**ABSTRACT**

**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 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.

**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 0.2 mL 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/µL 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. The amplified *amoA* genes were verified by visualizing the PCR products of all samples, including the negative controls, on a 2 % agarose gel. 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.

**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). Whereas, 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 quantification. 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/µL 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. 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. To investigate any PCR inhibition occurrences, we assessed the qPCR cycle-threshold (Ct) values between the samples and controls.

**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 …..