**Ammonia oxidizing archaea and bacteria respond dynamically to drought in rewetted fen peatlands**

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**Abstract**

The impact of drought on ammonia oxidizing microbes in peatlands remains unclear, despite their role as a rate-limiting step in nitrification and increasing drought prevalence. This study aims to identify trends in archaeal (AOA) and bacterial (AOB) ammonia oxidizer abundances and their feedbacks to summer drought in a rewetted percolation (PW) and coastal fen (CW) in northeastern Germany. We used unsupervised clustering to define drought conditions based on water table depth in the field. AOA and AOB abundances are quantified via *amoA* gene and transcript copies with reverse-transcription (RT-) qPCR from *in situ* peat soil bi-monthly between April and February. These results are supported by both metatranscriptomes and clade assignment of AOA amplicons. The magnitude of the nitrifying microbiomes’ drought response correlated to site hydrological stability. Both RT-qPCR and metatranscriptomics showed that PW had an increase in bacterial and archaeal *amoA* transcript abundance during drought. Additionally, there was evidence in the PW metatranscriptome for shifts in soil nitrogen sources, first from a decrease in nitrogen fixation after drought onset, then due to a late-drought increase in assimilatory nitrate reduction to ammonium. The dynamics of soil nitrogen sources in PW could be a biotic mechanism driving the peak of AOA and AOB, as a stable soil water content throughout the drought suggests the soil remained hypoxic despite the lowered water table. In contrast, CW had no significant shifts in either RT-qPCR *amoA* or nitrogen cycling mRNA gene abundances during the drought. There was also higher AOA clade diversity in PW (4 clades across 3 species) compared to CW (exclusively *Ca. Nitrosotaleales* clade Alpha). These results suggest that ammonia oxidizers react significantly to drought, responding to changes in soil nitrogen sources and amplifying shifts in nitrogen cycling gene transcription. As such extreme weather events occur more frequently, they will likely play pivotal roles in rewetted fens’ ecosystem functioning in a changing climate.

1. **Introduction**

Peatlands cover just 3.8% of the Earth’s land surface but are responsible for storing 600 billion tons of carbon [1]. However, this carbon storage function is threatened by the increasing nitrogen eutrophication of soil since the industrial revolution [2]. High volumes of nitrogen in peatland soils competitively disadvantage sphagnum in peatland flora communities, leading to lowered peat formation rates that could cost temperate peatlands 5 g C m⁻²a⁻¹ in carbon sequestration [3]. Further, increased nitrogen loads in degraded peat soils significantly increases nitrous oxide (N₂O) emissions and downstream waterway eutrophication [4, 5]. Nitrifiers are crucial actors in nitrogen cycling; globally, an average of 90% of plant-available nitrogen in soils is mineralized by nitrifying organisms [6]. In peatlands, rates of nitrogen mineralization by nitrifiers have been found to depend highly on both temperature and moisture [7]. Nitrous oxide emission rates in rewetted peatlands are accordingly highly variable and dependent on water table depth below the ground surface, varying from 2.3 - 27.4 kg N ha⁻¹a⁻¹ [4]. Therefore, it is of great interest to study how nitrifying microorganisms respond to changes in water table levels in rewetted peatlands, in order to understand how these changes will impact nitrogen mineralization rates and subsequent N₂O emissions, carbon storage capacity, and waterway eutrophication.

Nitrification is the step of nitrogen cycling which transforms ammonia to nitrate via ammonia oxidation to nitrite and subsequent nitrite oxidation to nitrate [8]. While denitrification is often considered the primary source of N₂O emissions in peatlands, there is also evidence to suggest that ammonia oxidation is a major source of N₂O under intermediate moisture levels [9]. Ammonia oxidation is mediated by both bacteria (AOB) and archaea (AOA) which use ammonia as their sole energy source, as well as complete ammonia oxidizing bacteria (CAOB or comammox) which perform both steps of the nitrification cycle [10]. Ammonia monooxygenase (*amo*) is the key enzyme for oxidizing ammonia to hydroxylamine and has been found to be a functional marker of AOB [11], AOA [12] and CAOB [13]. Therefore, comparisons of archaeal and bacterial *amo* genes (A-*amo* and B-*amo*, respectively) are useful proxies to quantify the presence and function of various ammonia oxidizing microbes in environmental samples. Although enzymatically similar, most AOA have higher substrate affinities than AOB or CAOB, which has been attributed to the higher surface-to-volume ratios of the archaea [14] and results in AOA having a competitive advantage in acidic environments [15–18]. Additionally, AOA were found to have both higher relative abundance [19] and contribution to gross nitrification [20] than AOB under low substrate conditions, but were outcompeted by AOB during ammonia influxes.

Wang et al. (2021)’s analysis of the fen peatlands suggested that seasonal dynamics played a minor role in prokaryotic community composition and functional guild activity (including nitrogen cycling), which was attributed to stable site hydrology [21]. This finding is supported by Berendt & Wrage-Moenning (2023), which found that in the same sites fluctuating water content in peat soils increased N₂O emissions [22]. Accordingly, during the drought of summer 2018, the prokaryotic and eukaryotic microbiomes at the sites displayed significant increases in stress-response functions, indicating that the seasonal dynamics of these fen microbiomes were sensitive to extreme weather changes [23, 24]. Although water table was not correlated with N₂O emissions in these sites, there was a positive correlation between N₂O emissions and soil ammonium concentration; additionally, N₂O fluxes increased after August 2018 during drought conditions [25]. The unclear trends in nitrous oxide emissions throughout the 2018 and 2019 drought cycles indicates the need to analyze the response of nitrogen-cycling microbiomes to these conditions, in order to better understand the links between climactic extremes, microbial communities and greenhouse gas emissions.

Central Europe was subject to extreme water table fluctuations during the drought of 2018, which caused a 112 Gt deficit in landscape water mass and a 50% decrease in ecosystem CO₂ uptake [9, 26]. While there was no comparison for the 2018 drought in paleo-reconstructions dating back to 1500 CE, projections indicate that such events could become normal as early as 2043 [27]. This was immediately confirmed by the subsequent 2019 drought, during which the landscape water deficit across central Europe was an even more extreme 145 Gt [26]. Rewetted peatlands in northeastern Germany experienced disruptions in ecosystem functioning from increased aerobic methanotroph abundance and a corresponding decrease in methane emissions [28], to increased vegetation growth that differentially altered CO2 fluxes across sites depending on respiration and photosynthetic uptake rates [29]. However, there has been no studies to date investigating the impact of these extreme drought cycles on nitrification in central European peatlands, which is vital to understand drought impacts on peatland ecosystem functions from carbon storage to greenhouse gas emissions.

Prior studies have been inconclusive in determining a standard drought response from ammonia oxidizing microbes; further, no studies to date have described such dynamics in rewetted temperate fens. Research across several landscapes indicates an increased abundance of AOB during drought conditions [30–33]. However, trends in AOA abundance during drought is less clear, with evidence for both increasing and decreasing abundance [30, 32–34]. There has been research to support an increase in ammonia oxidizers during drought in alpine peatlands on the Zoige Plateau; however, to date there has been no study differentiating between bacterial and archaeal *amo* in peatland ecosystems [35]. It is unclear how the response of archaeal and bacterial ammonia oxidizers differ under drought conditions in rewetted fens. The vast variation in nitrous oxide emissions across temperate rewetted fens (from 2.3 - 27.4 kg N ha⁻¹a⁻¹) indicates the close relationship between water table depth, soil pore oxygen content, and substrate availability [4]. Therefore, in order to understand the role of rewetted fens in greenhouse gas exchange during increasingly frequent drought events, it is necessary to understand dynamics in ammonia oxidizers as the key facilitators of nitrogen mineralization.

In this study, we addressed the annual seasonal dynamics of soil microbiome compositions in two pairs of drained and rewetted fen peatlands through 16S rRNA amplicon sequencing across the unprecedented 2018 summer drought. We used unsupervised clustering algorithms to neutrally define the drought period during this time based on site hydrological conditions. Specifically, we focused on how ammonia oxidizing microbial dynamics differ under drought conditions in drained and rewetted fens through quantitative analysis of AOA and AOB phylotypes and their *amoA* gene abundances during the 2018 drought cycle. We hypothesize that both AOA and AOB abundances will increase under drought conditions due to aeration in the soil as the water table lowers, facilitating the activities of obligate aerobe ammonia oxidizers. Further, ammonia oxidizing archaea will have a greater drought response than ammonia oxidizing bacteria because of their higher substrate affinity in acidic soils such as those at the fen sites.

1. **Methods**

**2.1 Sample collection**

Soil samples were collected from WETSCAPES project sites between April 2017 and October 2019. The WETSCAPES sampling sites and methods are extensively described in previous publications, and are briefly introduced here [21, 36, 37]. Samples were collected from a rewetted percolation fen (PW) and a rewetted coastal fen (CW) in Mecklenburg-Vorpommern, Germany. PW is in the catchment areas of the rivers Trebel and Recknitz and was deeply drained in the 20th century before rewetting in 1998 as part of an EU-Life initiative [36]. CW was first drained for agricultural purposes in 1850 and rewetted via dike removal in 1993; since then, it has been periodically been flooded by brackish water from the Bay of Greifswald on the Baltic Sea [36]. While CW is currently used for cattle pasture, the PW site is not utilized for agricultural purposes and is managed via biannual mowing [36].

Three peat cores were sampled from each of the sites bi-monthly between April 2018 and February 2019 (April, June, August, October, December and February). Samples were removed from each core at a depth of 05-10 cm, homogenized and stored on ice in the field before long-term storage at -20°C.

Site water levels and soil temperatures were monitored on a continuous basis between September 2017 and February 2020 with Campbell Scientific CR300 Dataloggers (Logan, USA) and HOBO Dataloggers (Bourne, USA), respectively. Additional data on regional precipitation and temperature were accessed on 31.03.2022 from the Deutscher Wetterdienst at weather station 1757 in Greifswald (54.0962, 13.4057). Information on soil moisture and physicochemical measurements is included in the supplementary information.

**2.2 K-means algorithm for drought definition**

Drought periods were neutrally defined by implementing an unsupervised k-means clustering algorithm on water table depths over a two year time period [38]. Water table depths below the ground surface from between September 2017 and February 2020 were separated between the four sites, due to the vast variations in between-site water table depths during this time period (Table 1). Optimal k-values (number of clusters) were identified via the clusGap function (K.max = 10, bootstraps = 500) from *cluster* (v2.1.4) [39]. The optimal Gap statistic (Gapₖ) was identified at k = 2 for each of the sites (values reported in Table 1) [40]. The optimal k was heuristically determined by optimizing the relationship between k and Gapₖ (i.e. at the elbow of the clusGap plot). Subsequently, the water table depth values for each site were clustered via the *kmeans* function in base R (k = 2). The k-means algorithm creates discrete clusters; when k = 2, a dividing value is identified that minimizes within-cluster distribution of the two clusters, and each water table value is assigned only to one group. Once water table depths continuously and consistently fell beneath this drought value, sites were assigned to the drought condition. As such, implementation of the k-means clustering algorithm enabled an unsupervised, hydrologically-defined drought period to be assigned based on site-specific drought thresholds. Because samples were collected bi-monthly, for the purposes of this study June, August and October 2018 were considered to be under hydrologically-defined drought conditions. Further details regarding the k-means algorithm implementation are available in the supplementary information.

Table 1. Values interpreted from k-means clustering algorithm in the CW and PW sites.

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| Site | Mean water table depth (cm) | (k = 2) | Drought threshold (cm) | 2018 drought dates | 2019 drought dates |
| PW | 0.08 | 0.35 | -5.45 | 25.05.2018-11.11.2018 | 24.07.2019-30.09.2019 |
| CW | -17.2 | 0.65 | -30.24 | 03.05.2018- 22.10.2018 | 08.04.2019- 04.10.2019 |

**2.3 Molecular methods**

The total RNA and 16S rRNA extraction protocols and amplicon sequencing, as well as the qPCR protocol for the 16S rRNA gene, have previously been described in detail and are included in the supplementary documentation [21, 36, 41]. The 515YF/B806R primer pair was used to amplify the 16S rRNA for both genome sequencing and qPCR [42]. Genome sequencing of the 16S rRNA gene was performed by LGC Genomics GmbH (Berlin, Germany) with the Illumina MiSeq 300 bp paired-end platform. The sequences were then filtered with the *dada2* pipeline (v1.8.0) in R v3.6.3 (maxEE = 2, truncQ = 2, maxN = 0) [43]. Amplicon sequence variants (ASVs) were assigned and counts were normalized with the metagenomeSeq’s CSS pipeline to combat uneven sampling depths [44]. Taxonomy was assigned against the Silva SSUref-NR\_128 database using the LCA algorithm from Megan5 [45, 46]. The absolute copy numbers of the 16S rRNA gene in each sample is multiplied with the relative abundance of each ASV in the 16S rRNA metagenome to proxy their absolute abundances.

Soil total RNA was extracted using the Rneasy PowerSoil Total RNA Kit (QIAGEN, Hilden, Germany) and libraries were prepared with NEBNext Ultra II RNA Library Prep Kit for Illumina (New England Biolabs, Ipswich, MA, USA). Total RNA was sequenced with a NextSeq 550 system (paired end, 2 x 150 bp) using one NextSeq 500/550 High Output Kit and one NextSeq 500/550 Mid Output Kit v2.5 each for 300 cycles (Illumina, San Diego, CA, USA). After merging the paired-end sequences with FLASH (min. overlap 10 bp), PrinseqLite was used to trim the poly-A/T tails (min. length 15 bp) and filter out sequences with a mean quality score less than 25 [47, 48]. The RNA fractions were categorized with SortMeRNA (v2.1), and SSU rRNA was assigned against the modified Silva 128 database using the LCA algorithm while putative mRNA were assigned to the NC-nr database with DIAMOND [49, 50].

Quantitative PCR of bacterial and archaeal *amo*A genes were performed with the *amoA*-1F/2R [11] and c*amoA*-19F [51, 52] /t*amoA*-629R [53] primer pairs, respectively. The 15 μl reactions included 7.5 μl innuMIX qPCR DSGreen Standard 2x (Analytik Jena), 0.75 μl each of the primer pairs, 5 μl nuclease-free water and 1 μl template diluted to a concentration of 5 ng/μl. Reactions were cycled on a qTOWER³G (Analytik Jena) according to the following protocol: denaturation at 95°C for 2 minutes (archaeal *amo*A) and 5 minutes (bacterial *amo*A); 40 3-step cycles of 30 seconds denaturation at 95°C, 45 seconds annealing at 55°C, and elongation and scanning for 45 seconds at 72°C; and finally melting from 60 to 95°C for 15 seconds with ΔT 1°C. The reactions were quantified based on serial dilution standard curves of 10⁸-10² gene copies for bacterial *amoA* from *Nitrosopira multiformis* [54] and 10⁷-10¹ gene copies for archaeal *amoA* from *Nitrososphaera viennensis* [55]. The mean qPCR correlation coefficients for both the archaeal and bacterial *amoA* reactions were 0.99; the average slopes were -3.60 and -3.44, respectively, for the archaeal and bacterial reactions; and the mean reaction efficiencies were 0.90 and 0.96.

The protocol for reverse transcription qPCR (hereafter RT-qPCR) with the RNA extractions used the same reaction cycles respective to bacterial and archaeal *amoA*, with the addition of an initial 10 minute reverse transcription step at 50°C. The 15 μl reactions again contained 0.75 μl each of the same forward and reverse primers and 5 μl nuclease-free water. For RT-qPCR, the mastermix additionally contained 0.02 μl BSA (20 mg/ml concentration), 1 μl RNA template, and the following components from the iTaq Universal SYBR Green One-Step Kit (BioRad, California, USA): 7.5 μl iTaqSYBRMix and 0.1875 μl iScript reverse transcriptase. To transcribe the standards, 20 ul reactions containing 1 ug template DNA, 1 mM each ATP, GTP, CTP and UTP, 2 ul 10x Transcription Buffer, 40 U T7 RNA Polymerase, 20 U RNase Inhibitor, and up to 20 ul nuclease-free water were incubated at 37°C for 2 hours. Then, the reaction was stopped by adding 2 ul EDTA and 2 ul lithium chloride and gently mixing before adding 75 ul 75% ethanol and incubating at -80°C for 30 minutes. The standards were centrifuged at maximum speed for 15 minutes at 4°C before discarding the supernatant and washing the pellet with 100 ul of 100% ethanol. The ethanol was then removed and the RNA pellet was left to dry on ice for 5 minutes before being resuspended in 20-50 ul nuclease-free water. Finally, the remaining DNA in the standards was digested with the DNase I kit (Zymo Research, California, USA). The RT-qPCR protocol for AOA resulted in an efficiency of 0.93 and slope of -3.52 (R² = 0.999); the results of the AOB protocol were an efficiency of 0.88 and slope of -3.65 (R² = 0.999).

**2.4 Biostatistics**

All statistics were conducted in R (v4.2.2) [56]. Non-metric multidimensional scaling (NMDS) of the resulting absolute abundances of each ASV were calculated with the *vegan* package (v2.6-4) [57]. The resulting group dispersal was verified with vegan’s Betadisper function (ANOVA method), and factor effects were tested with *adonis2* in *vegan* package (distance method ‘Bray’).

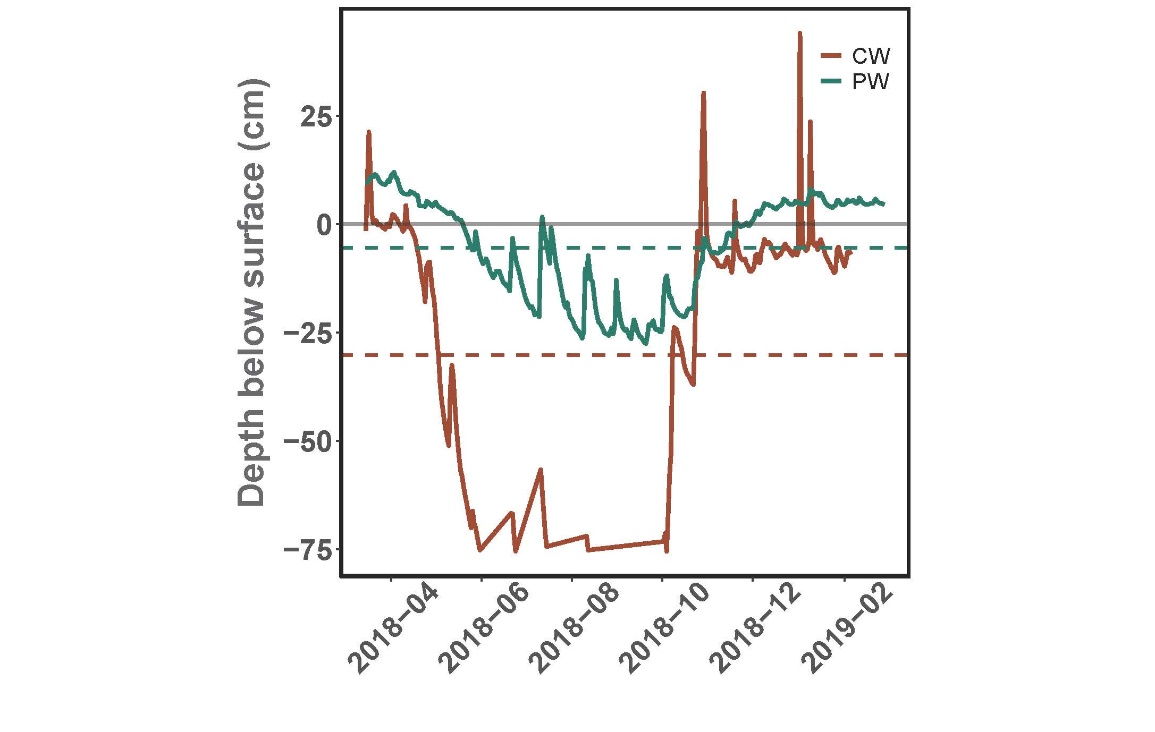
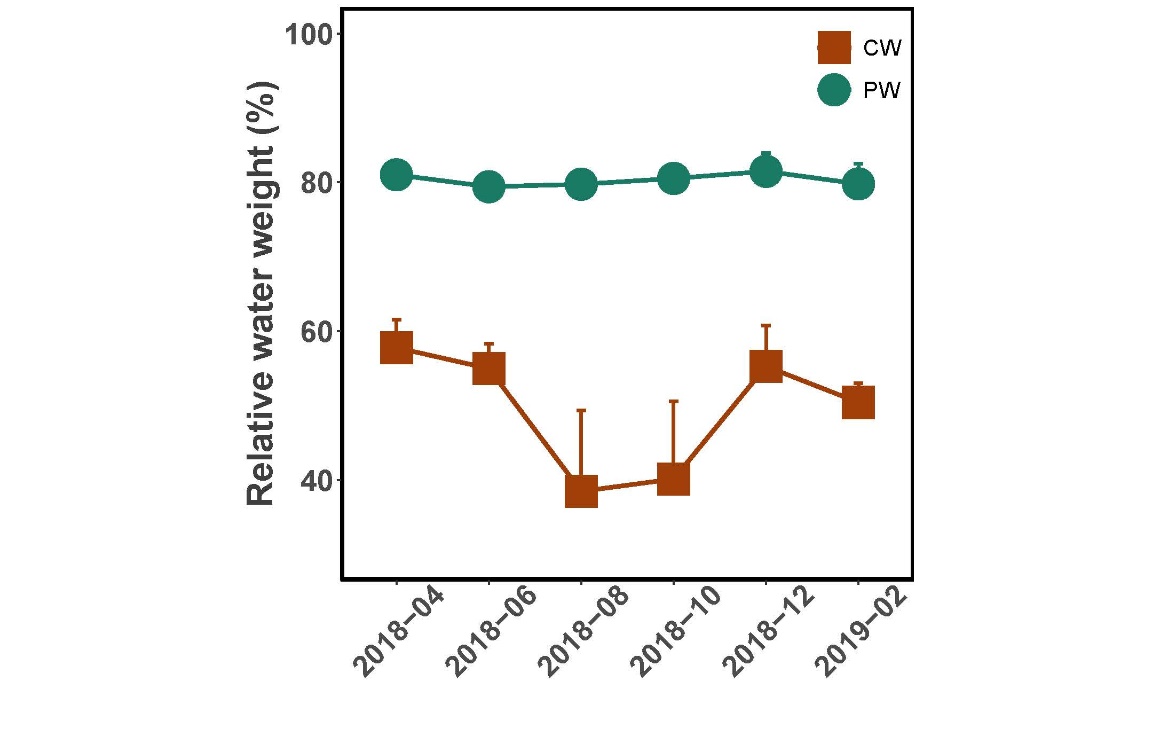
Due to the random sampling design, each time point at each site is treated as an independent sample with three replicates for statistical purposes. Therefore, comparisons in gene (via qPCR) and nutrient abundances between different time points were primarily calculated via the Kruskal-Wallis rank sum test (with a Bonferroni adjustment when k > 2) [58] in base R with a post-hoc Dunn’s test of multiple comparisons in *rstatix* (v0.7.2) [59, 60]. When the data met the standards of normality and equal variance (via Shapiro-Wilk [61] and Levene’s [62] tests via *rstatix*) were satisfied, statistics were analyzed with ANOVA in base R and a post-hoc Tukey HSD test [63, 64]. Visualizations (with the exception of the phylogenetic tree) were created in *ggplot2* (v3.4.1) [65].

**2.5 Phylogenetic tree construction**

To construct the phylogenetic tree of ammonia oxidizing archaea (AOA), potential AOA ASVs were initially identified from the larger 16S rRNA sequence dataset using the Faprotax database aerobic ammonia oxidation functional classification identified via the *microeco* (v0.14.1) wrapper in R, and filtered for sequences in the class Nitrososphaeria [66, 67]. These reads were then confirmed against the BLASTn database identity threshold of 93% [68]. To further verify the functional identity of these reads within AOA, a phylogenetic tree was constructed using a database linking 16S rRNA gene classifications to amoA taxonomy [41]. First, the BLASTn-curated 16S rRNA sequences were merged with the amoA database and aligned with MAAFT (v7) [69]. Then, the aligned sequences were trimmed to the 16S sequence bp length in AliView [70]. The phylogenetic tree was then constructed via IQTree [71] (v1.6.12) using the TIM3e+G64 model (BIC = 15778.75, identified via ModelFinder [72]) with 1000 ultrafast bootstraps via UFBoot2 [73] to create a maximum likelihood tree. This tree was then visualized using the iTOL platform [74].

1. **Results**

**3.1 Defining drought periods**

Drought periods in the region were defined via an unsupervised k-means clustering algorithm using site hydrological characteristics to the following periods: May to November 2018 and April to October 2019. During these drought periods, across all sites there was an average decrease in water table depth of 45.57 cm (p < 0.0001, ANOVA with post-hoc Tukey’s Range Test). Within individual sites, the average water table decreases were 23.85 cm in PW and 48.59 cm in CW (p < 0.0001).

b)

a)

*Figure 1a.* Time series of daily average water table depth below the surface between 2018-03-15 and 2019-02-28. Dashed lines indicate the drought threshold values for each site as calculated by the k-means clustering algorithm (-5.45 cm for PW and -30.24 cm for CW).

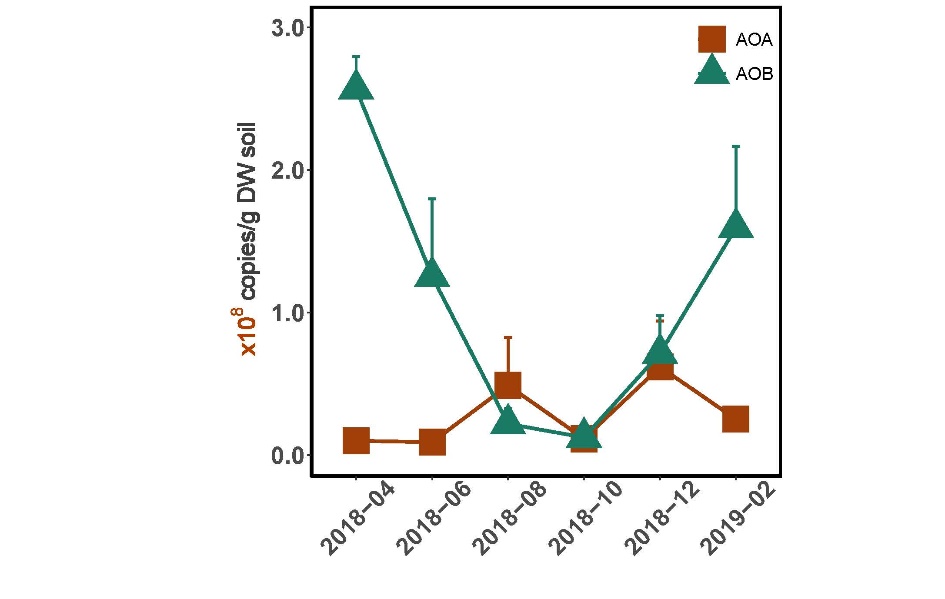
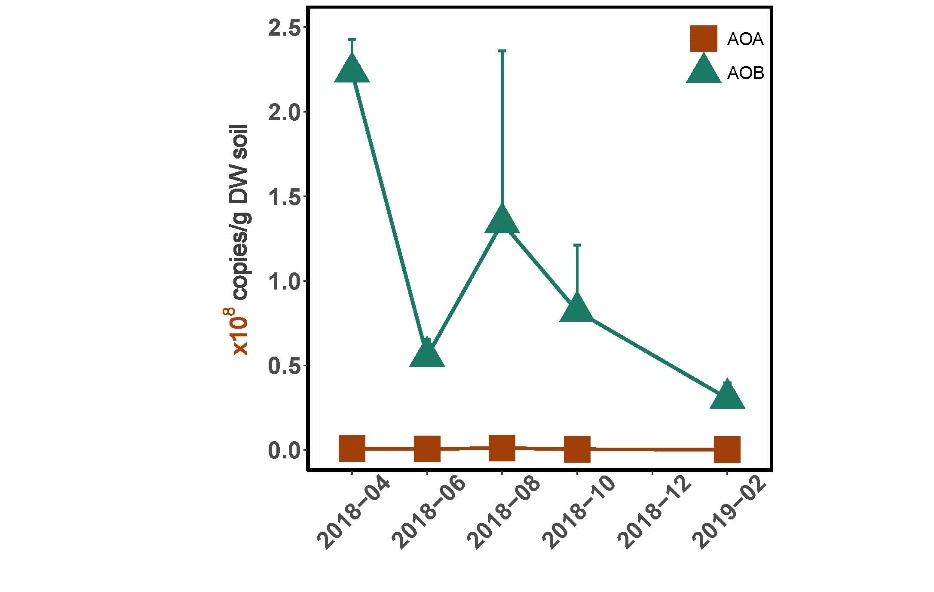
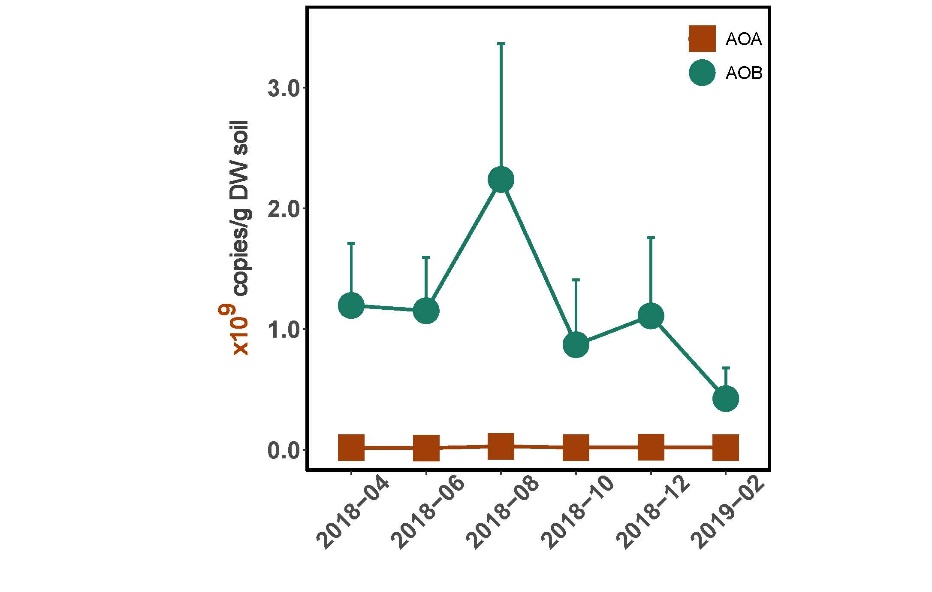
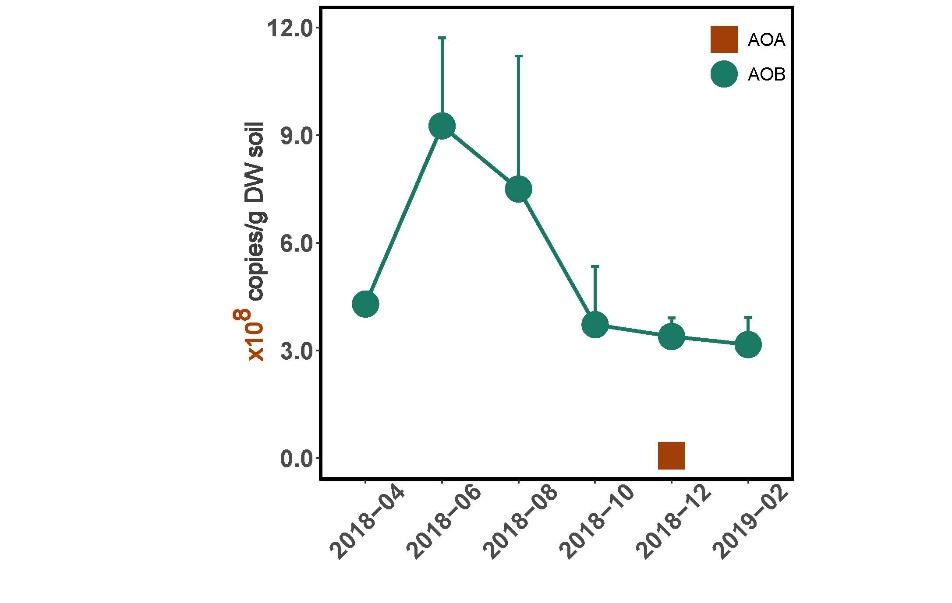
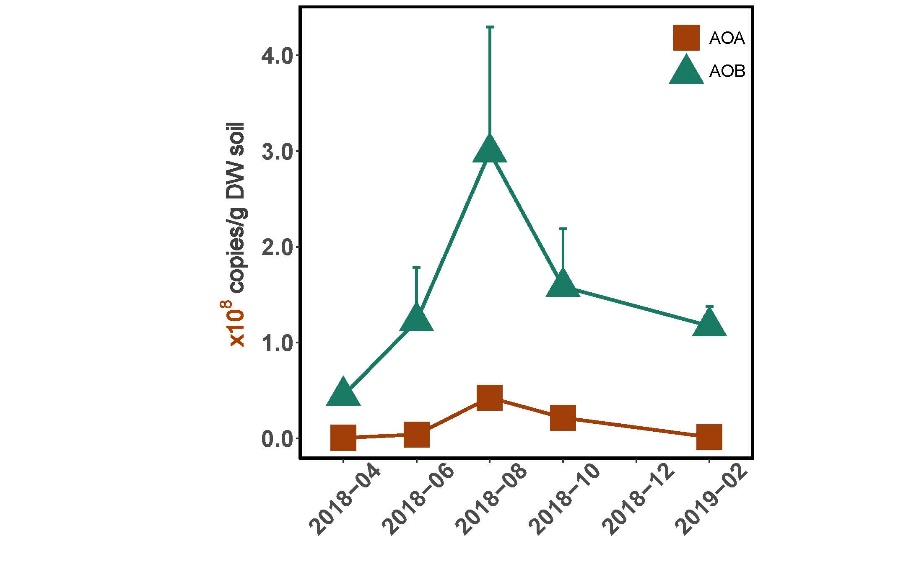
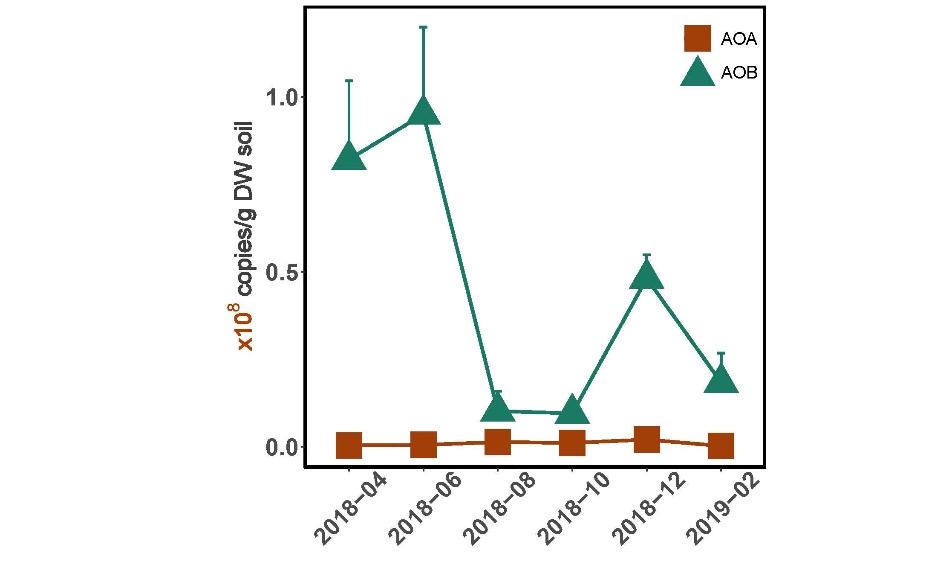
*Figure 1b.* Average soil water content expressed as percentage of weight in the PW and CW sites. Averages were calculated across all subsamples for the sampling period (n = 9) and are included with their respective standard errors.

Drought thresholds were defined by the site-specific lowermost water table value that clustered to the ‘normal’ hydrological conditions. For PW this value was -5.45 cm and for CW -30.24 cm (Fig. 1a).

In spite of the drastic water table depression in both sites, the water content in the PW topsoil remained stable, with an average of 80.32% of the soil weight from water (Fig. 1b). In contrast, there was a decrease in topsoil water content in the CW site, though this was insignificant (Kruskal-Wallis). Overall, the average water content in CW ranged from 25.80% to 64.30% of soil weight.

**3.2 Absolute abundance of AOA and AOB**

To investigate the temporal variability and impact of drought on ammonia oxidizing microbes in fen peatlands, both archaeal and bacterial ammonia oxidizers were considered. Although a third group known as complete ammonia oxidizing bacteria (comammox) within the *Nitrospira* genus has been shown to play a role in ammonia oxidation by fully converting ammonia to nitrate [75, 76], their presence was negligible in the sequencing data for these sites. Only 10 *Nitrospira* ASVs in the genomic dataset between April 2018 and February 2019 had over a 97.5% identification match when compared against 7 known comammox genomic sequences via nucleotide BLAST [77]. Of these, 6 ASVs were present in the PW site and 1 in the CW site. The potential comammox ASVs did not display significant temporal variation in either site (Kruskal-



RT-qPCR of *amoA* gene

*Figure 2.*

PW

CW

e)

qPCR of *amoA* gene

16S rRNA genomes

b)

a)

c)

d)

f)

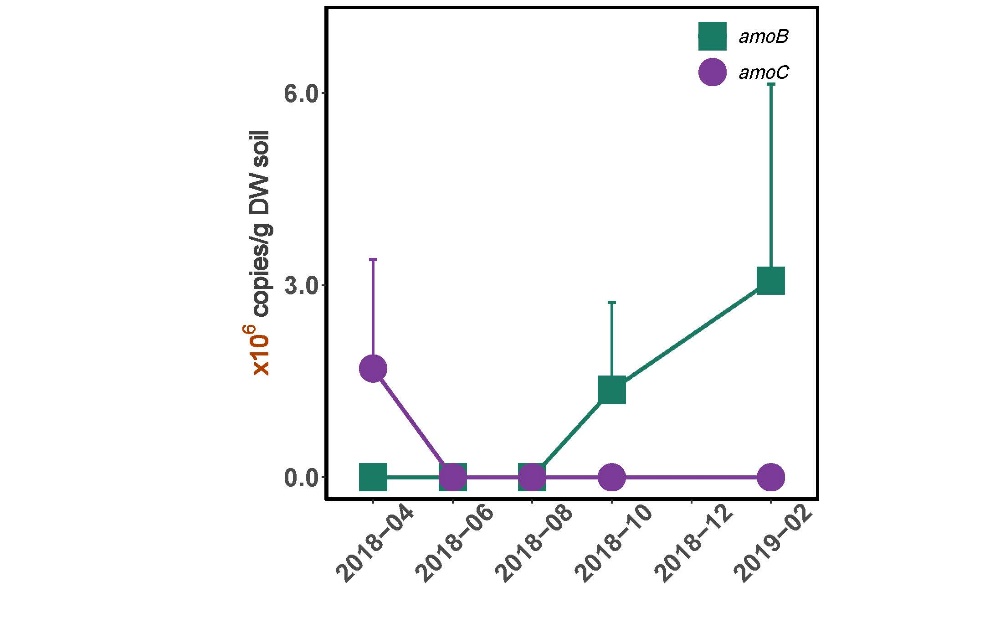
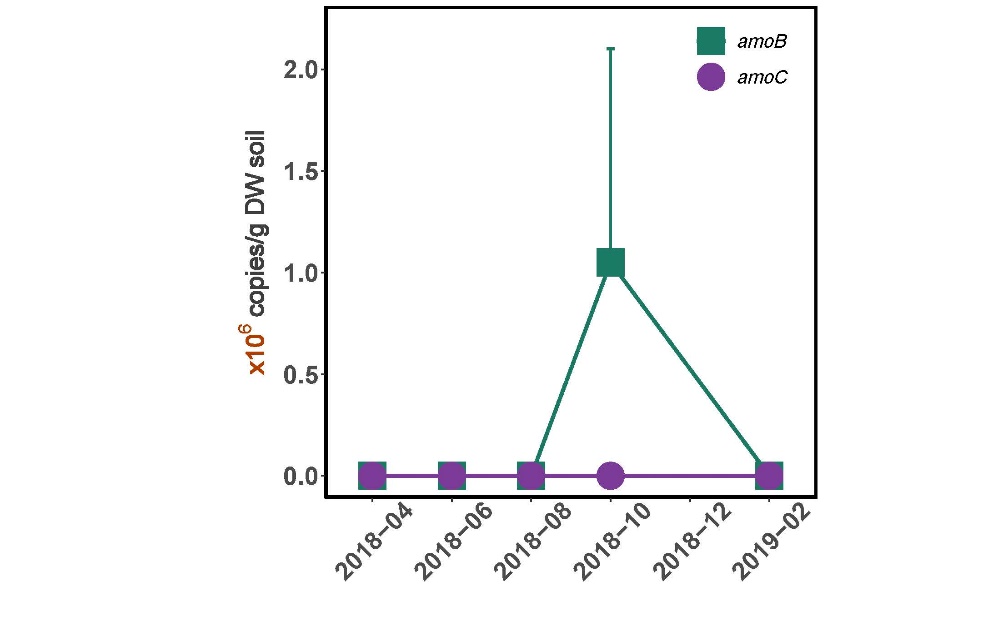
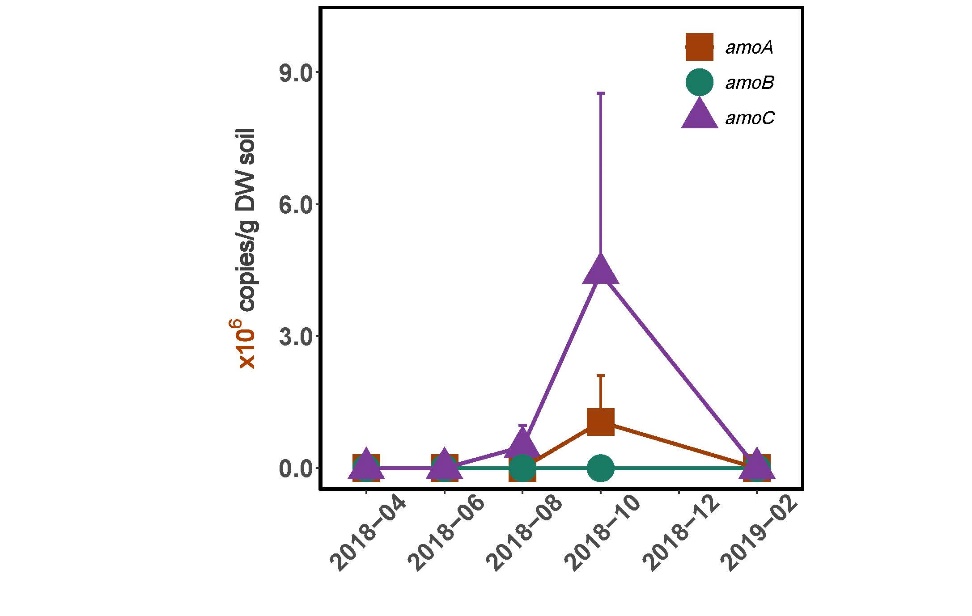
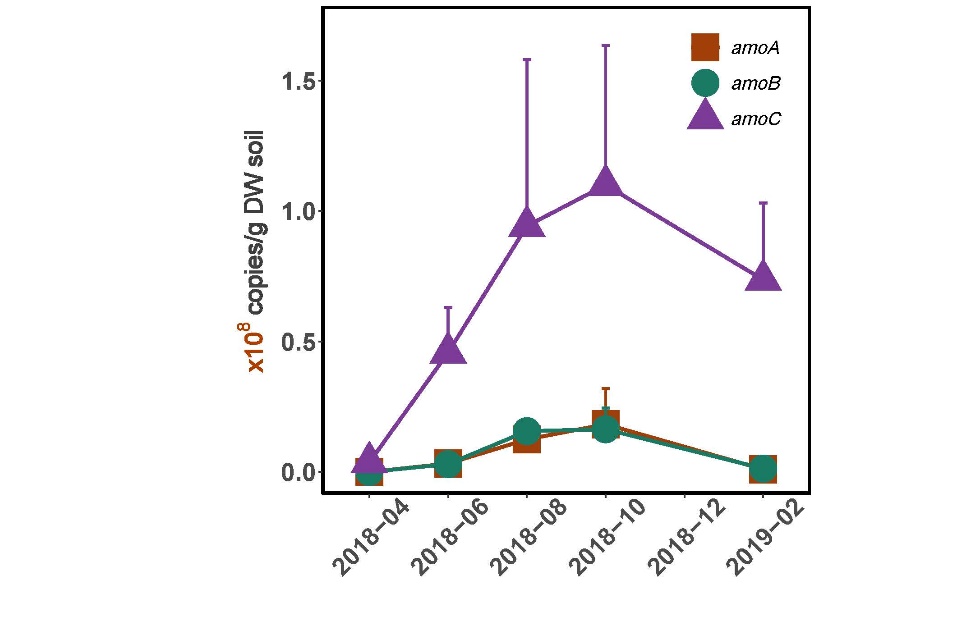
Wallis). Therefore, we considered the impact of potential comammox bacteria negligible on our central question of the impact of drought dynamics on ammonia oxidation in fen peatlands.

*Figures 2a,b.* Relative abundance of AOA- and AOB-identified 16S rRNA amplicon sequences, multiplied by copies of the 16S rRNA gene per gram dry weight soil (as determined via qPCR) to proxy the absolute copy number of AOA and AOB 16S rRNA amplicons per gram of dry weight soil. Mean values and standard errors are included for each time point (n=3).

*Figures 2c,d.* qPCR of the bacterial and archaeal *amoA* gene in both sites, represented in *amoA* copies per gram dry weight of soil, with mean values and standard errors (n=3).

*Figures 2e,f.* RT-qPCR of transcribed bacterial and archaeal *amoA* genes in both sites, with mean transcribed *amoA* copies per gram dry weight soil (n=3) and standard errors.

An initial investigation of AOA and AOB abundances was conducted using 16S rRNA amplicon sequences (Fig. 2a,b). Both AOA and AOB were significantly dynamic in PW throughout the drought cycle, with AOA peaking in August and decreasing after the drought end in December (KW, p<0.05). Additionally, AOB had higher copy numbers in June and August and a decrease in abundance by October (p < 0.01). Similarly, in CW AOB peaked in June before decreasing in October (p<0.005), albeit at abundances an order of magnitude lower than in PW. AOA was only identified in December at the CW site.

Quantification of AOA and AOB was implemented with both DNA-based quantitative PCR (qPCR) and RNA-based reverse-transcription qPCR (RT-qPCR) targeting *amoA*. Based on DNA, temporal dynamics of AOA abundances were insignificant in both sites (Kruskal-Wallis). Further, a comparison of drought AOA abundances compared to non-drought abundances were significant only in CW (Dunn, k = 2, p = 0.047). In contrast, AOB abundances displayed temporal variability in both CW and PW. PW displayed a decrease in AOB abundances during the drought period (p = 0.024), as well as sensitivity to temporal variability between sampling points (p = 0.02). Within this time span, there was a decrease in AOB abundance between April and October 2018 (Dunn with Bonferroni, p = 0.043). Finally, while AOB in CW demonstrated significant temporal variability throughout the sampling period (p = 0.019), there was no meaningful difference in the AOB abundance between drought and non-drought periods.

*Figures 3a,b.* Abundances of archaeal *amoABC* in the metatranscriptomes of 16S mRNA transcripts in both PW (a) and CW (b). Mean values of the transcript copy number per gram dry weight soil and standard errors thereof are included, and n=3 for each time point.

*Figures 3c,d.* Abundances of bacterial *amoABC* in the metatranscriptomes of 16S mRNA transcripts; bacterial *amoA* transcripts *were* not detected in either site. Each time point is represented with the mean transcript copy number per gram dry weight soil (n=3) and standard error.

d)

c)

b)

a)

AOB 16S mRNA

AOA 16S mRNA

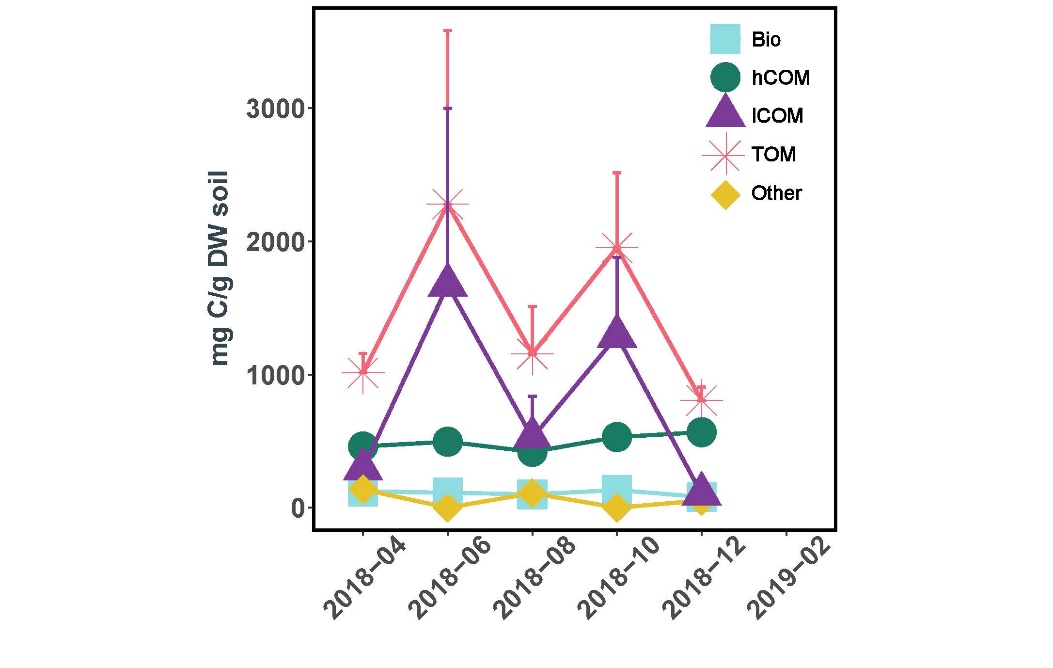
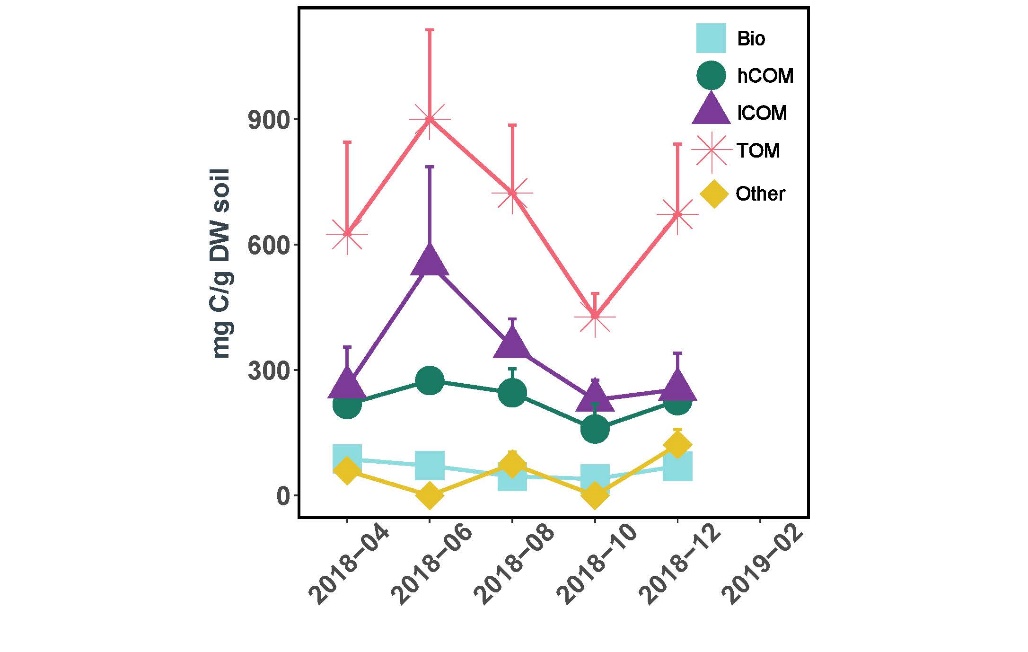
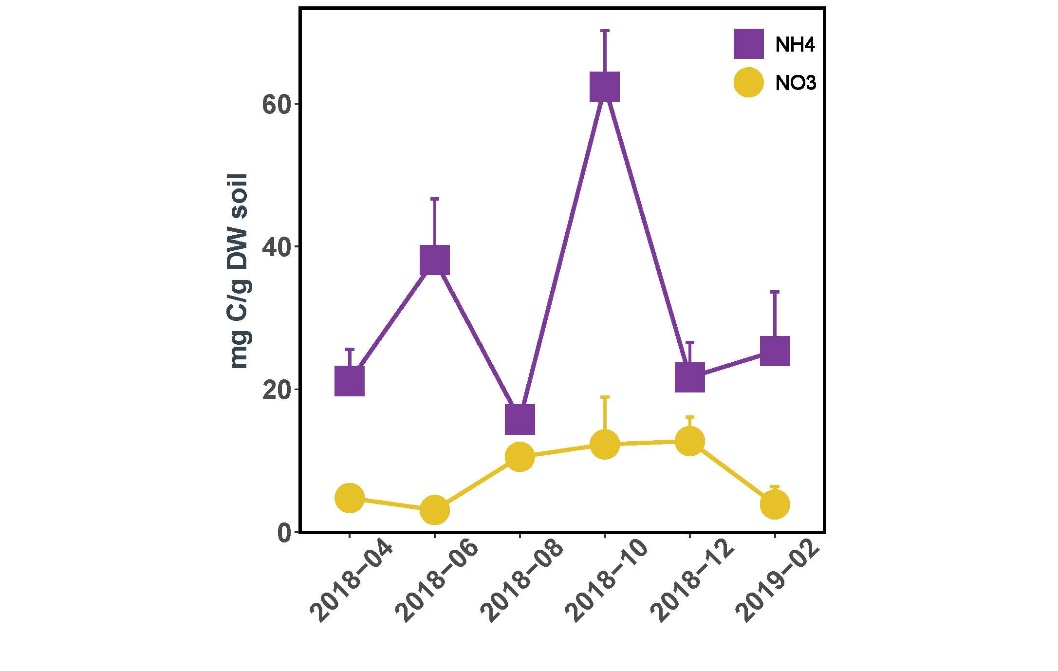
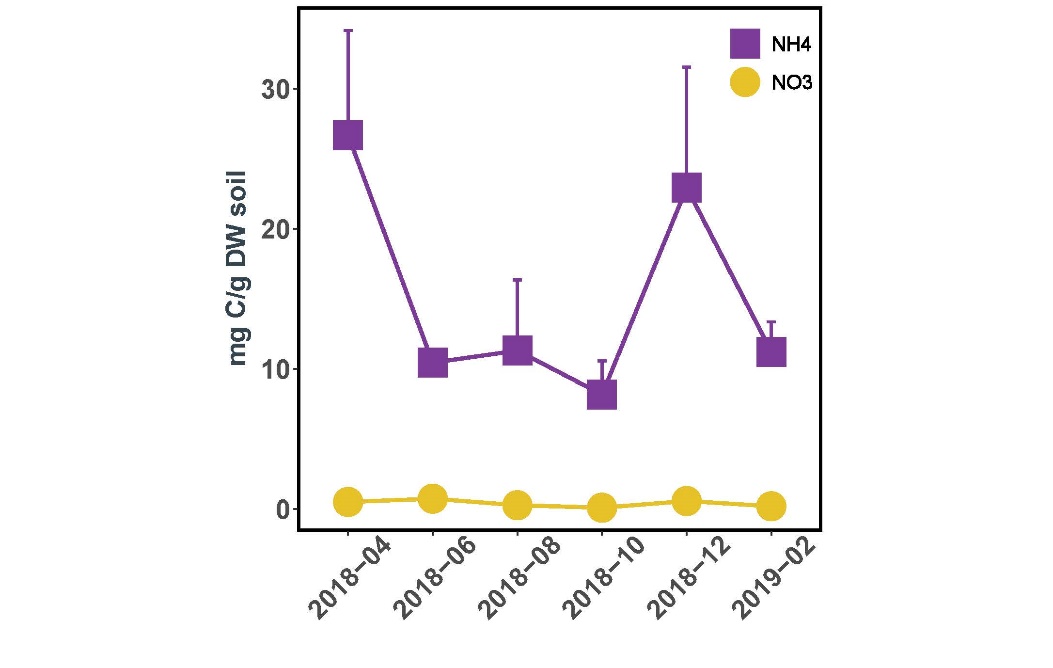
PW

CW

Interestingly, the RNA-based RT-qPCR method demonstrated divergent trends in AOA and AOB abundance when compared to the DNA-based qPCR results. RT-qPCR analysis showed an increase in abundance of PW AOB between April and August 2018 (p = 0.047), and a nearly-significant increase during drought periods (p = 0.059) (Fig. 2B). Similarly, AOA in PW also increased in abundance between April and August (p = 0.026), in addition to an increase during drought periods (p = 0.018). In contrast, CW samples displayed no significant temporal variability in either AOA or AOB abundances (Fig. 2B). Further, drought was not a corollary for either AOA or AOB in the CW site.

No subunit of archaeal or bacterial *amoABC* in the 16S mRNA metatranscriptomes varied over the drought period in either site (Figs. 3a-d). However, the PW abundances of archaeal *amoABC* were two orders of magnitude higher than that of the CW site (KW, p<0.0001). Interestingly, there was no bacterial *amoA* identified in either site. Bacterial *amoBC* was only identified in October in CW (Fig. 3d). In PW, bacterial *amoBC* was identified in half of the time points but was two orders of magnitude lower in abundance than archaeal *amoABC* (p<0.0001), in stark contrast to the other ammonia oxidation proxies previously discussed.

In addition to the quantification of archaeal and bacterial ammonia oxidation proxies, the samples were analyzed for soil dissolved organic carbon (DOC), as well as ammonium (NH₄⁺) and nitrate (NO₃⁻) volumes (Fig. 4). While no fraction of DOC was variable over the 2018 drought cycle, PW had a higher volume of organic matter than the CW site (Fig.4a-b, KW p<0.0001). Ammonium was dynamic in the PW site (ANOVA, p = 0.012), with a peak in October that was higher than other months (TukeyHSD, p < 0.05). Nitrate was not significantly dynamic across any time points in the PW site. The CW site displayed an opposing trend, with no meaningful shifts in ammonium content across the study period, but variation in nitrate loads (Kruskal-Wallis, p = 0.033). Both sites had a higher volume of ammonium than nitrate, and PW had both a higher nitrate and ammonium content than CW (KW, p < 0.01).



a)

*Figures 4a,b:* The dissolved organic carbon content of the topsoil samples (depth 5-10 cm) at the PW and CW sites. Values are averaged at each time points (n=3) and each have standard error bars. The fractions of soil carbon in milligrams of carbon per gram dry weight soil are as follows: biopolymers (Bio), humic substances (hCOM), low-weight molecular substances (lCOM), total dissolved organic carbon (TOM) and other (remaining TOM values after Bio, hCOM and lCOM were removed).

*Figures 4c,d:* The ammonium (NH₄⁺) and nitrate (NO₃⁻) nutrient contents in the topsoil samples (5-10 cm depth) at the PW and CW sites. Values represented are averages across subsamples with standard error bars (n = 3). The nutrient volumes are measured as milligrams of nitrogen per gram of dry weight soil.

d)

c)

Ammonium and Nitrate

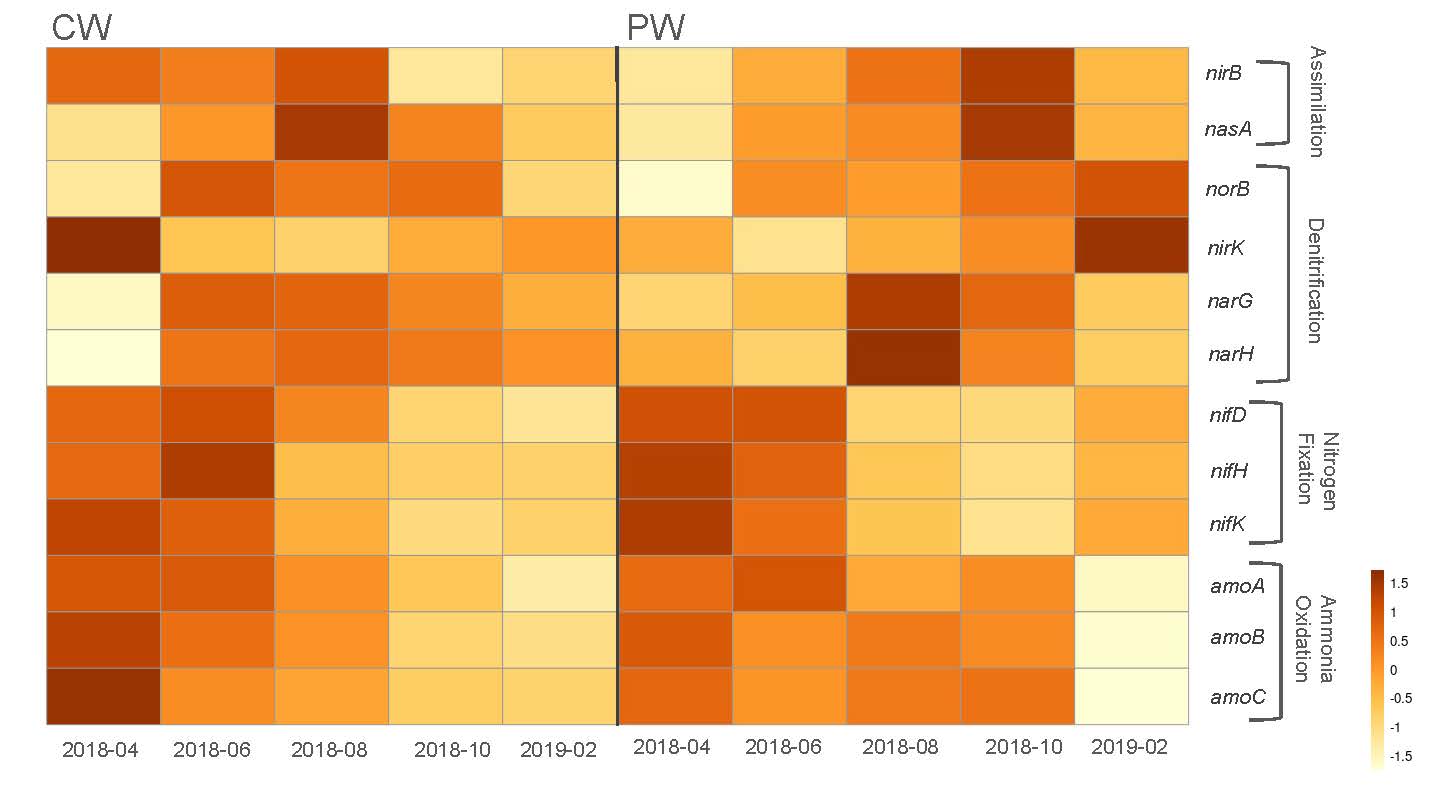
Dissolved Organic Carbon

PW

CW

b)

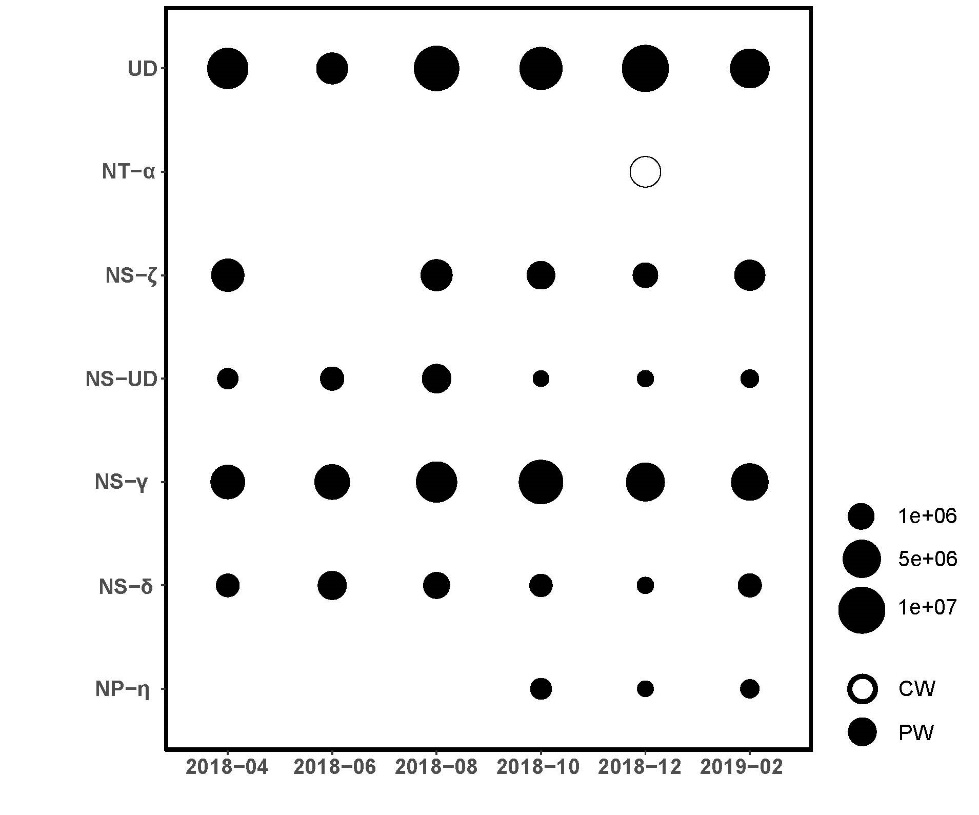
**3.3 Changes in nitrogen-cycling gene transcript abundances**

There was significant fluctuations in gene transcript abundance over the drought period for nitrogen fixation, nitrogen assimilation and ammonia oxidation in the PW site, as indicated by KEGG functional group assignment based on the metatranscriptome data Fig. 5). Nitrogen-assimilation indicator *nirB* was variable over time with a peak in October as compared to April (ANOVA p = 0.046, Tukey p = 0.038). A similar trend was evident in the related *nasA* gene for assimilatory nitrate/nitrite reduction to ammonium (ANRA) (Kruskal-Wallis p = 0.044, Dunn p = 0.040). Both *nifH* and *nifK* nitrogen fixation marker genes were dynamic over the drought cycle (Kruskal-Wallis, p = 0.002 and p =0.011), with a peak in April and decrease to a minimum in October. Of the ammonia oxidation-indicator genes in the KEGG database (*amoABC*), only *amoA* was significantly dynamic (ANOVA, p = 0.0006) with a peak in June as compared to February (Tukey p = 0.042). No denitrification-indicator genes varied meaningfully over time, though there was a slight increase in *narGH* in the August samples.

*Figure 5.* A heatmap of nitrogen-cycling functional group abundance (mRNA transcripts per gram dry weight soil) across the 2018 drought cycle in the CW and PW sites. Functional genes were assigned via the KEGG database, and all genes are standardized by site, with n=3 per time point. Red values indicate higher than average within-site gene transcript abundances across the time series, while yellow values indicate lower than average values.

In comparison to PW, CW had lower abundances on the scale of an order of magnitude across all marker genes (Kruskal-Wallis, p ≤ 0.01). Denitrification activity was also higher in PW than CW (p < 0.001), although the transcription rates were not dynamic in either site across the drought cycle. Of the analyzed marker genes, only the nitrogen-fixation associated *nifK* fluctuated meaningfully in CW between April 2018 and February 2019 (p = 0.035), with evidence for a slight decrease in August. While *nifH* also showed a slight decrease in August, none of the variation over time in this gene marker was significant.

**3.4** **AOA Phylogeny**

Based on the phylogenetic congruency between 16S rRNA and *amoA* gene in archaeal genomes, we could assign *amoA*-defined clades to their corresponding 16S rRNA gene counterparts (Wang et al., 2021). The *amoA*-defined clades provide a higher resolution on AOA classification and thus a better understanding on their functional diversity. Clades were assigned based on affinity to phylogenetic tree regions based on known AOA clades in the *amoA* database (Supplement Fig. 4) [41]. ASVs with ambiguous locations (i.e. between clades) were labeled as undefined (UD). Hereafter, *Ca.* Nitrosotaleales is denoted as NT, *Nitrosopumilales* as NP, *Ca.* Nitrosocaldales as NC and *Nitrosophaerales* as NS. PW had a higher overall diversity in AOA clades than CW (Fig. 4). The CW metagenome contained only one clade at one time point (NT*-*α in December 2018). In contrast, the PW metagenome contained AOA-identified ASVs across all time points, including taxonomic units assigned to NP (NP-η) and NS(NS-δ, NS-γ, NS-ζ and NS-UD), as well as additional unidentified AOA ASVs. There were no ASVs assigned to NC in either site. All AOA clade absolute abundances were stable over time (Kruskal-Wallis), with the exception of NT-α (only present in CW in December) and NP-η (which first appeared in October). The most prominent clades identified in the PW site were NS-γ, which showed a slight increase during the drought period.

*Figure 6.* Average absolute abundance of AOA clades at each location throughout the 2018 drought cycle. Absolute abundance of the respective clades for each site and time point is from 16S rRNA metagenome OTUs (i.e. relative abundance) multiplied by the total DNA copies per gram dry weight soil to calculate absolute abundance. Clade assignments are from the phylogenetic tree constructed with the *amoA* database described in Wang et al. (2021). UD indicates undetermined AOA taxonomy, NT is Ca. *Nitrosotaleales*, NS is *Nitrososphaerales* and NP is *Nitrosopumilales*.

1. **Discussion and Conclusion**

In this study, we investigated the temporal dynamics of ammonia oxidizing bacteria (AOB) and archaea (AOA) in response to extreme summer drought in two restored temperate fens. First, we used an unsupervised k-means clustering algorithm across 3 years of water table data to identify when the fens were under drought conditions. We then quantitatively compared abundances of bacterial and archaeal *amoA* across six time points during the 2018 drought cycle using both qPCR and RT-qPCR to investigate both presence and activity. This was supported by both amplicon and metatranscriptome sequencing of the 16S rRNA and mRNA regions, respectively. Microbial ammonia oxidation across all proxies was higher in the PW site than in CW. While temporal dynamics varied between proxies and sites, RT-qPCR of *amoA* revealed a significant increase of transcription activity from both bacteria and archaea in the PW site. This result was consistent with our hypothesis that obligate aerobes AOA and AOB would increase in abundance as water tables fell in response to drought, exposing the typically submerged fen topsoil to oxygen. Across most proxies, AOB abundance far exceeded that of AOA, contrasting our expectation that AOA would respond more strongly to drought-driven soil oxidation due to its higher substrate affinity. Additionally, while a phylogenetic tree of AOA 16S rRNA amplicons against an *amoA* database revealed cladic diversity across three phylums of AOA in PW, no clade demonstrated a strong drought response.

***4.1 Applying K-means clustering to define drought periods***

The unsupervised clustering method resulted in defining regionally-specific drought periods (May-November 2018 and April-October 2019) that largely coincided with previously published drought spans [9, 26, 27, 29]. Although ‘drought’ is often synonymous with ‘summer drought,’ this is not the case for events such as those in 2018 and 2019 where drought conditions extended into late autumn. Therefore, incorporating annual data rather than just spring and summer sampling points is necessary to capture the full scope of the drought cycle. While remote sensing methods of drought detection are better able to provide large-scale hydrological analysis [78, 79], applying k-mean clustering to *in situ* water table measurements has a comparable cost benefit on a regional scale while stillproviding a meaningful overview of site-specific drought resilience and recovery.

Further, this method allows direct comparison between multiple sites within a region via the drought threshold metric, which could be useful to assess their resistance to drought conditions. In the case of this study, the PW site has the highest water table drought threshold at 5.45 cm below the surface. This suggests that the PW site is more hydrologically robust, as a stable water table is desirable for maintaining mire landscapes [80]. In comparison, the CW site had a drought threshold of 30.24 cm below the surface. Correspondingly, while PW had an average water table drop of 23.85 cm and maintained a stable water content of 80.32%, CW’s water content fluctuated between 25.80 and 64.30% and had a water table decrease of 48.59 cm. This suggests that CW is insufficiently hydrologically connected to the surrounding watershed to be resistant against drought-forced drying and maintain a stable soil water content, which is a desirable condition for peat formation [81]. The identification of a drought threshold over multiple drought periods also provides the opportunity for identifying future droughts, indicating when sites experience a shift in hydrological states.

***4.2 Microbial ammonia oxidation proxies vary in reliability***

Ammonia oxidation was quantified via methods with both RNA- and DNA-based indicators, and proxy abundances and dynamics varied greatly across methods. The RT-qPCR results best fit our first hypothesis that drought-driven soil oxidation would lead to an increase in ammonia oxidation activity, with the RT-qPCR results best fitting our first hypothesis that drought-driven soil oxidation would lead to an increase in ammonia oxidation activity, with *amoA* transcript copy numbers increasing (particularly in PW) for both AOA and AOB. While the 16S rRNA sequence analysis method also displayed a similar temporal variation with a peak in AOB-identified genomes in August for both PW and CW, the abundances were an order of magnitude higher (on the scale of 10⁹) compared to all other proxies. It is well-established that molecular DNA abundance estimations are often inflated by relic and necromass biomolecules persisting in the soil environment [82, 83]. This inconsistency also had an evident impact on the DNA-based qPCR analysis of *amoA*, with high pre-drought abundances decreasing after drought onset in an opposite trend to the results of all other methods. Drought-driven soil oxidation likely increased the turnover of microbial necromass, causing the decrease in detected *amoA* copies with the qPCR method as fewer remnant biomolecules persisted in the soil. Further, variation in ammonia oxidation proxies from the 16S rRNA amplicon sequencing and qPCR do not necessarily correspond to shifts in transcription rates [84]. We attempted to limit the influence of sample compositionality by scaling relative amplicon abundance with copy numbers of the 16S rRNA region for each sample [85]. In spite of these efforts, it was clear that the DNA-based proxies were less reliable than RNA-based approaches to evaluate in situ temporal dynamics due to the instability of environmental factors influencing degradation rates.

It is therefore more meaningful to use RNA-based methods to study the temporal dynamics of ammonia oxidation functional groups in response to drought. However, the RT-qPCR of *amoA* transcripts and metatranscriptome of the 16S mRNA region had contrasting results, with bacterial *amoA* vastly outnumbering archaeal *amoA* in the RT-qPCR results but no bacterial *amoA* identified at all in the metatranscriptomes. Further, although *amoA* from both bacteria and archaea increased in the RT-qPCR results during the drought, both proxies were stable in the metatranscriptomes during the same time period. This is likely due to the low resolution of the KEGG database in identifying bacterial *amoABC* copies, likely due to their sequence similarity and taxonomic proximity to methanotrophic *pmoAB* genes [86]. Although RT-qPCR copies of archaeal *amoA* still significantly increased during the drought period in the PW site, the shift was much less drastic than in the more-abundant bacterial *amoA*. Therefore, it is likely that the inability to identify AOB SSUs in both the June and August samples subsequently decreased the resolution of metatranscriptomic *amoABC* analysis. Further improvement of database specificity to differentiate between bacterial *amo* and *pmo* could decrease the discrepancies between quantitative and metatranscriptomic methods in analyzing *amo* fluxes.

***4.3 amoA transcription increases during drought in fens***

In comparison to the April pre-drought system, by the middle of the drought in August there was a significant increase in both AOB and AOA transcription in the PW site, as evidenced by the results of the RT-qPCR. It is likely that both AOB and AOA responded positively to the influx of oxygen into the peatland topsoil as the water table fell, as both groups are obligate aerobes [52, 54, 55]. In both sites, bacterial *amoA* was transcribed at an abundance order of magnitude more than archaeal *amoA* (10⁸ vs. 10⁷ copies gˉ¹ DW soil). Both sites have a high ammonium supply (as per Ruetting et al. 2021 [20]), and the decrease of ammonium in August in PW corresponds to the peak of *amoA* transcription. AOB has been found to outnumber AOA in ammonium-rich environments, which could be explained either by a niche preference due to increased cell maintenance requirements of AOB, or a competitive advantage of AOB over AOA [20, 87]. The dominance of AOB in these rewetted fens is notable, as AOB produces the greenhouse gas N₂O at much higher rates than AOA during ammonia oxidation due to converting the intermediary product hydroxylamine or nitrifier-denitrification [88]. Therefore, the drought-period increase of AOB in fens implies a risk for higher N₂O emissions. Correspondingly, there was an increase in N₂O release in the PW site after August 2018, which the increased bacterial *amoA* transcription could have contributed to [25].

The ammonium and nitrate loads in the PW site support the observed increase in *amoA*, with a decrease in NH₄⁺ and increase in NO₃⁻ in August indicating higher nitrification activity. Further, in PW there was an increase in genes from the KEGG peptidoglycan degradation and biosynthesis pathway between April and June/August (ANOVA with TukeyHSD, p < 0.05). This indicates an increase in cell turnover via an increase in cell wall degradation enzymes [89]. This could explain the observed decrease in DNA copies of bacterial *amoA* qPCR, because increased cell turnover would result in less remnant DNA detected via qPCR. Cell turnover could also contribute to a novel soil ammonium source via muramic acid release, providing a substrate for ammonia oxidation metabolism that was less abundant pre-drought [90–92]. Although mineralization of nitrogen in the peat matrix can also be used for microbial ammonia oxidation, there is evidence that the rate of this process is unaffected by fluctuations in peat water content [7].

***4.4 Shifts in associated nitrogen-cycling microbes***

In April, before the onset of the 2018 drought, there was a peak in nitrogen fixation genes *nifK* and *nifH* in the PW site. Previous studies of nitrifying microbes in alpine fens of the Zoige Plateau found that *nifH* gene copies were positively correlated with soil water content, and that *nifD* gene copies decreased by 25% after the onset of an extreme drought [35, 93]. The evidence for a decrease in transcription of nitrogen fixation genes after drought onset in PW supports these previous findings. It further suggests a shift in soil nitrogen sources from atmospheric nitrogen to plant-microbe or microbe-microbe interactions after drought onset.

In October, the metatranscriptomes displayed a significant increase in the *nirB* and *nasA* genes. While the *nasA* gene is obligatory for nitrate assimilation [94], *nirB* codes for both dissimilatory and assimilatory nitrate/nitrite reduction [95]. However, due to the lack of shifts in other DNRA markers (particularly the DNRA-exclusive *nrfA* gene), it is likely that the observed increase in *nirB* corresponds to an uptick in ANRA rather than DNRA. Both *nirB* and *nasA* facilitate cytoplasmic nitrite and nitrate reduction (respectively) requiring the synthesis of a [4Fe-4S] cluster [96]. Drained fens that had been subject to soil desiccation often have large pools of iron upon rewetting; fluctuating water tables in these ecosystems facilitates iron-redox which has the potential to mineralize organic matter [97]. The increase in ANRA-related genes indicates that this iron fertilization after water fluctuations could also facilitate the synthesis of the sulfate-iron clusters required for nitrate assimilation. It is possible that nitrate concentrations in the fen soil was high enough that *nirB* activity contributed only to biomass synthesis, rather than producing ammonia that is available for further oxidation [98]. Further studies on ANRA activity in rewetted fens that fluctuate between oxic and anoxic conditions (particularly during increasingly frequent droughts) would be informative regarding the impact of ANRA on peatland nitrogen cycling.

***4.5 AOA clade diversity during drought***

We constructed a phylogenetic tree linking the archaeal *amoA* gene taxonomy to the 16S rRNA marker to further describe drought dynamics between clades and orders of AOA, as per Wang et al. 2021 [41]. Of the 57 amplicon sequences, 6 (11%) were not clustered within any one clade or order in the phylogenetic tree and were labeled ‘undetermined’ (UD). However, these six unidentified amplicons constituted 33.6% of the AOA abundance, second only to NS-γ, which made up 45.7%. The amplicon sequences identified within the class *Nitrososphaera* clustered with four of the five known orders of AOA (*Nitrosophaerales* or NS, Ca. *Nitrosotaleales* or NT and *Nitrosopumilales* or NP). Only amplicons in the order Ca. *Nitrosocaldales* were not identified within the sequences, which is sensible because they most often occur in thermal environments [99].

AOA amplicons were rare in the CW sequences, with amplicons only assigned to NT-α in December 2018. This could be attributable to sampling depth, because NT-α (and some NS-γ) amplicons were previously described in deeper soils, and only rarely appeared in topsoil in the CW site [41]. NT-α occurs both in sediments and freshwater, and are most often found in acidic environments [100]. Accordingly, the pH range for CW during the study period was slightly acidic and ranged from 5.75 to 6.89.

PW exhibited a higher diversity than CW across freshwater and soil environment AOA clades. The clades NS-γ and NS-δ have been found to constitute up to 66% of AOA variants in soils, and are both present within the PW site [100]. NS-γ was the most abundant of the identified clades, and was dynamic over the study period, with evidence for a slight increase during the drought period [100]. The other clades identified were NS-ζ and NP-η, which are both common in freshwater and soil microbiomes. Therefore, these soil amplicons could also be influenced PW’s freshwater source in the Trebeltal River. Similarly to in Wang et al.’s 2021 investigation, amplicons from undetermined clades in the NS class were abundant in PW, and could belong to the underrepresented NS-ε or NS-β clades [41].

***4.6 Percolation and rewetted coastal fens varied in drought response***

One of the notable differences in the study is between the dynamics and microbial profiles of the PW and CW site. Some of these differences are attributable to each sites’ mire type, with the PW site hydrologically linked to a river watershed, while CW is occasionally flooded with brackish water from the Greifswald Bay. These differences in hydrological qualities can explain the more static microbiome factors, such as the lack of overall AOA clade diversity and lower functional gene copy numbers in CW as compared to PW.

However, water quality alone is insufficient to resolve why the nitrifying microbial communities in PW are dynamic in response to drought conditions, whereas those in CW remain largely stable. This is likely attributable to the fact that during non-drought periods, the water table in PW is often above the ground level. Further, the PW site is only considered to be in drought conditions when the water table drops just below the sampling depth of 0-5 cm (-5.45 cm). In contrast, in the CW site a fluctuating water table is typical outside of drought periods due to flooding. This indicates that under typical precipitation regimes, the topsoil nitrifying microbes are exposed to a higher oxygen content in the soil than those in the PW site, where the water table is often above the surface of the soil. Therefore, drought conditions are a more extreme shift from a stable hydrological state for the PW microbiome compared to CW, resulting in a greater response from the nitrifying microbiome to the change from anoxic to oxic soil conditions.

A remaining source of uncertainty concerns the soil water content in both sites. Although the water table falls in CW and PW, the soil water content only decreases in CW. There seem to be dampening feedback mechanisms at work in PW that maintain topsoil moisture at approximately 80% even with a low water table. This could be a result of shrinking feedback in the peat structure to maintain the relationship of the peat surface to the water table surface, although this could not have compensated for the greatest water table depressions during the drought (Figure 1) [101, 102]. Given the history of drainage in the site, it is more likely that the hydraulic conductivity of the peat was still low despite rewetting measures after a history of compaction [101]. This feedback mechanism functions to maintain the water content in the substrate, as the reduced pore space and increased bulk density leaves less space for water evaporation and flow-through. In contrast, the soil water content in CW does decrease with the drought-driven water table depression. However, there is limited corresponding ammonia oxidation dynamics or shifts in nitrogen cycling gene copy numbers. This disparity suggests that the correlation between water content and oxygen content alone is not enough to explain the relationship between increased ammonia oxidation activity and drought in PW.

Notably, PW is characterized by sedge reed vegetation, and therefore the often-discussed sphagnum feedbacks to water table depressions are of little relevance [37]. The PW site had high biomass production throughout 2018, indicating that its carbon storage function was maintained even during drought [103]. Carex acutiformis covers 80% of the PW site, and has the ability to form intra-tissue gas chambers that allow them to transport oxygen into the root zone in flooded soils [103, 104]. There is a zone within 1mm of new roots where oxygen is radially diffused, which could provide a niche for obligate aerobes such as AOA and AOB, particularly given the evidence for increased root biomass production during the drought of 2018 [105]. However, root production was higher in CW than in PW during the 2018 drought [103], so this explanatory mechanism contradicts the stability (and low abundance) of AOA and AOB in CW.

***4.7 Conclusion***

This study provides evidence that ammonia oxidation functions increased in temperate fen soils in response to drought conditions. This trend was most clearly supported by RNA-based RT-qPCR of bacterial and archaeal *amoA* gene copies, while DNA-based qPCR was biased by the presence of remnant DNA, and metatranscriptomic data was biased by low database resolution between *pmo* and *amo* genes. The increase in ammonia oxidation functions was supported by overall dynamics of nitrogen cycling indicator genes in the metatranscriptome, with a decrease in transcription of nitrogen fixation genes *nifDHK* and an increase in that of nitrogen assimilation genes *nirB*/*nasA*. Shifts in the nitrogen cycling microbiome were more extreme in the PW site than the CW site across all proxies. This suggests that drought could have a greater impact on peatland microbiomes in ecosystems with a consistently high water table, likely because the drought-driven change in abiotic factors is further from the peatlands’ stable state. As temperate fens are increasingly impacted by drought conditions in the near future, it is crucial to consider the hydrological stable state of restored fen landscapes and its relationship to nutrient cycling functions such as ammonia oxidation. These feedbacks will determine the quality of the peat substrate and nutrient load in subsequent post-drought rewetting, mimicking on a shorter time scale the draining-rewetting process that is key to global peatland viability.

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1. **Conflicts of interest**

The authors declare no competing interests.

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