extracts (10-fold dilution), including RNase-free water with the plasmid DNA as positive controls. The specific T7 and SP6 primers were used for the inhibition test and no inhibition was detected in all samples.

2.6. Data analysis

Statistical analyses were conducted in R v.4.3.1 (R Core Team, 2023) with R Studio (Posit team, 2023). Microbial alpha and beta diversity metrics were calculated based on the rarefied ASV tables. To standardize the sampling efforts, rarefying (without replacement) to the lowest number of sequences was performed with 3832, 1282, and 5242 sequences per sample for AOA, AOB and comammox, respectively. The count of observed ASVs (richness) and the Shannon diversity index were calculated to analyze microbial alpha diversity using the *specnumber* and *diversity* functions, respectively from the *vegan* package (v.2.6.4) (Oksanen et al., 2022).

The significance of treatment effects (drought, cropping system, and timepoint) as well as their interactions on the *amoA* gene abundance, alpha diversity, gravimetric water content (GWC), ammonium (NH_4^+), nitrate (NO_3^-), and average N_2O flux was tested by three-way repeated-measures analysis of variance (ANOVA) using the *anova_test* function in the *rstatix* package (v.0.7.2) (Kassambara, 2023). We identified any outliers with *identify_outliers* function, and verified the normality and homoscedasticity of the data using Saphiro-Wilk and Levene's test with *saphiro_test* and *levene_test* functions, respectively implemented in the *rstatix* package. Data transformation of the response variables was performed when necessary, using log or square-root transformation. The difference within or between groups was conducted by pairwise comparisons using the estimated marginal means (P value ≤ 0.05) with the *rstatix* package using the *emmeans_test* function (Kassambara, 2023). The raw P values were corrected using the Benjamini-Hochberg method implemented in the *emmeans_test* function (Benjamini and Hochberg, 1995).

The ratio of AO groups to the total microbial communities (*amoA*:16S rRNA gene ratio), as well as the abundance of the total bacteria (16S rRNA gene) in bulk soil were tested by fitting linear mixed-effects models (LMM) using the *lmer* function in the *lmerTest* package (v.3.1.3), with drought (D), cropping system (C), and timepoint (T) as the fixed effects, while block and its combination with timepoint as the random factor to allow intercept to vary among block within time (Kuznetsova et al., 2017). The blocks were referred to as whole plots where different cropping systems were assigned, with control and drought plots as the experimental units within each block. Gene copy number and its ratios were log-transformed and arcsine square root-transformed when necessary. The residual diagnostic was performed using the DHARMA package (v.0.4.6) with *simulateResiduals* function to check the model residual distribution (Hartig, 2022). The pairwise comparisons were conducted to assess the difference in *amoA* gene abundance between drought and control for each timepoint within each cropping system using the *pairwise_t_test* function from the *rstatix* package with the Benjamini-Hochberg-adjusted P value.

Beta diversity analysis was calculated using Bray-Curtis dissimilarities using the *vegdist* function in the *vegan* package. Permutational multivariate analysis of variance (PERMANOVA) was performed to assess the effect of treatments on beta diversity using the *adonis2* function of the *vegan* package with 999 permutations. Similarities and dissimilarities between groups were assessed by unconstrained ordination using Principal Coordinates Analysis (PCoA) plot using the *cmdscale* function in the *stats* package (v.4.3.2). Constrained ordinations were obtained using Canonical Analysis of Principal Coordinates based on Discriminant Analysis (CAP) with the *CAPdiscrim* function in the *BiodiversityR* package (v.2.15–4) using *drought x cropping system* as the constraining factor, and estimating the reclassification success rate by permuting the dissimilarity matrix 9999 times (Anderson and Willis,

2003; Legendre and Anderson, 1999).

Ammonia-oxidizing community composition and relative abundance were assessed using the *phyloseq* package (v.1.44.0) (McMurdie and Holmes, 2013). We performed differential abundance analysis to identify ASVs abundance that changes significantly between control and drought treatment. We filtered the ASV tables by removing low-abundance ASVs ($<0.01\%$) and keeping ASVs that were found in at least 80 % of replicates for each treatment because dataset with high proportion of zero counts can increase the false positive number. We performed generalized linear mixed models (GLMMs) to model our microbiome abundance data that we assumed followed a Poisson distribution (Huet et al., 2023). We calculated an ASV abundance Y with parameter λ as $Y \sim P(\lambda)$, in any j replicates of any i treatment using the following model:

$$\log(A_{ij}) = o_{ij} + \mu + \alpha_i + Z_{ij}, Z_{ij, 1 \leq j \leq 12} \text{ iid} \sim N(0, \sigma^2)$$

We introduced offset (o) as the log of the sample read sum, α is the effect of the drought coded as a factor, and Z is the random sampling effect modeling the data overdispersion. $i = \{1, 2\}$ represents the water regime and $j = \{1, \dots, 4\}$ represents the replicates. The model was run using the *glmmTMB* function of the *glmmTMB* package (v.1.1.7) (Brooks et al., 2017). We run this model to compare ASVs abundance between control and drought within each cropping system. A post-hoc test with the *emmeans* function of the *emmeans* package (v.1.8.8) (Lenth, 2024) was then performed for single pairwise comparison between drought and control for each timepoint within cropping system.

We performed Mantel tests with the Spearman correlation method to analyze the correlations between the structure (beta diversity) of ammonia-oxidizing community with its alpha diversity, the abundance of *amoA* gene, as well as with mineral N pools and other measured soil properties. The correlation test was conducted for drought and control to compare between the two treatments using the *microeco* package (v.1.4.0) (Liu et al., 2021) and *ggcor* package (v.0.9.4.3) (Huang et al., 2024). The actual P values were corrected using the Benjamini-Hochberg (FDR) method.

3. Results

3.1. Drought affected soil water availability and mineral N pools

The drought treatment successfully reduced the soil water availability in all cropping systems, with an average decrease of more than 60% in GWC compared to the control (Fig. S1; Table S1). The effect of drought was still significant one week after rewetting, but not at the final timepoint of eleven weeks after rewetting (Fig. S1; Table S1). This effect of drought on GWC depended on the sampling timepoint but not on the cropping system (Table S1).

Large differences in NH_4^+ contents were observed in the control treatments between cropping systems with the BIODYN system exhibiting on average 82–85 % lower NH_4^+ content compared to the two conventional systems receiving mineral fertilizer (Fig. 1A; Table S1). Drought was also a strong driver of the NH_4^+ content, with significant impacts depending on both the cropping systems and the sampling timepoint (three-way repeated measures ANOVA, $P < 0.01$; Table S1). While drought consistently increased the average NH_4^+ content in the CONFYM and CONMIN systems by two to eleven times compared to the control, there were almost no significant effects for the BIODYN system (Fig. 1A). Eleven weeks after rewetting, no difference in NH_4^+ content between the drought and control treatments in both conventional systems were found (Fig. 1A).

Similarly to the NH_4^+ content, the effect of drought on NO_3^- content depended on the cropping systems as well as on the sampling timepoint (three-way repeated measures ANOVA, $P < 0.01$; Table S1). Drought led to an increase in NO_3^- content in the CONFYM and CONMIN systems by more than 100 % relative to the control across all timepoints, except at

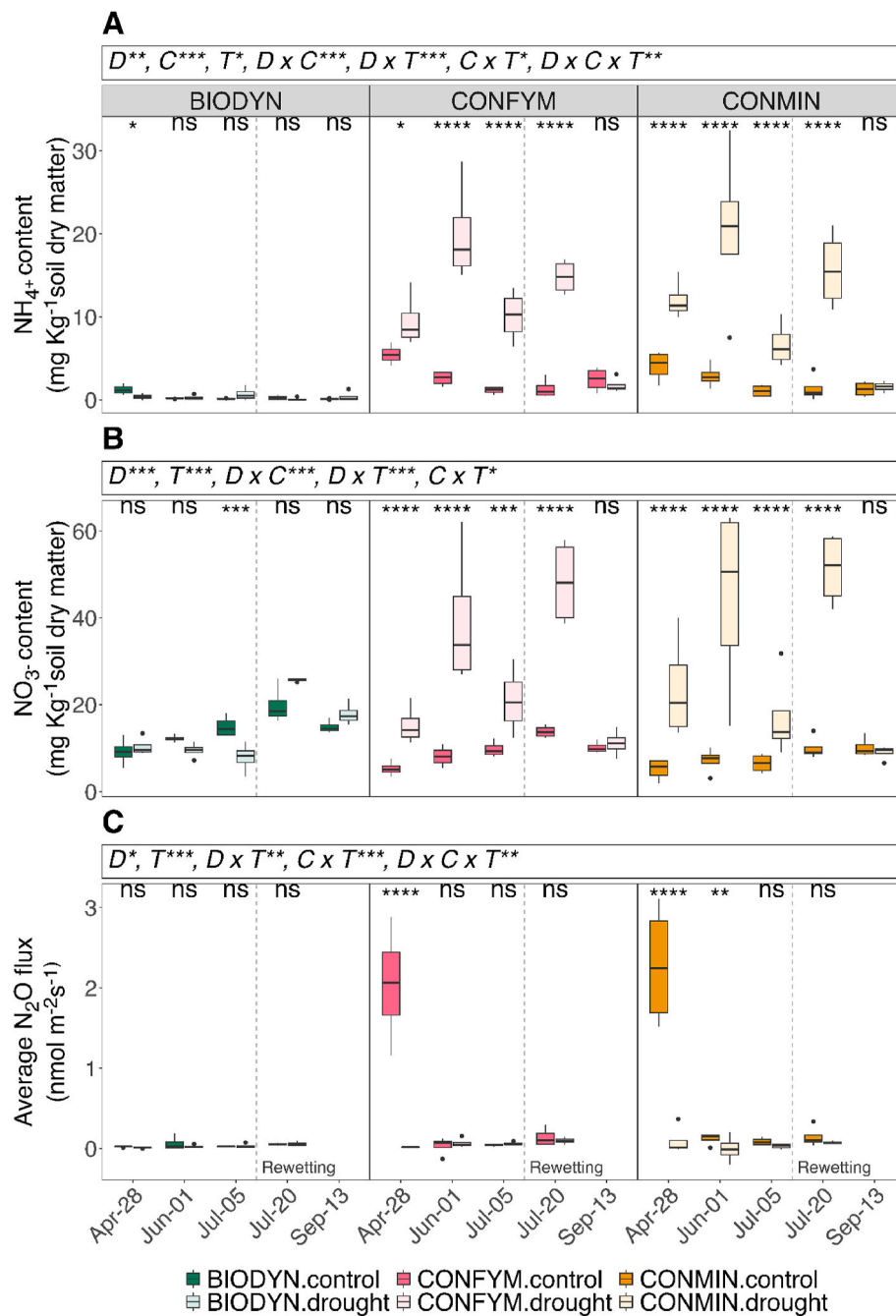


Fig. 1. Ammonium (NH_4^+) (A) and nitrate (NO_3^-) (B) contents, and the average N_2O flux (C) in the control and drought-induced plots. The effect of drought (D), cropping system (C), and timepoint (T), as well as their interactions was assessed by three-way repeated measures ANOVA. Pairwise comparison between control and drought for each timepoint within cropping system was assessed using the estimated marginal means with significant differences indicated by asterisks (**** $P < 0.0001$, *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$, ns = not significant). Boxplots show the median (center line), first and third quartiles (box limits), and smallest and largest values within 1.5x interquartile range (whiskers).

eleven weeks after rewetting, where the differences were not significant anymore (Fig. 1B; Table S1). In the BIODYN system, the effect of drought was only observed at the third sampling of the drought period with a slight decrease in the NO_3^- content, indicating that the overall drought effect was marginal (Fig. 1B).

Compared to the drought effect on NH_4^+ and NO_3^- contents, we detected a weaker but significant drought effect on the monthly average N_2O flux (three-way repeated measures ANOVA, $P < 0.05$; Fig. 1C–Table S1). In particular drought decreased N_2O in CONFYM and CONMIN systems at the beginning of the drought period. On the contrary, no drought effect was detected on the N_2O fluxes in the BIODYN

system, where fluxes were low. (Fig. 1C).

3.2. Differential responses of ammonia-oxidizing communities to drought across cropping systems

The AOB, AOA, and comammox communities were dominated by *Nitrosospora* (bulk soil: 84.56%, rhizosphere: 83.38%), *Nitrososphaerales* clade Delta (NS-Delta) (bulk soil: 73.51%, rhizosphere: 71.14%), and *Nitrosospora* clade B (bulk soil: 97.43%, rhizosphere: 96.85%), respectively. We found no notable shifts in the taxonomic composition of the ammonia-oxidizing communities in response to drought, although the

community compositions were largely different among cropping systems (Fig. S2). The alpha diversity of AOB, AOA and comammox was not affected by drought alone both in the bulk soil and rhizosphere (three-way repeated measures ANOVA, $P > 0.05$; Fig. S3G-L; Table S2). However, we found a significant interaction of drought \times cropping system for comammox alpha diversity in the bulk soil (three-way repeated measures ANOVA, $P < 0.05$; Table S2). Nonetheless, we could not identify any significant differences between drought and control within timepoint of each cropping system, indicating that the detected effect of drought on comammox alpha diversity was only marginal. Cropping system was an important driver of the ammonia-oxidizers alpha diversity, with significantly higher richness and Shannon index for the

comammox in BIODYN than in CONFYM and CONMIN (Fig. S3C and F). On the contrary, BIODYN had lower alpha diversity of the AOB compared to the two conventional systems (Fig. S3A and D).

The unconstrained PCoA plots based on Bray-Curtis dissimilarities showed a strong clustering by cropping system with 23 % (bulk soil) and 29 % (rhizosphere), 33 % (bulk soil) and 34 % (rhizosphere), and 49 % (bulk soil) and 48 % (rhizosphere) of the variance explained for AOB, AOA, and comammox, respectively (PERMANOVA, $P < 0.05$) (Fig. S4; Table S3). Due to a strong block effect (PERMANOVA, $P < 0.01$), we further investigated the effect of drought on the beta diversity of ammonia oxidizers by performing a constrained CAP analysis using drought \times cropping system as the grouping variable. Overall, there was a

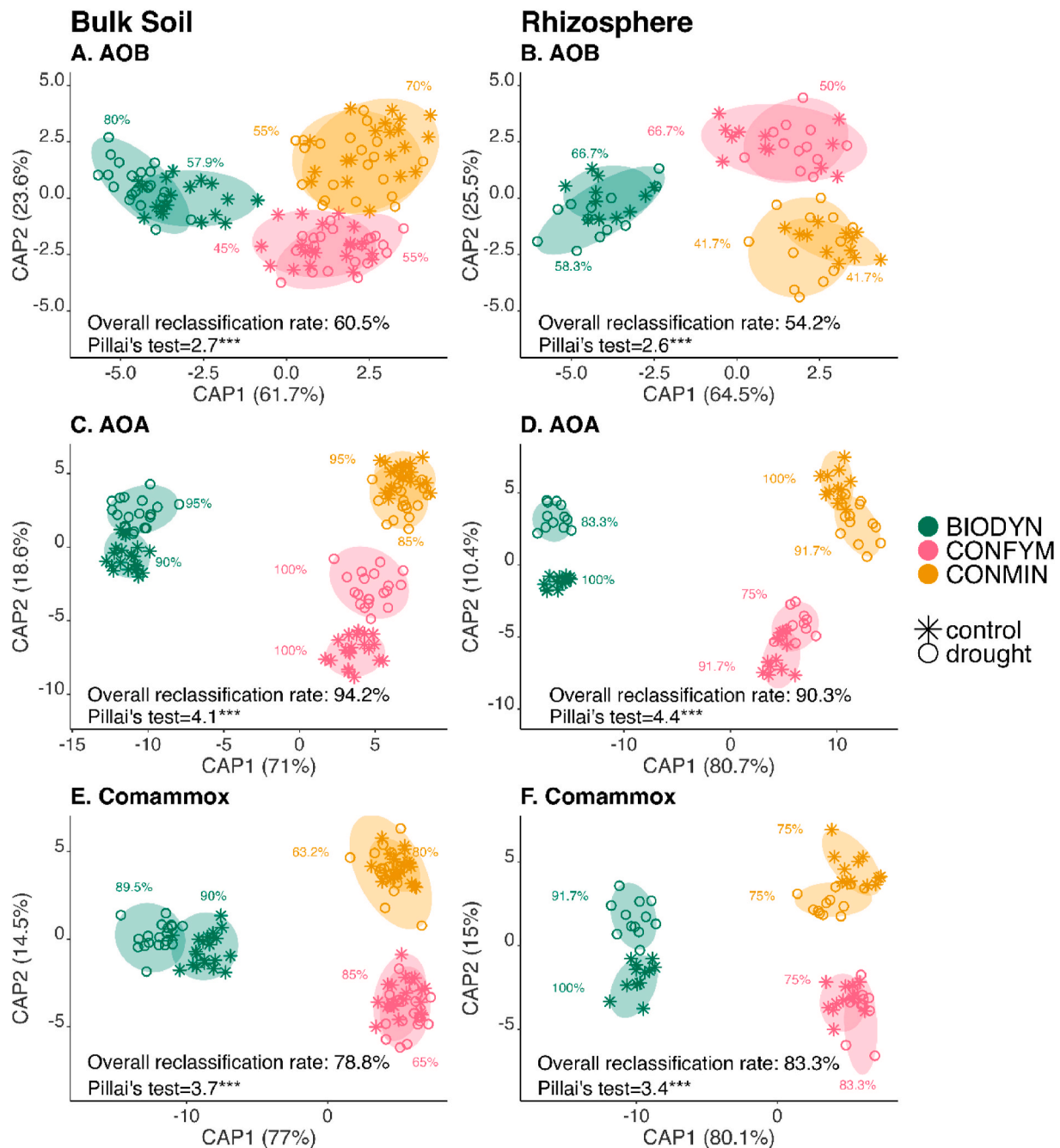


Fig. 2. Effects of drought and cropping system on the ammonia-oxidizer community structure as assessed by constrained canonical analysis of principal coordinates (CAP) of AOB (A and B), AOA (C and D), and comammox (E and F) in bulk and rhizosphere soil. Overall reclassification success rate represents the degree of discrimination between the grouping factors. The statistical significances are indicated by the Pillai's trace statistics (MANOVA, *** $P < 0.001$). Reclassification rates for drought and control within each cropping system are provided next to the respective ellipses.

distinct clustering by drought and by cropping system on the ordination of all groups of ammonia-oxidizing community by CAP analysis (MANOVA, $P < 0.001$) (Fig. 2). The AOA community exhibited the most robust compositional differences between the drought and the control treatments as demonstrated by high overall reclassification rates of 94.2 % and 90.3 % in bulk soil and rhizosphere, respectively. The effect of drought on the AOA community structure was also influenced by the cropping system with a better clustering by the drought treatment in the CONFYM and BIODYN cropping systems in the bulk soil and in the BIODYN in the rhizosphere (Fig. 2C and D). Distinct clustering by the drought treatment was also observed in the comammox community with higher reclassification rates in the BIODYN than the other two cropping systems regardless of the compartment (bulk soil and rhizosphere) (Fig. 2E and F). In contrast, the AOB community showed only marginal separations between drought and control within cropping system with lower overall reclassification rates of 60.5 % and 54.2 % in bulk soil and rhizosphere, respectively (Fig. 2A and B).

3.3. Several dominant ammonia-oxidizer ASVs were affected by drought

We performed a differential abundance analysis to identify ammonia-oxidizing ASVs exhibiting differences in relative abundances between drought and control in each cropping system. The ASVs that were significantly impacted by drought represented 44% and 35 % (AOB), 20% and 16 % (AOA), 23% and 25 % (comammox) of the ASVs after filtering out the rare one in bulk soil and rhizosphere, respectively

(Fig. 3B). Among the three ammonia-oxidizing groups, the AOB community had the largest number of affected ASVs in all samples (30 and 25 ASVs in bulk soil and rhizosphere, respectively) (Fig. 3A). Most of the affected AOB ASVs in bulk soil (70 %) exhibited a decrease in relative abundance with drought, while no clear pattern emerged for the AOA and comammox. The AOB, AOA, and comammox ASVs responsive to drought were mainly affiliated with *Nitrosospira* sp., Nitrososphaerales (*NS Delta Incertae sedis*), and *Nitrosospira* sp. clade B, respectively (Fig. 3A). Moreover, CONMIN exhibited less drought-affected AOA and comammox ASVs compared to BIODYN and CONFYM (Fig. 3).

3.4. Minor effects of drought on the abundance of ammonia oxidizers in bulk soil

Quantification of the abundances of ammonia-oxidizing communities showed that the effects of drought varied across cropping systems and ammonia-oxidizing groups (Table S4). Thus, in the bulk soil, a small but significant effect of drought alone was observed on the abundance of AOB and comammox clade B, but not on that of AOA and comammox clade A (three-way repeated measures ANOVA, $P < 0.05$, Fig. 4; Table S4). The abundance of comammox clade B was consistently lower in the drought treatment across cropping systems, with the strongest effects observed in the CONFYM system (Fig. 4D). In contrast, the effect of drought depended on the cropping system for AOB with decreases in abundance of up to 39 % relative to the control in the CONFYM system only. We also found that drought led to significant decreases in the

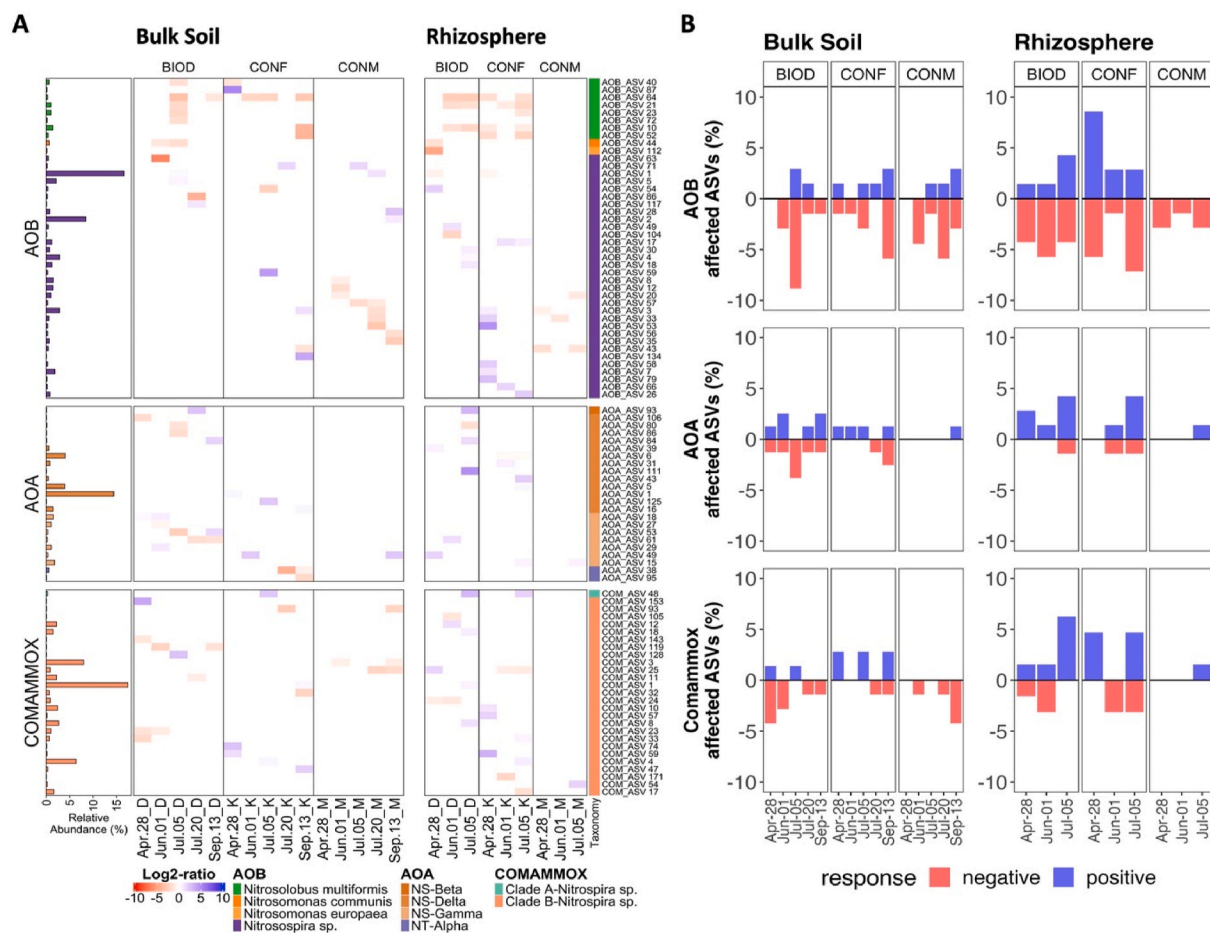


Fig. 3. Heat map showing amplicon sequence variants (ASVs) of AOB, AOA, and comammox that were significantly affected by drought in bulk soil and rhizosphere as assessed by differential abundance analysis using generalized linear mixed models ($P < 0.05$) (A) and the percentage of affected ASVs (B). Taxonomic affiliations are indicated by genus (AOB) and clade (AOA and comammox). The enriched and depleted ASVs are indicated in blue ($\log_2\text{-ratio} > 0$) and red ($\log_2\text{-ratio} < 0$), respectively. The relative abundance of each ASV is provided in the left side of the heat map. (BIOD= BIODYN, CONF= CONFYM, CONM= CONMIN).

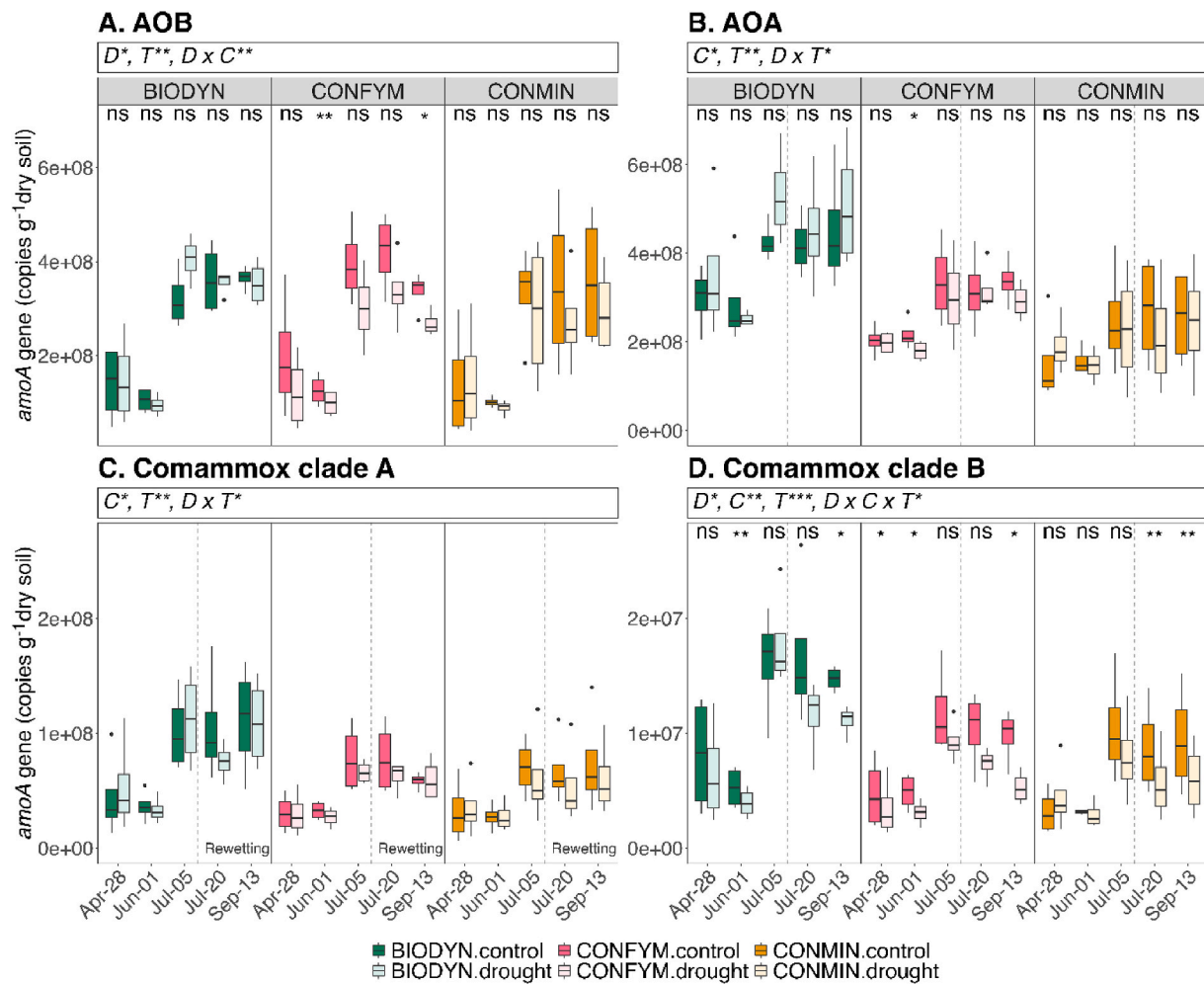


Fig. 4. *amoA* gene abundance of AOB (A), AOA (B), and comammox clade A (C) and B (D) in bulk soil. The effect of drought (D), cropping system (C), and timepoint (T), as well as their interactions was assessed by three-way repeated measures ANOVA. Pairwise comparison between control and drought for each timepoint within each cropping system was assessed using the estimated marginal means with significant differences indicated by asterisks (**** $p < 0.0001$, *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$, ns = not significant). Boxplots show the median (center line), first and third quartiles (box limits), and smallest and largest values within 1.5x interquartile range (whiskers).

proportion of AOB, and comammox B within the total bacterial community in the bulk soil (LMM, $P < 0.05$, Fig. S5; Table S5), while no significant effect was observed in the rhizosphere (three-way repeated measures ANOVA, $P < 0.05$, Fig. S5; Table S5). In contrast to the comammox community structure, we found that comammox clade A was dominating over comammox clade B, which is likely due to primer bias leading to preferential amplification. Overall, there was no effect of drought on the AOA/AOB ratio in bulk soil, but we identified a slight increase in AOA/AOB ratio in the CONFYM system in April (Fig. S6, Table S6). Increasing in AOA/AOB ratio in response to drought was also detected in rhizosphere, particularly in the BIODYN and CONMIN systems (Fig. S6).

3.5. Correlation between ammonia oxidizing community, N pools, and soil properties

We further investigated how the relationships between the diversity and composition of ammonia oxidizing communities with soil properties, including mineral N pools and N_2O emissions, were affected by drought (Fig. 5). Notably, we found that the NO_3^- content was correlated to the abundance and the beta diversity of all AO as well to the alpha diversity of AOA and comammox in the control treatment. In contrast, only the alpha diversity of AOB was positively correlated to the NO_3^- content in the drought treatment while a negative relationship was

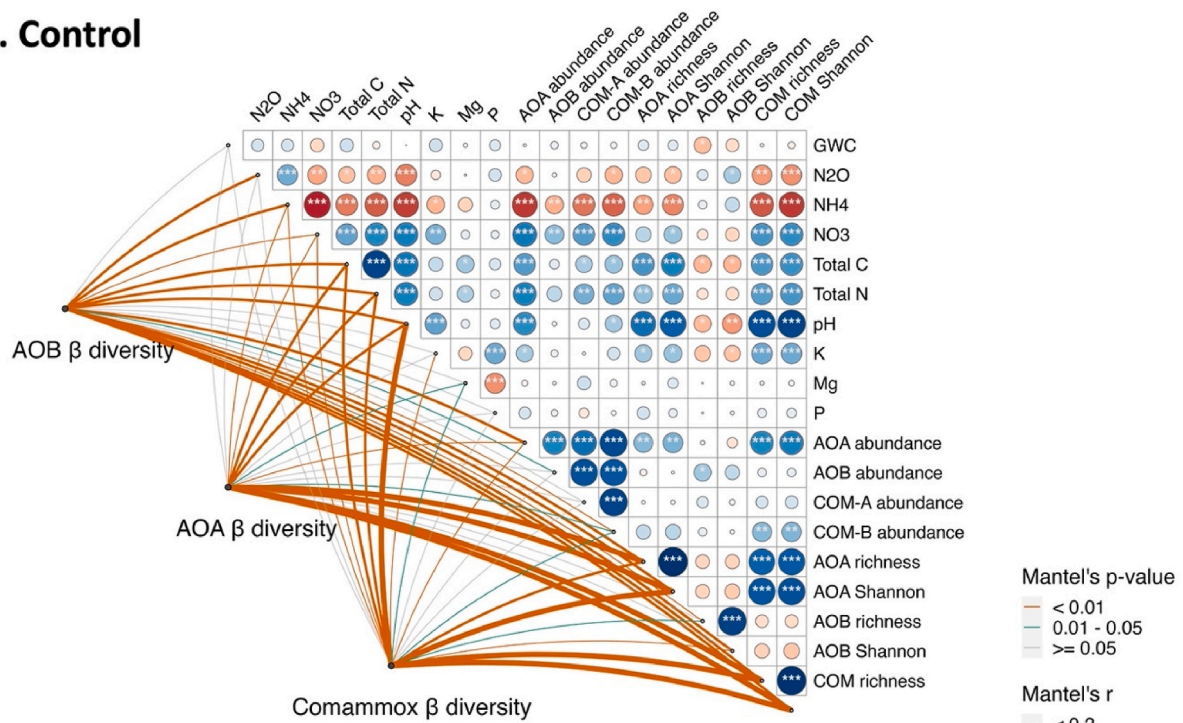
observed with the alpha diversity of comammox. Similarly, stronger correlations were found between the NH_4^+ content and AO communities in the control than in the drought treatment. Interestingly, all these correlations were negative except for the alpha diversity of AOB. Among all AO groups, only the beta diversity of AOB related to the N_2O flux, and this relationship was only found in the control. In the control, average N_2O fluxes were also negatively correlated to the abundance of AOA and comammox (clade B), as well as with their alpha diversity, and positively correlated with the alpha diversity of AOB. Overall, there was no significant relationship between the N_2O flux with AO communities in the drought treatment, except with the AOB abundance.

4. Discussion

4.1. The effects of drought on mineral nitrogen pools (NH_4^+ , NO_3^-) and N_2O fluxes are modulated by the cropping system

We found that drought strongly affected the mineral N pools with lower GWC resulting in large increases in the NH_4^+ and NO_3^- pools, particularly in the conventional systems (CONFYM and CONMIN). While some studies also reported that drought increased both NH_4^+ and NO_3^- pools in soil (Deng et al., 2021; A. A. Hartmann et al., 2013; Ullah et al., 2020), others found that the NO_3^- pools remained unchanged or even decreased in response to drought (Canarini et al., 2021; Séneca

A. Control



B. Drought

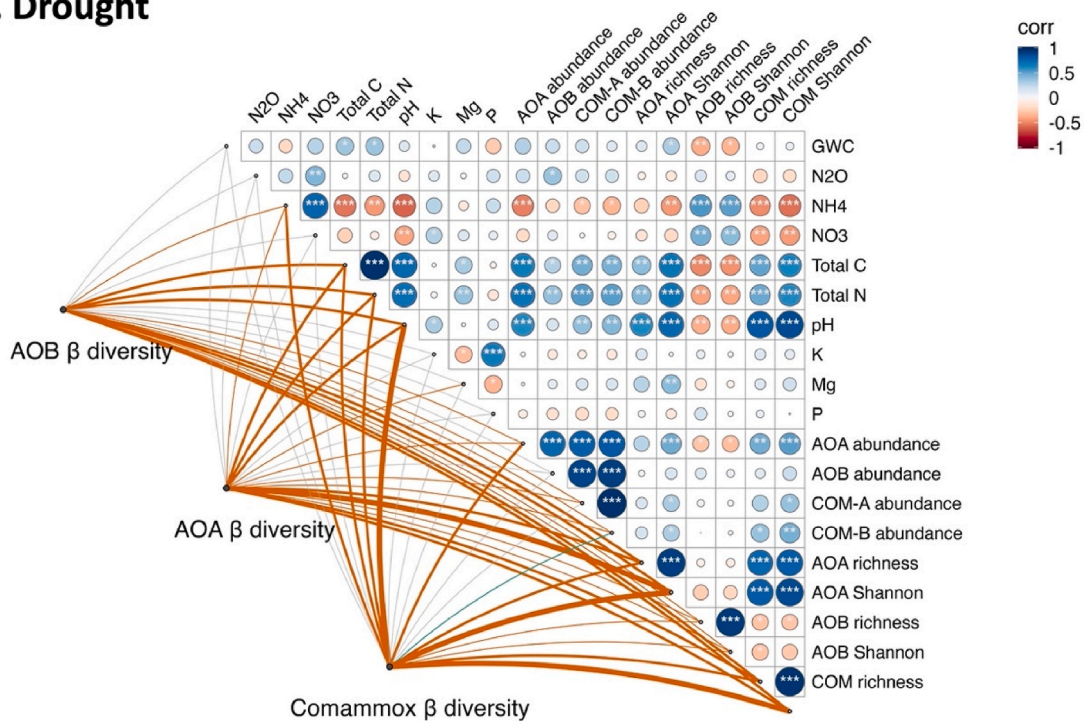


Fig. 5. Mantel's test for the correlation analysis between ammonia-oxidizing community beta diversity (Bray-Curtis dissimilarity) with mineral N pools (NH_4^+ , NO_3^-) and other soil properties, as well as the community alpha diversity and abundance in the control (A) and drought (B). The width and color of the edges represent the Mantel's R and P value, respectively. Thicker edges indicate stronger relationship. Spearman correlation coefficients among variables are indicated by the area of the circle with blue and red colors indicate positive and negative correlation, respectively. Significant correlation indicated by asterisks (** $p < 0.01$, *** $p < 0.001$, * $p < 0.05$).

et al., 2020). High NO_3^- accumulation under drought has been attributed to reduced denitrification and increased nitrification due to higher oxygen diffusion as well as to reduced NO_3^- leaching (Deng et al., 2021; A. A. Hartmann et al., 2013), while microbial death can contribute to increased NH_4^+ (Homyak et al., 2017). Alternatively, drought affects

plant growth by reducing the capacity for root N-uptake, which can consequently lead to a buildup of mineral N in soils (de Vries et al., 2016; Homyak et al., 2017). Interestingly, unlike in the conventional systems, the NH_4^+ and NO_3^- pools in the BIODYN system were mainly unaffected by drought, suggesting a stronger resistance of the underlying microbial

N-processes in this system (Fuchslueger et al., 2014). This suggests that differences in fertilization and agricultural management approaches between the organic and conventional systems can lead to diverging responses of mineral N to drought.

The control plots of the conventional cropping systems exhibited highest average N₂O flux after the application of mineral fertilizers early spring. Our findings align with previous studies reporting a strong reduction in N₂O flux in response to drought (Dobbie and Smith, 2001; Harris et al., 2021; A. A. Hartmann and Niklaus, 2012). This may be explained by the lower microbial activity under drought but also by the higher oxygen diffusion within the soil with drought resulting in decreased N₂O production by denitrification (Dobbie and Smith, 2001; Harris et al., 2021; X. Xu et al., 2024). The low N₂O fluxes in the BIODYN system were not affected by drought, which suggests that low mineral N concentrations rather than soil moisture was limiting the underlying microbial processes in this system (Skinner et al., 2019). Accordingly, previous studies reported that in mineral N-limited soils, drought had marginal effect on N₂O emissions (X. Xu et al., 2016, 2024). Overall, our findings highlight that the effect of drought on the mineral N pools and N₂O flux depends on agricultural management practices. It is important to mention that since the fluxes were only measured weekly, some N₂O peaks could have been missed as they can be highly variable in time and space (Butterbach-Bahl et al., 2013; Francis Clar and Anex, 2020).

We also examined the extent to which drought legacy effects were affecting mineral N-pools one and eleven weeks after rewetting. An impact of drought was still detected one week after rewetting in the conventional systems, but the effect was not significant anymore at the end of the rewetting phase. This mild legacy effect of drought indicates a strong resilience of the N-cycling processes. Accordingly previous studies showed that microbes-mediated nitrification can initiate rapidly when dry soil becomes wet (Parker and Schimel, 2011), as a result of increasing N mineralization and NH₄⁺ diffusion (Leitner et al., 2017; Schimel, 2018), as well as of available N flush (Homyak et al., 2014). Particularly, rewetting leads to a rapid transcriptional response by all groups of ammonia oxidizers despite months of drought-induced inactivation (Placella and Firestone, 2013).

4.2. The effect of drought on the diversity and abundance varied depending on the ammonia-oxidizing groups and the cropping system

While drought had no or minor impact on the alpha diversity of the ammonia-oxidizers, the CAP analysis revealed differences in beta diversity that were dependent on the AO group (Fig. 2). Particularly, the structure of the AOA community was less resistant to drought than that of AOB as previously described (Séneca et al., 2020; Thion and Prosser, 2014). Such differences in drought sensitivities between AO groups can be explained by the low tolerance of AOA to increasing ammonia concentrations during drought, but also by the higher sensitivity of AOA to osmotic stress when compared to AOB as demonstrated by Bello et al. (2019). Little is known on how comammox *Nitrospira* responds to drought and the niche specification of this group is still under debate (Sakoula et al., 2021; S. Xu et al., 2020). Here we found a small yet significant impact of drought on both the alpha- and beta-diversity of comammox, which were dependent on the cropping system. Differential abundance analysis indicated that on average more than a quarter of the ammonia-oxidizing AOB and more than 15 % of AOA and comammox ASVs (prevalence >0.01%) were affected by drought both in the bulk and rhizosphere soil. While, Lavalée et al. (2024) found that dominant microbial taxa were highly resistant to drought, our study showed that some of the drought-affected ASVs were among the most dominant taxa. Notably, the affected AOB ASVs belonged to the dominant *Nitrospira*, which has been described as a key player in ammonia oxidation with wide distribution across ecosystems (Krüger et al., 2021; Sanders et al., 2019). We didn't identify any ASVs exhibiting consistent shifts in relative abundance across timepoints, which suggests a dynamic response to drought without any clear resilience after rewetting. This indicates that

within the AO, the dominant taxa are not necessarily resistant to drought, and the period of drought in this study may have been severe enough to prevent the affected ASVs from recovering after the stress ended. The impact of drought on AO communities was very similar between the bulk and rhizosphere soil. In contrast, previous studies reported that rhizosphere microbiomes are more responsive to drought than bulk soil, due to its proximity with plant roots and greater influences of plant rhizodeposition (Kost et al., 2024; Santos-Medellín et al., 2017). As changes in root exudates play a key role in plant and microbial response to drought (Williams and de Vries, 2020), the lack of distinct responses of AO communities between the two compartments in our study could be explained by the fact AO are mostly autotrophs and thus less dependent on root exudates.

Quantification of the *amoA* gene copy numbers as a proxy of the AO abundance revealed minor effects of drought that were also depending on the AO group. Thus, the abundance of AOB and comammox clade B significantly decreased with drought alone, while the abundances of AOA and comammox clade A were affected by drought only in interaction with the sampling timepoint (Fig. 4). These findings are in accordance with previous studies assessing the effect of seasonal precipitation changes on the abundances of AO communities, and reporting the detrimental impact of drought (Kaurin et al., 2018; Wang et al., 2023). While niche differentiation between AOA and AOB has been reported in several studies (Prosser and Nicol, 2008, 2012; Verhamme et al., 2011), knowledge of the ecology of comammox bacteria is scarce (Li et al., 2023). However, a recent study suggest that differences may also exist between comammox bacteria with clade B having NH₄⁺ transporter with higher affinity than that in clade A (Koch et al., 2019). Our results showed that not only the abundance (Fig. 4) but also the proportion of AO within the total bacterial community (Fig. S5) generally decreased with drought, suggesting a lower resistance of this functional group to drought. Accordingly, it is assumed that phylogenetically and physiologically narrow functional groups such as the nitrifiers are more sensitive to disturbances than the broad functional groups (Griffiths and Philippot, 2013; Schimel, 2018).

These effects of drought on the AO communities also varied depending on the type of cropping system. For example, a significant difference in the AOB abundance was observed in response to drought in the CONFYM system only (Fig. 4A). Such differential effect of drought was not due to differences in GWC between cropping systems as no significant interaction effects (*cropping system (C) x drought (D)*) was observed (Fig. S1). Alternatively, the observed differences in the diversity, abundance, and structure of the AO communities between cropping systems (Fig. 2) may be responsible for these differential responses to drought. It is known that AO taxa vary in their sensitivity and strategies to soil water fluctuation (Lehtovirta-Morley, 2018; Séneca et al., 2020). This is supported by the work of Lavalée et al. (2024) who reported that land management could affect the drought response strategies of the dominant soil microbial taxa. For example, the studied cropping systems led to distinct soil pH, and pH is widely known as the major factor that regulates microbial community structure, as well as their functional activities, including N cycling (Nicol et al., 2008). Thus, Shu et al. (2023) found that pH moderates the resistance and the resilience of N-cycling to disturbance, with a greater resilience in more neutral soils. Taken together, these results indicate that cropping system can be an important factor determining AO response to drought. However, further studies are needed to determine the extent to which this impact of drought on AO is modulated by the soil type.

4.3. Drought influenced the relationship between soil properties, mineral N pools, and AO communities

Soil environmental conditions shape microbial communities and influence their functional response to disturbances, which in return can lead to modifications of their soil environment (Philippot et al., 2024). However, links between microbial community properties and

biogeochemical processes remain unclear despite being central to understanding how ecosystem functions are affected by climate change (Graham et al., 2016; Wallenstein and Hall, 2012). To better understand how drought affected the relationships between soil properties, mineral N pools, N₂O fluxes, and AO communities, we performed a Mantel test combined with Spearman's correlation analysis of these variables. Significant correlations were observed between several properties of the AO communities and the mineral N-pools. In particular, in the control treatment, strong correlations were observed between mineral N-pools and the abundances and diversity of AOA or comammox compared to AOB. This suggests that AOA and comammox rather than AOB were playing an important role in the fate of the mineral N pools in the studied cropping systems. In line with our findings, Ouyang et al. (2016) found that AOA dominated gross nitrification activity in moist agricultural soils. However, the contribution of the different AO groups to nitrification remains controversial (Yu et al., 2023). For example, using ¹⁵N-tracers and AO inhibitors, a recent study revealed a comparable contribution of AOB and AOA to gross nitrification under low NH₄⁺, but a higher contribution of AOB in a NH₄⁺-rich environment (Rütting et al., 2021). We also found that the NH₄⁺ pools were negatively correlated with the alpha diversity of AOA and comammox, while being positively correlated with AOB, which supports niche differentiation between AO groups (Prosser et al., 2020). Thus, AOA and comammox are described as oligotrophs (Kits et al., 2017) with higher NH₄⁺ affinity and that they thrive in NH₄⁺-poor conditions while AOB exhibits copiotroph life-style and are favored in high NH₄⁺ concentration (Verhamme et al., 2011).

Overall, we found that drought reduced the strength of correlations between N-pools and AO alpha and beta diversity as well as AO abundances. This is likely explained by drought reducing overall microbial activity, including nitrification, due to direct physiological stress (Schimel, 2018). In addition, the relationships between AO communities and NH₄⁺ pools could also be indirectly affected by drought due to diffusion-driven substrate limitation as shown by the reduction in nitrification by at least 50% with lower water potential (Stark and Firestone, 1995). Altogether, our findings demonstrate the pervasive consequences of drought affecting not only AO communities but also their complex relationships with soil properties and functions.

5. Conclusions

Our study revealed that the effect of drought on the structure and diversity, and abundance of AO was modulated by the cropping system, which was the predominant driver influencing AO communities. Our findings also highlight that the responses of AO communities to drought were taxa-specific, and depended on the measured variable. Specifically, the community structures of AOA and comammox were more strongly affected by drought than that of AOB, while the abundances of AOB and comammox clade B were more sensitive to drought. Moreover, the effect of drought on AO communities was largely similar between bulk soil and rhizosphere, indicating that host plants may have a minor influence on AO community likely due to its autotrophic lifestyle. This study highlights the importance of cropping systems in shaping the response of N-cycling processes and their associated microbial communities to drought, which is crucial for predicting the impact of climate change on soil functions. Furthermore, our work advocate for a more holistic approach considering not only crops but also microbial communities providing crucial soil services such as nutrient cycling for cropping system management aimed at mitigating the impacts of climate change.

CRedit authorship contribution statement

Ari Fina Bintarti: Writing – original draft, Visualization, Methodology, Investigation, Formal analysis. **Elena Kost:** Writing – review & editing, Methodology, Investigation. **Dominika Kundel:** Writing – review & editing, Investigation. **Rafaela Feola Conz:** Writing – review & editing, Investigation. **Paul Mäder:** Writing – review & editing, Project

administration, Methodology. **Hans-Martin Krause:** Writing – review & editing, Methodology. **Jochen Mayer:** Writing – review & editing, Project administration, Methodology. **Laurent Philippot:** Writing – review & editing, Writing – original draft, Supervision, Funding acquisition, Conceptualization. **Martin Hartmann:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization.

Data availability

The computational workflows for sequence processing and ecological statistics are available on GitHub (https://github.com/arifinabintarti/Bintarti_2024_Microservices). Raw sequence data of amoA gene of AOB, AOA, and comammox have been deposited in the Sequence Read Archive NCBI database under BioProject accession number of PRJNA1129138, PRJNA1129476, and PRJNA1129662, respectively.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix B. Supplementary data

Supplementary data related to this article can be found at <https://doi.org/10.1016/j.soilbio.2024.109658>.

Appendix A. Supplementary data

Supplementary data to this article can be found online at.

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