Impact of nitrogen fertilization on the soil microbiome and nitrous oxide emissions

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Chapter 5

Nitrification inhibitors effectively target N_2O-producing Nitrosospira spp. in tropical soil

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A version of this chapter was accepted as:

Cassman NA, Soares JR, Pijl A, Lourenco KS, van Veen JA, Cantarella H and Kuramae EE, February 2019. "Nitrification inhibitors effectively target N_2O-producing Nitrosospira spp. in tropical soil." *Environmental Microbiology*.

Abstract

The nitrification inhibitors (NIs) 3,4-dimethylpyrazole (DMPP) and dicyandiamide (DCD) effectively reduce N_2O emissions; however, which species are targeted and the effect on the nitrifying community is still unclear. Here we characterized the ammonia oxidizing bacteria (AOB) species linked to N_2O emissions and evaluated the effects of urea and urea with DCD and DMPP on the nitrifying community in a 258-day field experiment under sugarcane. Using an *amoA* AOB amplicon sequencing approach and mining a previous dataset of *16S rRNA* sequences, we characterized the most likely N_2O-producing AOB as a *Nitrosospira* spp. and identified *Nitrosospira* (AOB), *Nitrososphaera* (archaeal ammonia oxidizer) and *Nitrospira* (nitrite-oxidizer) as the main nitrifiers. The fertilizer treatments had no effect on the alpha and beta diversities of the AOB communities. Interestingly, we found three clusters of co-varying variables with nitrifier OTUs: the N_2O-producing AOB *Nitrosospira* with N_2O, NO_3^-, NH_4^+, WFPS and pH; AOA *Nitrososphaera* with NO_3^-, NH_4^+ and pH; and AOA *Nitrososphaera* and NOB *Nitrospira* with NH_4^+. These results support the co-occurrence of non-N_2O-producing *Nitrososphaera* and *Nitrospira* in the unfertilized soils and the promotion of N_2O-producing *Nitrosospira* under urea fertilization. Further, we suggest that DMPP is a more effective NI than DCD in tropical soil under sugarcane.

5.1 Introduction

Anthropogenic inputs of N fertilizers to agriculture have stimulated agricultural soils to contribute up to 59% of anthropogenic N_2O emissions [1-4]. Because N_2O has a global warming potential 298 times that of CO_2 [5] and diverts N that would otherwise be used by the crop, reducing N_2O emissions is a major target for sustainable management practices [6]. The N_2O emitted from a soil is the cumulative result of abiotic and biotic N_2O-generating pathways [7, 8]. The two main biotic processes contributing to N_2O in agricultural soils are nitrification (oxidation of NH_4^+ to NO_2^- to NO_3^-) and denitrification (anaerobic reduction of NO_3^- to NO_2^- to N_2O to N_2; reviewed in [9, 10]. Nitrification is carried out by a few bacterial and archaeal genera: ammonia oxidation is mediated by the ammonia-oxidizing archaea (AOA), such as the Thaumarchaeota *Nitrososphaera*, and the ammonia-oxidizing bacteria (AOB), such as the Betaproteobacteria *Nitrosomonas* and *Nitrosospira*; nitrite oxidation is carried out by nitrite oxidizing bacteria (NOB), including the Nitrospirae *Nitrospira* and the Alphaproteobacteria *Nitrobacter*. Denitrification is carried out by microorganisms widely dispersed over the bacterial, archaeal and fungal domains, and denitrification genes may also be carried by nitrifiers in what is termed nitrifier denitrification. Further, the process of complete nitrification by the recently discovered comammox bacteria, which have so far been found in the NOB *Nitrospira* genus, might also contribute to N_2O emissions [11].

Nitrification and denitrification processes are regulated by the abiotic factors temperature, oxygen availability, moisture, ammonia and nitrate availability, carbon availability and pH [12, 13]. These factors also affect the distribution and niche differentiation of nitrifiers; for example, the AOB numerically dominate in neutral soils with high NH_4^+ concentrations while the AOA dominate in acidic soils with low NH_4^+ concentrations [14, 15]. However, there are also exceptions to this general rule, for example the Gammaproteobacteria AOB Candidatus *Nitrosoglobus* recently isolated from acidic soils with survival in conditions down to pH 2 [16]. Within the domains there are also niche specializations, as the AOB *Nitrosomonas* is generally isolated from neutral pH soils while the AOB *Nitrosospira* is found in acid soils [17, 18]. Further, the nitrite oxidizer bacteria *Nitrobacter* and *Nitrospira* have optimal growth under higher and lower nitrite supplies, respectively, which is linked to their ecological niches [19, 20].

Nitrification is doubly implicated in N_2O production, either directly or indirectly by producing NO_3^- as the basis for denitrification, and has been shown to be the main process involved in N_2O emissions in sugarcane soils [21-27]. Thus, the addition of nitrification inhibitors with nitrogen fertilizers is currently being ex-

plored as a sustainable management practice in Brazilian sugarcane [24, 28, 29]. Nitrification inhibitors include dicyandiamide (DCD) and 3,4-dimethylpyrazole phosphate (DMPP), which are thought to be Cu-chelating agents acting on the ammonia monooxygenase enzyme [30]. The inhibitors have been shown to effectively reduce N_2O emissions by 40-95% in temperate and tropical soils [24, 31, 32]. This effect is generally restricted to the ammonia oxidizing bacteria, not affecting ammonia oxidizing archaea or the rest of the microbial community at coarse-grained levels [24, 33]. Evidence for the interdependence of ammonia and nitrite oxidizers as determined in unfertilized grassland soil suggests that the nitrifying community may be negatively affected under nitrogen fertilization with nitrification inhibitors [34]. It is yet unknown how the nitrification inhibitors DCD and DMPP might affect the nitrifying community in tropical soil under sugarcane.

Here our objectives were to identify the AOB species linked to N_2O emissions in a previous experiment and to compare the effects of urea fertilization with or without nitrification inhibitors on nitrifier abundances, with a focus on the ammonia-oxidizing bacterial community [24]. We sequenced *amoA* AOB amplicons from a 258-day field experiment encompassing treatments with urea and two nitrification inhibitors, DCD and DMPP, on soils growing ratoon sugarcane. We combined the *amoA* dataset with the 16S rRNA gene, nitrification and denitrification gene copy numbers and soil environmental variable datasets previously generated to test our hypotheses [24]. We hypothesized that the nitrification inhibitors would decrease the *amoA* AOB community diversity. Further, we hypothesized that the two nitrification inhibitors would have similar effects on the abundances of nitrifiers, including ammonia oxidizers (AOB and AOA) and nitrite oxidizers (NOB). To our knowledge, no studies to date have investigated the effect of nitrification inhibitors in urea fertilized soils on the nitrifier community growing in tropical soil.

5.2 Materials and Methods

5.2.1 Experimental design and sampling summary

A field experiment on Typic Hapludox soil (also known as Red Latosol) was set up at the Agronomic Institute in Campinas, Brazil at 22°52′15″ S, 47°04′57″ W, as described previously [24, 35]. Briefly, four treatments containing four replicate plots each were established in the 2013/2014 season on a third cycle of ratoon sugarcane (cultivar SP791011). The treatments were 1) no N fertilizer (control), 2) urea (UR), 3) urea with dicyandiamide (UR+DCD), 4) urea with 3,4-dimethylpyrazone phosphate (UR+DMPP). Urea was incorporated into the first 5

cm of soil and applied at a rate of 120 kg N ha^{-1}. The DCD (Sigma Aldritch) was added at 5% DCD-N per unit N from urea (v/v) while powdered DMPP (Sigma Aldritch) was added as 1% DMPP (w/w). Gas emission rates of CO_2, CH_4 and N_2O were measured daily to monthly using static chambers. Soil samples were taken of the top 10 cm of soil such that three subsamples were combined per plot. The soil samples were collected at eight time points: 7, 16, 18, 27, 35, 42, 82 and 158 days following fertilizer application and stored at -80 °C. Total DNA was extracted from the soil samples using a Power Soil kit from Mobio without modifications (Carlsbad, CA, USA). Further, pH, NO_3-N and NH_4-N were measured from the soil samples and water-filled pore space (WFPS) and temperature was previously calculated [24].

5.2.2 *amoA* AOB amplification and sequencing

Amplification of the partial *amoA* bacterial gene (491 bp) was performed using a two-step barcoding approach. The first PCR from the total DNA samples was carried out using forward primer H-AmoA1F-mod (5'-GCTATGCGC-GAGCTGCGGGGHTTYTACTGGTGGT-3') and reverse primer H-amoA2R (5'-GCTATGCGCGAGCTGCCCCCTCKGSAAAGCCTTCTTC -3') [36, 37]. In the second PCR, the *amoA* amplification products were amplified with primers that consisted of a 16 bp head sequence and included at the 5' end a library-specific 8 bp barcode [38]. Each PCR reaction (20 µl in first step, 50 µl in second step) consisted of 0.025 units of FastStart Taq DNA Polymerase (Roche), 1x reaction buffer with $MgCl_2$ (Roche), 0.5 mM dNTPmix (Fermentas), 0.125 µM of the forward and reverse primers, 0.1 mg/ml bovine serum albumin and 1 µl of DNA template. Thermocycler (C1000 Touch Thermal cycler, Biorad) conditions were as follows: 1) 5 minutes at 95 °C; 35 times 30 seconds at 95 °C, 30 seconds at 53 °C, 30 seconds at 72 °C; and 7 minutes at 72 °C and 2) 5 minutes at 95 °C; 10 times 30 seconds at 95 °C, 30 seconds at 53 °C, 1 minute at 72 °C; and 10 minutes at 72 °C. The first PCR reaction was performed in duplicate, screened by gel electrophoresis and pooled for use as a template in the second step, which used one primer (5'-BARCODE-HEAD-3'). Second step PCR products were checked by agarose gel electrophoresis and the concentration and quality determined using a fragment analyzer (Advanced Analytical). The bar-coded PCR products from all samples were normalized in equimolar amounts before sequencing. The *amoA* amplicon pool was sequenced using MiSeq V3 (2x300bp) technology (LGC, Germany). To complement the analysis of the *amoA* amplicon sequences, we mined the previously published dataset of 16S rRNA partial gene amplicons [24]. The *amoA* AOB

amplicons were thus, obtained from the same total DNA samples as the *16S rRNA* amplicons.

5.2.3 *amoA* AOB amplicon sequence processing

Bioinformatics steps were performed on a multi core server with 64 threads running Linux Ubuntu 16.04. Processing was accomplished through a Snakemake pipeline and bash and perl scripts. The *amoA* AOB sequences were clipped of primers and barcodes using bbduk (bbmap version 35.82) and the paired-ends were merged with the "join_paired_ends.py" script from ea_utils version 1.1.2-537. The AOB merged sequences were dereplicated and clustered into 97% AOB OTUs with minimum size of 2 using USEARCH version 9.2.64 (commands: derep_fulllength, and cluster_otus; Edgar, 2010). These parameters were chosen based on the recommendation found in the USEARCH manual (see also Figure S1). To confirm the functional potential of the OTUs as *amoA* (KEGG pathway K10944), the centroids were compared to the KEGG database (2014-03-17 version) using uproc-dna (UPROC v1.2.0; [39]). The table of OTU abundances across samples was created with the usearch global command based on 97% identity of sequences to the OTUs. Taxonomy was assigned to OTU centroids by diamond blastx v0.8.20 against the 2016-10-04 NCBI-nr database [40]. When this step yielded only classifications in the category "environmental samples," taxonomy was assigned instead by best blastn (e-value cutoff 0.02; blast v2.6.0) comparison against the custom *amoA* database described below.

To support the taxonomic classification results, a phylogenetic tree was created to depict the relationships between the 54 *amoA* OTUs and their closest matches in the custom *amoA* database. The latter was constructed as follows. High-quality *amoA* AOB sequences were downloaded from the FUNGENE RDP database (v9.4.1) with score above 350, HMM coverage above 80% and a minimum amino acid size of 270. Duplicates were reduced to one entry. The *amoA* OTU centroids and reference *amoA* AOB sequences along with an outgroup *amoA* sequence from *Nitrosococcus oceani* C-27 were aligned using ClustalW and used as input to make a phylogenetic tree in MEGA7 [41, 42]. The Maximum Composite Likelihood method was used to calculate phylogenetic distances, and bootstrap tests with 1000 replicates were performed [43]. The iTOL was used to create the final tree with bootstrap values of at least 90% depicted on the branches [44].

5.2.4 *amoA* AOB OTU processing and beta and alpha diversity analyses

Statistical analyses were carried out in R version 3.3.1 using R-Studio version 1.0.136. The R package phyloseq was used to handle the amplicon datasets. To remove outliers, the *amoA* AOB samples with less than 120 sequences were filtered out. To evaluate the sequencing coverage of the AOB communities, Good's coverage was calculated (package jfq3/QsRutils) and rarefaction curves were produced. Relative abundances of the *amoA* AOB OTUs were converted to absolute abundances by multiplying by sample the relative abundances by the relevant gene copy numbers previously obtained [24].

To ascertain the effect of treatment on the AOB community structure, we ordinated the *amoA* AOB samples using Bray-Curtis distances based on OTU relative abundance profiles. Multivariate homogeneity of dispersion was checked with function "betadisp" in the vegan R package. If dispersions were homogeneous, the effects of time point, treatment within time point, and time point within treatment were assessed through PERMANOVA analyses ("vegan" R package). Post-hoc tests of different pairwise group means were carried out using the "pairwiseAdonis" R package [45].

To determine the effect of treatment and time point, treatment within time point and time point within treatment on the AOB community alpha diversity, the data was first rarified to 120 sequences across samples using random seed 42. After confirming that all the data were not normal using the Shapiro-Wilk test and visual check of quantile plots, two-way crossed analyses of treatment and time point, and one-way analyses of treatment within time point and time point within treatment were evaluated using Kruskal-Wallis tests. These were supplemented with Dunn's post-hoc tests.

5.2.5 *16S rRNA* OTU processing and differential abundance and indicator species analyses

We supplemented the analyses of the *amoA* AOB dataset using the previously published 16S rRNA gene sequence dataset [24]. Good's coverage was calculated and rarefaction curves were produced as described for the *amoA* AOB OTU dataset. The *16S rRNA* OTU abundance dataset was processed as follows. Samples with less than 3000 sequences and *16S rRNA* OTUs with less than 23 sequences across all samples were filtered out. To determine significantly different nitrifier *16S rRNA* OTUs between treatments, differential abundance analysis was applied between treatment pairs considering all time points. The DeSeq2 package, which applies a negative binomial transformation of the filtered abundance data to

stabilize variances, was used for the differential abundance testing [46]. The Wald test with local model fit was applied to the *16S rRNA* data; orthogonal contrasts of the control and all other treatments, and of the urea against the treatments with a nitrification inhibitor, were carried out using Bonferroni-Hochberg correction for multiple tests. Significantly different *16S rRNA* OTUs with Bonferroni-adjusted p-values of less than 0.05 were identified.

The *16S rRNA* relative abundances were converted to absolute abundances using the *16S rRNA* copy numbers previously obtained by real-time PCR [24]. To examine the *16S rRNA* OTUs that were indicators of combinations of up to three treatments, we used the *multipatt* function from the "indicspec" R package to apply Legendre's indicator species analysis on the *16S rRNA* absolute abundances. Multiple comparison p-values were adjusted using the Benjamini-Hochberg correction.

5.2.6 Spearman correlations of *amoA* AOB and nitrifier *16S rRNA* OTUs with environmental variables

To reveal correlations between nitrifier OTU abundances and environmental variables, a subset of the previously published environmental data was employed [24]. Log transformations of the gene copy numbers obtained by qPCR (*nirS*, *nirK*, *amoA* AOB, *amoA* AOA, total Archaeal, *16S rRNA*) were carried out leaving the other variables (CO_2, N_2O, CH_4, soil NH_4–N, soil NO_3–N, soil pH and WFPS) untransformed (Figure S2). The nitrifier *16S rRNA* and *amoA* AOB OTU relative and absolute abundances, and the nitrifier *16S rRNA* normalized abundances, were correlated with the environmental variables using Spearman correlations. Significant correlations ($p<0.01$) were kept; for visualization the correlations were clustered using complete linkage clustering through the "corrplot" package.

5.3 Results

5.3.1 *amoA* AOB community sequencing coverage and composition

Processing of the *amoA* AOB amplicon data resulted in 68,211 sequences, which were clustered into 54 OTUs. The number of sequences ranged between 121 and 3,019 across the 127 samples (4 treatments X 8 time points X 4 replicates with one outlier sample removed). The samples had average Good's coverage of at least 94% (**Supplementary Table S1**), which was supported in the rarefaction curve results, with more sequences not adding more species in the samples with more sequences (**Supplementary Figure S3**). At the genus level, the AOB com-

munity was composed of unclassified Betaproteobacteria, *Nitrosomonas* and *Nitrosospira* (which included the *Nitrosovibrio* classification; **Figure 1A**). The phylogenetic tree of the *amoA* AOB OTUs with reference sequences indicated that these aligned with *Nitrosospira* (52/54 *amoA* OTUs) and *Nitrosomonas* (2/54 *amoA* OTUs) (**Figure 2**). In support of the low diversity of the *amoA* AOB communities, the 16S rRNA gene dataset revealed only two *Nitrosospira* OTUs (abundant OTU 30 and rare *16S rRNA* OTU 1102) and one *Nitrosomonas* OTU (rare *16S rRNA* OTU 2875). Further, the *Nitrosospira 16S rRNA* OTUs had similar absolute abundances as the *Nitrosospira amoA* AOB OTUs across the treatments (**Figure 1B and 1D**).

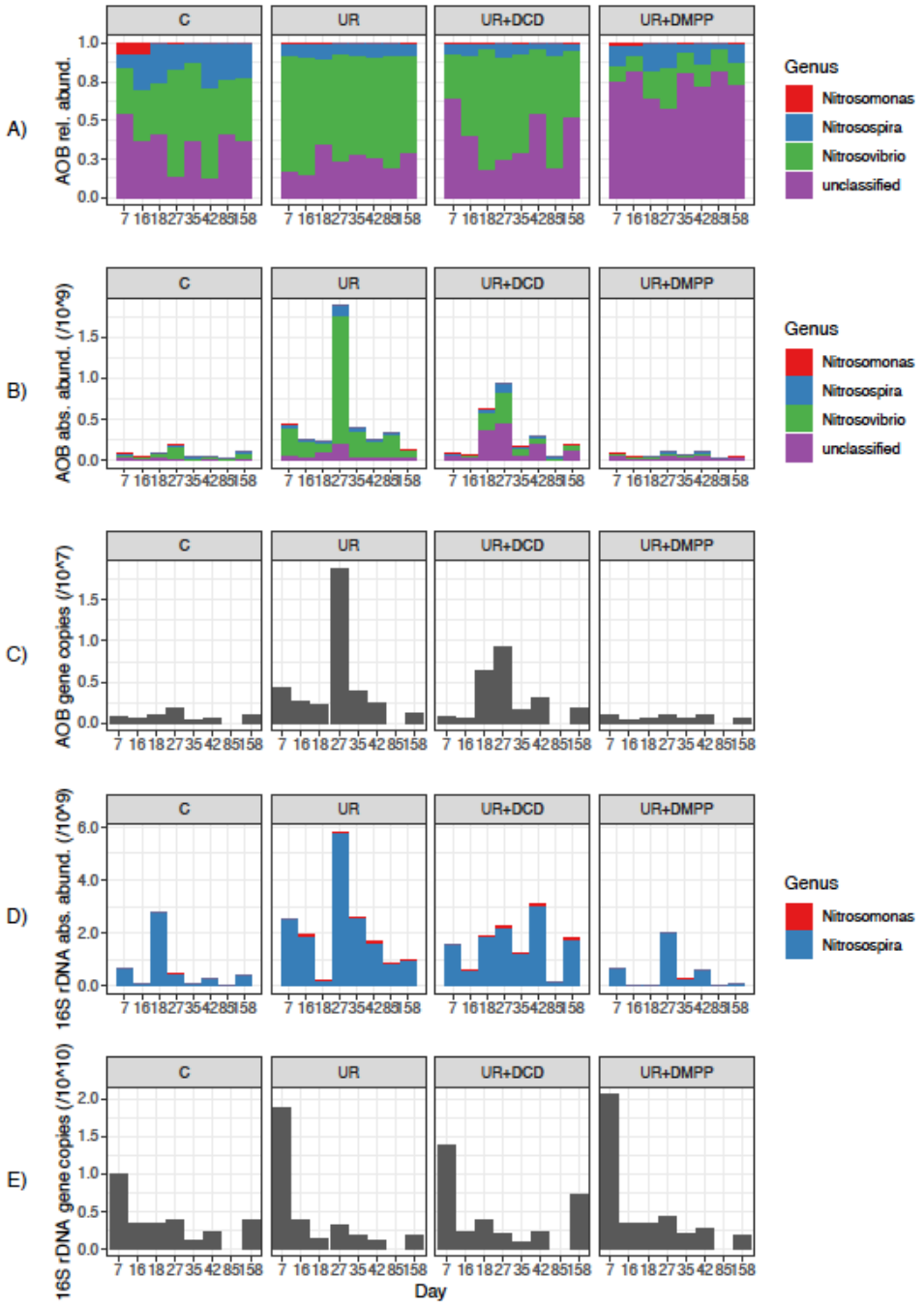


Figure 1. Taxonomic distributions of the *amoA* AOB amplicon samples by A) relative abundances or B) absolute abundances within genus, and C) the amoA AOB gene copy numbers. Also included are the D) taxonomic distributions of the *16S rRNA* amplicon samples by absolute abundances within the *Nitrosomonadaceae* family and E) the gene copy numbers of 16S rRNA gene sequences. Mean values within treatments and time points are shown. Treatments were the unfertilized control (C), urea (UR), urea with dicyanimide (UR+DCD) and urea with 3,4-dimethylpyrazole phosphate (UR+DMPP). Day = days after fertilization.

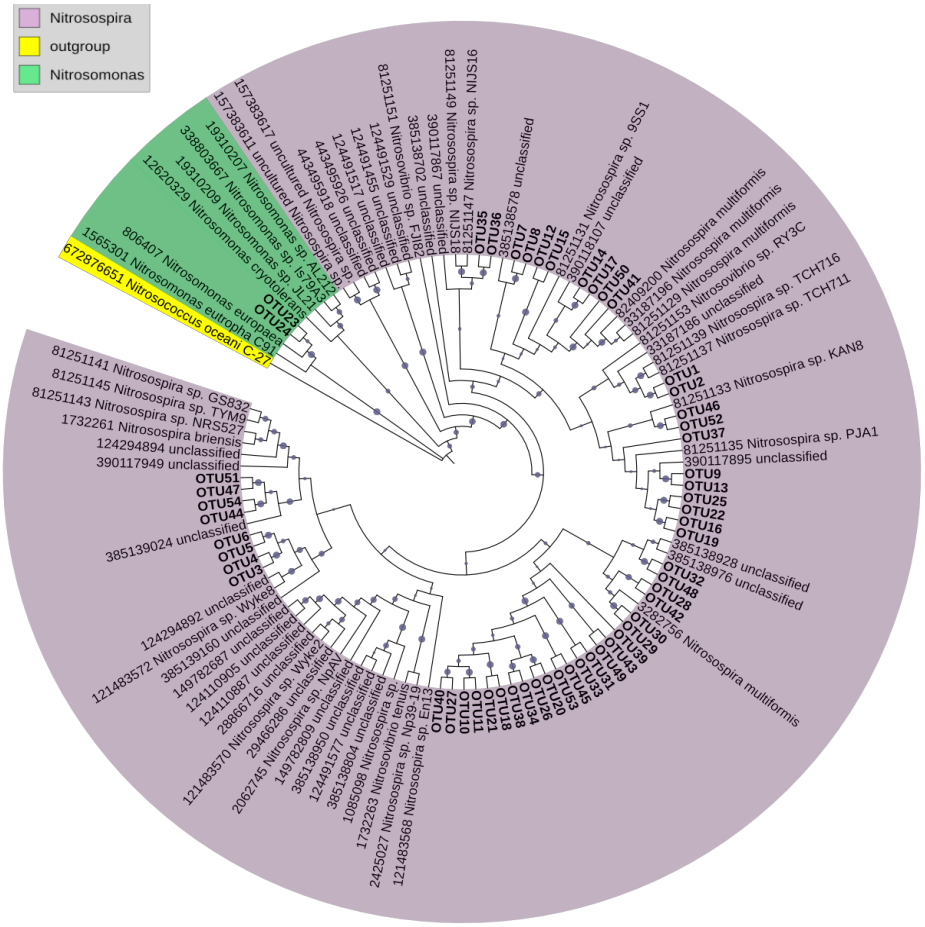


Figure 2. Phylogenetic analysis of *amoA* AOB OTUs and reference *amoA* sequences from the FUNGENE database based on the Maximum Likelihood distance method. Bootstrap values (1000 replicates) of less than 90% are depicted by the blue dots on the branches. The *Nitrosospira* are depicted with purple color bars, *Nitrosomonas* with green color bars, and the outgroup *Nitrososoccus* with the yellow color bar.

5.3.2 Treatment effects on *amoA* AOB community beta diversity

Beta dispersion analysis on all the samples revealed that treatment, but not time point, had a significant effect on the AOB community dispersions ($F=3.6529$, $P<0.05$). Subsequent beta diversity analysis revealed that time point, considering all treatments, had no effect on the AOB community structures (**Supplementary Table S2**). Ordination plots showed that the *amoA* AOB communities overlapped between treatments, considering all time points, according to 95% confidence intervals (**Figure 3A**). Within time points and treatments, the beta dispersions of the

amoA AOB communities were unaffected by treatment and time point, respectively. Treatment had a significant effect on the *amoA* AOB community structures only within days 7 and 16 (PERMANOVA; P<0.1; **Supplementary Table S2**). However, pairwise comparisons revealed that no *amoA* AOB community structures were significantly different between treatments within these time points. Time point had no effect on *amoA* AOB community structures within any treatment. Ordination plots within time point revealed that the *amoA* AOB communities did not cluster separately for treatments nor time points at 95% confidence intervals (**Figures 3C and 3D**).

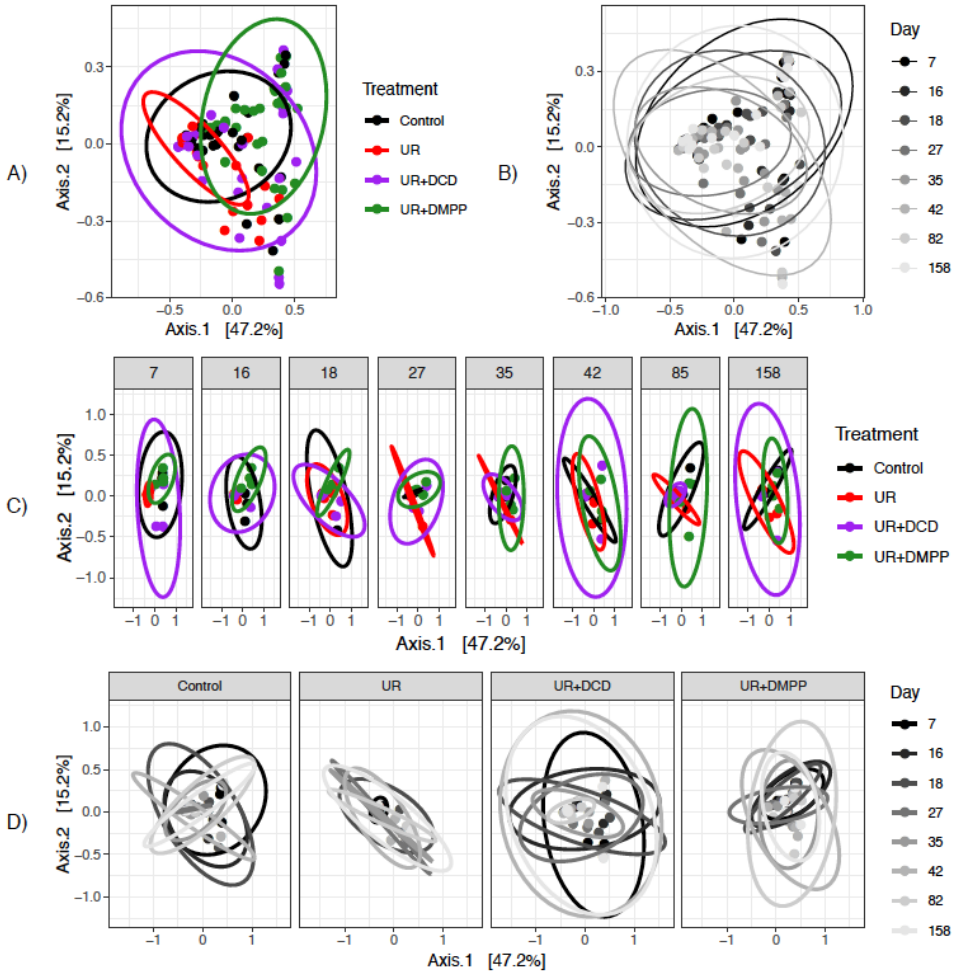


Figure 3. Ordination plots of the ammonia-oxidizing bacterial communities using PCoA on Bray-Curtis sample distances based on *amoA* AOB OTU relative abundances (n=127) A) across all time points (n=127) and B) across all treatments, C) by treatment within each time point (n=16) and D) by time point within each treatment (n=24). Time points were 7, 16, 18, 27, 35, 42, 82, and 158 days after fertilization. Treatments were unfertilized (Control, black), urea (UR, red), urea with dicyanimide (UR+DCD, purple), urea with 3,4-dimethylpyrazole phosphate (UR+DMPP, green). Confidence intervals of 0.95 are drawn around the treatments or days as ellipses.

5.3.3 Treatment and time point effects on *amoA* AOB community alpha diversity

The alpha diversities of the *amoA* AOB communities ranged from 1 to 3 based on Shannon index (**Figure 4**). Considering all time points, treatment had an effect on the alpha diversity of the *amoA* AOB communities (chi-squared value 33.884, p-value = $2.096e^{-07}$), but time point had no effect on the alpha diversities when considering all treatments. Post-hoc testing over all time points found that the *amoA* AOB communities in the DMPP treatment had higher alpha diversity compared to the other treatments (Dunn's test, $p < 0.05$; **Figure 4**). Within time point, treatment had an effect on the *amoA* AOB alpha diversities for days 7, 18, 27, with chi-squared values of 7.6103 (p-value 0.05479), 4.7792 (p-value 0.1887) and 6.7721 (p-value 0.07953), respectively. However, *post hoc* testing revealed no different pairs. Within treatment, time point had an effect on the *amoA* AOB community alpha diversities only for the Control treatment (chi-squared 12.534, p-value=0.08431); further, pairwise post hoc tests revealed no difference in alpha diversity between treatments.

5.3.4 Differential abundance of nitrifier *16S rRNA* OTUs and treatment group indicators

From the 16S rRNA gene sequence data, four genera of nitrifiers were represented: *Nitrosomonas* (1 OTU), *Nitrososphaera* (37 OTUs), *Nitrosospira* (2 OTUs) and *Nitrospira* (11 OTUs). The variance-stabilized trajectories of *Nitrosospira*, *Nitrososphaera* and *Nitrospira 16S rRNA* OTUs across the four treatments can be seen in Figure S4. The two *16S rRNA Nitrosospira* OTUs showed a similar trend across the treatments, with higher abundances in the urea and urea with DCD treatments compared to the control and the urea with DMPP treatments. The *16S rRNA Nitrososphaera* OTUs showed three trends, with OTUs 11 and 429 having lowest abundances in the control treatment and higher abundances in the treatments with urea, with the highest abundances in the urea with DMPP treatment; OTUs 40 and 45 having highest abundances in the control treatment, lower abundances in the treatments with urea, and the lowest abundance in the urea treatment; and OTUs 112 and 39 having highest abundances in the control and urea with nitrification inhibitor treatments and the lowest abundance in the urea treatment. The *16S rRNA Nitrospira* OTU followed the last trend with the highest abundances in the control and urea with nitrification inhibitor treatments and the lowest abundance in the urea treatment.

The differential abundance and indicator species analyses generally supported the abundance trajectories of the *16S rRNA* nitrifier OTUs. Differential abundance analysis revealed the nitrifier *16S rRNA* OTUs that were significantly over- and under-represented between pairwise comparisons of treatments based on variance-stabilized abundances (**Supplementary Table S3**). Of the *Nitrosospira 16S rRNA* OTUs, OTU 30 was an indicator of the control, urea and urea with DCD treatments, while OTU 1102 was an indicator of only the urea and urea with DCD treatments (adjusted p-value < 0.1; **Supplementary Table S3**). Of the *Nitrososphaera 16S rRNA* OTUs, OTU 45, OTU 112, OTU 40, OTU 39 and OTU 11 were indicators of the control, urea with DCD and urea with DMPP treatments. Of the *Nitrospira 16S rRNA* OTUs, OTU 79 was an indicator of the control, urea with DCD and urea with DMPP treatments.

5.3.5 Nitrifier *amoA* and *16S rRNA* OTU and environmental correlations

The correlations of the environmental variables with the gene copy numbers of AOB, AOA, *nirK*, *nirS*, *16S rRNA* total bacteria and total Archaea (**Supplementary Figure S5**) depict the positive links between AOB, N_2O, NO_3^-, NH_4^+, WFPS and pH, and AOA, *nirS*, *nirK*, total archaea and total bacteria; and the negative links between CO_2, CH_4^+ and WFPS, and AOA, total Archaea, NH_4^+, N_2O and NO_3^- (**Figure 5A**). As can be seen in Figure 5B which depicts correlations including the normalized abundances of *16S rRNA* OTUs, N_2O emissions were correlated with *amoA* AOB copy numbers, water-filled pore space (WFPS), NO_3^-, NH_4^+ and pH. Interestingly, the *16S rRNA* and *amoA* AOB OTU correlations clustered with the previous variables with the exception of NH_4^+ and pH, which nevertheless suggests that *Nitrosospira* (OTU 30 and OTU 1102) were the N_2O-producing AOB in these soils. Other interesting clusters were the *16S rRNA Nitrososphaera* OTUs 429 and 11 with NO_3^-, NH_4^+ and pH; the *nirS*, *nirK*, total archaeal and 16S rRNA gene copy numbers; and the *amoA* AOA, *16S rRNA Nitrospira* OTU 79, the *16S rRNA Nitrososphaera* OTUs 45, 112, 40 and 39. These clusters were found in all the correlations with absolute and relative abundances of the *amoA* AOB and the 16S rRNA gene sequence data (**Supplementary Figure S5**).

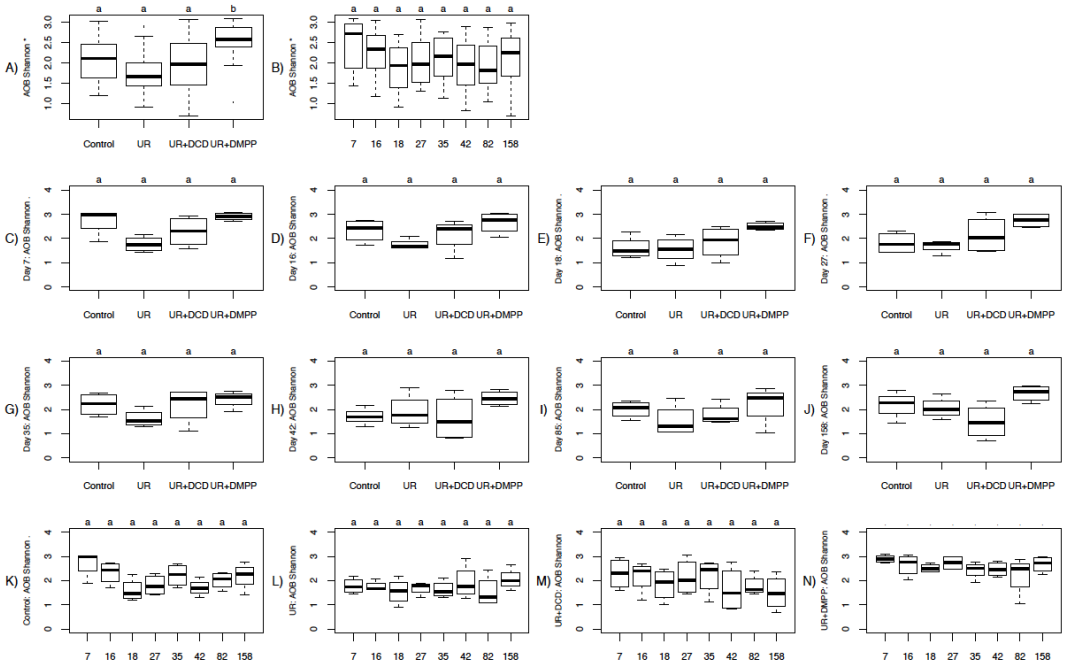


Figure 4. Alpha diversity of the amoA AOB communities as affected by A) treatment, for all time points, B) time point, for all treatments, C-J) treatment, within each time point, and K-N) time point, within each treatment. Treatments were unfertilized (C), urea (UR), urea with dicyanimide (UR+DCD) and urea with 3,4-dimethylpyrazole phosphate (UR+DMPP); time points were 7, 16, 18, 27, 35, 42, 82,158 days after fertilization. The y-axis label includes the result of a Kruskal-Wallis chi-squared test ("*" for $p<0.05$, "." for $p<0.10$); the letters above the plots represent the results of Dunn's post hoc tests at alpha < 0.05 in which similar letters denote no difference between groups.

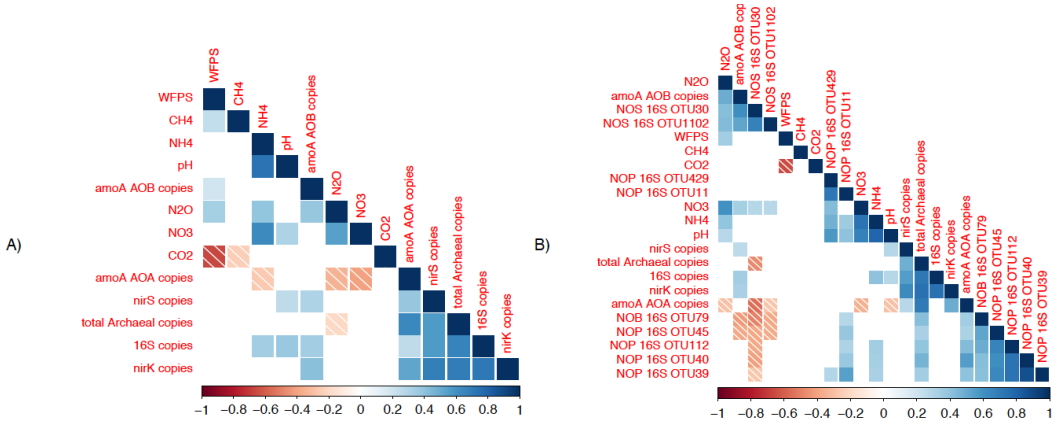


Figure 5. Cluster plots visualizing Spearman's correlations A) between environmental variables and gene copy numbers, and B) between environmental variables, gene copy numbers and the normalized abundances of the *16S rRNA* nitrifier OTUs. Normalization was carried out using DeSeq2. Only significant correlations are shown ($p<0.01$). Clusters were determined using complete linkage clustering. NOS = *Nitrosospira*, NOP = *Nitrososphaera*, NOB = *Nitrospira*.

5.4 Discussion

From our previous work, we found that bacterial *amoA* (AOB) but not archaeal *amoA* (AOA) nor denitrification gene copy numbers (*nirK*, *nirS*) were correlated with nitrous oxide emissions from tropical soil growing sugarcane [24]. Here we found evidence that the AOB responsible for the N_2O emissions was most similar to the *Nitrosospira* spp. (*Nitrosovibrio* RY3C), based on the decrease in abundance of these OTUs in soils with the nitrification inhibitors in comparison with the urea treatment and the correlation of these OTUs with N_2O emissions. The *Nitrosovibrio* RY3C species was originally isolated from avocado rhizosphere and its nitrifying activity was susceptible to DCD [47]. To our knowledge, just one other study has identified *Nitrosospira* spp. as the N_2O-generating AOB in tropical soil under sugarcane, and that study applied NH_4NO_3 as the N source [25]. The *Nitrosospira* in general are widespread spiral soil bacteria with generally low specificity for ammonia and thus found in soils under high levels of ammonia [15, 48, 49]. The only other AOB identified was *Nitrosomonas*, which was present in low abundance in the soils and was not linked to N_2O emissions. The *Nitrosomonas* are generally found in soils with high N inputs; moreover, *Nitrosomonas europaea* has a 3.5-fold higher Vmax compared to *Nitrosospira* sp., suggesting that these AOB do better in soils with consistently higher N [50]. We suggest that the conditions these soils encounter (generally low N with occasional high N inputs from fertilization) selects for the *Nitrosospira*, but that perhaps a *Nitrosomonas* species adapted to these conditions is present in low abundance.

The AOB are widely implicated in N_2O emissions under conditions favoring nitrification in tropical and temperate soils, in contrast to the AOA [51-55]. This is thought to be linked to the enzymatic capabilities of different AOB and AOA species, with the former generating higher amounts of N_2O through both abiotic (nitric oxide oxidation by O_2) and biotic (incomplete hydroxylamine oxidation and nitrifier denitrification) mechanisms, while the latter likely emits lower N_2O using only an abiotic (nitric oxidation by O_2) mechanism [56, 57]. While the AOB *Nitrosospira* was abundant in the soils under urea and urea with DCD treatments, we found that in the unfertilized and in the urea with DMPP treatment, the AOA *Nitrososphaera* were more abundant. More than 5 AOA *Nitrososphaera 16S rRNA* OTUs were identified compared to the two AOB *Nitrosospira 16S rRNA* OTUs; this supports the idea that the conditions in these unfertilized soils normally support the AOA *Nitrososphaera* rather than the AOB *Nitrosospira* or *Nitrosomonas* as the main ammonia oxidizers. Moreover, these native *Nitrososphaera* appeared to be non-N_2O-producing AOA. These results support observations that the AOA

Nitrososphaera is associated with low concentrations of ammonia linked to the stronger affinity of the archaeal ammonia monooxygenase for ammonia [48].

Interestingly, we identified two types of *Nitrososphaera* (AOA): one cluster of *Nitrososphaera* OTUs was more abundant in the soils with urea and DMPP, while the other cluster was more abundant in the unfertilized soils and co-varied with the NOB *Nitrospira*. The *Nitrospira* was the only nitrite-oxidizer found in our soils according to the 16S rRNA gene sequence data; interestingly, this was most abundant in the unfertilized soils and co-varied with AOA *Nitrososphaera* OTUs. The *Nitrospira* are thought to be adapted to low NO_2^- availability [20], which might explain their presence in our soils instead of *Nitrobacter* [19, 58]. Further, perhaps the *Nitrososphaera* and *Nitrospira* naturally interact in these unfertilized soils, as has been suggested for unfertilized grassland soils [34]. Future work could focus on this hypothesized interaction between non-N_2O-generating *Nitrososphaera* and *Nitrobacter*, which appears to be selected for by low levels of available substrate and might be enhanced by adding organic residues with high C:N [51, 59].

The inhibitors DCD and DMPP are both thought to inhibit ammonia monooxygenase by chelating the Cu co-factor in the enzyme [9].The limitation of *Nitrosospira* but not *Nitrososphaera* by DCD has been shown also in a paddy field soil and in microcosms of *Nitrosospira multiformis* but not *Nitrososphaera viennensis* [60, 61]. Based on gene copy numbers, the AOB but not the AOA were inhibited by DMPP in a sandy soil [62]; and the AOB but not the AOA were inhibited by DCD in a grazed grassland system [54]. In a Chinese vegetable soil, DMPP rather than DCD was revealed to be the more effective inhibitor of N_2O-producing AOB rather than AOA, although the N source urea was also amended with manure [63]. In studies of nitrification in agricultural soils, DMPP inhibited AOB expression under neutral pH conditions [64, 65]. The different success of the nitrification inhibitors appears to be a function of temperature, Cu-levels, and variation in abundance, genetic potential and/or expression levels of the targeted nitrifiers [9]. The different effects of DCD and DMPP on the abundance of the AOB *Nitrosospira* and the AOA *Nitrososphaera* found here suggests that evaluating the nitrification dynamics of these species in culture would be interesting for future work.

In contrast to our hypothesis that the nitrification inhibitor treatments would decrease the *amoA* AOB community alpha diversity, this diversity remained largely unchanged across treatments. There overall was low alpha diversity of the *amoA* AOB community, which was supported in both the *amoA* AOB and *16S rRNA* sequence results. Nitrifiers occupy a specific functional niche in the soil

environment, and the nitrifying functions are restricted to a handful of genera; new AOB are not likely to appear at least over the relatively short duration of this experiment (in total 258 days, subset presented here was 158 days). Moreover, the sugarcane plant competes with microbes for NH_4^+ and NO_3^- and these substrates are not likely to remain immobile long in this soil [66]. The highly weathered soils have high soil drainage capacity and have been under more than 20 years of sugarcane cultivation. Due to the long time of cultivation by sugarcane, likely the nitrifiers found in this soil are those that are adapted to the natural unfertilized conditions, to the brief high inputs of ammonia through urea fertilization, and to the competition with the sugarcane plant for ammonia. We speculate that the overall low nitrifier diversity and the selection of the nitrifiers that are present in these soils are driven by the generally low N levels.

While we inferred our results from the analysis of two different amplicon gene datasets, making our results more robust, some caveats to our methods should be acknowledged. The precision of the OTU classification was dependent upon the coverage of the databases used; for example, for our *16S rRNA* dataset we were only able to confidently classify to the genus level. This prevented us from directly comparing the classification results between the *amoA* AOB and *16S rRNA* datasets at the species level. However, we were reassured by the congruence of the *amoA* and *16S rRNA* sequence data relative to the absolute abundances of the *amoA* AOB at genus level. Further, the low diversity of the *amoA* bacterial communities was echoed in the *16S rRNA* data with just a few OTUs identified as *Nitrosospira* and only one as *Nitrosomonas*. Last, though the *16S rRNA* samples had high Good's coverage values between 85% and 99%, there is a possibility that the nitrifying subset of the community did not have such high coverage values. However, the focus of this paper was the *amoA* AOB community, although future studies could target in more depth and with more specificity the nitrifying network in these soils.

In summary, the nitrification inhibitors in our experiment were revealed to target the N_2O-producing bacterial ammonia-oxidizer *Nitrosospira* spp. in the soils. The low N availability appeared to drive the nitrifier community found in these soils. Treatment with urea and DMPP appeared to favor one functional type of AOA *Nitrososphaera* while the unfertilized soils revealed potentially interdependent AOA *Nitrososphaera* and NOB *Nitrospira*; it seems these species do not greatly contribute to N_2O emissions. Our results support the use of DMPP and especially DCD as inhibitors of N_2O-producing *Nitrosospira* spp. in tropical soils under sugarcane. The DMPP treatment may also increase the amount of NH_4^+ in the soil, allowing the sugarcane crop to uptake this N source while blocking the

N_2O from *Nitrosospira*. Furthermore, we provide evidence that the nitrification process in these soils is controlled by a few bacterial and archaeal species, driven mainly by the overall low N levels, and which have contrasting functional potentials for N_2O emission rates.

5.5 Declarations

Acknowledgements

This work was supported by NWO-FAPESP (The Netherlands Organization for Scientific Research NWO-729.004.003- Sao Paulo State foundation FAPESP-2013/50365-5). Publication number 6678 of the NIOO-KNAW, Netherlands Institute of Ecology.

Conflict of interest

The authors declare no conflict of interest.

Author's contributions

EEK and NAC designed the current study. JRS and HC designed the original experiment. JRS and KSL carried out the field work and DNA extractions. JRS and AP ran the qPCRs. AP prepared the *amoA* amplicon library for sequencing. NAC performed all bioinformatics and NAC and JRS performed analysis steps. NAC wrote the paper. JAV, HC and EEK contribute in the interpretation of the results and discussion of the paper. All authors read and approved the final version of the manuscript.

5.6 Supplementary Material

Table S1. Good's coverage of the *16S rRNA* (n=93) and *amoA* AOB (n=127) datasets. Treatments were unfertilized (C), urea (UR), urea with dicyanimide (UR+DCD) and urea with 3,4-dimethylpyrazole phosphate (UR+DMPP). Day: days after fertilization.

Treatment	Day	*16S rRNA* (avg; %)	*16S rRNA* (sd)	*amoA* (avg; %)	*amoA* (sd)
C	7	92.0	5.8	98.0	0.6
	16	96.4	1.9	96.7	1.1
	18	95.5	2.0	98.2	0.5
	27	93.9	3.2	98.1	0.3
	35	93.7	1.7	98.4	0.7
	42	91.3	8.0	99.2	0.6
	82	94.4	0.8	98.4	0.3
	158	90.9	4.6	98.4	0.8
UR	7	91.6	4.0	98.9	0.8
	16	96.7	1.0	97.9	1.0
	18	95.6	0.8	97.9	0.5
	27	95.2	0.1	98.1	1.2
	35	93.7	0.9	99.1	0.5
	42	94.7	1.3	99.0	0.7
	82	97.9	0.2	98.2	0.5
	158	91.9	2.1	96.6	2.2
UR+DCD	7	95.6	0.9	98.2	0.8
	16	94.4	2.5	98.0	1.1
	18	95.8	1.7	98.7	0.7
	27	96.4	1.9	98.0	1.9
	35	94.6	0.3	98.3	1.2
	42	90.2	2.3	99.0	0.5
	82	90.4	3.6	98.0	0.8
	158	88.7	0.9	98.5	0.8
UR+DMPP	7	94.7	0.4	95.6	1.9
	16	95.9	1.2	96.0	1.3
	18	96.4	0.6	98.6	1.0
	27	95.6	0.9	97.1	1.0
	35	96.2	0.5	97.6	1.3
	42	91.0	1.9	98.8	0.6
	82	93.4	1.0	97.9	0.9
	158	95.4	1.5	97.3	1.2

Table S2. Beta diversity results from PERMANOVA tests of the effects of treatment and time point on the AOB communities based on the Bray-Curtis distances between *amoA* OTU relative abundance profiles. Treatments were unfertilized (C), urea (UR), urea with dicyanimide (UR+DCD) and urea with 3,4-dimethylpyrazole phosphate (UR+DMPP). Days were 7, 16, 18, 27, 35, 42, 85 and 158 days after fertilization.

All treatments	df	F statistic	R^2	P-value
Day	7	0.8253	0.0463	0.711
Residuals	119		0.9537	
Within Day 7				
Treatment	**3**	**2.0553**	**0.33942**	**0.005 ****
Residuals	**12**		**0.66058**	
Within Day 16				
Treatment	**3**	**1.5286**	**0.29422**	**0.085 .**
Residuals	**12**		**0.70578**	
Within Day 18				
Treatment	3	0.9178	0.18663	0.623
Residuals	12		0.81337	
Within Day 27				
Treatment	3	1.2305	0.23525	0.207
Residuals	12		0.76475	
Within Day 35				
Treatment	3	1.1248	0.21948	0.272
Residuals	12		0.78052	
Within Day 42				
Treatment	3	1.1308	0.2204	0.271
Residuals	12		0.7796	
Within Day 82				
Treatment	3	1.4292	0.26324	0.134
Residuals	12		0.73676	
Within Day 158				
Treatment	3	1.0193	0.20308	0.410
Residuals	12		0.79692	
Within Control				
Day	7	1.1906	0.25775	0.157
Residuals	24		0.74225	
Within UR				
Day	7	0.95792	0.22573	0.584
Residuals	23		0.77427	
Within UR+DCD				
Day	7	0.81333	0.19174	0.821
Residuals	24		0.80826	
Within UR+DMPP				
Day	7	0.78015	0.18536	0.858
Residuals	24		0.81464	

Table S3. Differentially abundant nitrifier *16S rRNA* OTUs based on pairwise comparisons of *16S rRNA* OTU abundances between treatments. Treatments were unfertilized (C), urea (UR), urea with dicyanimide (UR+DCD), and urea with 3,4-dimethylpyrazole phosphate (UR+DMPP). The *16S rRNA* OTUs were included if the mean normalized abundance across all samples was greater than 4 and had log2 fold changes of more or less than 1. Values are significant log2 fold changes from DeSeq2 analysis (p adjusted < 0.05).

			Log_2 fold Change					
Genus	*16S rRNA* OTU Id	Mean Norm. Abund.	UR vs C	DCD vs C	DMPP vs C	DCD vs UR	DMPP vs UR	DMPP vs DCD
Nitrosospira	30	102	2.7	2.0			-2.5	-1.8
	1102	5	3.2	2.9			-2.1	
Nitrososphaera	11	97		2.0	1.7		1.5	1.0
	39	49	-1.1					
	40	44	-1.2					
	45	22	-3.2			2.0	2.1	
	112	15	-2.4				1.9	
	429	9	2.6	2.9	4.0		1.3	
Nitrospira	79	36	-1.3			1.1	1.6	

Table S4. Results of the indicator species analysis depicting indicator nitrifiers 16S rRNA gene sequence for groups of treatments (asterisk indicates adjusted p-value < 0.1) based on absolute abundances of *16S rRNA* OTUs. The OTUs were included if the mean normalized abundances were at least 4. Treatments were unfertilized (C), urea (UR), urea with dicyanimide (UR+DCD), and urea with 3,4-dimethylpyrazole phosphate (UR+DMPP). A "1" is in place if the *16S rRNA* OTU was an indicator of the treatment or, taken together, the group of treatments.

Max Order 3							
Genus	*16S rRNA* OTU Id	C	UR	UR+DCD	UR+DMPP	statistic	adj. p-value
Nitrosospira	30	1	1	1		0.95	0.025 *
	1102		1	1		0.82	0.015 *
Nitrososphaera	11		1	1	1	0.95	0.553
	39	1		1	1	0.92	0.459
	40	1		1	1	0.90	0.760
	45	1		1	1	0.94	0.033 *
	112	1		1	1	0.91	0.015 *
	429		1	1	1	0.93	0.009 *
Nitrospira	79	1		1	1	0.94	0.232
Max Order 2							
Genus	*16S rRNA* OTU Id	C	UR	UR+DCD	UR+DMPP	statistic	adj. p-value
Nitrosospira	30		1	1		0.88	0.010 *
	1102		1	1		0.82	0.010 *
Nitrososphaera	11			1	1	0.88	0.218
	39	1		1		0.77	0.825
	40	1		1		0.78	0.752
	45	1			1	0.78	0.854
	112	1			1	0.77	0.340
	429			1	1	0.87	0.022 *
Nitrospira	79	1			1	0.82	0.164
Max Order 1							
Genus	*16S rRNA* OTU Id	C	UR	UR+DCD	UR+DMPP	statistic	adj. p-value
Nitrosospira	30		1			0.66	0.058 *
	1102		1			0.63	0.096 *
Nitrososphaera	11				1	0.73	0.176
	39			1		0.59	0.836
	40	1				0.58	0.843
	45				1	0.55	0.971
	112	1				0.60	0.375
	429				1	0.76	0.036 *
Nitrospira	79				1	0.64	0.188

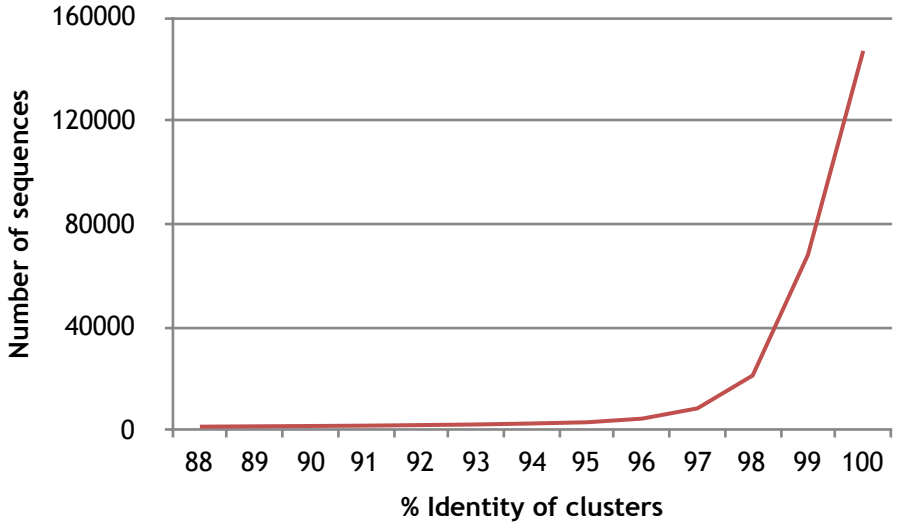


Figure S1. Line graph depicting the number of *amoA* AOB clusters (OTUs) at various percent identity levels from the USEARCH clustering algorithm. The percentage of 97% was chosen as per the recommendation of the USEARCH manual.

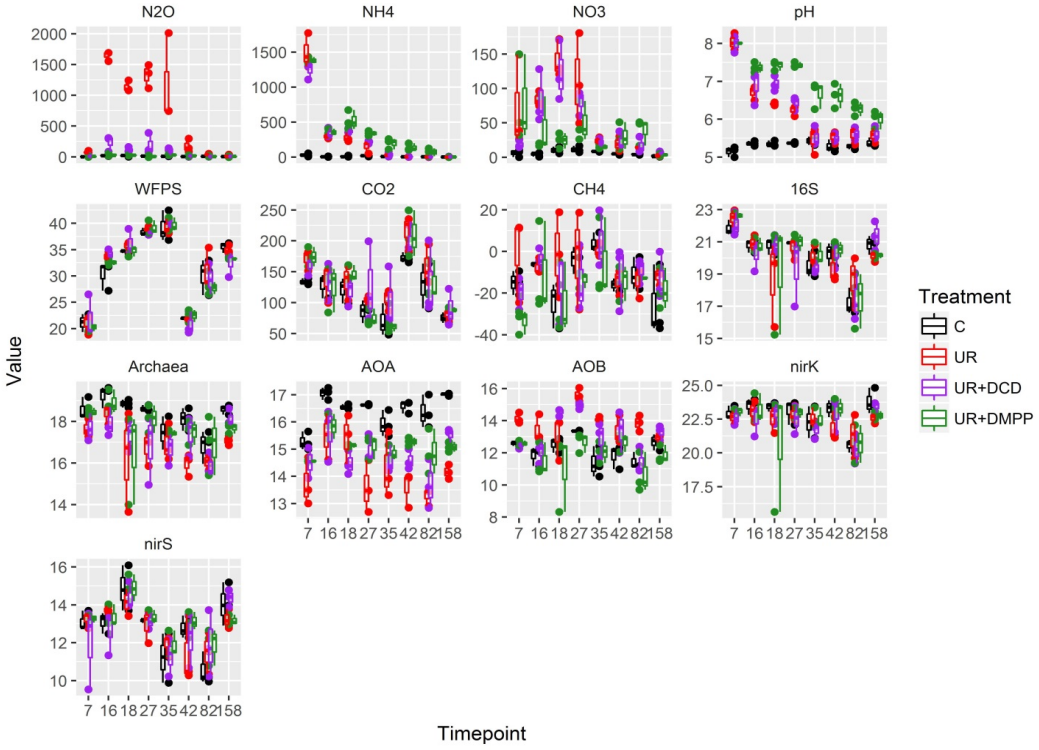
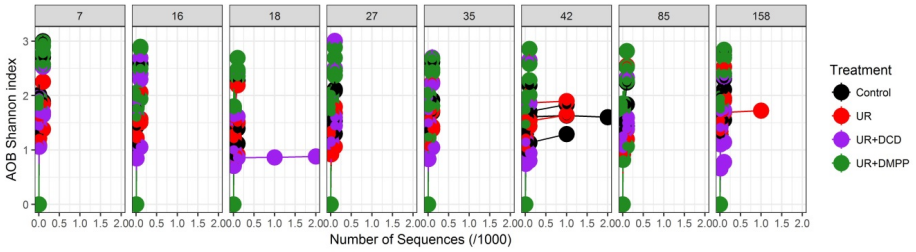


Figure S2. The previously published environmental variables and the gene copy numbers which were used in the current paper (Soares et al., 2016). Treatments were control (C; black), urea (UR; red), urea with dicyanimide (UR+DCD) and urea with 3,4-dimethylpyrazole phosphate (UR+DMPP). Time points were 7, 16, 18, 27, 35, 42, 82 and 158 days after fertilization. The gene copy numbers (qPCR) are presented as log values.

A)



B)

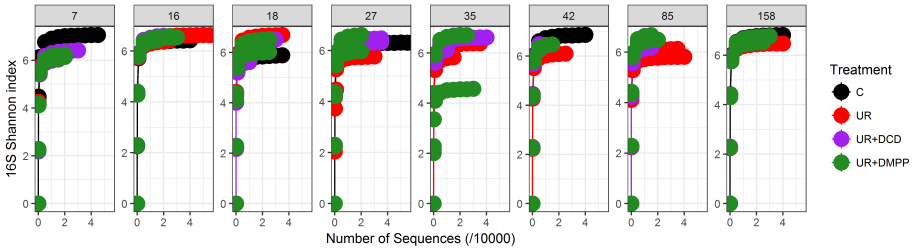


Figure S3. Rarefaction curves of the A) *amoA* AOB (n=127) and B) *16S rRNA* (n=93) datasets across time point. Treatments were unfertilized (C, black), urea (UR, red), urea with dicyanimide (UR+DCD, purple), and urea with 3,4-dimethylpyrazole phosphate (UR+DMPP, green). Days are 7, 16, 18, 27, 35, 42, 85 and 158 days after fertilization.

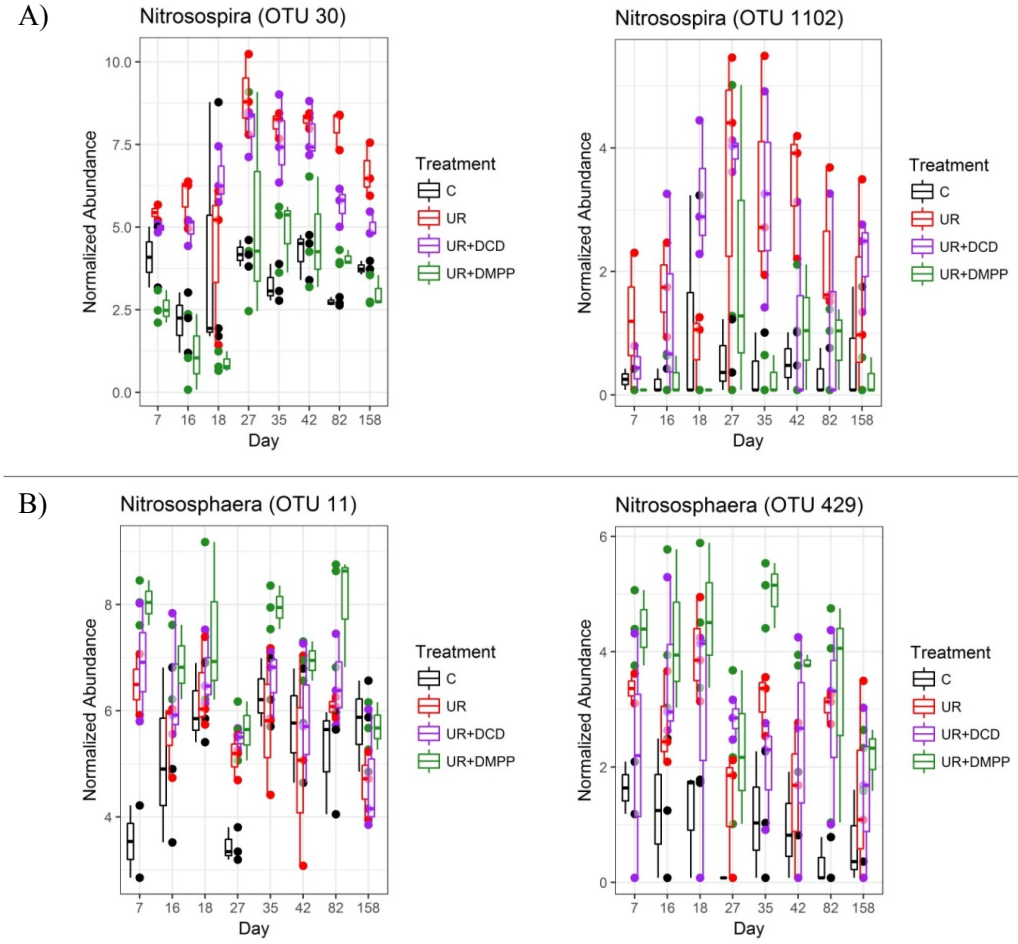


Figure S4. Normalized abundances of the A) AOB *Nitrosospira*, B) AOA *Nitrososphaera* and C) NOB *Nitrospira 16S rRNA* OTUs that were differentially abundant across the treatments based on DESeq2 analysis. Treatments were control (C), urea (UR), urea with DCD (UR+DCD) and urea with DMPP (UR+DMPP). Days are 7, 16, 18, 27, 35, 42, 85 and 158 days after fertilization. Note the different limits on the y-axes. Normalization was carried out with DeSeq2. Note the differing Y-axis limits.

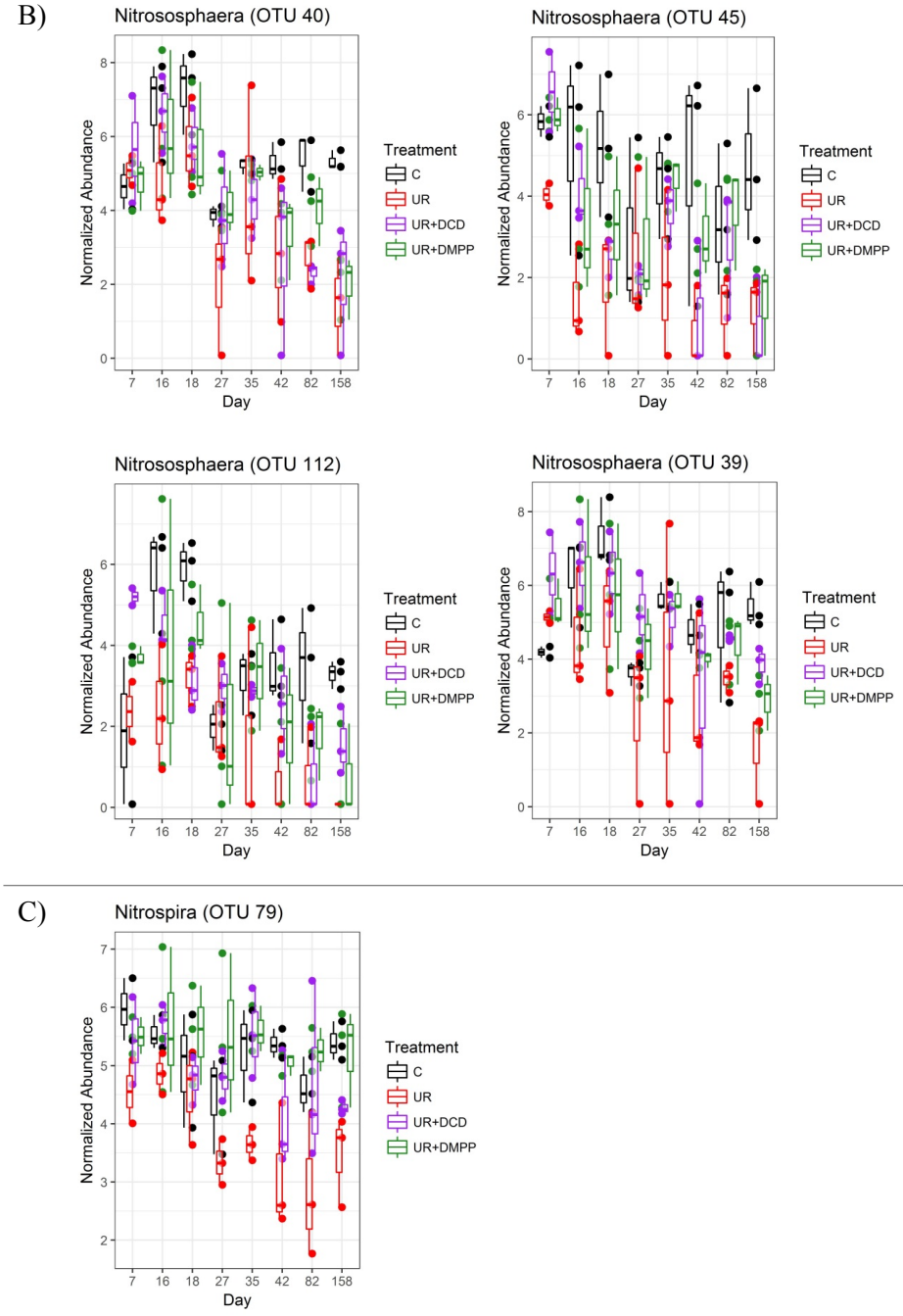
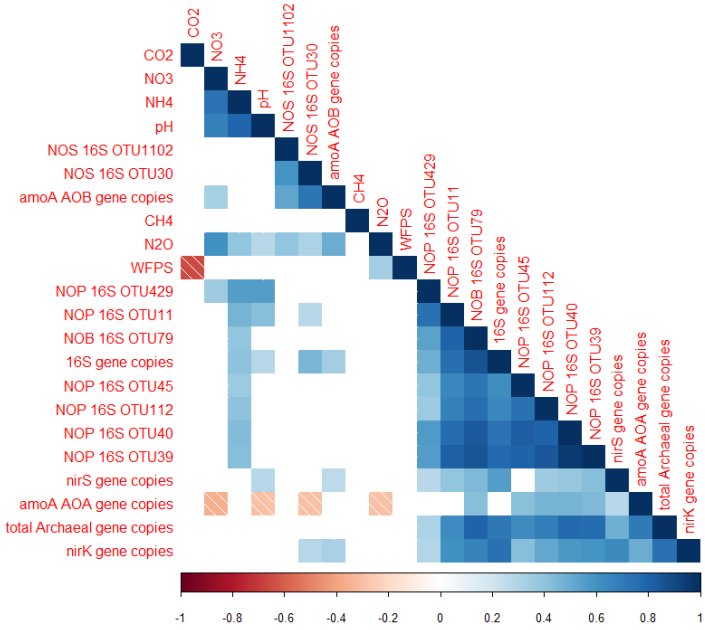
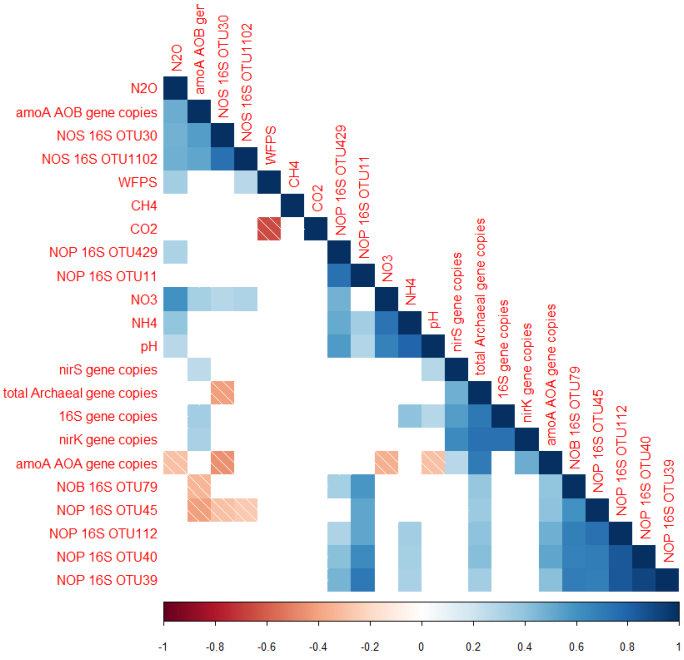


Figure S4 continued. Normalized abundances of the A) AOB *Nitrosospira*, B) AOA *Nitrososphaera* and C) NOB *Nitrospira 16S rRNA* OTUs that were differentially abundant across the treatments based on DESeq2 analysis. Treatments were control (C), urea (UR), urea with DCD (UR+DCD) and urea with DMPP (UR+DMPP). Days are 7, 16, 18, 27, 35, 42, 85 and 158 days after fertilization. Note the different limits on the y-axes. Normalization was carried out with DeSeq2. Note the differing Y-axis limits.

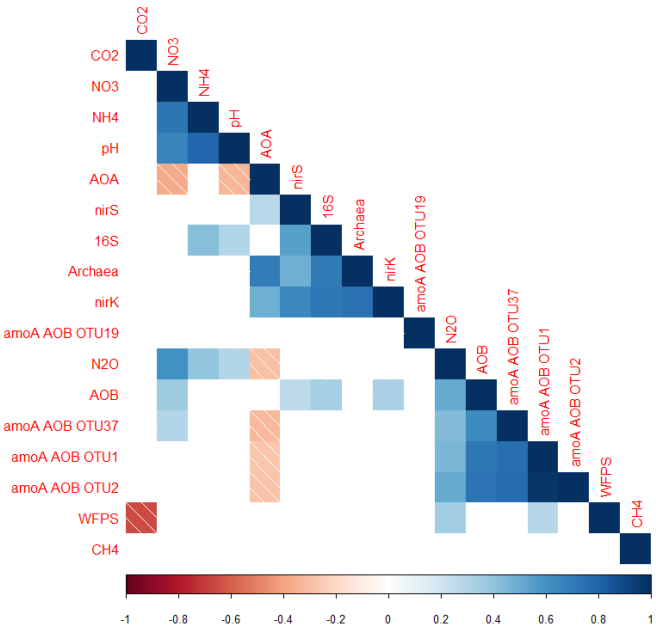


A)

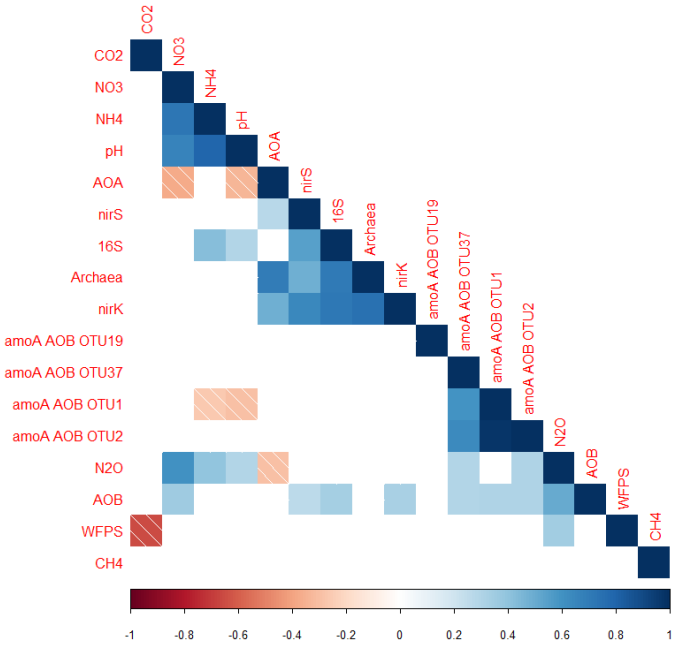


B)

Figure S5 cont'd. Cluster plots visualizing Spearman's correlations between environmental variables and the A) absolute abundances and B) relative abundances of the *16S rRNA* nitrifier OTUs. Only significant correlations are shown ($p<0.01$). Clusters were determined using complete linkage clustering.



C)



D)

Figure S5 cont'd. Cluster plots visualizing Spearman's correlations between environmental variables and the C) absolute abundances and D) relative abundances of the *amoA* AOB OTUs classified as the *Nitrosospira* spp. Nitrosovibrio RY3C. Only significant correlations are shown ($p<0.01$). Clusters were determined using complete linkage clustering.

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