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.