Cropping system modulates the effect of 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 comammox (complete ammonia oxidizers) respond to simulated drought in a rain-out shelter experiment in the DOK long-term field trial comparing different organic and conventional agricultural practices since 1978. This study is part of the MICROSERVICES (BiodivERsA) project aiming to understand and predict the effects of climate change on crop-associated microbiomes and their ecosystem functions. We monitored the diversity, the composition, and the abundance of ammonia-oxidizers for five months by Illumina-based amplicon sequencing and quantitative real-time PCR using the *amoA* gene as molecular marker. We found that the effect of drought varied depending on the ammonia-oxidizing community and also on the agricultural practices. The community structures of AOA and comammox were more strongly affected by drought than the AOB community structure. Drought also had a stronger impact on the community structure in the biodynamic (organic) cropping system than in both the mixed and mineral-fertilized conventional systems. The abundance of ammonia oxidizers was also influenced by drought, with comammox clade B exhibiting the strongest sensitivity to drought. The drought effect on the community abundance was more prominent in the biodynamic and mixed-conventional systems than in the mineral-fertilized conventional system. We further found a significant interaction between drought and agricultural practices on the abundance of all groups of ammonia-oxidizers except AOB. Overall, our study showed that the impact of drought on ammonia oxidizers was modulated by agricultural practices and varied with time as well as among members of ammonia-oxidizers. These results underscore the significance of agricultural management practices in influencing the response of nitrogen cycling and the corresponding communities to drought.

**INTRODUCTION**

**METHODS**

**Experimental design and soil sampling**

The rain-out shelter study was conducted in 2021 to 2022 at the DOK (bio-Dynamic, bio-Organic, and “Konventionell”) long-term experimental field at Therwill, Switzerland. The field has been established in 1978 under five cropping systems received different fertilization and pesticide management systems (Hartmann et al., 2015; Maeder et al., 2002). For this study, three cropping systems were chosen from the DOK trial: manured biodynamic (BIODYN), mixed-conventional (CONFYM), and mineral-fertilized conventional (CONMIN) plots, n (Hartmann et al., 2015). The study was performed using a strip-split-plot design, with 3 levels of cropping systems as the main plot and 2 levels of irrigation (control, drought) as the sub-plot (6 treatment combinations). The rain shelters were installed in each plot to exclude the rainfall to simulate the drought effect, while the control plots had no rain shelter installed. The study was performed in four replications for each treatment combination with total of 24 plots. The field was planted with a commercial variety of winter wheat (Wiwa) in October 2021 before the rain shelter installment in November 2021, when the crops were at the early vegetative stage to start the drought stress treatment. The rainout-shelters were then removed on July 2022. Agricultural practices (e.g. fertilization, irrigation, pesticides application, and weed management) were performed according to the assigned cropping system (Kost et al.,). .

Samplings were conducted at five timepoints, three samples were collected during drought period and two samples were collected after rewetting events (Kost et al.,). The first sampling was at the stem elongation stage in April 27-28th 2022 (stage 6, the first node of stem visible; n = 24 bulk soil, n = 24 rhizosphere). The second samples were collected at the flowering stage in June 1st (stage 10.5; n = 24 bulk soil, n = 24 rhizosphere). The third sampling was at the ripening stage in the beginning of July (July 5th) (stage 11; n = 24 bulk soil, n = 24 rhizosphere) before the rain shelters removal (July 6-7th) and rewetting process (July 14th). The fourth (n = 24) and fifth (n = 24) samplings were conducted on July 20th (one week after rewetting) and in September (eleven weeks after rewetting), respectively, by collecting only the bulk soils. A total of 120 of 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 of sieve to remove any plant debris and to achieve more homogenous soil particles. Root-attached rhizosphere soils were collected from within a plant row using an 8 cm soil auger. 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. ).

**Amplicon libraries preparation and sequencing of *amoA* genes**

Soil DNA were extracted from a total of 192 samples using DNeasy ® PowerSoil Pro Kit (Qiagen, Hilden, Germany) according to the manufacturer’s protocol from 0.25g homogenized rhizosphere and bulk soil. The quality and quantity of the DNA was assessed via UV/VIS spectrophotometry with the QIAxpert (Qiagen) and normalized to 10 ng/μL. The analysis of ammonia-oxidizing communities was conducted by sequencing of *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 *amoA*-1F (5’-GGGGTTTCTACTGGTGGT-3’) and *amoA*-2R (5’-CCCCTCKGSAAAGCCTTCTTC-3’) primer pair (Rotthauwe et al., 1997); and CrenamoA23f (5’-ATGGTCTGGCTWAGACG-3’) and CrenamoA616r (5’-GCCATCCATCTGTATGTCCA-3’) primer pairs (Tourna et al., 2008), respectively. The PCR conditions used to amplify the *amoA* genes of AOB and AOA 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 pair specific for each sample.

Comammox *amoA* genes were amplified using comamoA-F (5’-AGGNGAYTGGGAYTTCTGG-3’) and comamoA-R (5’-CGGACAWABRTGAABCCCAT-3’) primer pair (Zhao et al., 2019). The PCR amplifications were set up in duplicate 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/µL of template DNA. For comammox, the first-step PCR products were cleaned up using the SequelPrep™ 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 SequelPrep™ 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).

***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) developed by Lee et al.,. 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 or simply concatenating the non-overlapping forward and reverse reads for AOB and AOA. 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 amplicon size (452, 410, and 396 bp for AOB, AOA, and comammox, respectively) 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 (Buchfink et al., 2021). The AMOA database incorporated in this AMOA-SEQ pipeline was constructed 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), Lee et al.

**Quantificationof total microbial 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, 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 as described previously (Bru et al., 2011; Leininger et al., 2006;). 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 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, we tested the presence of PCR inhibitors in the DNA samples 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), and also into RNase-free water as positive controls. The specific T7 and SP6 primers were used for the inhibition test and no inhibition was detected in all samples.

**Ammonia-oxidizing community analysis**

Statistical analyses were conducted on R software (v.4.3.1) (R Core Team, 2023). Microbial alpha and beta diversity were calculated 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. Count of observed ASVs (richness) and Shannon diversity index were calculated to analyze microbial alpha diversity using the vegan package (v.2.6.4) (Oksanen et al., 2022).

The significance of treatment effects (drought, cropping system, and sampling date) as well as their interactions on the *amoA* gene abundance, alpha diversity, gravimetric water content (GWC), ammonium (NH4+), nitrate (NO3-), and on average N2O 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 and verified the normality and homoscedasticity of the data using Saphiro-Wilk and Levene’s test, respectively using the rstatix package. Data transformation of the response variables were performed when necessary, using log or cube 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 (Benjamini & Hochberg, 1995).

The *amoA*/16S rRNA gene ratio as well as the abundance of the total bacteria (16S rRNA) in bulk soil were tested by fitting the linear mixed-effects model (LMM) using the lmerTest package (v.3.1.3), with drought (I), cropping system (C), and sampling date (D) as the fixed effects, while block and its combination with sampling date as the random factor to allow intercept to vary among block within time (Kuznetsova et al., 2017). Gene copy number and its ratio were log-transformed and arcsine square root-transformed when necessary. The residual diagnostic was performed using the DHARMa package (v.0.4.6) to check the model residual distribution (Hartig, 2019). The pairwise comparisons were conducted to assess the difference in *amoA* gene abundance between drought and control for each sampling date within each cropping system using *emmeans\_test* function from the rstatix package with the Benjamini-Hochberg-adjusted *P* value.

Beta diversity analysis was calculated using Bray-Curtis distances using *vegdist* function in the vegan package. Permutational multivariate analysis of variance (PERMANOVA) was performed to assess the effect of treatments using the *adonis2* function of the vegan package. 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). We also performed constrained ordination using Canonical Analysis of Principal Coordinates based on Discriminant Analysis (CAP) with *CAPdiscrim* function in the BiodiversityR package (v.2.15-4) using drought x cropping system as the constraining factor, and estimating the classification success by permuting the distance matrix for 9999 times (Anderson & Willis, 2003; Legendre & Anderson, 1999). To further investigate the difference between drought ad control in each cropping system, we calculated Euclidean distance matrix from the positions of the sites provided by the discriminant analysis obtained from the CAP analysis using the *dist* function from the stats package, and we assessed the distance within and between groups using the *dist\_groups* function from the usedist package (v.0.4.0).

Ammonia-oxidizing community composition and relative abundance were assessed using the phyloseq package (v.1.44.0) (McMurdie & 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. We calculated an ASV abundance with parameter as , in any replicates of any treatment using the following model:

We introduced offset as the log of the sample read sum, is the effect of the irrigation treatment coded as a factor, and is the random sampling effect modeling the data overdispersion. represents the irrigation treatments and represents the replicates. The model was run using the glmmTMB function of the glmmTMB package (v.1.1.7) (Brooks et al., 2017). A post-hoc test with the *emmeans* function of the emmeans package (v.1.8.8) was performed for pairwise comparison between drought and control. We applied this analysis to compare ASVs abundance between control and drought within each cropping system.

We performed Mantel’s test with Spearman’s correlation method to analyse 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. 2020). The actual *P* values were corrected using the Benjamini-Hochberg (FDR) method (Benjamini & Hochberg, 1995).

**Data and code availability**

The computational workflows for sequence processing and ecological statistics are available on GitHub(..). 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 …..

**RESULTS**

**Drought affected soil water availability and mineral N pools**

As expected, drought severely affected the soil water availability in all cropping systems, with an average decrease of more than 40% in GWC compared to the control (Supplementary Fig. 1; Supplementary Table 1). The effect of drought was still significant one week after rewetting, but not at the final sampling date (eleven weeks after rewetting event) (Supplementary Fig. 1; Supplementary Table 1). This effect of drought on gravimetric water content depended on the sampling date but not on the cropping system (Supplementary Table 1).

Large differences in NH4+ content were observed in the control treatments between cropping systems with BIODYN system exhibiting in average 82−85 % lower NH4+ content compared to the other two conventional systems (Figure 1 A; Supplementary Table 1). Drought was also a stronger driver of the NH4+ content, with a significant impact dependent both the cropping systems and the sampling date (Three-way repeated measures ANOVA, P<0.01; Supplementary Table 1). Thus, drought increased the average NH4+ content in the CONFYM and CONMIN systems by two to eleven times compared to the control. While we observed a marginal decrease of NH4+ content at the first sampling date, overall, there were no significant effect was observed for BIODYN system (Figure 1 A). No difference in NH4+ content between the drought and the control treatments in both conventional systems were found eleven weeks after rewetting (Figure 1 A).

Similarly to the NH4+ content, the effect of drought on NO3- content depended on the cropping systems as well as on the sampling date (Three-way repeated measures ANOVA, P<0.01; Supplementary Table 1). Drought led to an increase in the NO3- content in the CONFYM and CONMIN systems by more than 100 % relative to the control across all sampling dates, except at eleven weeks after rewetting, where the differences were not significant (Figure 1 B; Supplementary Table 1). 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 NO3- content, indicating that the overall drought effect was marginal (Figure 1 B).

Compared to the drought effect on NH4+ and NO3- contents, we detected weaker but significant drought effect on the average of N2O flux (Three-way repeated measures ANOVA, P<0.05; Supplementary Table 1). Drought effect was found in CONFYM and CONMIN systems at the beginning of drought period with a strong effect at the first sampling dates. In the contrary, there was no drought effect detected in the BIODYN system (Figure 1 C).

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**Differential responses of ammonia oxidizing communities to drought**

The AOB, AOA, and Comammox communities were dominated by *Nitrosospira* (bulk soil: 84.56%, rhizosphere: 83.38%), *Nitrososphaerales* clade Delta (NS-Delta) (bulk soil: 73.51%, rhizosphere: 71.14%), and *Nitrospira* 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 (Supplementary Fig. 2). The alpha diversity of AOB, AOA and comammox was not affected by drought alone both in the bulk soil and in the rhizosphere (Three-way repeated measures ANOVA, P>0.05; Supplementary Fig. 3G-L; Supplementary Table 2). However, we found a significant interaction of *drought* × *cropping system* for Comammox alpha diversity in the bulk soil (Three-way repeated measures ANOVA, P<0.05; Supplementary Table 2). Nonetheless, we could not identify any significant difference between drought and control within sampling date 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 (Supplementary Fig. 3C and F). On the contrary, BIODYN led to a decrease in alpha diversity of the AOB compared to the two conventional systems (Supplementary Fig. 3A and D).

The unconstrained PCoA plots using Bray-Curtis dissimilarity distances showed a strong clustering by cropping system (PERMANOVA, P<0.05) with 34 % (bulk soil) and 43 % (rhizosphere), 74 % (bulk soil) and 76 % (rhizosphere), and 69 % (bulk soil) and 70 % (rhizosphere) of the variance explained by the first two axes for the AOB, AOA, and Comammox, respectively (Supplementary Fig. 4). 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 x cropping system as the grouping variable. Overall, there was a distinct clustering by drought and cropping system on the ordination of all groups of ammonia-oxidizing community by CAP analysis (MANOVA, P<0.001) (Figure 2). The AOA community exhibited the highest 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 BIODYN and CONFYM cropping system than in the CONMIN cropping system (Figure 2C and D). Distinct clustering by the drought treatment were also observed in the Comammox community with a higher reclassification rates in the BIODYN than the other cropping systems regardless of the compartment (bulk soil and rhizosphere) (Figure 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 (Figure 2A and B). The calculation of Euclidean distances between the drought and control treatments based on the discriminant analysis confirmed the stronger impact of drought on both the AOA and Comammox communities in the BIODYN cropping system (Supplementary Fig. 5).

**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 significant impacted by drought represented 44% and 35 % (AOB), 20% and 16 % (AOA), 23% and 25 % (Comammox) of the most dominant and prevalent ASVs in bulk soil and rhizosphere, respectively (Figure 3). Among the three ammonia-oxidizing groups, the AOB community has the largest number of affected ASVs in all samples (30 and 25 ASVs in bulk soil and rhizosphere, respectively). 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 *Nitrospira* sp. clade B, respectively (Figure 3). ~~Eight AOB ASVs (except the ASV 87) assigned to~~ *~~Nitrosolobus multiformis~~* ~~and one ASV of~~ *~~Nitrosomonas communis~~* ~~exhibiting a decrease in relative abundance were found in all cropping system, except in CONMIN. On the other hand, there were in total ten AOB ASVs in bulk soil and rhizosphere belonging to the genus~~ *~~Nitrospira,~~* ~~which were depleted by drought only in the CONMIN system, but not in the other cropping systems (Figure 3).~~ Moreover, CONMIN exhibited less drought-affected AOA and Comammox ASVs compared to BIODYN and CONFYM (Figure 3).

**Drought affected the abundance of ammonia oxidizers in bulk soil**

Quantification of theabundances of ammonia-oxidizing communities showed that the effects of drought were different depending on the ammonia-oxidizing group and the cropping system (Supplementary Table 3). In the bulk soil, a significant effect of drought 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, Figure 4; Supplementary Table 3). This effect of drought depended on the cropping system only for the AOB. Thus, drought led to a decrease in the AOB abundance in the CONFYM system only, with decreases of up to 39 % relative to the control. In contrast, 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 (Figure 4D). We also found that drought led to significant decreases in the proportion of AOB and comammox within the total bacterial community in the bulk soil (LMM, P<0.05, Supplementary Fig.6; Supplementary Table 4), while no significant effect was observed in the rhizosphere (Three-way repeated measures ANOVA, P<0.05, Supplementary Fig. 6; Supplementary Table 4).

**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 N2O emissions, were affected by drought (Figure 5). Notably, we found that the NO3- 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 the alpha diversity of AOB was positively correlated to the NO3- content in the drought treatment while a negative relationship was observed with the alpha diversity of comammox (Figure 5). Similarly, stronger correlations were found between the NH4+ content and AO communities in the control than in the drought treatment (Figure 5). Interestingly, all these correlations were negative except the alpha diversity of AOB. Among all AO groups, only the beta diversity of AOB that related to the N2O flux, and this relationship was only found in the control. We detected negative correlation between the N2O flux with the abundance of AOA and comammox (clade B), as well as with their alpha diversity, while also positively correlated with the alpha diversity of AOB in the control. Overall, there were no significant relationship between the N2O flux with AO communities, except with the AOB abundance, in the drought treatment (Figure 5). Additionally, we found a significant positive and negative correlation between soil water content (GWC) and the alpha diversity of AOA and AOB, respectively in the drought. While in the control treatment, GWC only correlated with the AOB richness (Figure 5).

**DISCUSSION**

**The effects of drought on mineral Nitrogen pools (NH4+, NO3-) and N2O fluxes are modulated by cropping system**

We found that drought strongly affected the mineral N pools with lower GWC resulting in large increases in the NH4+ and NO3- pools, particularly in the mixed- and mineral-conventional systems (CONFYM and CONMIN). While some studies also reported that drought increased both NH4+ and NO3- pools in soil (Deng et al., 2021; Hartmann et al., 2013; Ullah et al., 2020), others found that the NO3- pools remained unchanged or even decreased in response to drought (Canarini et al., 2021; Séneca et al., 2020). High NO3-accumulation under drought has been attributed to reduced denitrification and increased nitrification due to higher oxygen diffusion as well as to reduced NO3- leaching (Deng et al., 2021; Hartmann et al., 2013; ), while microbial death can contribute to increased NH4+ (Homyak et al., 2017). Alternatively, drought affects plant growth by reducing the capacity for root N-uptake, which can consequently leads to a buildup of mineral N in soil ( DOI 10.1007/s11104-016-2964-4; Homyak et al., 2017). Interestingly, unlike in the conventional systems, the NH4+ and NO3- 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). These diverging responses of mineral N to drought between organic and conventional systems might have been caused by differences in fertilization and agricultural management approaches between systems. ~~For example, manure amendment enhances soil organic matter which improves the water-holding capacity, potentially mitigating the harsh effect of drought (Ullah et al., 2020)~~.

s (ref)We found thattheseaverage Our findings align with previous studies ingstrong reduction inHarris et al 2021, Dobiie & Smith 2002is by higher oxygen diffusion within the soil with resulting in decreased N2O production by denitrification , XXX low were not affected by droughtwhich slow mineral N concentrations rather than soil moisture was limiting the underlying microbial processes in this systemAccordingly, piess

We also examined the extent to which drought legacy effects were affecting N-pools until 11 weeks after rewetting.

We found that the impact of drought decreased one week after rewetting in the conventional systems and was not significant anymore at the end of rewetting phase. This suggest a strong resilience of the N-cycling processes XXXX

Schimel??

nitrifiers and nitrification process. It is commonly known that nitrifiers are considered as slow growing microbes. Rewetting may stimulate and reactivate the growth of nitrifiers, hence increases nitrification process, reduces NH4+ substrate in soil, and omits the drought effect (Krüger et al., 2021).

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

*First paragraph:Intro first to avoid redundancy*

Drought can have affect microbial communities in many ways as water is crucial as resource, as solvent, and as transport medium (<https://doi.org/10.1146/annurev-ecolsys-110617-062614>). As such, several studies reported XXXXXX. However, studies are scarce on ammonia oxidizers and especiallly comammox despite…..Medium related to N-mineralization and N-cycling because water availability controls their growth and determines whether they will remain active or dormant in soil (Metze et al., 2023). Ammonia-oxidation is considered as the first and rate-limiting step of nitrification (Lehtovirta-Morley, 2018; Séneca et al., 2020) performed by ammonia-oxidizers, and any environmental perturbations may alter the whole process of nitrification.

While drought had no or minor impact on the alpha diversity of the ammonia-oxidizers, the CAP analysis revealed differences in the beta diversity that were dependent on the AO group (Fig. 2). Thus, the structure of the AOA community was less resistant to drought than than that of AOB as previously described (Thion & Prosser, 2014; Seneca 2021) . 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 significantly impact of drought on both the alpha diversity and beta-diversity of comammox, which were dependent on the cropping system. Differential abundance analysis indicated that in average more than a quarter of the dominant ammonia-oxidizing ASVs were affected by drought both in the bulk and rhizospheric soil and regardless of the taxa. In contrast to the CAP analysis showing a higher resistance of AOB, the percentage of affected ASVs belonging to AOA and comammox was lower than those belonging to the AOB. One possible explanation is that by filtering out the rarest and least prevalent ASVs for the analysis of differential abundance, we may have missed the impact of the drought treatment on less common members of AOA and comammox Notably, some the drought-affected ASVs were among the most prevalent taxa regardless of the AO group. For example affected AOB ASVs belonged to the dominant *Nitrosospira*, which has been described as a key player of 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 dates, which suggests a dynamic response to drought without any clear resilience after rewetting. REFS. *To be continued (1-3 sentences) in relation to resilience. While plant roots are affecting …here discuss the fact that the impact of drought is similar in bulk and rhizosphere.*

Qication of significant effects ofthat were also depending on thegroupus, thesignificantly alonethe abundances of were affected by drought only in the interaction with cropping systemsTheseies the abundances detrimental impact ofdrought(Kaurin et al. 2018; ; H. Wang et al., 2023While niche differentiation between AOA and AOB has been reported in several studies (ref), knowledge of the ecology of comammox bacteria is scarce. However, recent study suggest that differences may also exists between comammox bacteria with clade B having NH4+ transporter with higher affinity than that in clade A (Koch et al., 2019). Our results showed thatthe not only the abundance but also the proportion of AO within the total bacterial community decreased with drought, suggesting a lower resistance of this functional group to drought. Accordingly, it is believed that phylogenetically and physiologically narrow functional groups such as the nitrifiers are more sensitive to disturbances than the broad ones (Schimel, Philippot&Griffth).

These effects of drought on the AO communities also varied depending on the type of cropping system. For example, larger differences in beta diversity were found between drought and control treatments in the BIODYN system compared to the other cropping systems in particular for the AOA and comammox both in the bulk and rhizospheric soil. It is known that AO taxa vary in their sensitivity and strategies to soil water fluctuation (Hu, Seneca). Here, we found differences in the diversity, abundance and structure of the AO communities between cropping systems, which therefore may be responsible for these differential responses to drought. This is supported by the work of Lavelee, who reported that land management could affect the drought response strategies of the dominant soil microbial taxa. Moreover, the studied cropping systems exhibited distinct pH, which may also have contributed to the differences in sensitivity of AO taxa to drought. Thus, Shu AES found that pH moderates the resistance and the resilience of N-cycling to disturbance. *Expend 2-3 sentences?* Taken together, these results indicate that cropping system is an important factor determining AO response to drought.

**Drought on relationship between soil properties, mineral N pools, and AO community**

Soil environmental conditions shape microbial communities properties and influence their functional response to disturbances, which in return can lead to modifications of their soil environment (Philippot NRM 2024). However, links between microbial community properties and biogeochemical processes remain unclear despite being central for understanding how ecosystem functions are affected by climate change (Graham 2016; Wallenstein,and Hall,E.K doi:10.1007/s10533-011-9641-8). To better understand how the relationship between soil properties, mineral N pools, N2O fluxes and AO communities were affected by drought, we performed a correlation analysis. Notably, significant correlations were observed between several properties of the AO communities and the mineral N-pools. In particular, stronger correlations were observed in the control treatment 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 are playing an important role in the fate of the mineral N pools in the studied systems. The contribution of the different AO taxa to nitrification remains controversial *..expend with literature showing that either AOA or AOB are dominating…including papers using inhibitors*. Moreover, the NH4+ pools were negatively correlated to the alpha diversity of AO and comammox while being positively correlated to that of AOB. Literature , these observations support niche differentiation between AO with AOA and comammox being oligotrophs (Kits et al., 2017, etc…) while AOB copiotrophs ……Thus… However, we found that in overall drought weakened these 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 a direct physiological stress (Schimel). In addition, the relationships between AO communities and NH4+ pools can also be indirectly affected by drought due to diffusion-driven substrate limitation as shown by the reduction at least 50% in nitrification with lower water potential (Stark & Firestone 1995).

*(+ “concluding” sentence about the drought effect on relationship)*

**Conclusions**

Our study revealed that the effect of drought on the structure and diversity, and abundance of AO was modulated by cropping system, which is likely related to the availability of mineral N pools. Our findings emphasize that the response of AO communities to drought were taxa specific, and also depend on the measured variable. Specifically, the community structures of AOA and comammox were more strongly affected by drought than that of AOB, while the abundance of *amoA* genes of AOB and comammox clade B were more sensitive to drought. This study provides insights on the significance of agricultural management practices in influencing the response of N cycling and the corresponding communities to drought, which is fundamental for predicting potential changes and nitrification management in the future climates.

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FIGURE LABEL

*Figure 1. Ammonium (NH4+) (A) and nitrate (NO3-) (B) contents, and the average N2O flux (C) of control and drought-treated plots. The effect of drought (I), cropping system (C), and sampling date (D), as well as their interactions was assessed by three-way repeated measures ANOVA. Pairwise comparison between control and drought for each sampling date 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, \*<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).*

*Figure 2. Effects of drought and cropping system on the 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 soil and rhizosphere. Overall reclassification success rate represents the degree of discrimination between the grouping factors. Reclassification success rates for each cluster are provided next to the respective ellipses. The statistical significances are indicated by the Pillai’s trace statistics and asterisks (MANOVA, \*\*\*P<0.001).*

*Figure 3. Heat map showing ASVs of AOB, AOA, and comammox that are 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 (log2-ratio>0) and red (log2-ratio<0) respectively. The relative abundance of each ASV is provided in the left side of the heat map.*

*Figure 4. amoA gene abundance of AOB (A), AOA (B), and comammox clade A (C) and B (D) in bulk soil. The effect of drought (I), cropping system (C), and sampling date (D), as well as their interactions was assessed by three-way repeated measures ANOVA. Pairwise comparison between control and drought for each sampling date 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).*

*Figure 5. Mantel’s test for the correlation analysis between ammonia-oxidizing community beta diversity (Bray-Curtis distance) with mineral N pools (NH4+, NO3-) and other soil properties, as well as the community alpha diversity and abundance in control (A) and drought (B). The width and color of the edges represents the Mantel’s R and P value, respectively. Thicker edge indicates stronger relationship. Spearman correlation coefficients among variables are indicated by the area of the square with blue and red colors indicate positive and negative correlation, respectively. Significant correlation indicated by asterisks (\*\*\*p<0.001, \*\*p<0.01, \*p<0.05)*