

1 **Comammox bacterial preference for urea influences its interactions with aerobic nitrifiers.**

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32 **Abstract**

33 While the co-existence of comammox bacteria with canonical nitrifiers is well documented in  
34 diverse ecosystems, there is still a dearth of knowledge about the mechanisms underpinning their  
35 interactions. Understanding these interaction mechanisms is important as they may play a critical  
36 role in governing nitrogen biotransformation in natural and engineered ecosystems. In this study,  
37 we tested the ability of two environmentally relevant factors (nitrogen source and availability) to  
38 shape interactions between strict ammonia and nitrite-oxidizing bacteria and comammox bacteria  
39 in continuous flow column reactors. The composition of inorganic nitrogen species in reactors fed  
40 either ammonia or urea was similar during the lowest nitrogen loading condition (1 mg-N/L), but  
41 higher loadings (2 and 4 mg-N/L) promoted significant differences in nitrogen species composition  
42 and nitrifier abundances. The abundance and diversity of comammox bacteria were dependent on  
43 both nitrogen source and loading conditions as multiple comammox bacterial populations were  
44 preferentially enriched in the urea-fed system. In contrast, their abundance was reduced in response  
45 to higher nitrogen loadings in the ammonia-fed system likely due to ammonia-based inhibition.  
46 The preferential enrichment of comammox bacteria in the urea-fed system could be associated  
47 with their ureolytic activity calibrated to their ammonia oxidation rates thus minimizing ammonia  
48 accumulation to inhibitory levels. However, an increased abundance of comammox bacteria was  
49 not associated with a reduced abundance of nitrite oxidizers in the urea-fed system while a negative  
50 correlation was found between them in the ammonia-fed system; the latter dynamic likely  
51 emerging from reduced availability of nitrite to strict nitrite oxidizers at low ammonia loading  
52 conditions.

53

54 **Importance**

55 Nitrification is an essential biological process in drinking water and wastewater treatment systems  
56 for managing nitrogen and protecting downstream water quality. The discovery of comammox  
57 bacteria and their detection alongside canonical nitrifiers in these engineered ecosystems has made  
58 it necessary to understand the environmental conditions that regulate their abundance and activity  
59 relative to other better-studied nitrifiers. This study aimed to evaluate two important factors that  
60 could potentially influence the behavior of nitrifying bacteria, and therefore impact nitrification  
61 processes. Column reactors fed with either ammonia or urea were systematically monitored to  
62 capture changes in nitrogen biotransformation and the nitrifying community as a function of

63 influent nitrogen concentration, nitrogen source, and reactor depth. Our findings show that  
64 comammox bacterial abundance decreased and that of nitrite oxidizers increased with increased  
65 ammonia availability, while their abundance and diversity increased with increasing urea  
66 availability without driving a reduction in the abundance of canonical nitrifiers.

67 **Introduction**

68 Comammox bacteria are routinely detected alongside strict ammonia oxidizing bacteria (AOB)  
69 and nitrite oxidizing bacteria (NOB) in both drinking water and wastewater systems (Cotto et al.,  
70 2020; Fowler et al., 2018; Pinto et al., 2015; Poghosyan et al., 2020; Roots et al., 2019; K. J. Vilardi  
71 et al., 2022; Wang et al., 2017; Yang et al., 2020; Zheng et al., 2023) but insights into the factors  
72 influencing their abundance, activity, and interactions in these environments are still limited.  
73 Interactions between AOB and NOB have been extensively studied including the impact of process  
74 and environmental conditions such as oxygen supply, ammonia concentration, and temperature  
75 (Pérez et al., 2014; Seuntjens et al., 2018; Sliekers et al., 2005). However, the presence of  
76 comammox bacteria within these communities requires a re-evaluation of these interactions and  
77 the collective response of nitrifying consortia to changes in environmental and/or process  
78 conditions. Our understanding of the role and ecological niche of comammox bacteria within  
79 complex nitrifying communities is further restricted by limited physiological insights due to the  
80 existence of only a few cultured representatives and/or enrichments, all belonging to clade A1  
81 (Daims et al., 2015; Ghimire-Kafle et al., 2023; Sakoula et al., 2021).

82

83 Ammonia availability is likely an important factor governing interactions between strict AOB and  
84 comammox bacteria. For instance, comammox bacterial cultures and enrichments have shown  
85 significantly higher affinity for ammonia compared to strict AOB (Ghimire-Kafle et al., 2023; Kits  
86 et al., 2017; Sakoula et al., 2021). Thus, comammox bacteria may outcompete strict AOB in  
87 ammonia-limited environments such as drinking water systems. Further, different comammox  
88 bacteria may exhibit varying preferences for ammonia concentration ranges and these may be  
89 dictated not just by ammonia affinities, but also by potential inhibition at higher concentrations.  
90 For example, ammonia oxidation by *Ca. Nitrospira kreti* was partially inhibited at relatively low  
91 ammonia concentrations (25  $\mu$ M) (Sakoula 2021) which was not observed for *Ca. Nitrospira*  
92 *inopinata* (Kits 2017). Comammox bacteria may also exhibit clade/sub-clade dependent  
93 preferences for ammonia availability and/or environments. For example, clade A1 comammox  
94 bacteria associated with *Ca. Nitrospira nitrosa* are typically found at higher abundances than  
95 canonical nitrifiers in some wastewater systems (Cotto 2020, Wang 2018, Xia 2018, Zheng 2023)  
96 and sometimes as the principal aerobic ammonia oxidizers in a wastewater system (Vilardi and  
97 Cotto 2023). In the latter situation, ammonia oxidation dominated by comammox bacteria could

98 also adversely impact *Nitrospira*-NOB by limiting nitrite availability through complete  
99 nitrification to nitrate at low ammonia concentrations; however, the relationship between the two  
100 *Nitrospira* groups is not well understood.

101

102 Nitrogen source could also have a significant effect on interactions between nitrifiers. For instance,  
103 ureolytic activity may enable access to ammonia derived from urea in engineered systems (e.g.,  
104 wastewater treatment), as well as natural systems such as freshwater ecosystems (Solomon et al.,  
105 2010). Genes for urea degradation accompanied by a diverse set of urea transporters are  
106 ubiquitously found in genomes of all comammox bacteria (Palomo et al., 2018). Their ability to  
107 grow in urea is supported by the enrichment of multiple species of comammox bacteria urine-fed  
108 membrane bioreactors (J. Li et al., 2021) and enrichment of comammox bacteria when supplied  
109 with urea (J. Li et al., 2021; Zhao et al., 2021). Some *Nitrospira*-NOB are also capable of  
110 catalyzing ammonia production through urea degradation and thus, potentially regulating nitrite  
111 availability via cross feeding of ammonia to strict AOB (Koch et al., 2015); this could potentially  
112 influence competition between canonical nitrifiers and comammox bacteria.

113

114 The present work aimed to investigate comammox bacterial preferences and potential interactions  
115 with canonical nitrifiers subject to different nitrogen sources and loadings. We operated two  
116 continuous-flow laboratory-scale column reactors with granular activated carbon (GAC)  
117 containing all three nitrifying groups and supplied the reactors with either ammonia or urea at three  
118 different influent nitrogen loadings. Our goal was to infer (1) nitrogen source (i.e., ammonia and  
119 urea), species (i.e., urea, ammonia, nitrite), and concentration preferences of nitrifying groups and  
120 (2) their potential interactions by quantitatively measuring their differential sorting within column  
121 reactors over time in the context of their genome-resolved metabolic capabilities.

122

## 123 Materials and methods

124 **Reactor operation.** Two laboratory-scale column reactors (diameter = 1", height = 10") were  
125 packed with GAC (packed height = 3") and operated with an approximately 1" of water head  
126 above the GAC to ensure the media was fully saturated. The systems were each packed with 35 g  
127 of GAC from the City of Ann Arbor, Michigan Drinking Water Treatment Plant (DWTP). The  
128 two reactors were fed with synthetic groundwater media (Smith et al., 2002). Stock solution for

129 the inorganic compounds in the media was prepared with 3.88 g/L MgCl<sub>2</sub>, 2.81 g/L CaCl<sub>2</sub>, 13.68  
130 g/L NaCl, 6.90 g/L K<sub>2</sub>CO<sub>3</sub>, 17.75 g/L Na<sub>2</sub>SO<sub>4</sub>, and 0.88 g/L KH<sub>2</sub>PO<sub>4</sub>. The organic compound stock  
131 solution contained 3.75 g/L of glucose (C<sub>6</sub>H<sub>12</sub>O<sub>8</sub>) and a third sodium bicarbonate solution was  
132 prepared with 30 g/L of NaHCO<sub>3</sub>. Influent media was then prepared in 10-L autoclaved carboys  
133 with 1 mL/L of the inorganic and organic compound stock solutions and 10 mL/L of the sodium  
134 bicarbonate stock solution. The two reactors were fed influent amended with stock solutions of  
135 ammonium chloride (NH<sub>4</sub>Cl) or urea (CH<sub>4</sub>N<sub>2</sub>O). Influent media was pumped at 1.15 L/day with  
136 the peristaltic pump resulting in an empty bed contact time (EBCT) of approximately 48 minutes.  
137 Both reactors were fed influent at three different nitrogen concentrations over the experimental  
138 period. Columns reactors were maintained in conditions 1 (1 mg-N/L) and 2 (2 mg-N/L) for eight  
139 weeks and in condition 3 (4 mg-N/L ) for six weeks.

140

141 **Sample collection and processing.** Influent and effluent were sampled twice weekly, while five  
142 samples spaced approximately 0.5" apart along the depth of the GAC column were collected  
143 weekly to capture depth-wise nitrogen species concentrations. The five sections are defined as  
144 sections 0.5 (top), 1, 1.5, 2 and 2.5 (bottom). All aqueous samples were filtered through 0.22 µm  
145 filters (Sartorius Minisart NML Syringe Filter - Fisher Scientific 14555269). GAC media samples  
146 were collected at week 0 followed by weeks six, seven and eight for conditions 1 and 2, and week  
147 six for condition 3. GAC media samples (0.3 grams) were collected from three locations along the  
148 reactor bed: one within the top 0.5" of the reactor, one mid-filter depth (1.5"), and another at the  
149 bottom approximately 3" from the top of the reactor location and were stored for DNA extraction  
150 in Lysing Matrix E Tubes (MP Biomedical - Fisher Scientific MP116914100). After each sampling  
151 event, the amount of GAC taken was replaced with virgin GAC which was mixed with the  
152 remaining GAC by first fluidizing the filter media with 50 mL deionized water followed by  
153 backwashing with air for 5 minutes.

154

155 **Chemical Analysis.** Hach TNT Vials were used to determine concentrations of ammonia  
156 (TNT832), nitrite (TNT839), nitrate (TNT835), and total alkalinity (TNT870). All samples were  
157 analyzed on a Hach DR1900 photospectrometer (Hach—DR1900-01H). Influent and effluent pH  
158 was determined using a portable pH meter (Thermo Scientific™ Orion Star™ A221 Portable pH  
159 Meter – Fisher Scientific 13-645-522). A Shimadzu TOC-L (total organic carbon analyzer) with a

160 TNM-L attachment (total nitrogen unit) (Stubbins and Dittmar 2012) was used to measure total  
161 dissolved nitrogen in influent and effluent samples using certified DOC/TDN standards (deep  
162 seawater reference (DSR): low carbon seawater, LSW, deep seawater reference material) (Batch  
163 21 Lot 11-21, 1). Urea concentrations in samples collected from the urea-fed reactors were  
164 determined by subtracting the total inorganic nitrogen measured (i.e., sum of ammonia, nitrite, and  
165 nitrate) in each sample from influent urea concentration.

166

167 **Nitrogen biotransformation rate calculations.** Rates of nitrogen biotransformations were  
168 calculated from the concentration profiles of ammonia, NO<sub>x</sub> (nitrite plus nitrate), nitrate, and total  
169 inorganic nitrogen (sum of ammonia, nitrite, and nitrate) measured along the column reactor  
170 depths. Rates were calculated for six sections of the columns: 0-0.5, 0.5-1, 1-1.5, 1.5-2, 2-2.5, and  
171 section 2.5-3 inches. Volumetric rates (mg-N/L packed GAC/h) were obtained by multiplying the  
172 concentration differences between the profile layers by the influent flow rate and dividing by the  
173 volume of packed GAC between the profile layers (V=6.4 mL packed GAC in each layer).

174

175 **DNA extraction and qPCR assays.** DNA was extracted from all GAC samples (n=43) which  
176 included the inocula and samples collected at all time points and locations during each condition.  
177 Extractions were performed using Qiagen's DNeasy Powersoil Pro (Qiagen, Inc – Cat. No.  
178 47014) with a few modifications. GAC in lysing matrix tubes with 800 µL of CD1 was vortexed  
179 briefly and placed in a 65°C water bath for 10 minutes. After heating, 500 µL of  
180 phenol:chloroform:isoamyl Alcohol (25:24:1, v/v) (Invitrogen™ UltraPure™ - Fisher Scientific  
181 15-593-031) was added to the lysing tube and bead beating proceeded with four 40 second rounds  
182 on the FastPrep-24 instrument (MP Biomedical – Cat. No. 116005500) with lysing tubes placed  
183 on ice for two minutes between rounds. Samples were then centrifuged for one minute and 600 µL  
184 of the aqueous phase was used for DNA extractions on the Qiacube (Qiagen, Inc—Cat No.  
185 9002160) protocol for Powersoil Pro. A reagent blank was included in each round of extractions  
186 as a negative control. DNA concentrations were measured using a Qubit with the dsDNA Broad  
187 Range Assay (Invitrogen™ - Fisher Scientific Q32850). Extracted DNA was stored at -80°C until  
188 further processing.

189

190 qPCR assays were conducted using Applied Biosystems 7500 Fast Real-Time PCR instrument.  
191 Primer sets listed in supplemental table 1 were used to target the 16S rRNA gene of AOB  
192 (Hermansson and Lindgren 2001), 16S rRNA gene of *Nitrospira* (Graham 2007), *amoB* gene of  
193 clade A comammox bacteria (Vilardi 2022), and 16S rRNA gene of total bacteria (Caporaso 2011).  
194 The qPCR reactions were performed in 20  $\mu$ L volumes, which contained 10  $\mu$ L Luna Universal  
195 qPCR mastermix (New England Biolabs Inc., Fisher Scientific Cat. No. NC1276266), 5  $\mu$ L of 10-  
196 fold diluted template DNA, primers at concentrations listed in supplemental table 1 and  
197 DNA/RNAase free water (Fisher Scientific, Cat. No. 10977015) to make the remaining volume.  
198 Each sample was subjected to qPCR in triplicate. The cycling conditions consisted of initial  
199 denaturing at 95°C for 1 minute, 40 cycles of denaturing 95°C for 15 seconds, annealing times and  
200 temperatures listed in supplemental table 1, and extension at 72°C for 1 minute. Three different  
201 sets of gBlock standards (Integrated DNA Technology gBlocks® Gene Fragments 125-500 bp)  
202 targeting the 16S rRNA gene of total bacteria and *Nitrospira*, 16S rRNA gene of AOB and *amoB*  
203 gene of clade A comammox bacteria were used to establish a 7-point standard curve for each  
204 respective assay (supplemental table 2). The qPCR efficiencies for all assays are listed in  
205 supplemental table 1.

206

207 **16S rRNA gene amplicon sequencing and data analysis.** DNA extracts (triplicate per sample)  
208 from all samples were submitted for sequencing of the V4 hypervariable region of the 16S rRNA  
209 gene at the Georgia Institute of Technology Sequencing Core. The MiSeq v2 kit was used to  
210 generate 250 bp pair-end reads using the 515F (Parada et al., 2016) and 806R (Apprill et al., 2015)  
211 primers with overhang of Illumina adapters. Removal of adapter and primer sequences from the  
212 resultant sequencing data was carried out using cutadapt v4.2. Amplicon sequencing data  
213 processing and quality filtering was performed using DADA2 v1.22.0 (Callahan 2016) in R v4.1.2.  
214 to infer amplicon sequencing variants (ASVs) using the pipeline for paired-end Illumina MiSeq  
215 data. The SILVA nr v.138.1 database was used for taxonomic assignment of ASVs with a  
216 minimum bootstrap confidence threshold of 80. The ASV table was rarefied with  
217 ‘rarefy\_even\_depth’ function from the R package phyloseq v1.38.0 to the sample with the smallest  
218 library size. The relative abundance of ASVs in each sample were calculated by dividing ASV  
219 reads counts in the sample by the total number of samples read counts.

220 **Metagenomic sequencing, assembly, and binning.** DNA extracted from samples taken at week  
221 six from the top layer of the ammonia- and urea-fed reactors during condition 3 were submitted  
222 for sequencing on the Illumina NovaSeq platform with a SP flow cell at the Georgia Institute of  
223 Technology Sequencing Core. Similar workflows and tools utilized in Vilardi 2022 and Cotto 2023  
224 (Cotto et al., 2023; K. J. Vilardi et al., 2022) were applied here to assemble and characterize  
225 metagenome assembled genomes (MAGs). Briefly, raw paired-end reads were quality filtered  
226 using fastp (Chen et al., 2018) and further mapped to the Univec database to remove contaminated  
227 reads. Samtools (Danecek et al., 2021) was used to sort the resulting bam files and bedtools  
228 (Quinlan & Hall, 2010) was used to convert them to fastq files. Assemblies were generated for  
229 each sample separately using metaSpades (Nurk et al., 2017) with kmer sizes 21, 33, 55 and 77.  
230 The two resulting fasta assemblies were indexed with bwa index and filtered pair-ended reads  
231 were mapped to back to their respective assemblies with bwa mem (H. Li & Durbin, 2009). The  
232 subsequent sam files were converted to bam files using appropriate samtools flags to retain only  
233 mapped reads.

234

235 Metabat2 (Kang et al., 2019) was used to bin contigs longer than 2000 bp followed by CheckM  
236 (Parks et al., 2015) to determine completeness and contamination levels of metagenome assembled  
237 genomes (MAGs) which were then classified using the Genome Taxonomy Database Tool Kit  
238 with database release 207 (Chaumeil et al., 2019; Parks et al., 2018). Open reading frames of  
239 coding regions predicted using prodigal (Hyatt et al., 2010) were annotated against the KEGG  
240 database (Kanehisa et al., 2016) with kofamscan (Aramaki et al., 2020). Up-to-date Bacterial Core  
241 Gene pipeline (Na et al., 2018) was used to construct maximum likelihood trees using a set 92  
242 extracted and aligned single copy genes from assembled *Nitrospira*-like and *Nitrosomonas*-like  
243 MAGs and references. A set of dereplicated MAGs was generated from MAGs recovered from the  
244 ammonia- and urea-fed systems at an ANI threshold of 99% using dRep (Olm et al., 2017). Reads  
245 from the ammonia- and urea-fed samples were mapped to the set of dereplicated MAGs to  
246 calculate breadth of coverage (i.e., percent of genome covered by reads) and relative abundance  
247 using coverM (<https://github.com/wwood/CoverM>).

248

249 16S rRNA gene sequences of a minimum length of 500bp were reconstructed from the  
250 metagenomes of both samples using MATAM v1.6.1 (Pericard et al., 2018) to establish the

251 linkage between ASVs generated from 16S rRNA gene amplicon sequencing and MAGs  
252 associated with nitrifying bacteria. To achieve a more comprehensive reconstruction, recursive  
253 random subsampling of different depths, i.e., 1%, 5%, 10%, 25%, 50%, 75% and 100%, was  
254 performed, followed by dereplication at 99.9% identity using USEARCH v11.0.667 (Edgar, 2010;  
255 Song et al., 2022). Only the longest sequence from each cluster was retained for downstream  
256 analysis. Furthermore, ASVs were aligned against the MATAM recovered 16S rRNA gene  
257 sequences and extracted 16S rRNA genes from MAGs by Barrnap using BLASTn v2.13.0  
258 (Camacho et al., 2009), and only ASV hits of 100% identity and 100% coverage were considered  
259 as linkage candidates between ASV and MAG unless the alignment interrupted at the end of the  
260 reference sequence.

261

262 **Statistical Analysis.** All statistical analysis was performed in R v4.1.2 (RCore team., 2021). A  
263 significant difference in effluent concentrations of ammonia, nitrite, and nitrate in the ammonia-  
264 and urea-fed was tested using the non-parametric Wilcoxon rank sum test. Ratios of effluent NO<sub>x</sub> to  
265 influent nitrogen concentrations as a proxy for ammonia consumption in both systems was  
266 compared with the Student's T-test for conditions 2 and 3 while condition 1 required the systems  
267 be compared with the Welch's T-test due to unequal variance between the ammonia- and urea-fed  
268 systems. The data distribution and variance for all tests was checked with the Shapiro-Wilks and  
269 Levene test, respectively. We tested if the microbial community in GAC samples clustered  
270 significantly by nitrogen source, loading condition, and reactor depth using the Bray-Curtis  
271 dissimilarities calculated from the ASV abundance table and applying a PERMANOVA test using  
272 the adonis function in the R package vegan v2.6-4 (*Vegan: Community Ecology Package*, 2022).  
273 Correlations between microbial community composition and concentrations of ammonia, nitrite,  
274 and nitrate measured in the biofiltrations were calculated with the Mantel test. The mean relative  
275 abundances of nitrifier ASVs and qPCR-based relative abundances of comammox bacteria, strict  
276 AOB and *Nitrospira*-NOB were compared in both systems across the nitrogen loading conditions  
277 using ANOVA. Pearson correlation was used to statistically quantify and test the significance of the  
278 relationship between the abundance of comammox bacteria and *Nitrospira*-NOB in both systems.

279

280 **Data availability.** Raw fastq files for amplicon sequencing and metagenomic sequencing data,

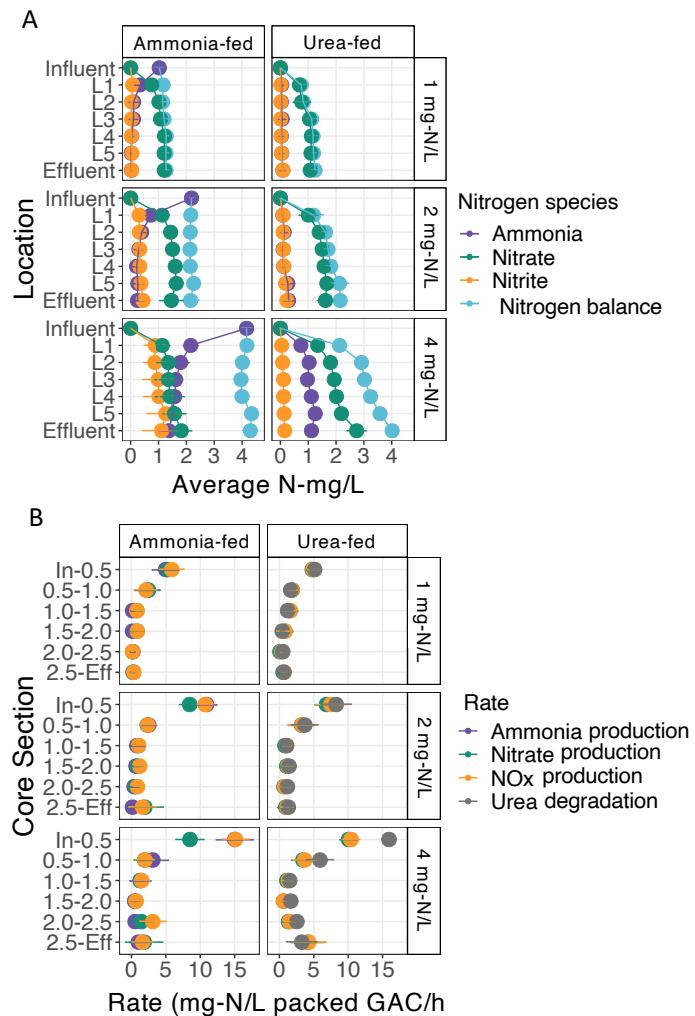
281 metagenomic assembly, and curated MAGs are available via NCBI bioproject submission number  
282 SUB13674413.

283

## 284 Results

285 **Nitrogen biotransformation in ammonia and urea fed systems.** Urea and ammonia fed systems  
286 had similar concentrations of ammonia, nitrite, and nitrate in the effluent (Wilcoxon  $p>0.05$ )  
287 (Supplemental Figure 1) and similar depth-wise distributions of inorganic nitrogen species (Figure  
288 1A) at lowest influent loading condition (condition 1 - 1 mg-N/L). Majority of the influent nitrogen  
289 (~70%) was complete oxidized to nitrate in the topmost portion of the reactors (section In-0.5)  
290 (Figure 1A and 1B). Rates of ammonia oxidation ( $4.99 (\pm 1.95)$  mg-N/L packed GAC/h) and urea  
291 degradation ( $5.13 (\pm 0.70)$  mg-N/L packed GAC/h) were highest in section In-0.5 and were nearly  
292 equal to the rate of nitrate production ( $5.31 (\pm 1.55)$  and  $4.79 (\pm 0.83)$  mg-N/L packed GAC/h,  
293 respectively) (Figure 1B). Increasing the influent nitrogen concentrations to 2 mg-N/L (i.e.,  
294 condition 2) led to significantly higher nitrite accumulation in the ammonia-fed compared to the  
295 urea-fed reactor (Wilcoxon  $p < 0.05$ ) due to an imbalance between ammonia oxidation ( $10.91 (\pm$   
296  $1.49)$  mg-N/L packed GAC/h) and nitrate production ( $8.51 (\pm 2.03)$  mg-N/L packed GAC/h) rates  
297 in section In-0.5 of the ammonia-fed system (Figure 1B). In contrast, average urea degradation  
298 rate ( $8.25 (\pm 2.21)$  mg-N/L packed GAC/h) was nearly equal to the nitrate production rate ( $6.85 (\pm$   
299  $1.67)$  mg-N/L packed GAC/h) in the top section of the urea-fed reactor. The nitrite accumulation  
300 was exacerbated at the higher influent nitrogen loading condition (condition 3: 4 mg-N/L) with  
301 significantly higher effluent nitrite concentrations in the ammonia-fed ( $1.00 (\pm)$  mg-N/L)  
302 compared to the urea-fed system ( $0.10 (\pm 0.04)$  mg-N/L). The average ammonia oxidation rate  
303 ( $15.02 (\pm 2.67)$  mg-N/L packed GAC/h) in the top section of the ammonia-fed system was 1.8  
304 times higher than the nitrate production rate ( $8.50 (\pm 2.03)$  mg-N/L packed GAC/h). The rates of  
305 nitrate production in section In-0.5 were similar between conditions 2 and 3 ( $8.56 (\pm 1.47)$  and  
306  $8.50 (\pm 2.03)$  mg-N/L packed GAC/h, respectively) in the ammonia-fed system indicating  
307 maximum rates of nitrate production had been reached. Interestingly, ammonia accumulation in  
308 the urea-fed systems resulted in similar effluent ammonia concentrations as the ammonia-fed  
309 systems for conditions 1 and 2 suggesting that both reactors had reached their ammonia oxidation  
310 capacity across the entire depth of the reactors.

311



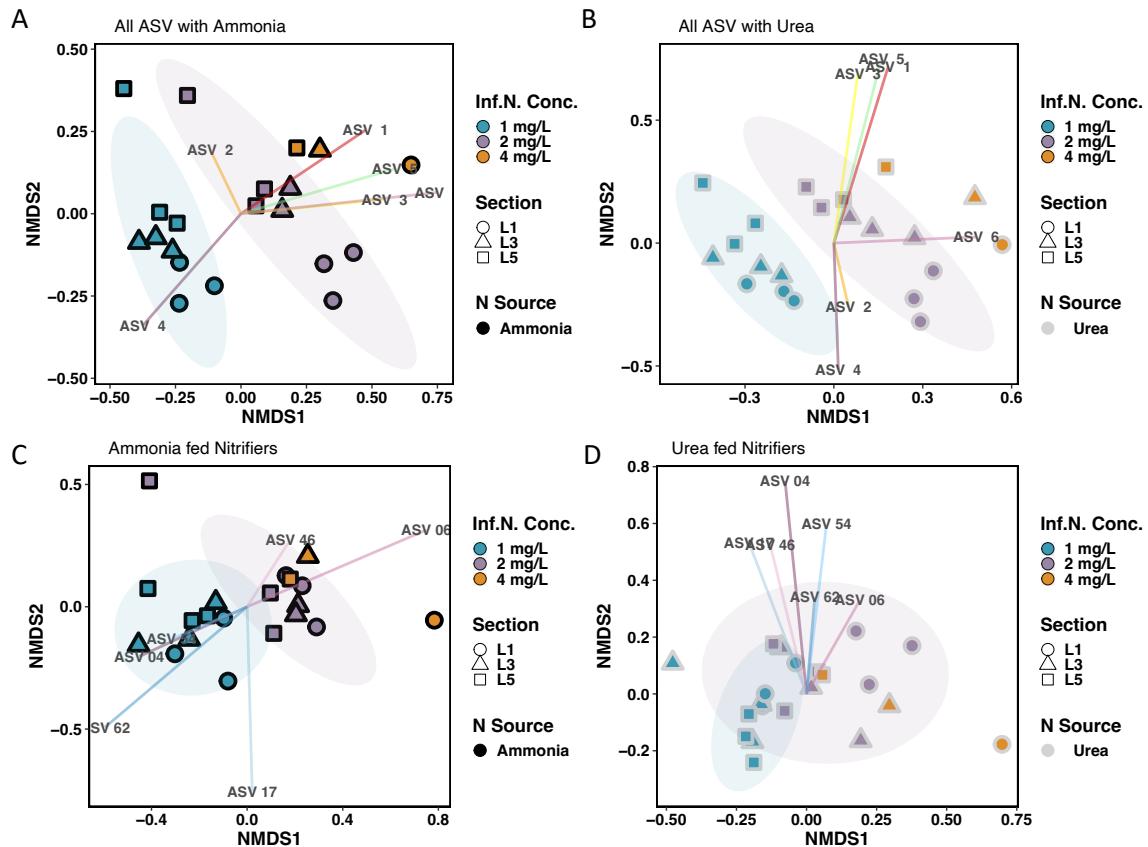
312  
313 **Figure 1:** (A) Concentrations of ammonia, nitrite, and nitrate measured at five depths along the reactors.  
314 Measurements were taken at 0.5", 1.0", 1.5", 2.0", and 2.5" from the top of GAC in the reactors. Data points  
315 represent the average concentration obtained from the different reactor depths along with error bars for  
316 standard deviation. (B) Rates of nitrogen biotransformation along the depths of the reactors. Rates are  
317 colored by the type of nitrogen biotransformation (purple = ammonia oxidation, green = nitrate production,  
318 orange = NOx production, and grey = urea degradation). Data points represent the average rates obtained  
319 from the different reactor depths along with error bars for standard deviation.  
320

321 **GAC microbial community composition.** Rarefaction to the smallest library size (n=70354  
322 reads) resulted in retention of 1738 ASVs out of 2400 constructed from the V4 hypervariable  
323 region of the 16S rRNA gene. ASVs with the highest relative abundance belonged to the class  
324 *Gammaproteobacteria* (5.09 ( $\pm$  1.96) % - ASV 1 and 4.62 ( $\pm$  2.96) % - ASV 2), *Vicinamibacteria*  
325 (3.70 ( $\pm$  1.49) % - ASV 3), *Nitrospirilia* (3.50 ( $\pm$  1.63) % - ASV 4 and 2.72 ( $\pm$  1.40) % - ASV 6),  
326 and *Alphaproteobacteria* (3.40 ( $\pm$  1.56) % - ASV 5). Microbial community composition was  
327 shaped significantly by nitrogen loading condition (ANOSIM R=0.628, p < 0.05), nitrogen source

328 (ANOSIM  $R = 0.134$ ,  $p < 0.05$ ), and reactor section (i.e., GAC sampling point, top (L1), middle  
329 (L3), bottom (L5)) (ANOSIM  $R = 0.163$ ,  $p < 0.05$ ) (Supplementary Figure 2A). Nitrogen source  
330 (i.e., ammonia-fed vs urea-fed) played a more significant role in shaping the overall microbial  
331 community for condition 2 (PERMANOVA  $R = 0.224$ ,  $p < 0.05$ ) as compared to condition 1  
332 (PERMANOVA  $R = 0.104$ ,  $p > 0.05$ ) or condition 3 (PERMANOVA  $R = 0.412$ ,  $p > 0.05$ ).  
333 Community composition of the two reactors may not exhibit a strong difference for condition 1  
334 due to very similar depth-wise nitrogen species profiles (Figure 1A and 1B), while differences  
335 during condition 3 may not be flagged as significant due to the limited data points. In the ammonia-  
336 fed system (Figure 2A), the microbial community separated into distinct clusters based on nitrogen  
337 loading condition (ANOSIM  $R = 0.614$ ,  $p < 0.05$ ) but not by reactor depth (ANOSIM  $R = 0.077$ ,  $p$   
338  $> 0.05$ ). In contrast, both nitrogen loading (ANOSIM  $R = 0.665$ ,  $p < 0.05$ ) and reactor depth  
339 (ANOSIM  $R = 0.204$ ,  $p < 0.05$ ) were significantly associated with differences in microbial  
340 community composition for the urea-fed system (Figure 2B).

341

342 Compositional differences in nitrifier communities were evaluated with ASVs classified as  
343 *Nitrospira*- (9 ASVs) and *Nitrosomonas*-like (5 ASVs) bacteria; this is in line with our previous  
344 work which found nitrifiers belonged to only these genera in GAC samples from the same  
345 biofiltration system (Vilardi 2022). Nitrifier communities in the ammonia- and urea-fed systems  
346 were significantly dissimilar during conditions 1 (PERMANOVA  $R = 0.319$ ,  $p < 0.05$ ) and 2  
347 (PERMANOVA  $R = 0.368$ ,  $p < 0.05$ ) (Supplementary Figure 2B). Nitrogen source explained a  
348 greater variance between communities in condition 3 (PERMANOVA  $R = 0.406$ ) but was found  
349 to be insignificant potentially due to fewer data points. In both the ammonia- and urea-fed systems,  
350 nitrifier community composition was most dissimilar between conditions 1 and 4 (PERMANOVA  
351  $R = 0.607$  and  $0.536$ ,  $p < 0.05$ ) (Figure 2C, D). Collectively, our results show that composition of  
352 both the whole community and nitrifiers were significantly shaped by nitrogen source and  
353 availability. The largest impacts were consistently observed when comparing the lowest and  
354 highest nitrogen loadings conditions.



355

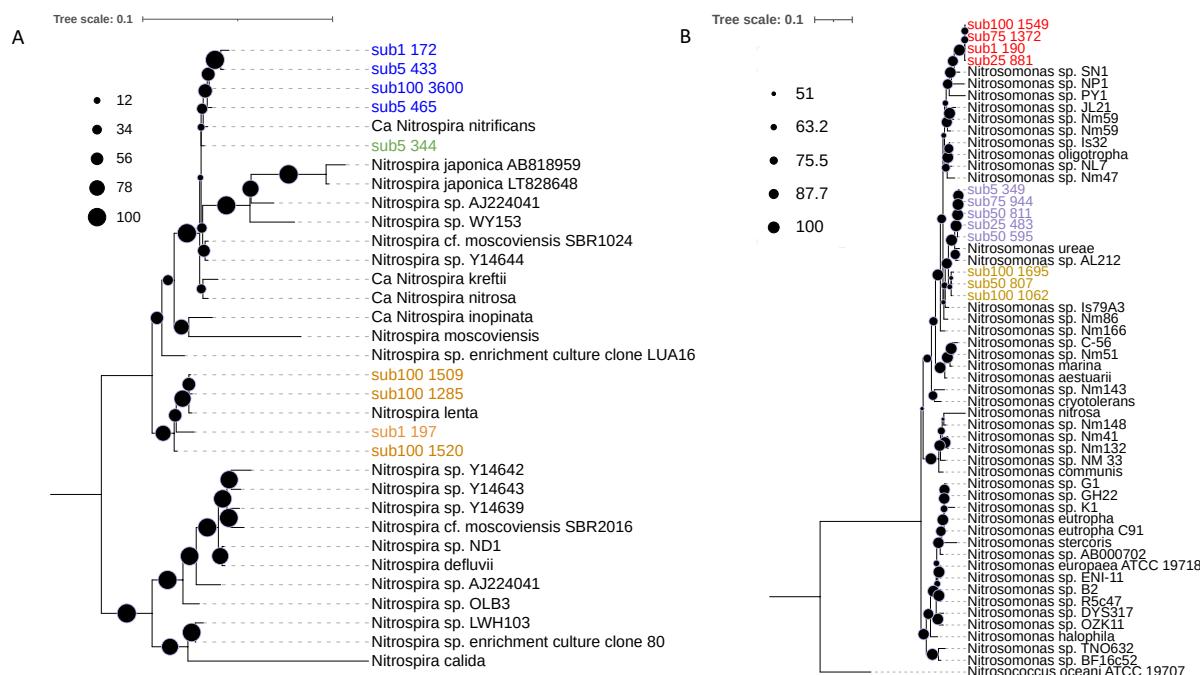
356 **Figure 2:** NMDS plots constructed with the abundance tables of all ASVs in the (A) ammonia-fed system,  
 357 and (B) urea-fed system and nitrifier ASVs in (C) the ammonia-fed and (D) in the urea-fed system (D). Blue-  
 358 , purple- and orange-colored points are GAC samples collected during conditions 1, 2, and 4, respectively.  
 359 Shape symbolizes the reactor depth the GAC samples were taken from (L1 = top (circle), L3 = middle  
 360 (triangle), and L5 = bottom (square)). The outline color of shapes represents the system the GAC was  
 361 collected from (gray = urea-fed, black = ammonia-fed).

362

363 **Impact of nitrogen source and loading conditions on nitrifying bacteria.** In the ammonia and  
 364 urea-fed systems, four *Nitrospira*-like (ASVs 4, 6, 46, 236) and three *Nitrosomonas*-like ASVs  
 365 (ASVs 17, 54, and 62) were detected. Mapping ASVs 4, 6, and 46 against *Nitrospira* reference  
 366 genomes and full length 16S rRNA sequences indicated ASV 4 was a *Nitrospira lenta*-like strict  
 367 NOB (100% ID to NCBI accession number KF724505) and ASV 6 and 46 were *Nitrospira nitrosa*-  
 368 like comammox bacteria (both 100 % ID to NZ\_CZQA00000000). All *Nitrosomonas*-like ASVs  
 369 shared high sequence similarity (>98% ID) with 16S rRNA gene sequences within *Nitrosomonas*  
 370 cluster 6a (*Nitrosomonas ureae* (NZ\_FOFX01000070) and Is79 (NC\_015731)).

371

372 To further evaluate the classification of the *Nitrospira* ASVs, 16S rRNA gene sequences were  
373 reconstructed using short reads obtained from metagenomic sequencing of samples taken from the  
374 top of the ammonia- and urea-fed reactors. ASVs 6, 46, and 4 uniquely mapped with 100%  
375 sequence identity to one, four, and four reconstructed 16S rRNA gene sequences, respectively.  
376 Phylogenetic analyses clustered all sequences corresponding to ASVs 6 and 46 with comammox  
377 bacterial species *Ca. Nitrospira nitrificans* while all ASV 4 matches clustered separately with  
378 *Nitrospira lenta* (Figure 3A). This further supports that two of the dominant *Nitrospira* ASVs (6  
379 and 46) belonged to comammox bacteria and ASV 4 was strict *Nitrospira*-NOB. Dominant  
380 *Nitrosomonas*-like ASVs 17, 54, and 62 uniquely mapped with 100% sequence identity to five,  
381 four, and three reconstructed 16S rRNA gene sequences, respectively. Phylogenetic placement of  
382 the matches revealed those associated with ASV 17 clustered with *Nitrosomonas ureae* and sp.  
383 AL212 which suggests ASV 17 belongs to a lineage of urease-positive strict AOB (Figure 3B).  
384 ASV 62 matches were a part of the same main branch but formed a separate cluster with  
385 *Nitrosomonas* Is79A3 and sp. Nm86. All ASV 54 matches clustered with uncultured  
386 *Nitrosomonas*.

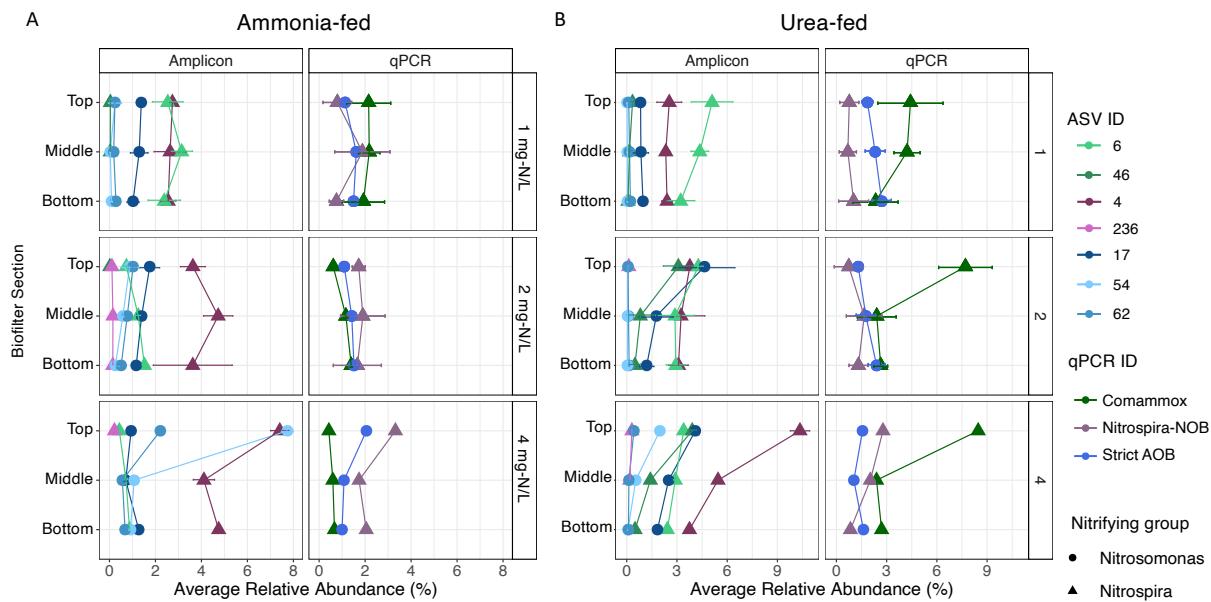


387  
388 **Figure 3:** Maximum likelihood tree for 16S rRNA gene sequences for (A) *Nitrospira* and (B) *Nitrosomonas*.  
389 Color denotes the ASV matches: ASV 46 (Blue), ASV 6 (Green), ASV 4 (Orange), ASV 54 (Red), ASV 17  
390 (Purple), and ASV 62 (Yellow).  
391

392 In the ammonia-fed system, the average relative abundance of *Nitrospira lenta*-like ASV 4  
393 increased with increasing nitrogen loadings (1 mg-N/L: 2.63 ( $\pm 0.45$ ) %, 2 mg-N/L: 3.89 ( $\pm 1.23$ )  
394 %, 4 mg-N/L: 5.40 ( $\pm 1.75$ ) %) with its abundance significantly higher in condition 3 compared to  
395 condition 1 (ANOVA/Tukey,  $p < 0.05$ ) (Supplementary Figure 3). In contrast, the average relative  
396 abundance of comammox-like ASV 6 decreased with increased nitrogen loadings (1 mg-N/L: 2.67  
397 ( $\pm 0.70$ )%, 2 mg-N/L: 1.16 ( $\pm 0.38$ )%, 4 mg-N/L: 0.67 ( $\pm 0.22$ )) with its the abundance  
398 significantly higher during condition 1 compared to both conditions 2 and 4 (ANOVA/Tukey,  $p <$   
399 0.05). Further, the abundance of ASV 6 did not change with depth in the ammonia-fed reactor  
400 during all conditions whereas the abundance distribution of ASV 4 appeared to be dependent on  
401 nitrogen availability (Figure 4A). The abundance of ASV 4 was positively associated with  
402 concentrations of ammonia ( $R=0.344$ ,  $p < 0.05$ ) and nitrite ( $R = 0.304$ ,  $p < 0.05$ ), but the opposite  
403 was observed for the abundance of ASV 6 which had a negative association with both ammonia  
404 ( $R=0.367$ ,  $p < 0.05$ ) and nitrite ( $R = 0.346$ ,  $p < 0.05$ ) (Supplemental Figure 4A, B). The relative  
405 abundance of *Nitrosomonas*-like ASV 17 remained consistent between conditions (1 mg-N/L:  
406 1.228% ( $\pm 0.313$ ), 2 mg-N/L: 1.436% ( $\pm 0.393$ ), 4 mg-N/L: 0.969% ( $\pm 0.282$ )) ( $p < 0.05$ )  
407 (Supplemental Figure 3) with similar relative abundances found in each section of the reactor  
408 regardless of nitrogen loading condition (Figure 3B). ASV 54 replaced ASV 17 as the dominant  
409 *Nitrosomonas*-like ASV as its average relative increased 27-fold between condition 1 (0.119% ( $\pm$   
410 0.189)) and 4 (3.233% ( $\pm 3.884$ )). The abundance of both ASVs 54 and 62 were positively  
411 correlated with the concentration of ammonia ( $R = 0.503$  and  $R= 0.474$ ,  $p < 0.05$ ) (Supplemental  
412 Figure 4A), indicating their abundance increased in response to higher ammonia concentrations in  
413 the ammonia-fed system.

414  
415 While the abundance of *Nitrosomonas*-like ASV 54 increased 40-fold with increased influent  
416 nitrogen concentration in the urea-fed system, ASV 17 remained the dominant ASV with its  
417 relative abundance increasing from 0.875% ( $\pm 0.310$ ) in condition 1 to 2.450% ( $\pm 1.923$ ) in  
418 condition 3. In contrast, *Nitrosomonas*-like ASV 62, which increased in abundance proportional  
419 to ammonia concentration in the ammonia-fed system, demonstrated no significant change  
420 between any of the urea conditions ( $p > 0.05$ ) and remained at low relative abundance suggesting  
421 it was outcompeted in the urea-fed system. While ASVs 4 and 6 were still dominant *Nitrospira*-  
422 like ASVs in the urea-fed system, another *Nitrospira*-like ASV (46) increased in abundance from

423 0.184% ( $\pm 0.144$ ) to 1.414% ( $\pm 1.361$ ) to 1.934% ( $\pm 1.761$ ) for conditions 1, 2, and 3, respectively;  
424 ASV 46 was only detected in condition 1 and 2 in the ammonia-fed system at extremely low  
425 abundance (<0.006%) thus showing a clear preference for the urea-fed systems (Supplemental  
426 Figure 3). Its abundance also changed with reactor depth during conditions 2 and 4 in the urea-  
427 fed system where its abundance was higher in the top section compared to lower portions (Figure  
428 4B). Consistent with the ammonia-fed system, the abundance of *Nitrospira lenta*-like ASV 4 was  
429 positively associated with ammonia concentrations ( $R = 0.289$ ,  $p < 0.05$ ) (Supplemental Figure  
430 4C) and that of ASV 6 was negatively associated with nitrite concentrations ( $R = 0.398$ ,  $p < 0.05$ )  
431 in the urea-fed system (Supplemental Figure 4D).



432  
433 **Figure 4:** Relative abundance of nitrifier ASVs and qPCR-based relative abundance of comammox  
434 bacteria, strict AOB and *Nitrospira*-NOB in the (A) ammonia-fed (A) and (B) urea-fed systems at 1 (top  
435 panels), 2 (middle panels), and 4 (bottom panels) mg-N/L. *Nitrosomonas*- and *Nitrospira*-like populations  
436 are represented by circles and triangles, respectively. Data points for ASVs are the average relative  
437 abundance of nitrifier ASVs calculated in the top, middle and bottom sections of the reactors during each  
438 nitrogen loading condition with error bars for standard deviation. Data points for qPCR assays are the  
439 average relative abundances of comammox bacteria, strict AOB and *Nitrospira*-NOB in the top, middle and  
440 bottom sections of the reactors during each nitrogen loading condition with error bars for standard deviation.  
441

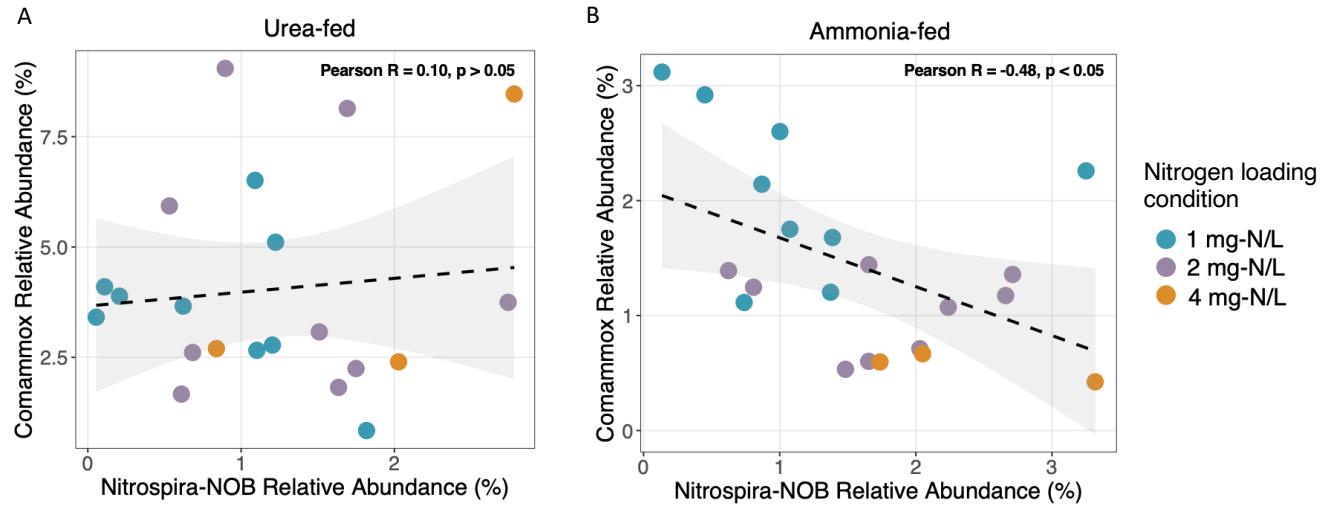
442 Comammox bacterial abundance based on qPCR assays was significantly lower in conditions 2  
443 (1.06 ( $\pm 0.35$ ) %) and 3 (0.56 ( $\pm 0.13$ ) %) compared to its abundance during condition 1 (2.08 ( $\pm$   
444 0.71)) % (ANOVA/Tukey,  $p < 0.05$ ) in the ammonia-fed system (Figure 4A). Additionally, the  
445 relative abundance of comammox bacteria displayed minimal change along sections of the  
446 ammonia-fed reactor during all conditions which aligns with the trends observed for ASV 6. The

447 relative abundance of *Nitrospira*-NOB assessed by qPCR increased with each nitrogen loading  
448 condition (1 mg-N/L: 1.14 ( $\pm$  0.88) %, 2 mg-N/L: 1.76 ( $\pm$  0.73) %, 4 mg-N/L: 2.37 ( $\pm$  0.84) %)  
449 though its abundance was not significantly different between each of them (ANOVA/Tukey, p >  
450 0.05). However, its relative abundance was highest overall (3.32%) during condition 3 in the top  
451 section where ammonia and nitrite availability was considerably higher compared to the other two  
452 nitrogen loading conditions. Thus, *Nitrospira*-NOB likely benefited from increased availability of  
453 ammonia and nitrite during higher nitrogen loading conditions whereas comammox bacteria  
454 preferred the lowest nitrogen condition with limited ammonia availability in the ammonia-fed  
455 system. The overall highest abundance of strict AOB and *Nitrospira*-NOB occurred in the top  
456 section of the reactor during condition 3.

457

458 qPCR-based abundance of comammox bacteria was significantly higher in all urea-fed conditions  
459 compared to its abundance in any of the ammonia-fed conditions (Figure 4A, B) which was also  
460 observed for comammox-like ASVs. The combined abundance of comammox-like ASVs was  
461 strongly correlated with the qPCR-based abundance of comammox bacteria (Pearson R = 0.92, p  
462 < 0.05) (Supplemental Figure 5). Further, qPCR and ASV based abundances agreed that  
463 comammox bacteria were the dominant ammonia oxidizers regardless of nitrogen loading  
464 condition in the urea-fed system. The qPCR-based abundance of *Nitrospira*-NOB in the urea-fed  
465 system was lower than that of comammox bacteria during each condition (Figure 4B). However,  
466 increased abundance of comammox bacteria did not result in decreased abundance of *Nitrospira*-  
467 NOB (Pearson R = 0.10, p > 0.05) (Figure 5A). This is in contrast to the ammonia-fed system  
468 where comammox bacteria and *Nitrospira*-NOB populations demonstrated a significant negative  
469 association (Pearson R = -0.48, p < 0.05) (Figure 5B). No significant associations between the  
470 abundance of comammox bacteria and strict AOB, and *Nitrospira*-NOB and strict AOB in either  
471 the ammonia- or urea-fed systems (Supplemental Figure 6A-D).

472



473

474 **Figure 5:** (A) Lack of any relationship between the abundance of comammox bacteria and *Nitrospira*-NOB  
475 in the urea-fed system contrasts with (B) significant negative association between the abundance of  
476 comammox bacteria and *Nitrospira*-NOB in the ammonia-fed system.

477

478 **Phylogeny and metabolism of nitrifier metagenome assembled genomes (MAGs).** 200 and 172  
479 MAGs were recovered from the metagenomic assemblies from the ammonia- and urea-fed  
480 systems, respectively. The nitrifier community in the ammonia-fed system was comprised of three  
481 *Nitrosomonas*-like MAGs and two *Nitrospira*-like MAGs (one classified as *Nitrospira*\_F and one  
482 classified as *Nitrospira*\_D) which aligns with the number of dominant nitrifier ASVs in the  
483 ammonia-fed system (Table 1). Nitrifier MAGs assembled from the urea-fed system also mirrored  
484 the number of dominant nitrifier ASVs with three *Nitrospira*-like (two *Nitrospira*\_F, one  
485 *Nitrospira*\_D) and three *Nitrosomonas*-like MAGs. While additional *Nitrosomonas*-like MAG was  
486 assembled from the urea-fed GAC sample, it was extremely low quality (completeness < 10%).

487 Phylogenetic analysis with 91 single copy core genes clustered *Nitrospira*\_F1\_A with clade A  
488 comammox bacteria (Figure 6A) and it showed high sequence similarity (~94% ANI) with  
489 *Nitrospira* sp Ga0074138 which is a comammox bacteria MAG previously assembled by Pinto et  
490 al. (2015) from GAC obtained from the same reactor. *Nitrospira*\_F1\_A shared extremely high  
491 sequence similarity (> 99% ANI) with *Nitrospira*\_F1\_U assembled from the urea-fed system,  
492 suggesting the two MAGs were likely the same population (Supplemental Figure 7). Another  
493 *Nitrospira* MAG (*Nitrospira*\_F2\_U) assembled from the urea-fed system sample was placed  
494 within comammox clade A, but clustered separately with other drinking water related comammox  
495 MAGs (*Nitrospira* sp. ST-bin4 and SG-bin2). This MAG shared less than 80% ANI with all other

496 *Nitrospira* MAGs in this study. Phylogenetic placement of hydroxylamine oxidoreductase (HAO)  
497 gene sequences present in all comammox MAGs in this study were grouped into clade A2  
498 comammox bacteria (data not shown). The remaining two *Nitrospira* MAGs (*Nitrospira\_D1\_A*  
499 and *Nitrospira\_D1\_U*) clustered with *Nitrospira*-NOB belonging to lineage II (Figure 6A) with  
500 *Nitrospira lenta* and other *Nitrospira*-NOB obtained from a drinking water system (*Nitrospira* sp.  
501 ST-bin5) and rapid sand filter (RSF 13 and CG24D). *Nitrospira\_D1\_A* and *Nitrospira\_D1\_U* from  
502 this study shared over 99% sequence similarity indicating they are the same population  
503 (Supplemental Figure 7).

504

505 **Table 1:** Quality statistics for nitrifier MAGs assembled from GAC taken from the ammonia- and  
506 urea-fed reactors.

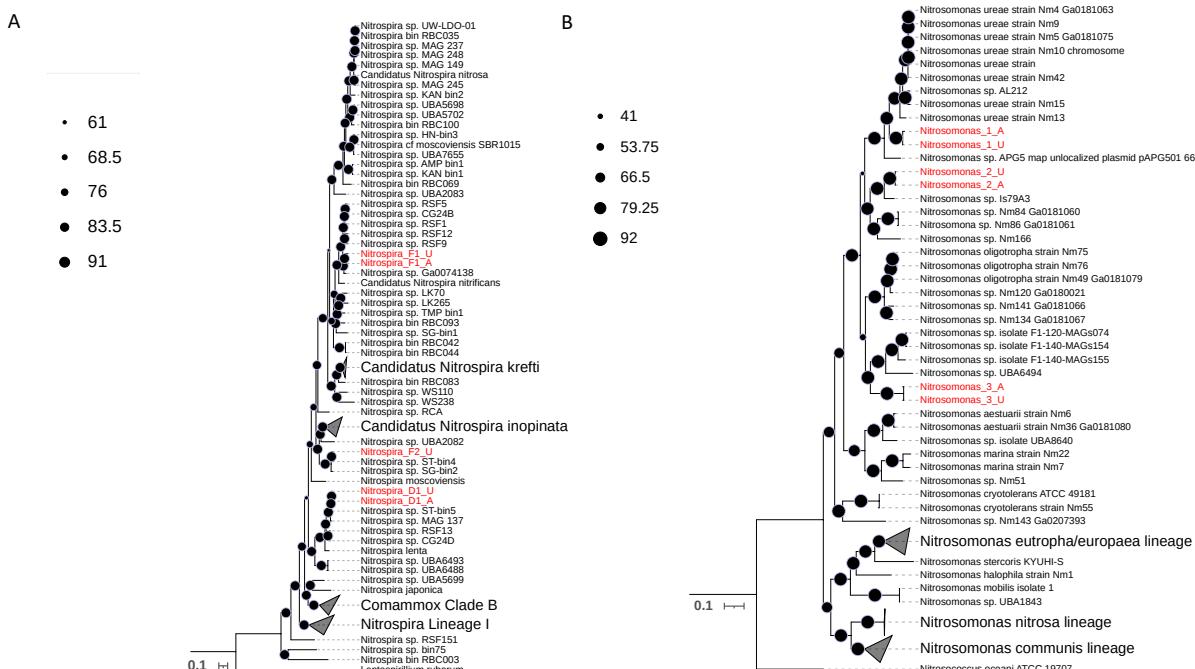
MAG name	Classification	Reactor	Completeness (%)	Redundancy (%)
<i>Nitrospira_D1_A</i>	<i>Nitrospira_D</i> sp002083555	Ammonia	92.27	3.91
<i>Nitrospira_F1_A</i>	<i>Nitrospira_F</i>	Ammonia	88.88	3.69
<i>Nitrosomonas_1_A</i>	<i>Nitrosomonas</i>	Ammonia	80.3	0.48
<i>Nitrosomonas_2_A</i>	<i>Nitrosomonas</i> sp016708955	Ammonia	97.38	0.51
<i>Nitrosomonas_3_A</i>	<i>Nitrosomonas</i>	Ammonia	92.34	0.03
<i>Nitrospira_F1_U</i>	<i>Nitrospira_F</i>	Urea	92.11	71.93
<i>Nitrospira_D1_U</i>	<i>Nitrospira_D</i> sp002083555	Urea	94.09	4.82
<i>Nitrospira_F2_U</i>	<i>Nitrospira_F</i> sp002083565	Urea	84.65	6.41
<i>Nitrosomonas_1_U</i>	<i>Nitrosomonas</i>	Urea	98.72	0.48
<i>Nitrosomonas_2_U</i>	<i>Nitrosomonas</i> sp016708955	Urea	93.38	0.51
<i>Nitrosomonas_3_U</i>	<i>Nitrosomonas</i>	Urea	93.54	0.03
<i>Nitrosomonas_4_U</i>	<i>Nitrosomonas</i>	Urea	6.22	0

507

508 ANI comparisons between the *Nitrosomonas*-like MAGs assembled from the ammonia-  
509 (*Nitrosomonas\_1\_A*, *Nitrosomonas\_2\_A*, *Nitrosomonas\_3\_A*) and urea-fed (*Nitrosomonas\_1\_U*,  
510 *Nitrosomonas\_2\_U*, *Nitrosomonas\_3\_U*) systems revealed the same set of three *Nitrosomonas*-  
511 like MAGs were assembled from both samples (Supplemental Figure 8). Within sample ANI  
512 comparisons showed that the three *Nitrosomonas*-like MAGs shared less than 95% ANI suggesting  
513 they were separate species. Phylogenomic placement of *Nitrosomonas* MAGs in this study  
514 affiliated them with *Nitrosomonas* cluster 6a which are known for their oligotrophic physiologies

515 (Koops 2001). *Nitrosomonas\_1\_A* and *Nitrosomonas\_1\_U* (ANI > 99%) clustered with  
 516 *Nitrosomonas ureae* while *Nitrosomonas\_2\_A* and *Nitrosomonas\_2\_U* (ANI > 99%) grouped with  
 517 *Nitrosomonas Is79A3* (Figure 6B). *Nitrosomonas\_3\_A* and *Nitrosomonas\_3\_U* (ANI > 99%)  
 518 clustered with uncultured *Nitrosomonas* MAGs that were still within the cluster 6a grouping. Out  
 519 of all reference comparisons, *Nitrosomonas* MAGs from this study shared the highest similarity to  
 520 *Nitrosomonas ureae* strain Nm5 Ga0181075 101 (ANI = 83%, *Nitrosomonas\_1\_A* and  
 521 *Nitrosomonas\_1\_U*), *Nitrosomonas Is79* (ANI = 89%, *Nitrosomonas\_2\_A* and  
 522 *Nitrosomonas\_2\_U*), and *Nitrosomonas* sp. Nm141 Ga0181066 101 (ANI = 79%,  
 523 *Nitrosomonas\_3\_A* and *Nitrosomonas\_3\_U*).

524



525 **Figure 6:** Maximum likelihood trees for (A) *Nitrospira* and (B) *Nitrosomonas* based on a set of bacterial  
 526 single copy core genes. MAGs assembled in this study are labelled in red and reference genomes and  
 527 MAGs are black.  
 528

529

530 Dereplication of MAGs from urea- and ammonia-fed systems resulted in three *Nitrosomonas*-like  
531 MAGs, one *Nitrospira*-NOB MAG and two comammox bacteria-like MAGs. Filtered reads from  
532 the ammonia-fed system were mapped to the set of dereplicated MAGs, revealing all nitrifier  
533 MAGs had 99% breath of coverage (i.e., percent of genome covered by reads) in both systems.  
534 However, the comammox MAG that was assembled only from the urea-fed sample  
535 (*Nitrospira* F2 U) had very low relative abundance (0.065%) in the ammonia-fed system which

536 could explain why it was not assembled. Comparably, relative abundances of comammox bacteria  
537 MAGs, *Nitrospira\_F1\_A*/*Nitrospira\_F1\_U* and *Nitrospira\_F2\_U*, were approximately 12 and 5-  
538 fold higher in the urea-fed system (~7.81% *Nitrospira\_F1\_A*/*Nitrospira\_F1\_U*, 0.30%  
539 *Nitrospira\_F2\_U*) than in the ammonia-fed system (~0.66 *Nitrospira\_F1\_A*/*Nitrospira\_F1\_U*,  
540 0.065% *Nitrospira\_F2\_U*). These results align with both the qPCR-based abundance of  
541 comammox bacteria and abundance of comammox-like ASVs 6 and 46 in the urea-fed system  
542 being substantially higher than their abundance in the ammonia-fed system. Thus, based on  
543 abundance trends of the comammox-like ASVs 6 and 46 and comammox MAGs, we associate  
544 ASV 6 with the comammox bacterial population belonging to *Nitrospira\_F1\_A*/*Nitrospira\_F1\_U*  
545 while ASV 46 is associated with *Nitrospira\_F2\_U*.

546

547 Similar to our previous study, comammox MAGs (*Nitrospira\_F1\_A*, *Nitrospira\_F1\_U*,  
548 *Nitrospira\_F2\_U*) contained genes for urea degradation (*ureCAB*) and transportation  
549 (*urtACBCDE*). Strict AOB MAGs *Nitrosomonas\_1\_A* and *Nitrosomonas\_1\_U* also possessed  
550 these genes for ureolytic activity which aligns with their sequence similarity to and clustering with  
551 *Nitrosomonas ureae*. The other *Nitrosomonas* MAGs (*Nitrosomonas\_2\_A*, *Nitrosomonas\_2\_U*,  
552 *Nitrosomonas\_3\_A*, *Nitrosomonas\_3\_U*) only encoded a single urea accessory gene (*ureJ*) and  
553 gene encoding for urea carboxylase. An unbinned *Nitrosomonas*-associated *ureC* gene was found  
554 in the metagenome assembly from the urea-fed system suggesting another urease-positive strict  
555 AOB MAGs could have been present in the system. *Nitrospira* MAGs (*Nitrospira\_D1\_A* and  
556 *Nitrospira\_D1\_U*) did not contain urease genes; however, unbinned genes for *ureA* with 100%  
557 sequence ID match to strict NOB *Nitrospira lenta* were detected in the metagenome assembly for  
558 both samples, suggesting *Nitrospira*-NOB were urease-positive.

559

## 560 DISCUSSION.

561 **Nitrite accumulation in ammonia-fed but not urea-fed system may be associated with NOB**  
562 **inhibition and with the rate of ammonia production from urea.** Strict AOB and *Nitrospira*-  
563 NOB were the dominant nitrifiers in the ammonia fed systems and particularly at higher ammonia  
564 concentrations with the ammonia oxidation rates being consistently higher than the nitrite  
565 oxidation rates leading to nitrite accumulation. While nitrite accumulation occurred in the  
566 ammonia-fed reactor for condition 3, *Nitrospira*-NOB were more abundant than both AOB and

567 comammox bacteria. It could be possible that despite their high abundance, *Nitrospira*-NOB were  
568 impacted by higher ammonia concentrations of conditions 2 and 3. Fujitani et al. (2020) observed  
569 that the average  $K_m$  value for nitrite (0.037 mg/L) attributed to a *Nitrospira*-NOB strain originating  
570 from a drinking water treatment plant increased five-fold to approximately 0.18 mg-N/L  $\text{NO}_2^-$  in  
571 the presence of free ammonia concentrations around 0.85 mg NH<sub>3</sub>-N/L. Thus, decreased nitrite  
572 affinity could have impacted the ability of this *Nitrospira* strain to oxidize low nitrite  
573 concentrations depending on the concentration of free ammonia. Further, in wastewater systems,  
574 suppression of strict NOB activity can be achieved at ammonia concentrations were higher than 5  
575 mg-N/L (Poot et al., 2016). Here in the ammonia-fed system, average ammonia concentrations  
576 observed in the top section of the reactor during condition 2 (0.89 mg NH<sub>3</sub>/L) and 4 (2.64 mg  
577 NH<sub>3</sub>/L) were in line with free ammonia concentrations shown to impact nitrite affinity of  
578 *Nitrospira*-NOB strain KM1 in Fujitani et al. (2020), thus explaining nitrite accumulation. In the  
579 urea-fed system, urease-positive nitrifiers, including comammox bacteria and *Nitrospira*-NOB,  
580 regulated ammonia production and thus potentially controlled ammonia availability. While  
581 ammonia did accumulate during the highest nitrogen loading condition in the urea-fed reactor,  
582 however, unlike the ammonia-fed reactors, comammox bacterial abundance did not decrease and  
583 there was no nitrite accumulation. This is likely because the highest ammonia concentrations in  
584 the urea-fed reactor were consistently lower than the highest concentrations in the ammonia-fed  
585 reactors and thus comammox bacteria were not outcompeted by AOB and both comammox and  
586 *Nitrospira*-NOB were likely not inhibited.

587

588 **Increased ammonia availability in ammonia-fed reactor detrimentally impacted comammox**  
589 **bacterial populations.** Consistent with reported higher ammonia affinity (i.e., lower  $K_m(\text{app})$ ) of  
590 comammox bacteria compared to strict AOB (Kits 2017, Sakoula 2020, Ghimire-Kafle 2023),  
591 comammox bacteria did indeed dominate over AOB only during the lowest nitrogen loading  
592 condition in the ammonia-fed system. Though strict AOB were affiliated with *Nitrosomonas*  
593 cluster 6a characterized with higher ammonia affinities ( $K_m(\text{app})=0.24\text{--}3.6 \mu\text{M}$  (Koops et al.,  
594 2006)) compared to other AOB, the reported ammonia affinity for comammox bacteria is still  
595 substantially higher for comammox bacteria ( $K_m(\text{app})=63 \text{ nM}$ ). In addition to ammonia affinity,  
596 it is very likely that ammonia tolerance played a role as partial inhibition of ammonia oxidation  
597 activity by *Ca Nitrospira kretii* has been reported at ammonia concentrations as low as 0.425 mg/L

598 which is within the range of ammonia concentrations observed in the ammonia-fed reactor (0.25-  
599 2 mg-N/L) during conditions 2 and 3. However, ammonia sensitivity resulting in partial inhibition  
600 of ammonia oxidation has not been observed for *Ca Nitrospira inopinata* (Kits 2017), and *Ca*  
601 *Nitrospira nitrosa*-like comammox bacteria in a wastewater systems were ammonia concentrations  
602 are higher (Cotto et al., 2020, 2023; K. Vilardi et al., 2023). Comammox bacteria in this study may  
603 be adapted to low ammonia concentrations and were most similar to other clade A2 comammox  
604 bacteria obtained from low ammonia environments. Thus, continuous exposure to elevated  
605 ammonia concentrations could be responsible for the observed reduction in abundance of  
606 comammox bacteria via inhibition.

607

608 **Increase in urea concentration favored *Nitrospira* bacteria including comammox bacteria.**  
609 Comammox bacteria were the dominant nitrifier across all conditions in the in the urea-fed system  
610 with its overall abundance significantly higher in the urea-fed system compared to its abundance  
611 in the ammonia-fed system. Urea preference of comammox bacteria was also supported by the  
612 highest abundances of comammox bacteria consistently observed in the top of the urea-fed reactor  
613 where urea was most available and the emergence of a second low abundance comammox  
614 population in the urea-fed reactor. Our observation is similar to other reports of enrichment of very  
615 different comammox populations at much higher urea concentrations (J. Li et al., 2021; Zhao et  
616 al., 2021). Though we are unable to identify the exact reason for comammox bacterial preference  
617 for growth on urea, it could be a combination of metabolic traits associated with urea uptake and  
618 utilization. Specifically, comammox bacteria may balance the rate of ammonia production from  
619 urea with its ammonia oxidation rate, thus maximizing ammonia availability while also  
620 maintaining ammonia concentrations at non-inhibitory levels. Further, additional urea transporters  
621 are found in comammox genomes that are absent in other *Nitrospira* including an outer-membrane  
622 porin (*fmdC*) for uptake of short-chain amides and urea at low concentrations and a urea  
623 carboxylase-related transport (*uctT*) (Palomo 2018). Thus, the enhanced ability to uptake urea and  
624 regulate its conversion to ammonia balanced with its ammonia oxidation rates may underpin  
625 comammox bacterial preference for urea. Estimating the kinetic parameters such as comammox  
626 bacteria's affinity for urea and uptake rate relative to other nitrifiers and ammonia production  
627 relative to its own ammonia oxidation rates would be extremely useful for assessing their overall  
628 preference for urea.

629 **Nitrogen source drives potential competitive and co-operative dynamics between aerobic**  
630 **nitrifiers.** In these continuous flow reactors and our previous batch microcosm experiments (K.  
631 J. Vilardi et al., 2022), we observed nitrogen source-dependent dynamics between the abundance  
632 of comammox bacteria and *Nitrospira*-NOB. We hypothesized that tight metabolic coupling exists  
633 between strict AOB and *Nitrospira*-NOB when urea is supplied due to reciprocal feeding mediated  
634 by the two groups. Here, the production of nitrite can be controlled by urease-positive *Nitrospira*-  
635 NOB via cross feeding ammonia to strict AOB, who in turn provide nitrite at a rate at  
636 which *Nitrospira*-NOB can consume it. This dynamic between canonical nitrifiers substantially  
637 contrasts with their relationship when only ammonia is provided as *Nitrospira*-NOB are fully  
638 dependent on strict AOB to provide them nitrite. Therefore, a negative relationship between  
639 comammox and canonical NOB *Nitrospira* when only ammonia is available may reflect  
640 comammox bacteria limiting *Nitrospira*-NOB access to nitrite (produced by AOB) by performing  
641 complete ammonia oxidation to nitrate. In contrast, at high ammonia concentration, comammox  
642 bacteria may in fact be a source of nitrite for *Nitrospira*-NOB as their ammonia oxidation rates are  
643 faster than their nitrite oxidation rates, and their affinities for nitrite are lower than that of  
644 *Nitrospira*-NOB (Daims et al., 2015; van Kessel et al., 2015). Supplementation with urea  
645 eliminates this potential comammox-NOB negative association as both nitrifiers are urease-  
646 positive and potentially produce ammonia themselves for different purposes (i.e., comammox  
647 produce their own ammonia, strict NOB provide ammonia to strict AOB). Competition for urea  
648 would then be determined by the urea affinity and uptake rates which are currently unknown.  
649 However, in this study, we show that increased abundance of comammox bacteria did not result  
650 in decreased abundance of *Nitrospira*-NOB in the urea-fed system. This suggests that the apparent  
651 competitive dynamics between these nitrifiers is reduced when an alternative nitrogen source is  
652 available compared to ammonia which induced a competitive relationship.

653

654 In this study, the impact of nitrogen source and availability on nitrifying communities was  
655 evaluated in continuous flow column reactors supplied either ammonia or urea and operated over  
656 three different nitrogen loading conditions. Consistent with our previous batch microcosm  
657 experiments (Vilardi et al, 2022), we show that different nitrogen sources and loadings distinctly  
658 shape the nitrifying community. Direct supply of ammonia favored a combination of AOB and  
659 NOB particularly as the nitrogen loadings were increased, with decrease in comammox bacterial

660 abundance was likely associated with ammonia-based inhibition. Ammonia availability has been  
661 considered an important niche differentiating factor between comammox bacteria and strict AOB,  
662 and here we show it may also be a significant factor for shaping populations of comammox  
663 bacteria. In contrast, the urea provision promoted the abundance of multiple comammox  
664 populations along with strict AOB and *Nitrospira*-NOB. With urea as a nitrogen source,  
665 nitrification can be initiated by urease-positive nitrifiers controlling ammonia production and its  
666 availability which in turn significantly impacted nitrification process performance.

667

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