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”) experimental field at Therwill, Switzerland. The field has been investigated long-term since 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, due to their contrasting treatments (Table 1) (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 crops in each plot were managed and irrigated according to the cropping system until ripening stage according to the specific timeline (Timeline: Table 2) (Kost et al.,).

Samplings were conducted at five timepoints, three samples were collected during drought period and two samples were collected after rewetting events (Timeline: Table 2) (Kost et al.,). The first sampling was at the stem elongation stage in April 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 (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. 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. ).

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

Soil DNA of the total 192 samples were extracted using DNeasy ® PowerSoil ® Pro Kit (Qiagen, Hilden, Germany) and QIAcube (Qiagen) 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 10ng/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; 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. 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**

We successfully obtained a total of 1 806 442 AOB, 1 528 985 AOA, and 1 924 171 comammox quality-filtered reads from 120 bulk soil and 72 rhizosphere samples. The construction of ASVs of AOB, AOA, and comammox resulted in 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.

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).

We performed separated statistical analysis for each type of sample (bulk soil and rhizosphere). The significance of treatment effects (drought, cropping system, and sampling date) as well as the interactions on the *amoA* gene abundance, alpha diversity, gravimetric water content (GWC), as well as ammonium (NH4) and nitrate (NO3) 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. Response variables were log-transformed when necessary. 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) 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), as well as by 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 (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 lest 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**

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 (Fig. X: Soil GWC, Table X: statistical analysis)). Drought effect was still observed one week after rewetting, but it recovered at the final sampling date (eleven weeks after rewetting event) as indicated by the non-significant difference between drought and control (Fig. X; Table X pairwise comparisons). Gravimetric water content was significantly affected by the interaction of *drought* × *sampling date* (Table X: statistical analysis), suggesting that the effect of drought was different by date.

Large differences in NH4 content were observed between cropping systems with BIODYN system having 82−85 % and 96.7 % lower average NH4 content compared to the other two conventional systems in the control and drought treatment, respectively (Fig. X: NH4 content). The effect of drought was varied with cropping systems, as well as sampling dates as we found a significant interaction of *drought* × *cropping system* × *sampling date* for the NH4 content (Table X: statistical analysis). Drought strongly influenced the NH4 pool in the CONFYM and CONMIN systems by increasing the average NH4 content two to eleven times higher than the control (Table X: statistical analysis). There was no difference in NH4 content between drought and control in both conventional systems eleven weeks after rewetting process. In other hand, while we observed a marginal decrease of NH4 content in the drought-induced BIODYN system at the first sampling date, the drought effect was absent at the remaining sampling dates (Fig. X: NH4 content; Table X statistic).

The effect of drought on NO3 content differed between cropping systems as indicated by significant interactions of *drought* × *cropping system*, as well as *drought* × *sampling date* (Table X statistic). Drought greatly increased 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 difference was insignificant (Fig. X: NO3 content; Table X statistic). In the BIODYN system, the effect of drought was only observed at the third sampling of the drought period with a slight decrease in NO3 content, indicating that the overall drought effect was marginal (Fig. X: NO3 content).

**The community alpha diversity was marginally affected by 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. X: Relative abundance bar plot). Drought treatment did not affect the alpha diversity of AOB and AOA (Table SX: alpha diversity statistical analysis). However, we found a significant interaction of *drought* × *cropping system* for Comammox alpha diversity in bulk soil (Table SX: alpha diversity statistical analysis), suggesting that the drought effect was varied depending on the cropping system. Nonetheless, we could not identify the difference between drought and control within sampling date of each cropping system, indicating that the detected effect of drought on alpha diversity was only marginal. Cropping system was also a notable driver of the ammonia-oxidizers alpha diversity, with the average Comammox richness in BIODYN was 50−56.75 % (control) and 42−50 % (drought) higher than that in the two conventional systems. Shannon index of Comammox in the BIODYN system was also higher (control: 31 %, drought: 24−28 %) compared to the conventional cropping systems (Fig. X: alpha diversity, Table SX: alpha diversity statistical analysis). On the contrary, BIODYN had 27.67−31 % (control) and 38−43 % (drought) lower AOB richness, and 10−11 % (control) and 12 % (drought) lower AOB Shannon diversity than that in the CONFYM and CONMIN systems (Fig. X: alpha diversity, Table SX: alpha diversity statistical analysis). Overall, no effect of drought was observed on the alpha diversity of ammonia-oxidizing community in rhizosphere, but cropping system was found to be an important factor shaping the community alpha diversity (Table SX: alpha diversity statistical analysis).

The unconstrained PCoA plots using Bray-Curtis dissimilarity distances showed distinct separation by cropping system 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 of AOB, AOA, and Comammox, respectively (Supplementary Fig. X: unconstrained PCoA plots & PERMANOVA). Meanwhile, the effect of drought was only apparent within block due to a strong block effect (Supplementary Fig. X: unconstrained PCoA plots). To further investigate the effect of drought on the beta diversity of ammonia oxidizers, we performed constrained analysis using CAP. The differences on the community composition between drought and control within each cropping system become more evident (Fig. X: 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. The effect of drought on the 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 (Fig. X: Constrained CAP Plots). 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. The clustering by drought in the Comammox community was also higher in the BIODYN than the other cropping systems, indicated by higher reclassification rate of 89.5 % (drought) and 90 % (control), and 91.7 % (drought) and 100 % (control) in bulk soil and rhizosphere, respectively (Fig. X: Constrained CAP Plots). 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. X: Constrained CAP Plots). To further quantify the impact of drought, we calculated the Euclidean distances between the drought and control treatments based on the discriminant analysis. The response to drought was dependent both on the ammonia-oxidizing community and on the cropping system. Thus, the highest differences in community structure between treatment (drought vs control) were observed for the AOA and Comammox communities within the BIODYN cropping system (Fig. X: The distance boxplot calculated from the discriminant CAP analysis).

**Most of the ammonia-oxidizer ASVs were resistant to 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. We identified ASVs with significant changes in abundances by drought, representing 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 (Fig. X: DAA). 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 %) were decreased by drought, while in AOA and Comammox, the number of ASVs with negative and positive responses were comparable (Table X). We identified 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 (Fig. X: DAA). Eight AOB ASVs (except the ASV 87) assigned to *Nitrosolobus multiformis* and one ASV of *Nitrosomonas communis* exhibiting a decrease in abundance were found in all cropping system, except in CONMIN. On the other hand, there were total ten AOB ASVs in bulk soil and rhizosphere belonging to the genus *Nitrospira* which depleted only in the CONMIN system, but not in the other cropping systems (Fig. X: DAA). Moreover, CONMIN tended to have less ASVs at 16 (AOB), 8 (AOA) and 6.67 % (Comammox) of the total affected ASVs in rhizosphere compared to BIODYN and CONFYM (Table X). For the AOA community in bulk soil, we identified higher number of drought-responsive ASVs in the BIODYN (10 ASVs) and CONFYM (6 ASVs) compared to the CONMIN system (1 ASV). Similarly, in bulk soil, the number of drought-affected Comammox ASVs in the CONMIN system were 50-60 % lower than those in the BIODYN and CONFYM. All of the altered Comammox ASVs in CONMIN system exhibited a decrease in abundance by drought treatment (Fig. X: DAA).

**Drought affects *amoA* gene abundance in bulk soil**

The abundance of ammonia-oxidizing communities measured by *amoA* genes quantification were affected by drought (Table S.X: stat). However, the effects of drought were different depending on the ammonia-oxidizing group and cropping system. Overall, we observed a similar trend of sampling date effect on *amoA* gene abundance of all ammonia-oxidizing groups in bulk soil with lower abundance in the initial sampling (first and second dates), and higher for the remaining sampling dates (July 5th – September 13th) (Fig. X; Table S.X: stat). This trend was explained by a large difference in total number of bacterial communities (16S rRNA gene copies) between those specific sampling dates within all cropping systems, regardless the irrigation treatments (Fig. X; Table S.X: stat).

*AOA and AOB*

We found a significant effect of drought, as well as *drought* × *cropping system* on AOB *amoA* gene abundance, indicating that the abundance of AOB *amoA* gene was influenced not only by drought, but also by cropping system (Table S.X: ANOVA stat). Drought decreased the abundance AOB *amoA* gene in the CONFYM system, with the highest decrease of 38.8 and 24 % relative to the control at the first and last drought periods, respectively (Fig. X). There was no a significant drought effect on AOB abundance within dates in BIODYN and CONMIN, however we observed an overall decrease (except at the first sampling) of AOB abundance in the CONMIN system (Fig. X). A significant interaction of *drought* × *sampling date* was detected on AOA *amoA* gene abundance, although we could not detect the difference between irrigation treatment within date, indicating that the effect was marginal (Fig. X; Table S.X: ANOVA stat). However, there was a strong effect of cropping system, with BIODYN had the highest AOA abundance than in the conventional systems, regardless of the irrigation treatments (Fig. X).

*Comammox A and Comammox B*

Comparably, we detected *drought* × *sampling date* being significant for *amoA* gene abundance of Comammox clade A, but there were no observed differences between control and drought within sampling date (Fig. X; Table S.X: ANOVA stat). On the other hand, drought strongly affected the Comammox clade B abundance, and the effect of drought was modulated by cropping system and sampling date, as indicated by a significant interaction of *drought* × *cropping system* × *sampling date* (Fig. X; Table S.X: ANOVA stat). Overall, there was a decrease on Comammox clade B abundance due to drought in all cropping system with CONFYM system had the highest effect. The changes in Comammox clade B abundance within CONFYM system were observed in almost all sampling dates, with the average decrease by 32 % compared to the control (Fig. X). Meanwhile, in BIODYN and CONMIN, the effect of drought was merely observed in certain sampling dates with the average of 16 and 22 % lower than control, respectively (Fig. X; Table S.X: summary). We also observed that BIODYN system had approximately two times higher abundance of Comammox clade B than that in the conventional systems, regardless of the irrigation treatments (Table S.X: summary).

*amoA/16S rRNA gene ratio*

Drought affected the *amoA* gene abundance within the total microbial community in all bacterial groups of ammonia-oxidizing communities (AOB and Comammox) (Table S.X: LMM stat). There was a decreasing trend in AOB *amoA*/16S rRNA gene abundance under drought in all cropping systems, even though the highest difference was only detected at the first drought and first rewetting sampling in BIODYN and CONFYM, respectively (Fig. X; Table S.X: LMM stat). Similarly, drought altered the abundance of Comammox clade A amoA/16S rRNA gene ratio in BIODYN and CONFYM cropping system (Fig. X; Table S.X: LMM stat). Comammox clade A/16S rRNA gene ratio in BIODYN decreased in response to drought by 37 and 34 % than the control at the first drought and rewetting sampling, respectively (Table S.X: summary). On the contrary, in the CONFYM system, drought increased the Comammox clade A/16S rRNA gene ratio at the first and last sampling by 41 and 43 % than the control, respectively (Table S.X: summary). A significant interaction of *drought* × *cropping system*, as well as *drought* × *cropping system* × *sampling date* was found for the Comammox clade B/16SrRNA gene ratio (Table S.X: LMM stat). The drought effect on Comammox clade B/16S rRNA gene abundance ratio exhibited different magnitudes among cropping systems (Fig. X; Table S.X: LMM stat). While drought decreased the Comammox clade B/16SrRNA gene ratio in all cropping system, BIODYN experienced the highest decrease by an average of 34 % compared to the conventional systems (16 %) (Fig. X; Table S.X: summary).

**Correlation between ammonia oxidizing community, N pools, and soil properties**

Correlations between the diversity and composition of ammonia oxidizing communities with soil properties, including mineral N pools were varied between drought and control treatment (Fig. X). For example, there was a significant positive correlation between soil water content (GWC) with the abundance ammonia-oxidizing groups in the drought, but not in the control treatment. On the other hand, correlation analysis indicated that the abundance of all ammonia-oxidizing groups correlated negatively with soil dry matter (TS) only in the drought treatment. These results supported the previous findings of the observed drought effect on the *amoA* gene abundance. Soil water content also correlated positively with AOA Shannon diversity, while negatively with AOB observed richness under drought treatment. Moreover, we found that *amoA* gene abundance of all ammonia-oxidizing groups correlated negatively with NH4 with different magnitudes between drought and control, with the stronger relationship was found in control treatment. Ammonium content was also a driver for the community alpha and beta diversity, however, the relationships differed by irrigation treatment as well as by ammonia-oxidizing group. Specifically, we found a stronger negative relationship between AOA and Comammox alpha diversity with NH4 content in the control rather than in the drought treatment. On the contrary, NH4 content had a positive correlation with AOB alpha diversity, but it was only observed in the drought treatment (Fig. X; Table SX).

Other notable observation was a positive relationship between NO3 content and the beta diversity and abundance of ammonia-oxidizing community, which only apparent in the control treatment. Nitrate content correlated positively with AOA Shannon diversity and Comammox alpha diversity in the control treatment. Meanwhile, in the drought treatment, NO3 correlated positively with AOB alpha diversity and negatively with Comammox alpha diversity (Fig. X; Table SX). While soil pH, total C, and N primarily connected with *amoA* gene abundances and beta diversity in both treatments, we observed a stronger correlation under drought condition. In general, regardless of the irrigation treatment, the beta diversity of ammonia-oxidizing community was largely associated with their alpha diversity and *amoA* gene abundance (Fig. X; Table SX).