Comammox *Nitrospira* bacteria outnumber canonical nitrifiers irrespective of electron donor mode and availability.

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Abstract

Complete ammonia oxidizing bacteria coexist with canonical ammonia and nitrite oxidizing bacteria in a wide range of environments. Whether this coexistence is due to competitive or cooperative interactions between the three guilds, or a result of niche separation is not yet clear. Understanding the factors driving coexistence of nitrifying guilds is critical to effectively manage nitrification processes occurring in engineered and natural ecosystems. In this study, microcosms-based experiments were used to investigate the impact of electron donor mode (i.e., ammonia and urea) and loading on the population dynamics of nitrifying guilds in drinking water biofilter media. Shotgun sequencing of DNA from select time points followed by co-assembly and re-construction of metagenome assembled genomes (MAGs) revealed multiple clade A2 and one clade A1 comammox bacterial populations coexisted in the microcosms alongside *Nitrosomonas-like* ammonia oxidizers and *Nitrospira*-like nitrite oxidizer populations. Clade A2 comammox bacteria were likely the primary nitrifiers within the microcosms and increased in abundance over canonical ammonia and nitrite oxidizing bacteria irrespective of electron donor mode or nitrogen loading rates. This suggests that comammox bacteria will outnumber nitrifying communities sourced from oligotrophic environments irrespective of variable nitrogen regimes. Changes in comammox bacterial abundance were not correlated with either ammonia or nitrite oxidizing bacterial abundance in urea amended systems where metabolic reconstruction indicated potential cross feeding between ammonia and nitrite oxidizing bacteria. In contrast, comammox bacterial abundance demonstrated a negative correlation with that of nitrite oxidizers in ammonia amended systems. This suggests that potentially weaker synergistic relationships between ammonia and nitrite oxidizers might enable comammox bacteria to displace nitrite oxidizers from complex nitrifying communities.

Importance

A deeper understanding of the interactions between nitrifying microorganisms, including comammox bacteria, is crucial to effectively managing nitrogen in engineered and natural systems. Despite their ubiquitous detection, our understanding of interactions between these coexisting nitrifying groups is limited. Here, we investigate the influence of differing sources of electron donor and loadings on a mixed nitrifier community which includes canonical ammonia and nitrite oxidizers, as well as comammox bacteria. Our results indicate that comammox bacteria will dominate nitrifier communities sourced from low nitrogen environments (e.g., drinking water) irrespective of fluctuations in nitrogen availability or source without directly competing with canonical ammonia oxidizers. However, canonical nitrite oxidizers are likely to get outcompeted by comammox bacteria in situations where metabolic interactions between ammonia and nitrite do not include cross feeding.

Introduction

Nitrification, the biological transformation of ammonia to nitrate via nitrite, is an important process in engineered and natural ecosystems. While nitrification mediated by ammonia oxidizing microorganisms (AOM) (1, 2), including ammonia oxidizing bacteria (AOB) and archaea (AOA), and nitrite oxidizing bacteria (NOB) (3) has been extensively investigated, complete ammonia oxidation (comammox) performed by comammox bacteria is understudied in large part due to its recent discovery. All known comammox bacteria belong to *Nitrospira* sub-lineage II (4-6) and are currently divided into two clades, A and B, with clade A further separated into sub-clades A1 and A2 (7). Due to close phylogenetic relatedness, comammox-*Nitrospira* cannot be distinguished from *Nitrospira*-NOB based on the 16S rRNA gene sequence or the marker genes for nitrite oxidation (*nxrAB*) (4). Thus, characterization of comammox bacteria has been largely enabled by shotgun DNA sequencing followed by reconstruction of assembled genomes (6, 8-12) and the development of primers targeting subuntis of comammox bacteria ammonia monooxygenase (*amo*) gene (13-18).

Within the engineered water cycle, clade A1 comammox bacteria have been primarily detected in wastewater treatment plants while clade A2 and B are been associated with drinking water treatment and distribution systems (7). It is unclear if this translates into physiological differences between the clades/sub-clades since there is only one comammox isolate and an enrichment enrichments whose kinetics have been characterized. To date, kinetic parameters of comammox bacteria are confined to two clade A representatives, cultured *Candidatus* N. inopinata and an enrichment of *Candidatus* N. kreftii (19, 20). Both demonstrate a high affinity for ammonia, with half-saturation constants orders of magnitude lower than strict AOB. Comparatively, the

109 *Candidatus* N. kreffti enrichment exhibited a higher affinity for nitrite compared to *Candidatus* N.
110 inopinata and partial inhibition of ammonia oxidation even at low ammonia concentrations (20).
111 This suggests that clade-specific comammox bacterial niche, if applicable, may be arise from a
112 combination of factors ranging from affinity to inhibition. Beyond clade specific traits, identifying
113 the potential environmental and physiological factors driving the coexistence of comammox
114 bacteria with canonical nitrifiers is also important to better understand comammox bacteria role in
115 complex nitrifying communities (21-29). Comammox bacteria have been detected along with their
116 canonical nitrifying counterparts in wastewater treatment plants (18, 25, 30-32), drinking water
117 systems (6, 9, 15, 33, 34) and soils (22, 28, 35, 36) at varying abundances over a wide range of
118 ammonium concentrations. While there is currently no quantitative estimate of the contribution of
119 comammox bacteria to nitrification compared to AOB and NOB, several studies have investigated
120 comammox bacterial dynamics in the context of mixed nitrifying communities. For instance,
121 DNA/RNA stable isotope probing combined with transcriptional analysis provided support for
122 comammox *Nitrospira* contributing to ammonia oxidation in lab-scale biofilters exposed to very
123 low ammonium concentrations (21). Soil microcosm amended with high ammonia concentrations
124 were enriched in AOB compared to those with lower ammonia concentrations where clade B
125 comammox bacteria proliferated (24, 28). Interestingly, in a lab-scale partial nitrification-
126 anammox reactor operating with incrementally increased ammonia loadings, comammox bacteria
127 initially dominated over strict AOB but its abundance significantly declined as loadings were
128 further increased (29).
129
130 Comammox bacteria may also acquire ammonia via urea degradation. Specifically, genes
131 encoding for urea transport and the urease enzyme are distributed among many *Nitrospira*

132 populations (37), including most comammox populations (38). While this may diversify potential
133 nitrogen sources for comammox bacteria (3), this could be a potential advantage for canonical
134 nitrifiers involved in a reciprocal feeding strategy as observed with co-cultured *Nitrospira*
135 *moscoviensis* converting urea to ammonia for *Nitrosomonas europaea* (37). The tight interplay
136 between canonical nitrifiers is well established; however, our understanding of comammox
137 competition (or lack thereof) with AOM and its impact on strict NOB in mixed communities is
138 limited. To better understand the comammox bacterial role within these complex nitrifying
139 communities, we investigated their population dynamics across two electron donor modes
140 (ammonia or urea) at three total nitrogen dosing strategies. The objectives of this study were (1)
141 to determine if comammox bacteria and canonical nitrifiers exhibit concentration and nitrogen
142 source dependent dynamics when subject to repeat nitrogen amendments and (2) to determine if
143 these dynamics are consistent or variable at the clade or population within each functional guild.
144
145 **Results**
146 **Microbial community composition in microcosms and nitrogen biotransformation potential.**
147 Microcosms consisting of granular activated carbon (GAC) from drinking water biofilters were
148 subject to intermittent amendments of nitrogen using two sources of electron donor (ammonia or
149 Urea) across three nitrogen concentrations (14, 3.5, and 1.5 mg-N/L). The conditions used in these
150 experiments are denoted as 14A, 3.5A, 1.5A, 14U, 3.5U and 1.5U where A or U represents
151 ammonia or urea amendments, respectively, and the number represents the concentration of
152 electron donor spike in mg/L as nitrogen. Two microcosms were sacrificed on a weekly basis over
153 the dotation of a eight week experiment (n=96 total microcosms). Extracted DNA from the inocula
154 and weeks four and eight were subject to shotgun DNA sequencing (n=13).

Initial assessment of taxonomic diversity in the samples based on analyses of metagenomic reads mapping to the small subunit rRNA database (SILVA SSU NR99 version 138.1) indicated that the GAC inocula largely consisted of bacteria with archaea and eukaryota constituting a small proportion of the overall metagenomic reads (~0.002%). The bacterial community was primarily composed of Gammaproteobacteria (30-20%), Alphaproteobacteria (25-31%) and Nitrospirota (8-15%) (Figure 1A). *Nitrospira* and *Nitrosomonadaceae* were the only nitrifiers identified and constituted 9-15% of the overall microbial community in samples. Full length 16S rRNA gene sequences were assembled from each sample (n=13) resulting in a total of eight sequences with closest matching SILVA database hits to uncultured *Nitrospira* bacteria (Accession numbers: MF040566, AY328760, JN868922). Clustering of all eight *Nitrospira* 16S rRNA gene sequences at 99% identity resulted in two *Nitrospira* operational taxonomic units (OTUs) with one cluster composed of six sequences (Nitrospira OTU 1) and the other cluster with two sequences (Nitrospira OTU 2). Phylogenetic placements of these OTUs revealed both clustered within *Nitrospira* sublineage II (supplementary figure S1A). Diversity of *Nitrospira* was likely underrepresented as full length *Nitrospira* 16S rRNA gene sequences could not be assembled from some samples despite a large portion of extracted 16S rRNA gene reads mapping to *Nitrospira* references in the SILVA database. Limited assembly of these reads could be due to several closely related *Nitrospira* species/strains coexisting in the samples making re-construction of full length sequences difficult. For canonical AOB, Nitrosomonas sp. AL212 (CP002552) was the closest matching database hit to one assembled sequence while another six had hits closet to *Nitrosomonadaceae* (Accession numbers: FPLP01009519, KJ807851, FPLK01002446) but could not be further classified at the genus or species level. Phylogenetic placement of the single

177 Nitrosomonas OTU affiliated it with *Nitrosomonas* sp. AL212 and *Nitrosomonas ureae* (Figure
178 S1B).
179
180 Following co-assembly of metagenomic reads, predicted protein coding genes from scaffolds
181 associated with the nitrogen metabolism were taxonomically classified (Figure 1B). The majority
182 of methane/ammonia monooxygenase (*pmo-amo*) like genes (KEGG orthology: K10944, K10945,
183 K10946) were associated with either nitrifiers (i.e., *Nitrospira* or *Nitrosomonas*) or methanotrophs
184 (i.e., *Methylocystis*) (Figure 1C). While some *amoCAB* genes could not be classified to the genus
185 level using kaiju software, blastp searches against the NCBI non-redundant protein database
186 indicated these were closely related to *Nitrosomonas*. All retrieved *hao* sequences (KEGG
187 orthology: K10535) were associated with Nitrospira which is likely due to the low relative
188 abundance of *Nitrosomonas*-like populations and the resulting inability to assemble their *hao*
189 genes. Potential for ureolytic activity was detected across four phyla based on the urease alpha
190 subunit (*ureC*). *ureC* sequences associated with Nitrospirota and Gammaproteobacteria could be
191 classified at the genus level as *Nitrospira* and *Nitrosomonas*. Sequences identified as nitrate
192 reductase/nitrite oxidoreductase alpha and beta subunits (K00370, K00371) were subject to further
193 classification to differentiate between nitrite oxidoreductase genes belonging to NOB from nitrate
194 reductases belonging to other community members. Phylogenetic placement of most *Nitrospira*
195 *nxrA* sequences found in this study cluster within a branch containing both comammox and
196 *Nitrospira*-NOB species (Candidatus *N. inopinata*, Candidatus *N. nitrosa* and *N. defluvii*) (Figure
197 1D). While other sequences clustered on a separate branch with Candidatus *N. nitrificans*, a single
198 *Nitrospira nxrA* sequence clustered closely within a branch containing only *Nitrospira*-NOB
199 belonging to sublineage II.

Phylogenomic placement of nitrifying populations and their metabolism.

To further define the composition of nitrifiers, metagenome assembled genomes (MAGs) were obtained from GAC microcosms after dereplication of MAGs from three binning approaches. All 204 MAGs were classified as bacteria, with 145 MAGs exhibiting completeness greater than 70% and contamination less than 10% (Table S1). Approximately 62% of the metagenomic reads mapped to these MAGs. Nine MAGs in total were classified as nitrifying bacteria belonging to *Nitrosomonas* and *Nitrospira* (Table S2). Genome annotation confirmed that four *Nitrospira* MAGs had key ammonia (ammonia monooxygenase and hydroxylamine oxidoreductase) and nitrite (nitrite oxidoreductase) oxidation genes (Figure S2). Quality assessment for these comammox MAGs indicated two high (Bin_49_2_2 and Bin_49_4) and one medium quality (Bin_260) (Table S1) according to (39). A fourth comammox MAG (Bin_13) was assembled with high completeness (89%) but also possessed high redundancy (18%) that could not be improved with further manual refinement. The remaining two *Nitrospira* MAGs (Bin_7_1 and Bin_188), which were likely strict NOB due to lack of ammonia oxidation genes, were less complete (38.04% and 48.25%) with low redundancy (8.76% and 8.46%). The low completeness was likely not due to their lower abundance, but potentially high level of strain heterogeneity which may have affected the assembly of reads associated with *Nitrospira*-NOB. For example, RPKM-based relative abundance estimated using all reads (total RPKM) showed the two *Nitrospira*-NOB MAGs exhibited similar relative abundance to comammox bacteria MAGs Bin_49_2_2 and Bin_49_4 (~7-10 total RPKM), but the CheckM estimated strain heterogeneities for Bin_7_1 and Bin_188 were 40 and 75, respectively, compared to 0 for both Bin_49_2_2 and Bin_49_4. Two

MAGs classifying as *Nitrosomonas* were deemed high (Bin_83) and medium quality (Bin_168); however, a third *Nitrosomonas* MAG was considered low quality.

A maximum likelihood tree based on 91 single copy core genes confirmed all *Nitrospira* MAGs affiliated with sublineage II (Figure 2A). Four of the *Nitrospira* MAGs from this study clustered within clade A comammox *Nitrospira* (Bin_49_2_2, Bin_49_4, Bin_260 and Bin_13) but were separated into distinct groups on the phylogenomic tree; namely, forming three clusters with MAGs obtained from tap water, drinking water filters, and freshwater. *amoA*-based phylogenetic analysis corroborated their placement into clade A (Figure 2B); however, *hao*-based phylogeny distinguished three of comammox MAGs (Bin_49_2_2, Bin_49_4, Bin_260) as clade A2 (Palomo et al. 2019) while one clustered within clade A1 (Bin_13) (Figure 2C). Consistent across all trees, Bin_49_2_2 and Bin_260 cluster closely with comammox MAGs *Nitrospira* sp. SG-bin2 and ST-bin4 (ANI ~ 92%) derived from tap water metagenomes (9). Bin_49_4 clustered closely with Nitrospirae bacterium Ga0074138 (ANI ~ 99%), which was previously detected in GAC from the same drinking water treatment plant (6), along with other tap water and groundwater-fed rapid sand filter MAGs (8, 9). Bin_13 associated with comammox MAGs obtained from freshwater, UBA5698 and UBA5702 (40) (ANI ~ 90%); however, its high contamination (18%) likely renders ANI comparison less accurate. Overall, the MAGs demonstrated less then 95% ANI to other reference comammox bacterial MAGs (Figure S3) suggesting comammox bacteria detected in GAC microcosms are distinct from one another and previously published comammox MAGs; as a result, they are likely novel *Nitrospira* species. The two remaining *Nitrospira* MAGs, Bin_7_1 and Bin_188, clustered with strict *Nitrospira*-NOB MAGs recovered from tap water, Nitrospira_sp_ST-bin5 (9) (ANI ~ 94%), and a rapid sand filter, Nitrospira CG24D (ANI ~ 87%) (8) (Figure 2A and S3). Only two strict AOB MAGs (Bin_83 and Bin_168) from this study were

used for phylogenomic analysis due high redundancy and low completeness of the third (Bin_195). Both Bin_83 and Bin_168 originate from *Nitrosomonas* cluster 6a and clustered closely with *Nitrosomonas ureae* and *Nitrosomonas* sp. AL212 (Figure 2D). Bin_168 shares a high sequence similarity to *N. ureae* (ANI ~ 98%) while Bin_83 shares less than 83% ANI to any of the references on the tree including Bin_168.

All comammox MAGs demonstrated the potential for ureolytic activity with the presence of the *ureABC* operon in addition to most genes for urease accessory proteins (Figure S2). *Nitrospira*-NOB MAGs did not contain genes encoding for urease; however, two *ureC* sequences found on assembled scaffolds that were classified as *Nitrospira* but were not binned into any of the Nitrospira MAGs. Queries of these *ureC* genes against the NCBI non-redundant database revealed one sequence shared the highest percent identity to *Nitrospira lenta* and *Nitrospira moscoviensis* while top hits for the second sequence belonged to an unclassified *Nitrospira*. One *Nitrospira*-NOB MAG (Bin_7_1) did harbor genes for the urea transport system permease proteins (*urtBC*), urea transport system substrate-binding proteins (*urtA*) and urea transport system ATP-binding proteins (*urtDE*). This suggests that the two unbinned *ureC* genes likely belonged to *Nitrospira*-like NOB bacteria. *Nitrosomonas* MAGs Bin_168 and Bin_83 each contained the *ureCAB* operon and some genes for urease accessory proteins and urea transport. A third *ureC* sequence found in the metagenome classified as *Nitrosomonas* but was not binned into any *Nitrosomonas* MAGs.

The impact of nitrogen amendments on nitrifying populations.

To address concentration and nitrogen source-dependent dynamics of the three nitrifier populations detected in our metagenomic analysis, qPCR-assays were used to estimate their

abundances over time in the nitrogen amended microcosms. In the high ammonia amendment (14A), strict AOB relative abundance increased 2.4-fold from weeks 1-3 but remained below 2% of total bacteria for the duration of the experiment whereas comammox relative abundance increased markedly over time reaching 2.8% of total bacteria by end of the experiment (Figure 3B). Similar to strict AOB, *Nitrospira*-NOB relative abundance increased early on but thereafter reduced from 4% at its peak in week two to 1.8% by week eight. Weekly measurements for nitrogen concentrations taken alongside biomass samples indicated the presence of residual ammonia and accumulated nitrite concentrations were highest during the first three weeks of the experiment but gradually reduced over time with most inorganic nitrogen present as nitrate (Figure S4). While comammox bacteria were always dominant, the abundance of strict AOB as a portion of AOM was significantly higher when ammonia and nitrite accumulated in weeks 1-3 as compared to weeks 5-8 (Welsh's t-test, p-value < 0.05) (Figure 3A).

The qPCR data was in concordance with metagenomic estimation of MAG abundance with clade A2 comammox (Bin_49_2_2, Bin_49_2 and Bin_260) highly abundant compared to strict AOB (Bin_83, Bin_168 and Bin_195) and clade A1 comammox (Bin_13) in the inocula and at weeks four and eight in the high ammonia amendment (Figure 3C). In particular, clade A2 MAGs Bin_49_2_2 and Bin_49_4 were the most dominant comammox populations while strict AOB was dominated by Bin_83 at each time point. *Nitrospira*-NOB MAGs had comparable abundance to clade A2 comammox MAGs but displayed limited variation in abundance in the high ammonia amendments. This contrasts with the qPCR data, where *Nitrospira*-NOB were significantly more abundant than comammox bacteria at earlier timepoints and then demonstrated a significant decrease in abundance over time. This is likely due to the fact that the two assembled Nitrospira-

NOB MAG's do not represent the entirety of NOB diversity in the microcosms and the fact that metagenomic data is only available for select timepoints as compared to qPCR data.

Nitrifier populations in mid and low ammonia amendments displayed similar dynamics to those observed in high ammonia with comammox relative abundance increasing to 3% and 2.2% of total bacteria by week eight, respectively. Interestingly, Bin_260, the least abundant clade A2 comammox MAG in the inocula, demonstrated significant increase in abundance in the low ammonia amendment over the course of the experiment compared to its abundance in the other ammonia amendments. Consistent with the ammonia amended microcosms, strict AOB in urea amended microcosms increased in relative abundance only at earlier time points followed by low but stable relative abundance (~2% of total bacteria). In the high urea amendment, relative abundance of comammox bacteria remained largely unchanged at earlier time points followed by an increase in abundance. Despite this, mean relative abundance of comammox bacteria compared to strict AOB was still approximately 2-fold greater in all urea amendments. Similar to the ammonia amendments, *Nitrospira*-NOB relative abundance did increase initially followed by a decline in all urea amendments. Interestingly though, the relative abundance of comammox bacteria and *Nitrospira*-NOB were similar in the later weeks of the experiment after *Nitrospira*-NOB's initial rise in urea amendments. Clade A2 comammox MAG Bin_260 was consistently lower in abundance than Bin_49_2_2 and Bin_49_2 in the urea amendments except for mid urea. Abundance of the clade A1 comammox MAG remained lower than all clade A2 MAGs and displayed minimal enrichment in all the urea amendments which was consistent with ammonia amended microcosms. Bin_168, which showed high sequence similarity to *Nitrosomonas ureae*, did not exhibit enrichment in any of the urea amendments and remained low in abundance with all other strict AOB MAGs.

There was no significant difference in mean relative abundance of strict AOB or *Nitrospira*-NOB between the high ammonia (14A) and urea amendments (14U) (Welch t-test, $p > 0.05$) but the mean relative abundance of comammox bacteria was significantly greater in high ammonia than in the high urea amendment (Welch t-test, $p < 0.05$). Comparatively, out of all nitrogen amendments, mean relative abundance of comammox bacteria was the lowest in high urea (1.8% of total bacteria). Comparisons between the mid ammonia (3.5A) and urea amendments (3.5U) as well as the low ammonia (1.5A) and urea (1.5U) amendments revealed no significant difference in mean relative abundance for any of the nitrifier populations (Welch t-test, $p > 0.05$). Additionally, no significant differences were detected when testing the mean relative abundance of the three nitrifier populations between high, mid, and low concentrations within each amendment type (ANOVA, $p > 0.05$).

The relative abundance of the nitrifying groups were used to examine potential correlations between the different populations in each of the nitrogen amendments. The ratio of comammox bacteria as portion of AOM to comammox bacteria as a portion of total *Nitrospira* revealed a strong positive relationship in all amendments (Pearson $R = 0.75\text{-}0.87$, $p < 0.001$) (Figure S5A), however, the change in relative abundance of comammox bacteria was not directly correlated with that of strict AOB in any of the nitrogen amendments (Figure S5B). Strict AOB and *Nitrospira*-NOB abundances were strongly correlated for all urea amendments and high (14A) and mid ammonia (3.5A) (Pearson $r = 0.58\text{-}0.82$, $p < 0.05$, Figure 4A) but exhibited a weaker relationship in low ammonia (Pearson $r = 0.42$, $p > 0.05$). Interestingly, while comammox bacteria abundance was significantly and negatively correlated with that of *Nitrospira*-NOB in ammonia amendments

(Pearson r = -0.37 to -0.61) ($p < 0.05$), there was no significant association between them in the urea amendments ($p > 0.05$) (Figure 4B).

Discussion

Key nitrifiers encompassing Nitrospira and Nitrosomonas-like bacteria share ureolytic potential

16S rRNA gene sequences assembled from short reads indicated *Nitrospira*- and *Nitrosomonas*-like populations were the only nitrifiers present in the microcosms. The proportion of 16S rRNA gene reads mapping to *Nitrospira*-like populations in this study suggested that they were highly abundant in the inocula and nitrogen amendments. Surveys of other DWTP biofilters using 16S rRNA gene amplicons have indicated that sublineage II *Nitrospira* account for a dominant portion of the bacterial community (41) with further investigation confirming high contributions to its abundance were from comammox-*Nitrospira* (8, 33). The strict AOB OTU found in this study was affiliated with oligotrophic *Nitrosomonas* cluster 6a which exhibit maximum growth rates at ammonia concentrations similar to the ones used for high and mid nitrogen amendments (42, 43). Despite this, the proportions of SSU reads mapping to *Nitrosomonas*-like populations in all nitrogen amendments were consistently low. Taxonomic classification of nitrogen cycling genes revealed metabolic potential for nitrification processes were confined to *Nitrospi*ra- and *Nitrosomonas*-like populations corroborating with assembled 16S rRNA gene sequences. Additionally, phylogeny of *amoA* sequences found in the metagenome indicated ammonia oxidation could be mediated by both *Nitrospira* and *Nitrosomonas*. Consequently, nitrate made available from nitrification processes would be available for nitrate reduction by other community members or as a source of nitrogen for biomass synthesis in oligotrophic environments.

We assembled a total of nine nitrifier MAGs which included comammox-*Nitrospira* (n=4), *Nitrospira*-NOB-like (n=2), and *Nitrosomonas*-like (n=3) populations. Three of the four comammox MAGs assembled were identified as clade A2 based on phylogenetic analyses of hydroxylamine dehydrogenase (*hao*) which has previously been shown to dominate drinking water biofilters along with comammox clade B (7). The remaining comammox MAG assembled from biofilter media in this study was affiliated with clade A1 based on *hao* gene phylogeny, which while atypical for drinking water biofilters is consistent with previously published metagenome from the Ann Arbor drinking water filters (6). Similar coexistence of clade A1 and A2 comammox bacteria with canonical nitrifiers has been observed in tertiary rotating biological contactors treating municipal wastewater with low ammonium concentrations (44). However, phylogenomic placement of clade A sub-groups in this study separated the comammox MAGs into distinct clusters associated with freshwater (Bin_13, clade A1), groundwater biofilters (Bin_49_4, clade A2) and tap water (Bin_260 and Bin_49_2_2, clade A2). Maintenance of high functional redundancy for the complete ammonia oxidation pathway may rely on coexisting comammox populations avoiding direct competition through distinct physiological niches. Additionally, the innocula were sourced from low substrate conditions which may also allow for the coexistence of multiple comammox populations. Strict AOB MAGs obtained in this study associated with low ammonia adapted *Nitrosomonas* cluster 6a (45) which is consistent with the inocula source being an oligotrophic environment (i.e., DWTPs). Furthermore, close relatives of *Nitrospira*-NOB MAGs obtained in this study originated from a tap water source where *Nitrospira*-NOB also coexisted with strict AOB and comammox bacteria under oligotrophic conditions (9). Our findings, consistent with previous studies, confirm the nitrifier community encompassed multiple populations capable of single and two-step nitrification within a single system. Further, assessment

of metabolic versality revealed initiation of nitrification through urea degradation was possible by all three nitrifying guilds. Though ureolytic activity is a widespread trait among cultured comammox-*Nitrospira* representatives and curated MAGs, the capability is confined to only some *Nitrospira*-NOB and *Nitrosomonas* species (37, 43). Here in particular, this a would allow *Nitrospira*-NOB to play a role in nitrite production in urea microcosms by crossing feeding ammonia from urea degradation to strict AOB, a mutualistic strategy which may not be active in ammonia amended microcosms.

Comammox bacterial abundance increased irrespective of nitrogen source or loading but may compete with NOB depending on nitrogen source type.

We tested the impact of nitrogen source and loading rates on temporal dynamics of a mixed nitrifying community to determine whether comammox bacteria are outcompeted at higher concentrations and/or favored in urea amendments due to their ureolytic activity. qPCR-based abundance tracking revealed comammox bacteria demonstrated a preferential enrichment over strict AOB in the nitrogen amendments irrespective of electron donor source or availability. Additionally, strict AOB abundance did not exhibit any significant difference across the nitrogen amendment types. This is in contrast to previous work in soil microcosms where AOB abundance increased in response to high ammonia amendments (28). However, strict AOB populations in these soil microcosms were primarily *Nitrosospira* compared to oligotrophic *Nitrosomonas* cluster 6a which were the primary AOB in this study. Here, both comammox bacteria and strict AOB demonstrated increased abundance in all amendments during the earlier weeks of the experiment. Ultimately, while comammox bacteria were enriched over time our findings demonstrated this increased abundance was not associated with a decrease in the abundance of strict AOB in any of the nitrogen amendments. This suggests a lack of direct competition between the two comammox

and strict AOB which could be attributable to the two ammonia oxidizers occupying separate nitrogen availability niches (19, 46). Stable abundances of strict AOB compared to enrichment of comammox could be due to a combination of factors ranging from (1) higher abundances of comammox bacteria in the inocula and (2) significantly higher biomass yields per mole of ammonia oxidizers for comammox bacteria compared to AOB (19).

Clade A2 associated comammox bacterial MAGs were dominant in the inocula and over the course of the experiment showed increased abundance in all amendments. In contrast, comammox bacteria belonging to clade A1 were lower in abundance and did not demonstrate significant change over time in any amended microcosm. Though physiological differences between comammox bacteria clades/sub-clades have yet to be established, earlier studies of DWTP biofilters have observed higher abundances of clade B (15) or alternatively both clades found at the same DWTP but within separate rapid sand filters, where clade B was more abundant in the secondary filters receiving lower ammonia concentrations (34). In this study, the lack of clade A1 enrichment may also indicate distinct physiological niches within clades (i.e., subclade-level niche differentiation). Future research is necessary to develop a clearer understanding of physiological differences between comammox bacteria at the clade/sub-clade level. Since cultivability of comammox bacteria remains an ongoing challenge, integrating multiple 'omics techniques (i.e., metatranscriptomics and metaproteomics) may be an appropriate strategy for examining ammonia utilization and the expressed metabolisms of multiple coexisting comammox bacteria populations alongside canonical nitrifiers.

The negative association between comammox bacteria and canonical NOB observed in ammonia amendments could be a result of nitrite limitation resulting from complete nitrification driven by

437 comammox bacteria. Nitrite limitation driven competition between comammox bacteria and NOB
438 is supported by the fact the negative associations between the groups were stronger at medium (3.5
439 mg-N/l) and low (1.5 mg-N/l) nitrogen availability as compared to the high ammonia amendments
440 (i.e., 14 mg-N/l). In contrast, there was no significant association between the abundance of
441 comammox bacteria and Nitrospira-NOB in the urea amended systems irrespective of nitrogen
442 loading. We hypothesize that this variable observations between ammonia and urea amended
443 systems likely emerge from the extent of metabolic coupling between AOB and NOB and the
444 resultant ability of comammox to outcompete NOB. Specifically, while the rate of nitrite
445 availability for NOB in ammonia amended systems is largely dictated by ammonia oxidation
446 activity of AOB it is likely that nitrite availability in urea amended systems would be dictated by
447 a combination of both AOB activity and indirectly by NOB. In this case, the production of nitrite
448 could be mediated by *Nitrospira*-NOB capable of ureolytic activity by crossing feeding ammonia
449 to strict AOB who in turn provide nitrite at a rate at which *Nitrospira*-NOB. This tight coupling
450 between AOB and NOB is supported by stronger and more significant correlation between AOB
451 and NOB abundance in urea amended systems as compared to ammonia amended systems. Thus,
452 it appears that while comammox bacteria may outcompete *Nitrospira*-NOB in systems where AOB
453 abundances are low and nitrite availability is largely dictated by AOB activity, this competitive
454 exclusion may be limited in scenarios with established AOB-NOB cross feeding via urea where
455 nitrite availability is governed not only by AOB's ammonia oxidation rate but also by NOB's
456 ureolytic activity.
457
458 Altogether, our study demonstrates that comammox bacteria will dominate over canonical
459 nitrifiers in communities sourced from nitrogen limited environments irrespective of electron

460 donor type or loading rate without directly competing with canonical AOB. Further, our study also
461 indicates comammox bacteria and AOB may occupy independent niches in communities sources
462 from low nitrogen environments. Interestingly, we see evidence of potential competitive exclusion
463 of NOB by comammox bacteria governed by nitrogen source dependent metabolic coupling
464 between AOB and NOB.
465
466 **Materials and Methods**
467 **Experimental design and execution**: Granular activated carbon (GAC) with coexisting AOB,
468 NOB, and comammox bacterial populations from biofilters at the drinking water treatment plant
469 (DWTP) in Ann Arbor, (AA) Michigan was used as the inoculum for this experimental work (6).
470 Microcosms consisted of 3 grams of GAC supplemented with 10 mL of filter influent from AA
471 DWTP in 40 mL pre-sterilized glass vials (DWK Life Sciences – Fisher 033395C). A total of 96
472 glass microcosms were prepared such that two biological replicates for each of the three nitrogen
473 concentrations (1.5, 3.5 and 14 mg-N/L) for the two nitrogen sources (i.e., ammonium and urea)
474 were harvested weekly for analyses over the period of the 8-week experiment. Ammonium was
475 spiked in at 0.1, 0.25 and 1 mM (in the form of ammonium chloride solution), corresponding to
476 1.5, 3.5 and 14 mg-N/L. For urea, 0.05, 0.125 and 0.5 mM (in the form of urea solution) were used
477 to ensure similar concentrations of total nitrogen as the ammonium microcosms. Microcosms were
478 maintained by removing approximately 10 mL of spent filter influent and subsequently
479 replenishing them with 10 mL of fresh influent and the respective electron donor spike every two
480 days. Once a week, two microcosms per condition (i.e., nitrogen concentration and nitrogen
481 source) were sacrificed and two 0.5 g GAC samples from each microcosm were transferred to
482 Lysing Matrix E tubes (MP Biomedical Lysing Matrix E – Fisher MP116914100) and stored at -

483 80°C until further processing. Spent filter influent was filtered using 0.2 μM filtration (Sartorius
484 Minisart NML Syringe Filter – Fisher Scientific 14555269) for subsequent chemical analyses.
485 Hach Company Test n' Tube Vials were used to determine concentrations of ammonia-N (Hach,
486 Cat No. 2606945), nitrite-N (Hach, Cat No. 2608345) and nitrate-N (Hach, Cat No. 2605345) in
487 microcosms. All samples were analyzed on a Hach DR1900 photospectrometer (Hach – DR1900-
488 01H). Alkalinity of filtered liquid samples were measured using Hach Alkalinity Total TNTplus
489 Vials (Hach – TNT870).
490
491 **DNA extraction and qPCR:** GAC samples were subjected to DNA extraction using the DNAeasy
492 PowerSoil kit (Qiagen, Inc – Cat No.12888) on the QIAcube (Qiagen, Inc – Cat No. 9002160)
493 following manufacturer's instructions with a few modifications. Specifically, the lysing buffer
494 from the PowerBead tubes were transferred to the Lysing Matrix E tubes and C1 buffer was added.
495 Prior to bead beating, an equal volume of chloroform was added (610 μL). Bead beating consisted
496 of four rounds of 40 seconds on a FastPrep-24 instrument (MP Bio – 116005500) with bead
497 beading tubes placed on ice for two minutes between each bead beating. Samples were then
498 centrifuged at 10,000 g for 1 minute and 750 μL of aqueous phase used to purify DNA using the
499 QIAcube Protocol for the DNeasy PowerSoil Kit. Each round of extractions included a reagent
500 blank as a negative control. After extraction, DNA concentration was determined using a Qubit
501 instrument with the dsDNA Broad Range Assay (ThermoFisher Scientific – Cat No. Q32850)
502 (Table S3). DNA was stored in a -80°C freezer until future use.
503
504 qPCR assays were conducted using QuantStudio 3 Real-Time PCR System (ThermoFisher
505 Scientific – Cat. No. A28567). Primer sets targeting the 16S rRNA gene of AOB (47), 16S rRNA
506 gene of *Nitrospira* (48), *amoB* gene of clade A comammox bacteria (18) and 16S rRNA gene for

total bacteria (49) were used (Table S4). Previously published primer set for the *amoB* gene of clade A comammox bacteria was updated based on metagenomic data generated as part of this study (18). Based on alignments of *amoB* gene sequences from the comammox MAGs assembled in this study, the previously published forward primer for comammox clade A *amoB* from Cotto et. al 2020 had one mismatch with one of our bins. Thus, this forward primer was further modified by changing the 13th position from G to a degenerate base S (seven base pairs from 3′-end). The use of the modified primers resulted in increased abundance of comammox bacteria in this study as shown in supplementary Figure S5, indicating the ability to capture comammox *amoB* gene sequences not amplified by previous primer set.

The qPCR reactions were carried out in 20 µL volumes, which included 10 µL Luna Universal qPCR Master Mix (New England Biolabs, Inc., Cat. No. NC1276266), 5 µL of 10-fold diluted template DNA, primer concentrations are outlined in Table S4 and DNAse/RNAse free water (Fisher Scientific, Cat. No. 10977015) to make up the remaining volume to 20 µL. Each sample per assay was subject to qPCR in triplicate and qPCR plates were prepared using the epMotion M5073 liquid handling system (Eppendorf, Cat. No. 5073000205D). The cycling conditions used in this study were as follows: initial denaturing at 95°C for 1 minute, 40 cycles of denaturing at 95°C for 15 seconds, annealing temperatures and time used are listed in Table S4 and extension at 72°C for 1 minute. qPCR analysis proceeded with a negative control and 7-point standard curve ranging from 10^3-10^9 copies of 16S rRNA gene of *Nitrosomonas europaea* for total bacteria quantification, 10^2-10^8 copies of 16S rRNA genes of *Nitrosomonas europaea* and *Ca* Nitrospira inopinata for AOB and *Nitrospira* quantification, respectively, and 10^2 – 10^8 copies of *amoB* gene of *Ca* Nitrospira inopinata for the quantification of comammox bacteria. The primer used to detect

530 the 16S rRNA gene of *Nitrospira* would inclusively track both comammox-*Nitrospira* and
531 *Nitrospira*-NOB. Thus, *Nitrospira*-NOB abundance was estimated by subtracting the copy number
532 of comammox bacteria *amoB* from the copy number of 16S rRNA gene of *Nitrospira*.
533
534 **Metagenomic analyses**:
535 A subset of samples were selected for metagenomic analysis including DNA extracted from the
536 initial GAC inoculum and samples from weeks four and eight (n=13) for all nitrogen dosing and
537 sources. DNA extracts from duplicated microcosms for each time point were pooled by in equal
538 mass proportion before sending DNA templates for sequencing at the Roy J. Carver Biotechnology
539 Center at University of Illinois Urbana-Champaign Sequencing Core. Two lanes of Illumina
540 NovaSeq were used to generate paired-end reads ranging from 29 to 68 million per sample (2x150-
541 bp read length) (Table S5). Raw paired-end reads were trimmed and quality filtered with fastp (50)
542 (Table S5). Filtered reads were mapped to the UniVec Database (National Center for
543 Biotechnology Information) using BWA (51) to remove potential vector contamination.
544 Subsequent unmapped reads were extracted, sorted and indexed using SAMtools v1.3.1 (52) then
545 converted back to FASTQ using bedtools v2.19.1 (53).
546
547 Small subunit rRNA sequence reconstruction from quality filtered short reads was carried out
548 using the Phyloflash pipeline v3.4 (54). Briefly, bbmap was used to map short reads against the
549 SILVA 138.1 NR99 database with the default minimum identity of 70% followed by assembly of
550 full-length sequences with Spades (kmers = 99,111,127) and detection of closest-matching
551 database sequences using usearch global within VSEARCH at a minimum identity of 70%. For
552 read pairs, taxonomic classification was performed by taking the lowest common ancestor of the

taxonomic strings of database hits using SILVA taxonomy (55). Assembled sequences from all samples belonging to nitrifying bacteria were clustered at 99% identity using vsearch v2.15.2 (56). Reference *Nitrospira* and *Nitrosomonadaceae* 16S rRNA reference sequences were obtained from ARB-SILVA and aligned with assembled sequences using muscle v3.8.1551 (57). Construction of 16S rRNA phylogenetic trees for *Nitrospira* and *Nitrosomonadaceae* was performed using IQ-TREE v1.6.12 (58) with model finder option (59) selecting TIM3+F+I+G4 and TPM2u+F+I+G4 as models for respective trees.

Quality filtered paired-end reads from all samples were co-assembled with metaSPAdes v3.11.1 (60) with k-mers lengths 21, 33, 55, 77, 99, and 119, and phred off-set of 33. Quality evaluation of the assembled scaffolds was performed using Quast v5.0.2 (61) (Table S6). Open reading frames (ORF) on scaffolds were predicted using Prodigal v2.6.2 (62) with the "meta" flag and functional prediction of resulting protein sequences were determined by similarity searches of the KEGG database (63) using kofamscan (64). Taxonomic classification of scaffolds harboring nitrogen cycling genes was performed using kaiju v1.7.4 (65) against the NCBI nr database with default parameters. CoverM v0.5.0 (www.github.com/wwood/CoverM) was used to calculate reads per kilobase million (RPKM) of these scaffolds as a metric for estimating relative abundance in each sample.

Scaffolds were binned into clusters and manually refined using Anvi'o (v5.1 and 5.5) (66) with three binning algorithms including CONCOCT (67), Metabat2 v2.5 (68) and Maxbin2 v2.2.7 (69). DAS_tool v1.1.2 (70) was used to merge bins from the three approaches to generate final metagenome assembled genomes (MAGs). Completeness and contamination of the final set was

576 determined using CheckM v1.0.7 (71) followed by taxonomic classification using the Genome
577 Taxonomy Database Toolkit v1.2.0 with release 89 v04-RS89 (72). CoverM was used to calculate
578 RPKM for each bin. Similar to the annotation of the metagenome, functional prediction of bin
579 ORFs were determined by similarity searches against the KEGG database using kofamscan. The
580 annotation of genes of interest were further confirmed by querying protein sequences against the
581 NCBI-nr database using BLASTP. MAGs were also annotated using Prokka as a secondary
582 annotation method (73). The Up-to-date Bacterial Core Gene pipeline (UBCG) (74) with default
583 parameters was used to extract and align a set of 92 singe copy core genes from *Nitrospira* and
584 *Nitrosomonas* references genomes (Table S7) and nitrifier MAGs for phylogenomic tree
585 reconstruction. Maximum likelihood trees were generated based on the nucleotide alignment using
586 IQ-TREE with model finder selecting the GTR+F+R10 and GTR+F+R4 models for *Nitrospira*
587 and *Nitrosomonas* trees, respectively, with 1000 bootstrap iterations. For outgroups, two
588 *Leptospirillum* and three *Nitrosospira* genomes were used for *Nitrospira* and *Nitrosomonas* trees,
589 respectively. Pairwise alignments of comammox *amoA* and *hao* and Nitrospira *nxrA* protein
590 sequences were created using muscle. Maximum likelihood trees were inferred by IQ-TREE with
591 model finder selecting LG+G4 for the *amoA* tree and LG+I+G4 for *hao* and *nxrA* trees with 1000
592 bootstrap iterations for each tree. The *amoA* and *hao* protein sequences from *Nitrosomonas*
593 *europaea* and *Nitrosomonas oligotropha* were used as the outgroup for comammox trees. All trees
594 were visualized using the Interactive Tree of Life (itol) (75). Pairwise comparisons of average
595 nucleotide identity of 38 *Nitrospira* and 15 *Nitrosomonadaceae* genomes (Table S4) with nitrifier
596 MAGs obtained in this study was determined using FastANI v1.31 (76).
597
598 **Statistical analysis**

The relative abundance of each nitrifier population was tested to determine if significant differences existed between concentration or source of electron donor types using ANOVA and Welch t-tests, respectively, with R version 4.0.4. Shapiro Wilks tests were used to confirm normality prior to these statistical tests. Linear regression and correlation analysis were used to examine the relationship between the abundance of nitrifying guilds in each of the nitrogen amendments over time.

Data availability

Raw sequence reads, metagenome assembly, and MAGs are available on NCBI at Bioproject number PRJNA764197.

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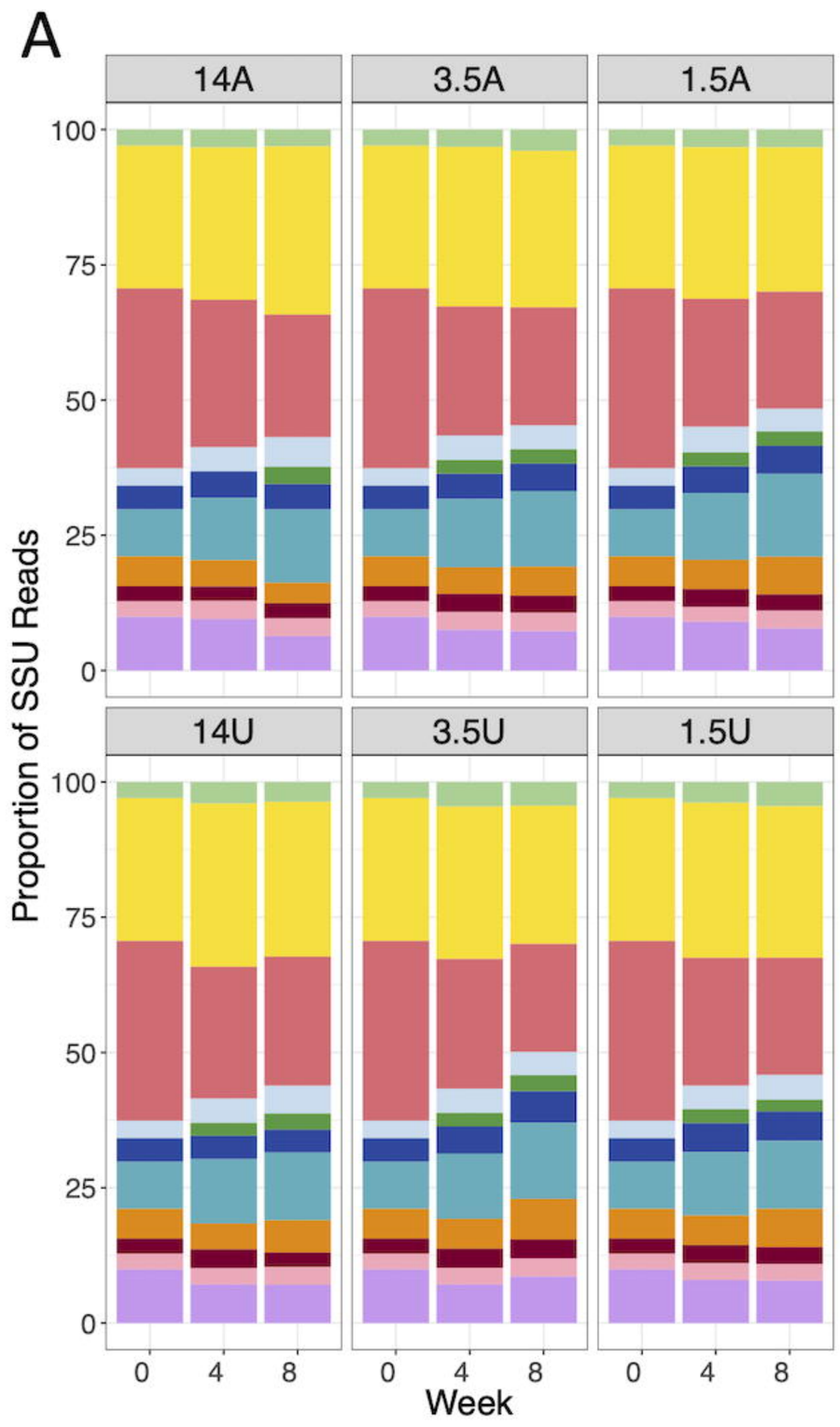
List of Figures.

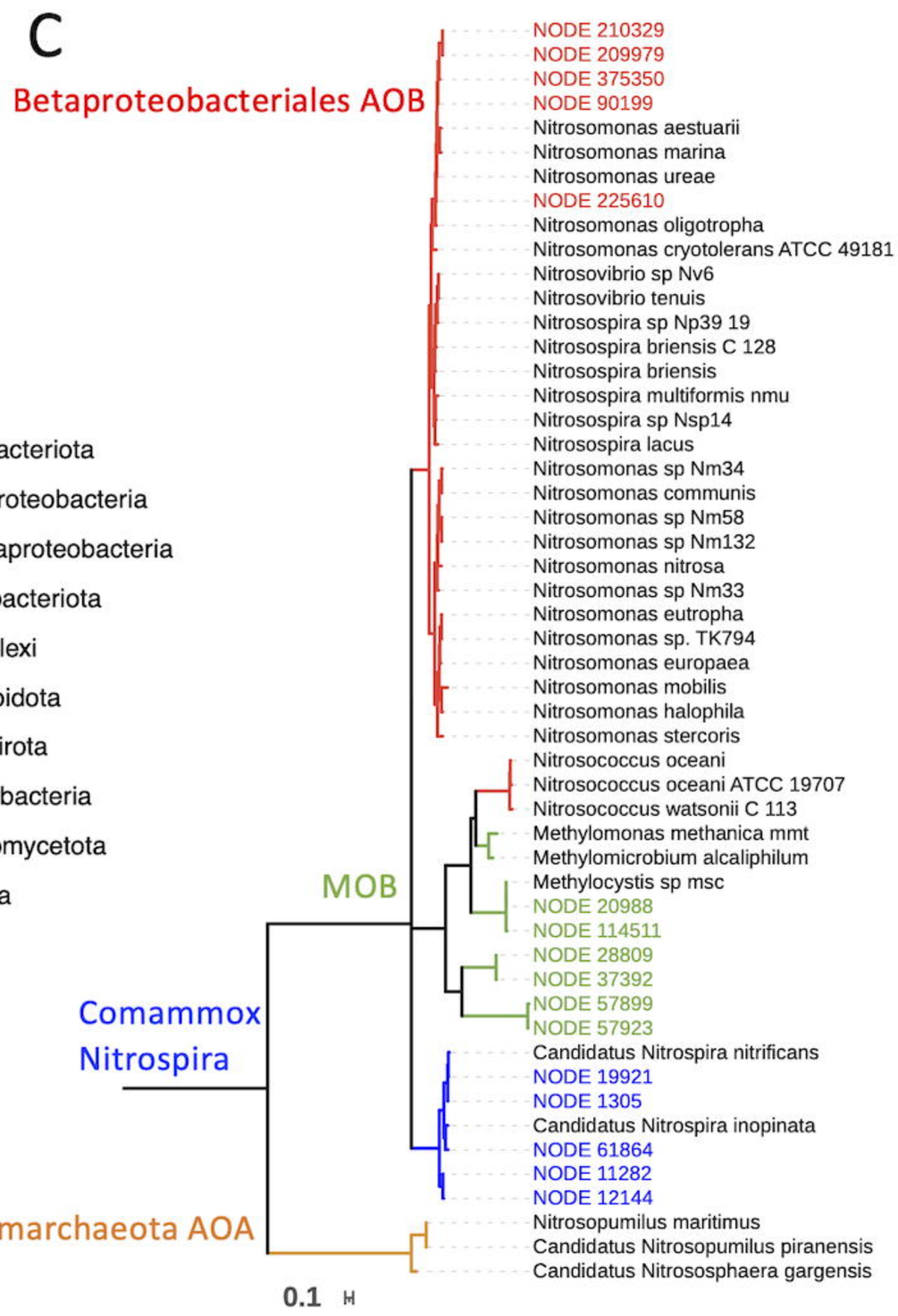
Figure 1: A) Proportion of SSU reads mapping to the SILVA SSU NR99 database (version 138.1) from the inocula and treated samples at weeks four and eight. The community primarily consisted of Gammaproteobacteria, Alphaproteobacteria and Nitrospirota. Proteobacteria is broken down by classes, Alphaproteobacteria and Gammaproteobacteria, though a small portion of Proteobacteria reads could not be classified further. B) Taxonomic classification of genes for nitrogen biotransformation present in the metagenome at the phyla level with Proteobacteria presented by classes, Alphaproteobacteria and Gammaproteobacteria. C and D) Phylogenetic placement of amoA-pmoA like sequences (C), and nxrA sequences (D) detected in the metagenomes. Both maximum likelihood trees were constructed based alignments of protein sequences of the respective genes. Sequences identified in this study are colored according to their phylogenetic placement (red, green, blue and orange) while references are black. AOB = ammonia oxidizing bacteria, MOB = methane oxidizing bacteria.

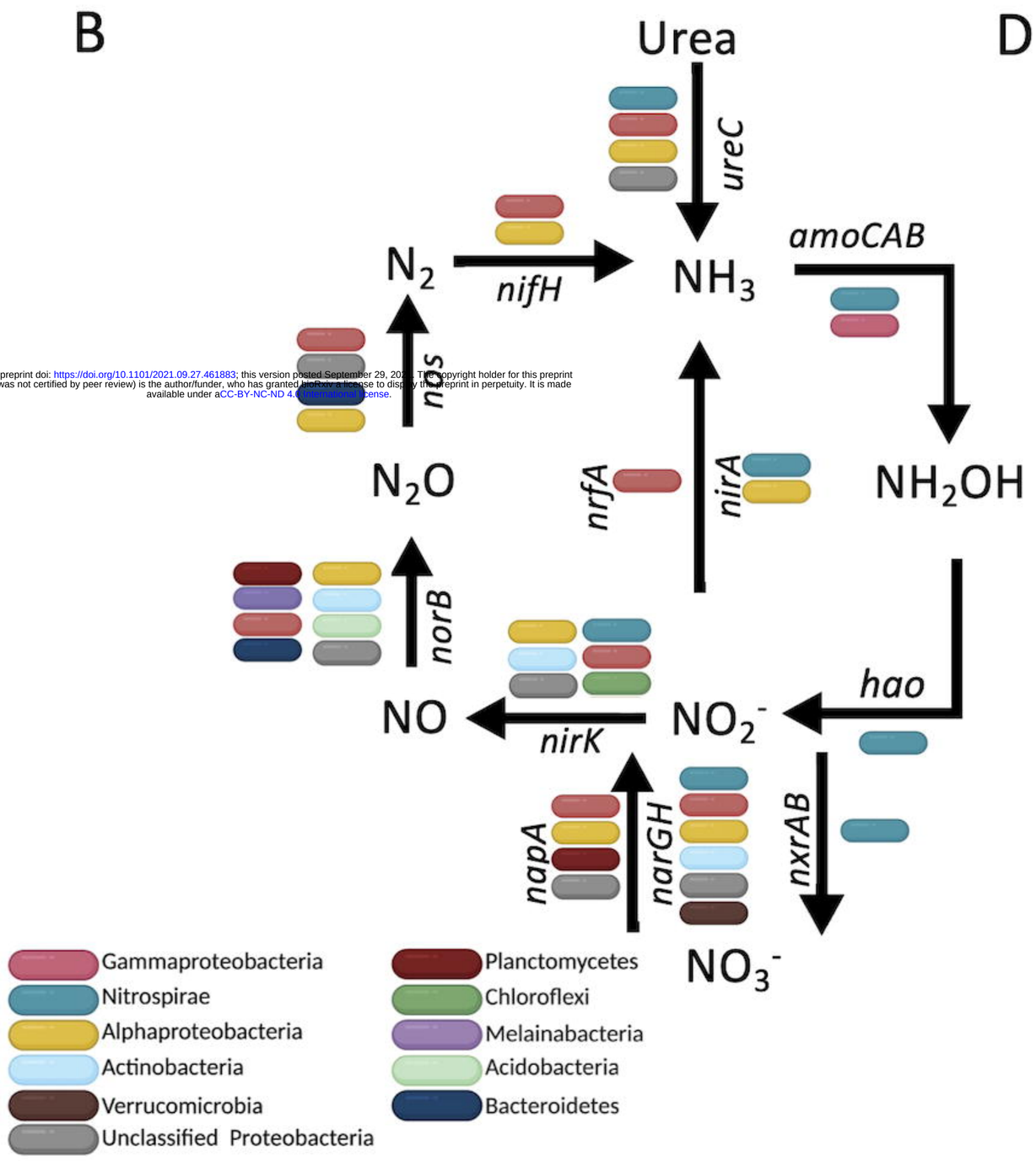
Figure 2: Phylogenomic tree for Nitrospira MAGs (blue and green) obtained in this study and 32 reference genomes (black). Blue label = comammox, green label = NOB. Branch colors represent different Nitrospira sublineages. Two Leptospirillum reference genomes were used as the outgroup for maximum likelihood tree construction. B) Maximum likelihood tree based on ammonia monooxygenase subunit A (amoA) sequences from comammox-Nitrospira. C) Maximum likelihood tree based on hydroxylamine oxidoreductase (hao) sequences of comammox-Nitrospira. For B and C blue labels represent amoA/hao gene sequences found in comammox MAGs from this study, while black labels are reference sequences. Environment of origin is denoted with

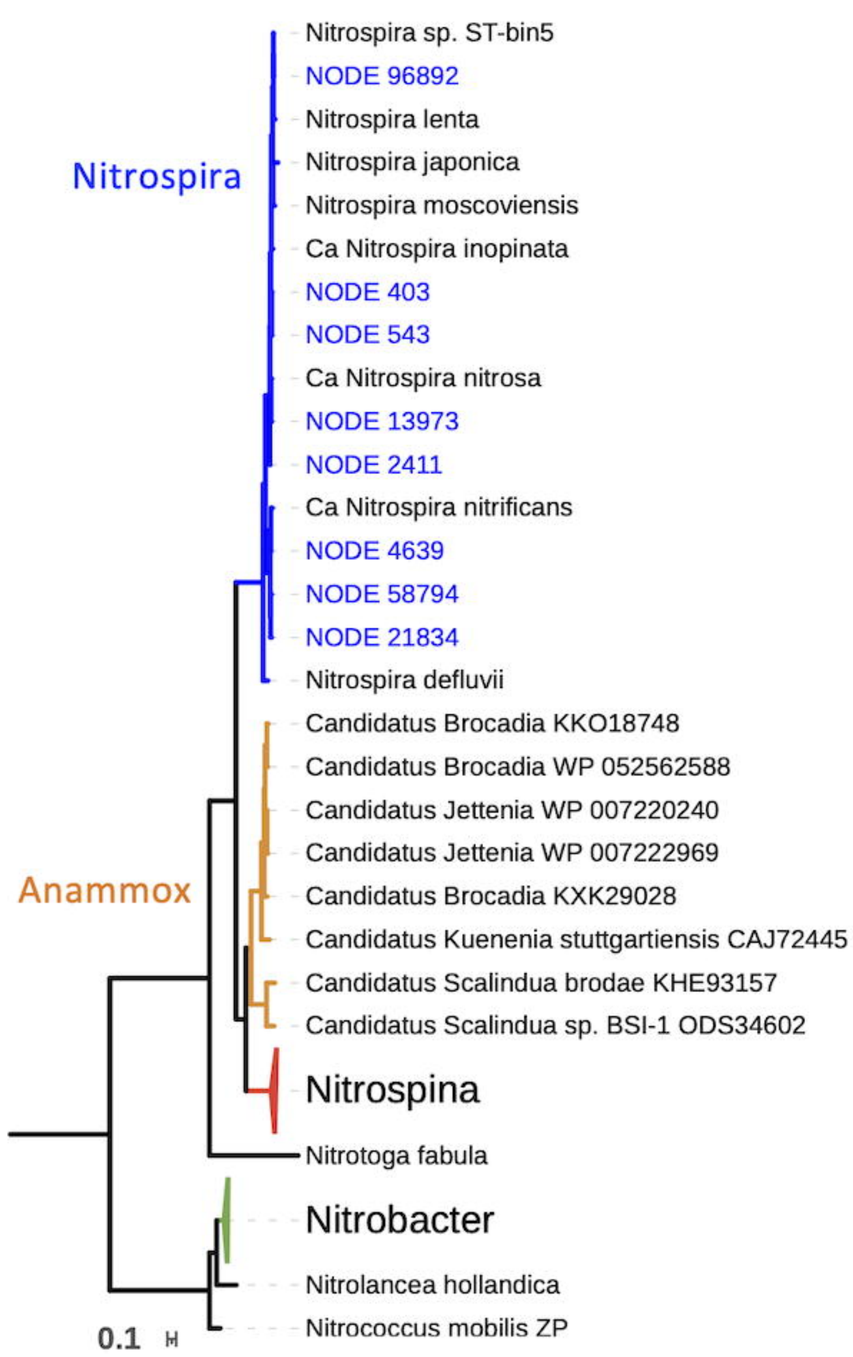
883 colored squares to the left of each tree. amoA and hao protein sequences from Nitrosomonas
884 europaea and Nitrosomonas oligotropha were used as the outgroup for comammox trees in B and
885 C, respectively. D) Phylogenomic tree for strict AOB MAGs (yellow) obtained in this study and
886 10 Nitrosomonas reference genomes (black). Three Nitrosospira genomes were used as the
887 outgroup.
888
889 **Figure 3**: A) The relative abundance of comammox bacteria (blue) and strict AOB (red) as a
890 proportion of all ammonia oxidizing microorganisms calculated using copy number of 16S rRNA
891 and amoB genes of strict AOB and comammox bacteria, respectively and dividing by the
892 combined copy number to represent total AOM for each time point. Data points are the average
893 ratio between duplicate samples. B) Relative abundance of comammox bacteria (blue), Nitrospira-
894 NOB (orange) and strict AOB (red) as a proportion of total bacteria using the ratio of copy number
895 of the respective nitrifier genes to copy number of total bacteria averaged between duplicate
896 samples. Initial abundances of comammox bacteria, Nitrospira-NOB, and strict AOB were 1.2, 1.7
897 and 0.9% of total bacteria, respectively. Panels display relative abundances of the nitrifiers subject
898 to varying nitrogen amendments. C) Reads per kilobase million (RPKM) calculated for all MAGs
899 identifying as comammox bacteria (blue), Nitrospira-NOB (orange) and strict AOB (red) at
900 selected time points.
901
902 **Figure 4**: A) Significant positive correlation between changes in AOB concentration and that of
903 Nitrospira-NOB as a proportion of total bacteria were found in most treatments expect low
904 ammonia (1.5A). B) Negative associations between changes in comammox bacteria concentration
905 and that of Nitrospira-NOB as a proportion of total bacteria existed in ammonia amendments with

906 statistically significance detected in 3.5A and 1.5A while no association existed in urea
907 amendments.
908









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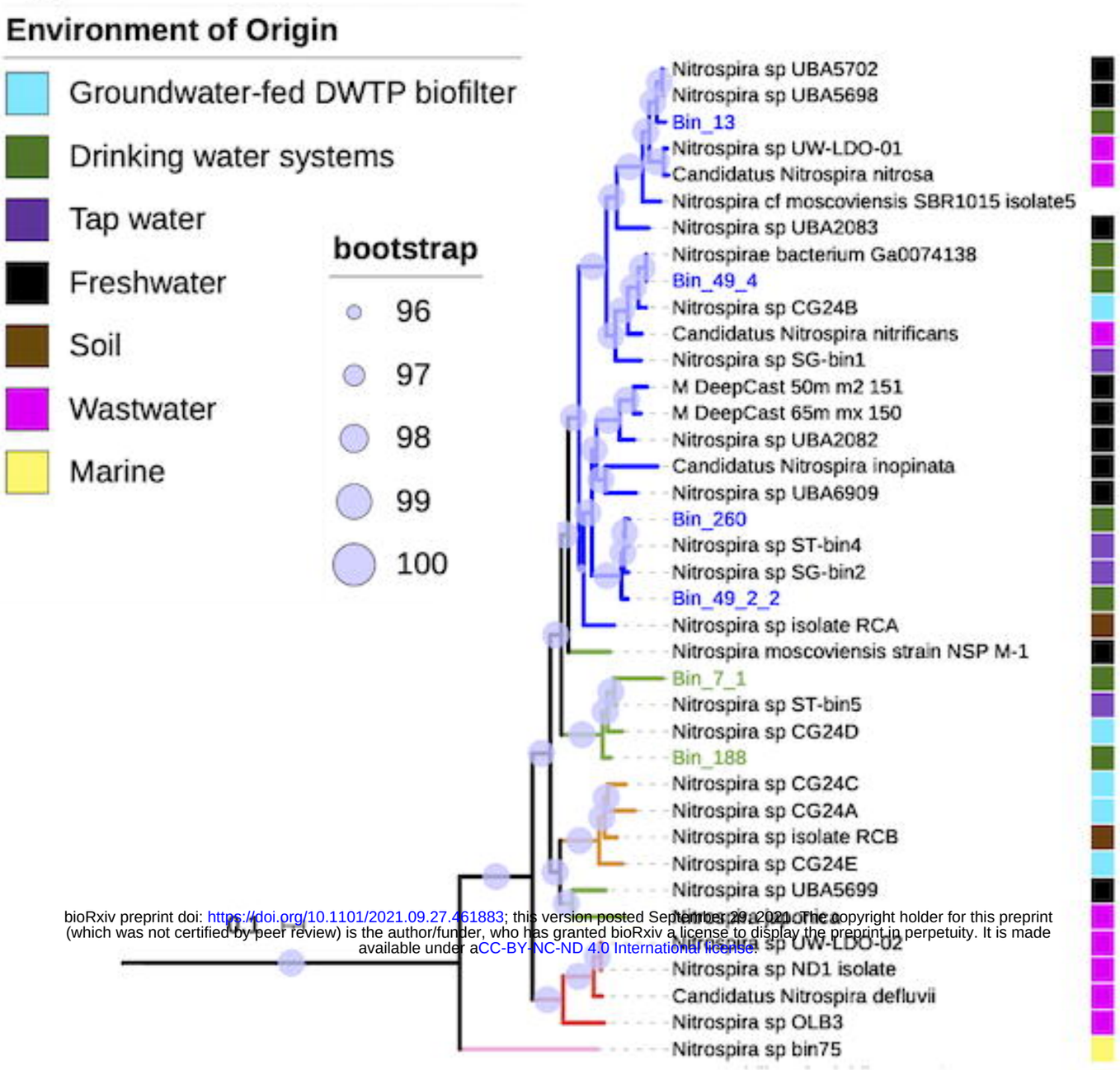

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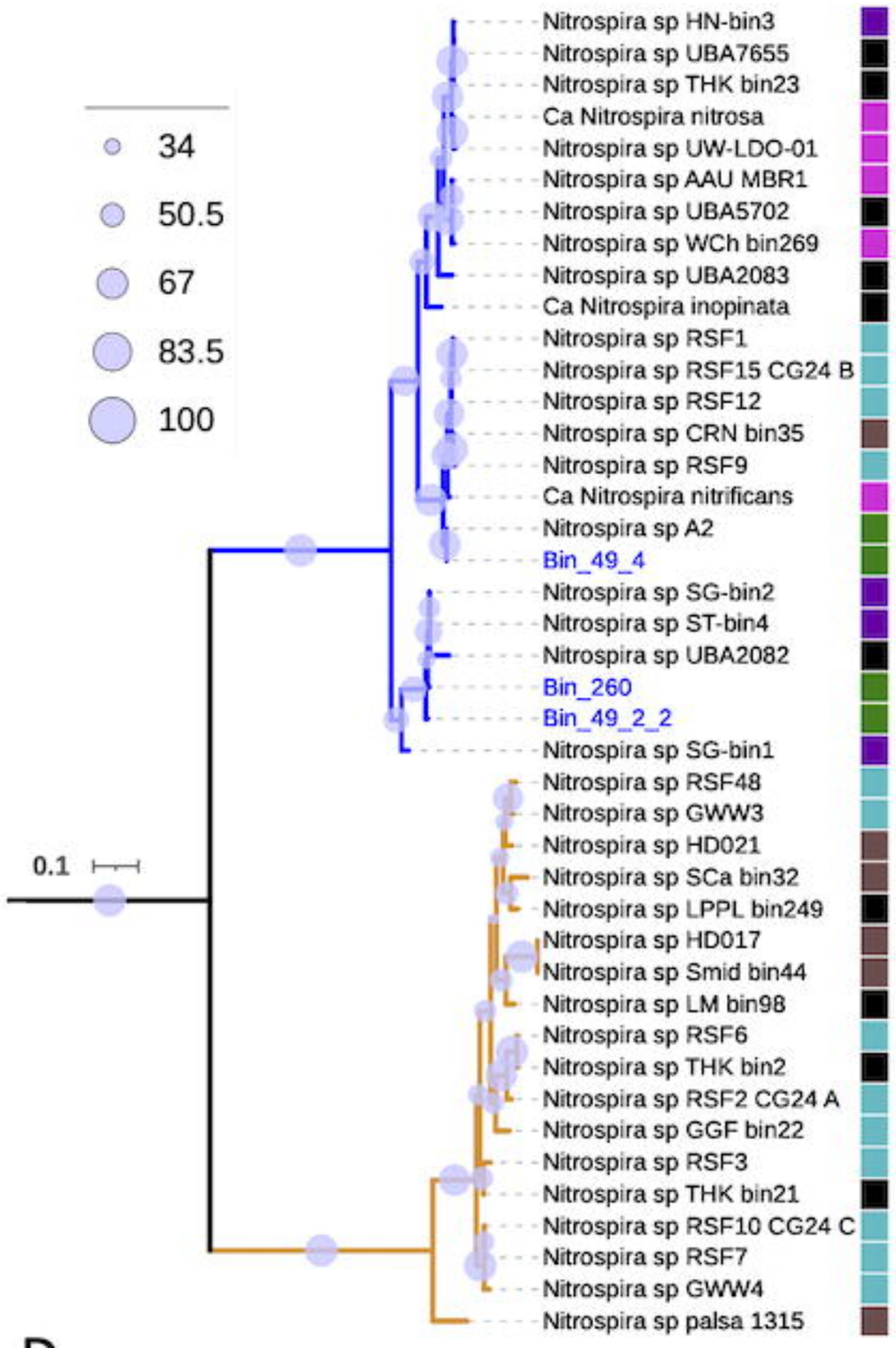

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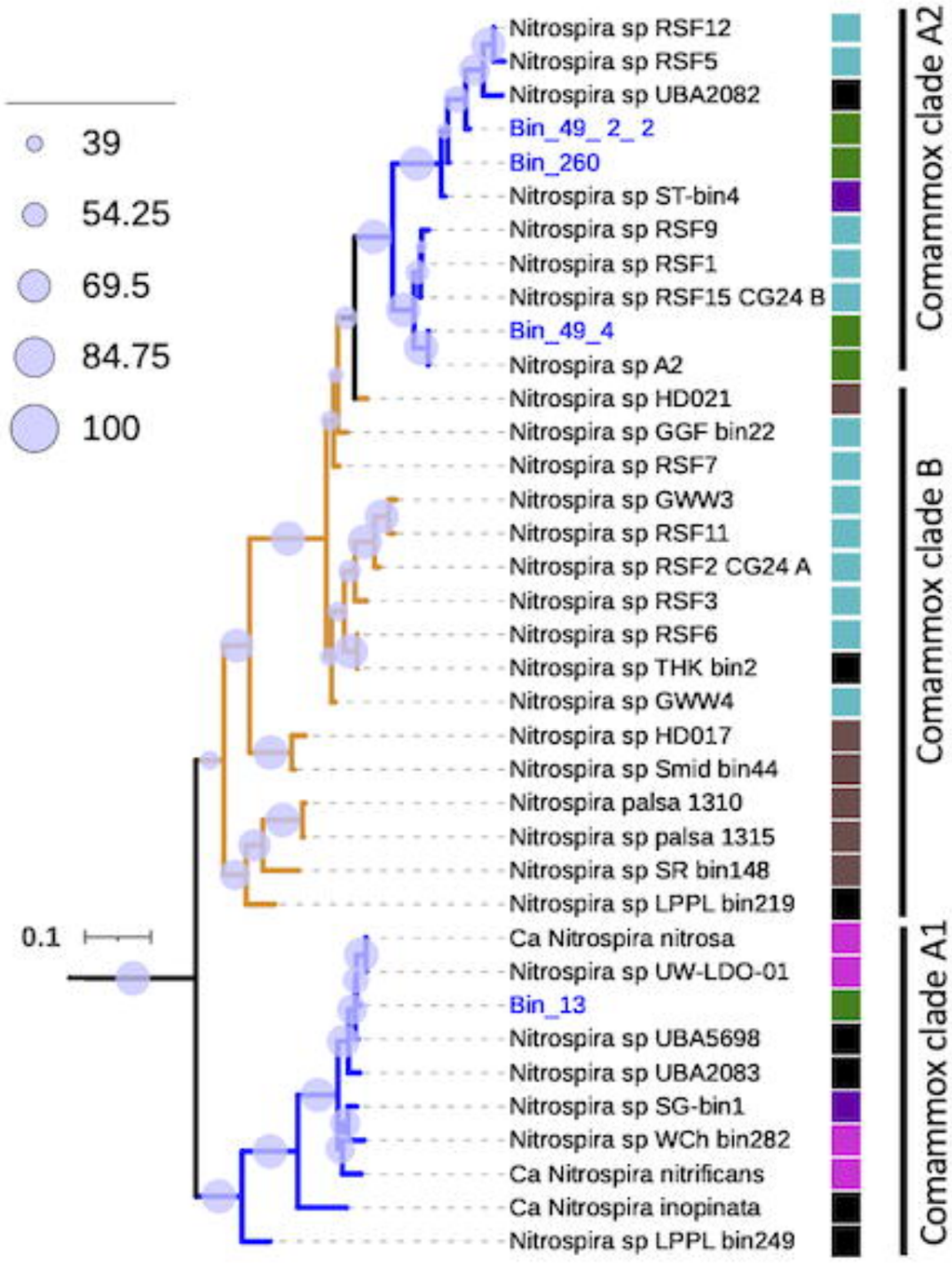

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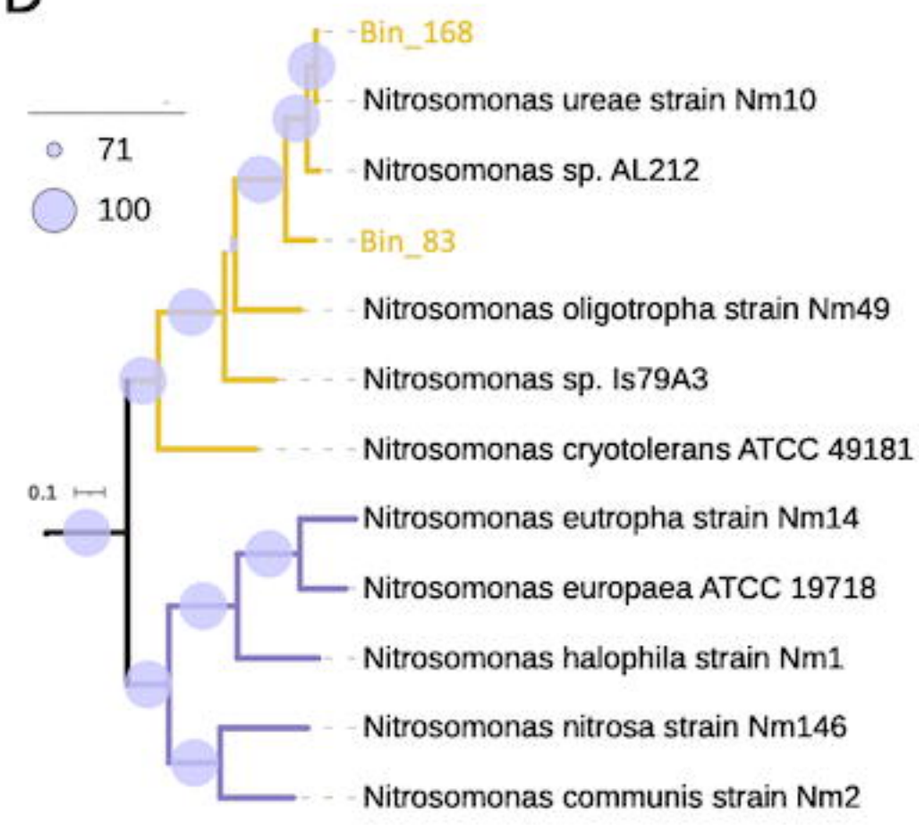
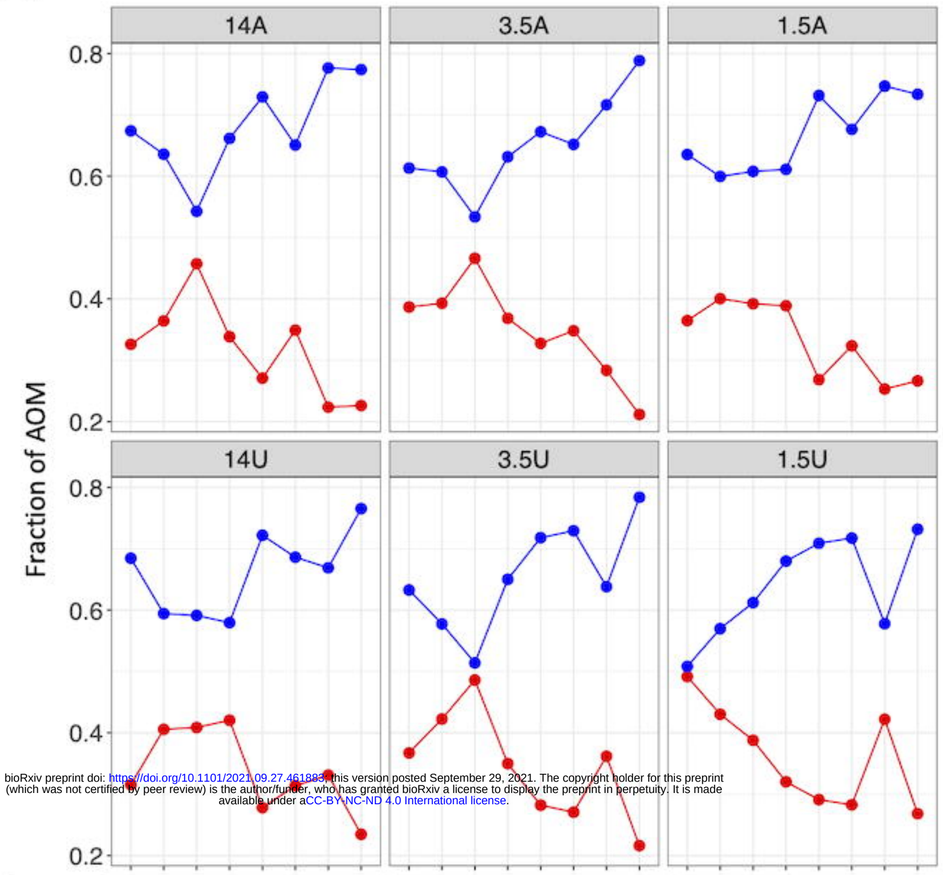


Sublineage II clade A comammox-*Nitrospira*
Sublineage II *Nitrospira*-NOB
Sublineage II clade B comammox-*Nitrospira*
Sublineage I *Nitrospira*-NOB
Sublineage IV *Nitrospira*-NOB
Nitrosomonas cluster 6a
Nitrosomonas cluster 7

A



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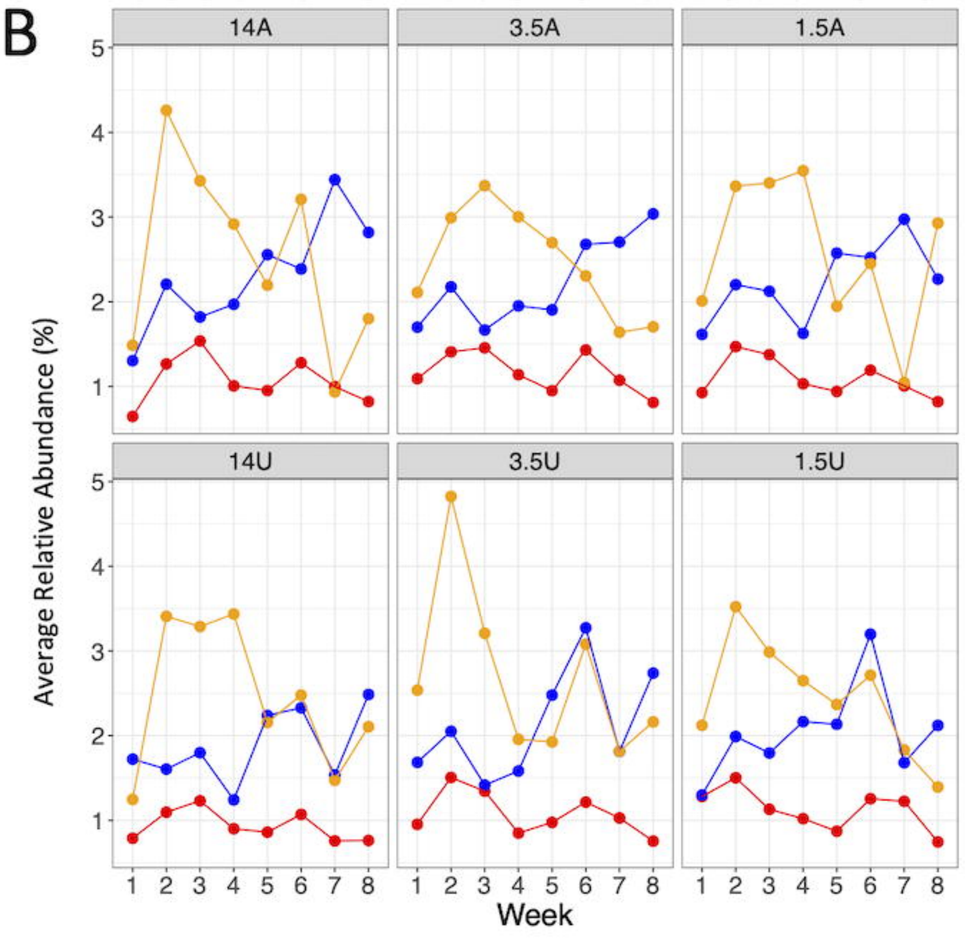

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