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 study was conducted in 2022 at the DOK (bio-Dynamic, bio-Organic, and “Konventionell”) experimental field at Therwill, Switzerland. The field has been investigated long-term since 1978 under five different fertilization and pesticide management systems (Hartmann et al., 2015; Maeder et al., 2002). For this study, three fertilization and pesticide management methods were chosen from the DOK trial: manured biodynamic (D), manured conventional (K), and mineral-fertilized (M) plots, due to their contrasting treatments (Table 1) (Hartmann et al., 2015). The study was performed using a strip-split-plot design, with three levels of farming system (D, K, M) as the main plot and 2 levels of irrigation (control, drought) as the sub-plot. 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. There were four replications for each treatment combination, so there was a total of 24 plots. The field was planted with a commercial variety of winter wheat (Wiwa) before the rain shelter installment and the shelters were set up when the crop was at the early plant growth stage in April to start the drought stress treatment. The wheat crops were maintained and irrigated until harvesting at the ripening stage in July according to the procedure (Table 2) (Kost et al., not yet published).

Bulk soils and rhizosphere soils were sampled gradually over the course of the experiment. The first sampling was at the stem extension stage on April 28th (stage 6, the first node of stem visible; *n* = 24 bulk soil, *n* = 24 rhizosphere). The second was at the heading stage on June 1st (stage 10.5, flowering; *n* = 24 bulk soil, *n* = 24 rhizosphere). The third was at the harvesting/ripening stage on July 5th (stage 11; *n* = 24 bulk soil, *n* = 24 rhizosphere) before the rain shelters were removed from the plots and rewetting was performed. The fourth (*n* = 24) and fifth (*n* = 24) samplings were conducted on July 20th and September 13th, respectively, after the removal of the rain shelters and the rewetting process by collecting only the bulk soils. A total of 120 of bulk soil and 72 of rhizosphere soil samples were collected. Bulk soil and rhizosphere samples were collected according to (Kost et al., not yet published). Briefly, 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 for further analyses. Soil physiochemistry analyses were performed for each bulk soil sample. The measured soil parameters including soil water content, soil dry matter, pH, mineral nitrogen content (NO3, NH4), total soil nitrogen and carbon, and plant available potassium (K), magnesium (Mg), and phosphorus (P) content (Supplementary Table 1: soil properties results; Kost et al. (2024)).

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

Soil DNA of the total 192 samples were extracted, quantified, and normalized to 10 ng/µL according to Kost et al., not yet published. 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. Each sample was conducted in duplicate and were pooled to be used as a template for the second-step PCR. 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., not yet published. 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., not yet published.

**Sequencing Summary**

We successfully obtained a total of 1 806 442, 1 528 985, and 1 924 171 quality filtered reads of AOB, AOA, and comammox *amoA* genes, respectively, from 120 bulk soil and 72 rhizosphere samples. Amplicon Sequence Variants of AOB, AOA, and comammox generated from the rarefied reads were 1 222, 592, and 632 ASVs, respectively. Rarefaction curves of all samples reached asymptotes and showed sequencing depths were sufficient to capture all of the representative communities in the samples (Supplementary Fig. 1: rarefaction curves).

**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; Ochsenreiter et al., 2003). 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; 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 DNA. Tenfold serial dilutions (101–108 gene copies/µL) 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. Add PCR efficiencies. 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…….

**Ammonia-oxidizing community analysis**

Ecological and 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 for AOA (3832 sequences per sample), while AOB and comammox were rarefied at 1282 and 5242 sequences per sample, respectively. Any sample below the specified rarefaction depth were removed from the dataset. In this study, only one sample was removed from the AOB (S11) and comammox (S52) dataset. Count of observed ASVs (richness), Inverse Simpson, and Shannon diversity index were calculated to analyze microbial alpha diversity using the vegan package (v.2.6.4) (Oksanen et al., 2022). Differences in alpha diversity between irrigation treatments and among farming systems for each type of sample (bulk soil, n = 120 and rhizosphere, n =72) were determined by fitting the linear mixed-effects models (LMMs) using the lmerTest package (v.3.1.3) (Kuznetsova et al., 2017). 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 (v.0.7.2) (Kassambara, 2023). Post-hoc analysis was conducted by pairwise comparisons between groups 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).

Beta diversity analysis was calculated using Bray-Curtis and Weighted UniFrac distances. We visualized the distance matrices using Principal Coordinates Analysis (PCoA) plot to analyze the similarities and dissimilarities between groups. We fitted the measured environmental variables into the PCoA plot and tested their significance correlations using a permutation test implemented in the enfvit function of the vegan package (Oksanen et al., 2022). Permutational multivariate analysis of variance (PERMANOVA) was conducted to assess changes in ammonia-oxidizing community structure between groups using the adonis2 function of the vegan package. We performed pairwise comparisons between irrigation treatment using the pairwise.adonis function of the pairwiseAdonis package (v.0.4.1) (Martinez Arbizu, 2020). Ammonia-oxidizing community composition and relative abundance were analyzed 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 rarefied ASV tables by removing low-abundance ASVs (< 0.01 %) and keeping ASVs that were found in at lest 75 % of replicates for each treatment. 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 comparisons. We adjusted raw p-values using the false discovery rate (FDR) method (Benjamini & Hochberg, 1995). We applied this analysis to compare ASVs abundance between control and irrigation treatments within each farming system of different sampling data.

**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 affects soil water availability and mineral N pools**

Drought severely affects the soil water availability in all cropping systems (Fig.1: Soil GWC; Kost et al. (2024)). We observed that drought-treated soil has lower gravimetric water content compared to the control over the course of drought treatment, and the water availability recovered at the final sampling time (eleven weeks after rewetting event) (Fig.1 A: Soil GWC). The total mineral N content, including the ammonium (NH4+) and nitrate (NO3+) contents, were strongly affected by drought in the mixed- (CONFYM) and mineral fertilized (CONMIN) conventional systems, but not in the biodynamic (BIODYN) cropping system (Fig. 1 B: Ammonium, nitrate, and total mineral N contents, Table S1: N pools statistical analysis). Drought altered the mineral N pools, where the ammonium and nitrate increased during drought treatment, and recovered back to the control levels after the removal of the rain-out shelter and rewetting event (Fig. 1 B: Ammonium, nitrate, and total mineral N contents).

**The effects of drought on the ammonia-oxidizers diversity and composition were marginal**

Overall, there were no differences of the observed richness and Shannon diversity index between drought and control of the AOB and AOA communities in both bulk soil and rhizosphere (Fig. 2: Alpha diversity, Table S2: alpha diversity statistical analysis). Meanwhile, the observed richness of the Comammox community was marginally affected by drought in the bulk soil, but not in the rhizosphere (Table S2: alpha diversity statistical analysis). In contrast, we found that cropping system was a strong driver of the ammonia-oxidizers alpha diversity, with higher AOA and Comammox, but lower AOB alpha diversity in the BIODYN system than in the CONFYM and CONMIN cropping systems (Fig. 2: alpha diversity, Table S2: alpha diversity statistical analysis).

The unconstrained PCoA plots using Bray-Curtis dissimilarity distances showed distinct separation by cropping system on the first axis, meanwhile, the effect of drought was only apparent within block due to a strong block effect (Supplementary Fig. 2: unconstrained PCoA plots). The results of the whole plot PERMANOVA supported the effect of cropping system in bulk soil and rhizosphere (*P*=0.001), and we could not detect the effect of drought on the ammonia oxidizers composition. However, the restricted permutations PERMANOVA showed the effect of drought on the composition of the AOB (*P*= 0.028, bulk soil; *P*=0.007, rhizosphere) and Comammox (*P*=0.042, bulk soil; *P*=0.001, rhizosphere) communities, but not on the AOA community (*P=*0.08,bulk soil and rhizosphere). To further observe the effect of drought on the beta diversity, we performed constrained analysis using CAP, and the differences on the community composition between drought and control within each cropping system become more evident (Fig. 3: Constrained CAP Plots). The AOA community has the highest compositional differences between drought and control as demonstrated by high overall reclassification rates of 94.2 % and 90.3 % in bulk soil and rhizosphere, respectively. Distinct clustering by the drought treatment were also observed in the Comammox community with overall reclassification rates of 78.8 % and 83.3 % in bulk soil and rhizosphere, respectively. 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. Evaluation of the Euclidean distances calculated from the positions provided by the discriminant analysis showed that the highest differences between treatment (drought vs control) were within the BIODYN cropping system, particularly in the AOA and Comammox communities (Fig. 3: The distance boxplot calculated from the discriminant CAP analysis). Meanwhile, for the AOB community in the bulk soil, CONMIN system has the largest distance between drought and control (Fig. 3: The distance boxplot calculated from the discriminant CAP analysis).

**Most of the ammonia-oxidizer ASVs were resistant to drought**

Mean relative abundance of the ammonia-oxidizing taxa revealed that AOB, AOA, and Comammox communities were dominated by genus *Nitrosospira* (bulk soil: 84.56%, rhizosphere: 83.38%), lineage *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 that there were no notable shifts of taxonomic composition of the ammonia-oxidizing communities in response to drought, although the community compositions were largely shifted among cropping systems (Supplementary Fig.1: Relative abundance bar plot). We performed differential abundance analysis to investigate ammonia-oxidizing ASVs exhibiting differences in abundances between drought and control in each cropping system. We detected a relatively small number of ASVs that were altered by drought, while most of the ammonia-oxidizing ASVs remained unchanged in response to drought (Fig.4: DAA). Among the three ammonia-oxidizing groups, the AOB community has the largest number of altered ASVs in all samples (17 ASVs in total), and most of them belonged to the dominant genera of *Nitrosospira* and *Nitrosolobus*. The majority of the drought-altered AOB ASVs were negatively impacted, indicating that these ASVs decreased in abundances in response to drought, and these ASVs responded to drought toward the end of sampling time (the last day of drought period or the third sampling, one week (fourth sampling), and eleven weeks (fifth sampling) after rewetting). Another notable observation was that differences in AOB ASVs abundance due to the effects of drought were mainly found in bulk soil samples, rather than rhizosphere, in the conventional cropping systems (CONFYM and CONMIN) (Fig.4: DAA). On the other hand, only a few AOA and Comammox ASVs (less than ten) were identified as sensitive to drought, and they also belonged to the dominant lineages of *Nitrososphaerales* and *Ca. Nitrosotaleales*, and Comammox *Nitrospira* clade B, respectively (Fig.4: DAA).

**Shifts in the abundance of *amoA* genes of ammonia-oxidizers in response to drought**

Even though the effect of drought on the ammonia-oxidizers diversity and composition was relatively marginal, the abundance of ammonia-oxidizing communities measured by *amoA* genes quantification were largely affected by drought (Table S3: ammonia-oxidizers abundance statistical analysis). However, the effects of drought were different depending on the ammonia-oxidizing group, cropping system, as well as sampling date. Drought affected the AOB *amoA* gene abundance within the total microbial community which tended to decrease, and the drought effect was found in bulk soil, specifically in the BIODYN and CONMIN systems (Fig.5: AOB/16S Ratio). The Comammox clade B *amoA* gene abundance in bulk soil was also negatively affected by drought with an overall decrease in *amoA*/16S rRNA gene ratio in all cropping systems (Fig.5: Comammox B/16S Ratio). On the contrary, we could not detect any effect of drought on the AOB and Comammox *amoA* gene abundances in rhizosphere (Table S3: ammonia-oxidizers abundance statistical analysis). The effect of drought on the AOA *amoA* gene abundance was more pronounced in the rhizosphere than bulk soil, and interestingly, the drought-treated rhizosphere soils tended to have increased AOA *amoA* gene abundance (Table S3: ammonia-oxidizers abundance statistical analysis; Fig.5: AOA/16S Ratio). While the drought effect on the AOA and Comammox clade A *amoA*/16S rRNA gene abundance ratio was not significant, we detected a significant interaction effect of drought and cropping system in bulk soil of both groups, as well as the Comammox clade B (Table S3: ammonia-oxidizers abundance statistical analysis). Further pairwise comparisons revealed that drought decreased the AOA and Comammox clade A *amoA*/16S rRNA gene abundance ratio in the BIODYN system, whereas it tended to increase in the CONFYM system (Fig.5: AOA/16S Ratio; Comammox A/16S Ratio). Shifts in *amoA* gene abundances in response to drought were mainly observed either in the beginning of drought period (first sampling) or after rewetting event (fourth and fifth sampling). Statistical analysis showed that sampling time was also found to be associated with drought and cropping system as indicated by significant interaction effects, specifically within bulk soil samples of the AOA and Comammox clade B (Table S3: ammonia-oxidizers abundance statistical analysis).

**Relationship between environmental factors and ammonia-oxidizing communities**

Correlation analysis showed that in general, all of ammonia-oxidizers beta diversity in bulk soil positively correlated with ammonium (NH4+) content, soil pH, total C and N, as well as magnesium (Mg) content (Fig. 6: correlation figure; Table S4: correlation statistical analysis), where the communities had strongest correlation with soil pH. Meanwhile, we could not identify any correlations between ammonia-oxidizers diversity with nitrate (NO3+) content or soil water content (GWC). The AOB and Comammox communities also demonstrated to have marginal correlation with phosphorus (P) and potassium (K) content, respectively (Table S4: correlation statistical analysis). Moreover, we found that *amoA* gene abundance of all ammonia-oxidizing groups negatively correlated with NH4+ with different magnitudes, with NH4+ being the strongest driver for the AOA abundance. By contrast, there were no significant correlations between NO3+ content with ammonia-oxidizers abundance. While soil pH, and total C and N primarily connected with *amoA* gene abundances, soil pH was not a significant driver for the AOB abundance. Importantly, we found a positive correlation between the community abundance with soil water content, which supported the previous findings of the observed drought effect on the *amoA* gene abundance. Furthermore, correlation analysis indicated that the abundance of all ammonia-oxidizing groups was negatively correlated with soil dry matter (TS) (Fig. 6: correlation figure; Table S4: correlation statistical analysis).