1 **Low diversity and microdiversity of comammox bacteria in wastewater systems suggests**

2 **wastewater-specific adaptation within the *Ca.* Nitrospira nitrosa cluster.**

3

4 Irmarie Cotto[1], Katherine J. Vilardi[1], Linxuan Huo[2], Emily C. Fogarty[3], Wendell Khunjar[4],

5 Christopher Wilson[5], Haydee De Clippeleir[6], Kevin Gilmore[7], Erika Bailey[8], Sebastian Lücker[9],

6 Ameet J. Pinto[2*]

7

8 1 Department of Civil and Environmental Engineering, Northeastern University, Boston, MA,

9 USA

10 2 School of Civil and Environmental Engineering, Georgia Institute of Technology, Atlanta, GA,

11 USA

12 3 Committee on Microbiology, The University of Chicago, Chicago, IL, USA

13 4 Hazen and Sawyer, Inc., New York, NY, USA

14 5 Hampton Roads Sanitation District, Norfolk, VA, USA

15 6 DC Water, Washington DC, USA

16 7 Department of Civil and Environmental Engineering, Bucknell University, Lewisburg, PA,

17 USA

18 8 City of Raleigh Public Utilities, Raleigh, NC, USA

19 9 Department of Microbiology, RIBES, Radboud University, Nijmegen, the Netherlands

20

21 *Corresponding author: ameet.pinto@ce.gatech.edu

22

23

24

25

26

27

28

29

Abstract

Studies have found *Ca.* Nitrospira nitrosa-like bacteria to be the principal or sole comammox bacteria in nitrogen removal systems for wastewater treatment. In contrast, multiple populations of strict ammonia and nitrite oxidizers co-exist in similar systems. This apparent lack of diversity is surprising and could impact the feasibility of leveraging comammox bacteria for nitrogen removal. We used full-length 16S rRNA gene sequencing and genome-resolved metagenomics to compare population-level (i.e., species) diversity of comammox bacteria with that of strict nitrifiers in full-scale wastewater treatment systems and assess whether these observations were consistent or diverged at the strain-level. Full-length 16S rRNA gene sequencing indicated that while *Nitrosomonas*-like bacteria exhibited higher population-level diversity, the effective microdiversity of most *Nitrospira*-like bacteria were comparatively higher except for one *Nitrospira* Lineage II population. Comammox bacterial metagenome assembled genomes (MAGs) were associated with *Ca.* Nitrospira nitrosa. The average amino acid identity between comammox bacterial MAGs (93% ± 3) across systems was significantly higher than that of the *Nitrosomonas*-like ammonia oxidizers (73%±8) and the *Nitrospira*-like nitrite oxidizer MAGs (75%±13), suggesting that the same comammox population was detected in all systems. Comammox bacteria and some ammonia oxidizers MAGs were significantly less microdiverse than most ammonia and nitrite oxidizers. Interestingly, strain-resolved analysis also indicates that different nitrogen removal systems harbor different comammox bacterial strains within the *Ca.* Nitrospira nitrosa cluster. These results suggest that comammox bacteria associated with *Ca.* Nitrospira nitrosa have low species- and strain-level diversity in nitrogen removal systems and may thus harbor specific adaptations to the wastewater ecosystem.

2

Introduction

Aerobic nitrification processes for nitrogen removal from wastewater are largely centered around biotransformation and growth kinetics of strict ammonia oxidizing bacteria (AOB) and nitrite oxidizing bacteria (NOB) (1). Since their discovery (2–5), several studies have detected comammox bacteria in a wide range of environmental and engineered systems (2–9), including nitrogen removal processes in laboratory and full-scale wastewater treatment bioreactors (9–20). Compared to oligotrophic engineered systems such as drinking water (9,21) and even tertiary treatment systems at full-scale wastewater treatment plants (WWTPs) (16) with low nitrogen concentrations, most studies have identified *Candidatus* Nitrospira nitrosa-like comammox bacteria as the principal or sole comammox bacteria in laboratory or full-scale secondary treatment systems (11,22,23). This apparent lack of diversity of comammox bacteria in secondary treatment processes is not only surprising but has the potential to impact treatment strategies centered around comammox bacteria (24,25) as functional outcomes reliant on low diversity communities may be less resilient to environmental fluctuations and perturbations.

Nitrosomonas-like AOB and *Nitrospira*-like NOB have been studied extensively in wastewater treatment systems and studies often report the coexistence of multiple populations (26–28) likely occupying different ecological niches (i.e., adapted to different conditions) within a complex community. Functional redundancy of multiple co-exiting populations can confer stability to microbial ecosystems ensuring the long-term persistence (29,30). Despite the apparent low population diversity of comammox bacteria compared to strict nitrifiers, they have exhibited remarkable stability in secondary treatment systems (11,15,31). One plausible reason for their temporal persistence could be that comammox bacteria may harbor intra-population (i.e., strain)

3

76 level diversity as compared to population level (i.e., species). Previous literature has demonstrated

77 that microdiversity within populations can not only allow them to adapt to rapidly changing

78 conditions, but also contribute to their persistence (7,32). Further, small variations in metabolic

79 capacity may underpin metabolic diversity and thus enable the co-existence of multiple closely

80 related strains within a single population. *Nitrospira*-like bacteria have been shown to harbor high

81 levels of microdiversity in drinking water (33,34) and wastewater (35) systems. Moreover, studies

82 have reported that closely related co-existing *Nitrospira* strains can exhibit differences in substrate

83 affinities, and utilization rates (35). Since, both levels of diversity (i.e., population and intra-

84 population) have been associated with microbial persistence and ecosystem stability, it is plausible

85 that the persistence of comammox bacteria may be associated with higher levels of microdiversity;

86 this would be consistent with observations for *Nitrospira*-like bacteria (35).

87

88 To test this hypothesis, we used a combination of full-length 16S rRNA gene sequencing and

89 hybrid assembly and binning approaches to systematically explore the population- and strain-level

90 diversity of nitrifiers in three full-scale nitrogen removal systems with different process

91 configurations. While 16S rRNA gene sequencing cannot provide information on genome content

92 or metabolic potential, the single nucleotide resolution across the full length 16S rRNA gene can

93 help determine differences in (micro)diversity among nitrifying populations (35,36). Nevertheless,

94 16S rRNA gene sequences cannot be used to reliably distinguish comammox bacteria from strict

95 *Nitrospira*-NOB. One approach to differentiate between the different guilds within *Nitrospira*

96 bacteria and to analyze their diversity at high resolution is to leverage a genome resolved approach

97 to obtain high quality metagenome assembled genomes (MAGs). Various methods exist to

98 quantify diversity (e.g., average nucleotide identity [ANI] and average amino acid identity [AAI])

99   and microdiversity (e.g., average nucleotide identity from reads and nucleotide diversity from

100 single nucleotide polymorphisms [SNPs]) within populations. Nevertheless, their applicability

101 depends on the quality of MAGs which can be impacted when relying on short read sequencing

102 (e.g., Illumina) in the presence of closely related strains (37); long-read sequencing on the

103 Nanopore platform can help mitigate this constraint (38,39). However, this requires high levels of

104 coverage to obtain polished consensus sequences with lower error rates compared to the raw data

105 (37). Here, we utilize a hybrid metagenomic assembly approach, including long- and short-read

106 data in an effort to assemble high quality MAGs for subsequent microdiversity analyses (37).

107

108 Thus, the overall objective of this study was to test the hypothesis that the widespread distribution

109 and persistence of nearly identical comammox bacterial populations in multiple secondary

110 wastewater treatment systems was due to the co-existence of multiple strains (i.e., high

111 microdiversity). To accomplish this, we used a combination of full-length 16S rRNA gene

112 sequencing and hybrid metagenome assembly and binning approach to systematically explore the

113 population and strain level diversity of nitrifiers in three full-scale nitrogen removal systems with

114 different process configurations. In doing so, we also aimed to assess factors that may influence

115 the inter- and intra-population diversity of co-existing nitrifiers.

116

117 **2.0 Materials and Methods**

118 **2.1 Samples selection and processing**

119 Samples were selected from three nitrogen removal systems with different process configurations

120 (i.e., sequencing batch reactor (SBR), Integrated Fixed Film Activated Sludge (IFAS), and

121 Bardenpho 4-stages activated sludge system) with high concentrations of comammox bacteria in

5

122   our previous study (31). From this sample archive collected in 2017-2018, a total of thirty-three,

123   twenty, and six samples, respectively, were selected for full-length 16S rRNA gene sequencing on

124   the PacBio Sequel IIe platform, and short and long read metagenomic sequencing on the Illumina

125   NovaSeq and Nanopore MinION platforms, respectively (Table 1). Sample collection and

126   processing (including DNA extraction) and process data collection were described previously (31)

127   and are outlined in the supplementary text.

128   **Table 1: Overview of system process type and sub-type, operational scale and samples included in**

129   **this study and sample-specific sequencing strategy.**

Site code	Process type	Process sub-type	Treatment stream	Selected samples (sampling month/sampling year)
GRE	ND[1]	SBR[2]	Mainstream	06/17[+], 07/17*[+†], 08/17[+], 09/17*[+], 10/17*[+], 11/17[+], 12/17*[+], 02/18*[+], 03/17[+], 04/18*[+†], 05/18[+], 06/18*[+]
JAMMSM	ND[1]	IFAS[3]	Mainstream	07/17[+], 09/17*[+], 10/17[+], 11/17*[†], 12/17[+], 01/18*[+†], 02/18[+], 03/18*[+], 04/18*[+], 06/18*[+]
NEU	ND[1]	Four/five stage	Mainstream	06/17[+], 07/17*[+], 08/17[+], 09/17*[+], 10/17*[+†], 11/17*[+†], 12/17[+], 01/18*[+], 02/17[+], 03/18*[+], 04/17[+], 05/18*, 06/18[+]

130   [1]Nitrification-denitrification, [2]Sequencing batch reactor, [3]Integrated Fixed-film Activated Sludge
131   *Samples selected for Illumina NovaSeq sequencing
132   [+]Samples selected for PacBio full-length 16S rRNA gene sequencing
133   [†]Samples selected for nanopore sequencing on the MinION platform
134
135

136   **2.2 PacBio full-length 16S rRNA gene sequencing and data analysis**

137   Sample DNA extracts (Table 1) were sent for full-length 16S rRNA gene sequencing at the Roy J.

138   Carver Biotechnology Center (Sequencing Core, University of Illinois Urbana-Champaign). The

139   full-length 16S rRNA gene amplicons were generated with universal barcoded primers (27F and

140   1492R)(40), PCR products were subject to library preparation and sequenced on the PacBio Sequel

141   IIe using the circular consensus sequencing (CCS) mode. Raw reads were demultiplexed and CCS

142   analyses were performed to obtained consensus reads. Further details on PCR, library preparation,

143   and sequencing are provided in the supplementary text. Downstream data processing was

144   performed on a system-by-system basis using DADA2 v1.19.2 (41) in R v4.0.4. using the sample

145   inference method for full-length 16S rRNA gene with single-nucleotide resolution (42). Table S1a

6

146   summarizes the reads per samples at different stages of data processing in DADA2. ASVs with

147   ≥100 reads in any of the three systems (∼>0.01% relative abundance) were clustered into

148   operational taxonomy units (OTUs) using the function 'IdClusters' in DECIPHER v2.16.1. (43)

149   at 98.7% sequence identity, a previously recommended threshold for clustering full-length 16S

150   rRNA gene sequences at the species level (44,45). The most abundant ASV within each OTU was

151   used as representative sequence and its taxonomic affiliation was used as the consensus taxonomy

152   for the OTU. Additional details on ASV to OTU data processing are provided in the supplementary

153   text. Representative ASV sequence from all OTUs were aligned with MUSCLE v3.8.1551 (46),

154   and the phylogenetic tree was constructed and visualized using IQ-TREE v2.0.3 (47) and iTOL

155   v2.1.7 (48), respectively. Principal Coordinates Analysis (PCoA) was performed with the weighted

156   and unweighted UniFrac distance metric (49) using the 'ordinate' function of phyloseq v1.32.0

157   and the 'plot_ordination' function of ggplot2 v3.3.5 (50).

158

159   ASVs classified at the genus level as *Nitrospira* and *Nitrosomonas* (the only two nitrifying genera

160   detected in this study) were extracted from the rarefied ASV table (rarefied to the sample with

161   lowest read count) and reference 16S rRNA gene sequences for the two genera were obtained from

162   the SILVA SSU 138.1 database (Tables S2 and S3). Comammox bacterial 16S rRNA genes were

163   extracted from references genomes downloaded from NCBI (Table S4). MUSCLE v3.8.1551 was

164   used to align ASVs sequences from each genus with their respective references, and a maximum

165   likelihood phylogenetic tree of each species was generated using IQ-TREE v2.0.3 and visualized

166   in iTOL v2.1.7. To assess the population diversity (i.e., genus level) the Shannon diversity was

167   calculated per sample from the relative abundance of the OTUs classified as the same genus (e.g.,

168   *Nitrospira*) as the exponential of the Shannon index. To estimate intra-population diversity, the

7

169   effective microdiversity of each OTU was calculated on a per sample basis from the relative

170   abundance of its ASVs as the exponential of the Shannon index, which is analogous to the effective

171   number of strains (i.e., ASVs) within a population (i.e., OTU) (36).

172

173   **2.3 Illumina and Oxford Nanopore sequencing, co-assembly and hybrid assembly**

174   Seven samples from GRE and NEU and six samples from JAMMSM were sent for sequencing on

175   the Illumina NovaSeq 6000 platform, and two samples from each system on the Oxford Nanopore

176   Technologies GridIONx5 (Table 1) to the Roy J. Carver Biotechnology Center at the University

177   of Illinois Urbana-Champaign Sequencing Core. Library preparation and sequencing details are

178   provided in the supplementary text. These runs resulted in 1.78 billion paired-end short reads

179   (2×150 nt reads) and 45.3 Gbps of long read data (Table S1b and c). Raw short reads were filtered

180   using fastp v0.20.0 (51) and the Univec database was used to remove contamination from the

181   filtered reads as previously described (31). The resulting bam files were sorted using SAMtools

182   v1.9 (52) and converted into fastq files with bedtools v2.29.0 (53). All reads from the same system

183   were co-assembled into contigs using metaSpades v3.13.0 (54). Co-assemblies were performed

184   with kmer sizes of 21, 33, 55 and 77. Contigs smaller than 500 bp were removed from the co-

185   assemblies using the Anvi'o v6.1 command 'anvi-script-reformat-fasta' (55). The reformatted

186   assembly fasta files were indexed with bwa index v0.7.17 (56) and the paired end reads from each

187   metagenome were mapped to the respective co-assembly using bwa mem v0.7.17. The resulting

188   sam files were converted to bam files using 'samtools view -F 4 -bhS' to retain only mapped reads.

189   Hybrid metagenomic assemblies were performed using OPERA-MS v0.9.0., which combines the

190   advantages of short and long-read technologies to improve genome assemblies (37). The OPERA-

191   MS inputs were short-read metagenomic assemblies to provide a good representation of the

8

192   sequences in the metagenome, and long and short reads to identify connections between the contigs

193   and obtain contiguous assemblies with low base-pair error. OPERA-MS was executed with the

194   flags --no-ref-clustering and --long-read-mapper minimap2. Both sets of co-assemblies were

195   evaluated using QUAST v5.0.2 (57) (Table S5).

196

197   **2.4 Recovery, annotation, refining and dereplication of metagenome assembled genomes**

198   **(MAGs)**

199   Binning was performed separately, with co-assemblies from Illumina short reads only and hybrid

200   assemblies from each system using MetaBAT2 v2.12.1 (58), CONCOCT v1.1.0 (59), and

201   MaxBin2 v2.2.7 (60) using contigs greater than 2000 bp. Pilon v1.23 (61) was used for polishing

202   to improve the draft bins obtained from the hybrid assemblies. The quality and taxonomy of the

203   resulting bins were determined with CheckM v1.1.2 (62) and the Genome Taxonomy Database

204   Toolkit (GTDB-Tk 1.1.1, database release r86 v3) (63), respectively. Bins were subject to gene

205   calling using Prodigal v2.6.3 (64) and gene annotation against the KEGG database (65) using

206   kofamscan v1.2.0 (66). Only bins that were taxonomically assigned to known nitrifying genera or

207   those containing genes associated with nitrification (i.e., *amoA* [KO number K10944], *amoB*

208   [K10945], *amoC* [K10946], *hao* [K10535], *nxrA* [K00370], *nxrB* [K00371]) were retained for

209   manual refinement with Anvi'o v6.1. DAStool v1.1.2 (67) was used to combine and curate the

210   refined bins from the three binning methods and generate a non-redundant set of bins from each

211   co-assembly (i.e., one set of bins per system for the short-read only and for the hybrid assemblies).

212   In total, 43 nitrifiers bins from the short-read co-assemblies and 30 from the hybrid assemblies

213   were obtained. These bins were de-replicated using drep v2.5.4 at 95% ANI with completeness

214   and contamination thresholds set to 50% and 10%, respectively. This resulted in 44 nitrifier MAGs

9

215   with completeness and redundancy estimates higher than 50% and lower than 10%, respectively,

216   of which 21 contained genes associated with nitrification (i.e., *amoA*, *amoB*, *amoC*, *hao*, *nxrA*,

217   and/or *nxrB*).

218

219   **2.5 Taxonomy aware re-assembly using nitrifying bacterial MAGs**

220   We performed re-assemblies from reads mapped to the 44 nitrifying bacterial MAGs to improve

221   MAG quality. First, contigs associated with *Nitrospira* (including comammox) and *Nitrosomonas*

222   MAGs from each system (i.e., GRE, JAMMSM, NEU) were collated into a single fasta file per

223   system. The resulting fasta files were indexed using bwa index v0.7.17 and short reads from the

224   respective system were mapped using bwa mem v0.7.17. The resultant bam file was split into

225   *Nitrospira* and *Nitrosomonas* specific bam files per system and respective fastq files were

226   generated per using samtools fastq. Long reads were also recruited by mapping the reads from

227   Nanopore sequencing to the fasta files with 'bwa mem -x ont2d'. Short reads mapping to the

228   contigs of the MAGs classified as *Nitrospira* and *Nitrosomonas* were re-assembled using

229   metaSpades v3.13.0 with kmers 21, 33, 55 and 77 on a system-by-system basis. The new genus-

230   specific assemblies and fastq files of mapped long reads were used as input for OPERA-MS to

231   perform hybrid metagenomic re-assemblies (n=9). Quality assessment, binning, taxonomy

232   annotation, manual refining and gene calling were performed as described before. This resulted

233   in 36 nitrifier MAGs with completeness and redundancy estimates higher than 50% and lower than

234   10%, respectively, of which 24 contained genes associated with nitrification (*amoA*, *amoB*, *amoC*,

235   *hao*, *nxrA*, and/or *nxrB*). The abundance of each MAG per sample (reads per kilobase million,

236   RPKM) and proportion of genome covered was calculated with coverM (68). Four low abundant

237   MAGs (3 AOB and 1 *Nitrospira*-NOB) with less than 50% genome coverage in any of the samples

10

238 were removed from subsequent analyses. The entire workflow for assembly and re-assembly of

239 MAGs is outlined in Figure S1. The nitrifier MAGs were subsequently phylogenetically placed in

240 the context of reference *Nitrospira* (Table S4) and *Nitrosomonas* (Table S6) genomes

241 (supplementary text).

242

243 **2.6 Diversity and micro-diversity analysis of nitrifiers**

244 FastANI v1.3 (69) was used to calculate the pairwise average nucleotide identity (ANI) between

245 MAGs within each functional group (i.e., AOB, *Nitrospira*-NOB, and *Nitrospira*-comammox).

246 ANI represents the mean nucleotide identity of the orthologous genes shared between two genomes

247 offering a robust resolution between similar or identical species (i.e., ~80-100% ANI) (69). Since

248 ANI values lower than 80% are not reported, we also calculated the average amino acid identity

249 (AAI) to estimate similarity between two genomes at the amino acid level using compareM (70).

250 To determine intra-population diversity (i.e., microdiversity), we calculated the average nucleotide

251 identity from reads (ANIr) (32) with 90% read identity threshold, as recommended for intra-

252 population comparisons. inStrain v1.3.9 (71) was used to determine the nucleotide diversity for

253 each MAG in each sample and their population average nucleotide identity (popANI) between the

254 samples where they were detected. Nucleotide diversity is a measurement of genetic

255 (micro)diversity at every position along the genome using mapped reads, while popANI is a unique

256 ANI calculation performed by inStrain that considers both major and minor alleles. This is

257 different from the traditional ANI (called consensus ANI [conANI] in inStrain), which only

258 considers major alleles to call (or not) a substitution. Details on parameters and procedures

259 associated with the implementation of ANIr and inStrain are presented in the supplementary text.

260

261 **2.7 Statistical Analyses**

262 Statistical tests were performed using R v4.0.4. All correlations were performed using a linear

263 regression model. The Spearman's rank correlation coefficients between MAG abundances were

264 performed with the 'rcorr' function of the R package Hmisc v4.5.0. Pair-wise significances were

265 calculated with the Kruskal-Wallis test, a nonparametric approach to the one-ANOVA. Principal

266 coordinates analysis (PCoA) with the weighted and unweighted UniFrac distance metrics was used

267 to compare the community composition among systems.

268

269 **3.0 Results**

270 **3.1 Community composition in nitrogen removal systems**

271 Three nitrification-denitrification systems with high absolute abundance of comammox bacteria

272 were selected for this time-series study on the basis of our previous findings (31) since high

273 sequencing coverage is critical for evaluation of sub-population level diversity. Specifically, over

274 the sampling period (June 2017 to June 2018) comammox bacteria constituted approximately 0.77

275 ($\pm$ 0.32), 4.7 ($\pm$ 4.21), and 0.45 ($\pm$ 0.24) % of total bacterial 16S rRNA gene abundances at GRE,

276 JAMMSM, and NEU, respectively.

277

278 The PacBio full-length 16S rRNA gene sequencing resulted in 2,317,019 total reads (Table S1a)

279 with 7501, 5783, and 8260 ASVs at GRE, JAMMSM and NEU, respectively. Each ASV set was

280 rarefied to the sample with the smallest library size per system resulting in 7040, 4349, and 5749

281 ASVs from GRE, JAMMSM, and NEU, respectively, and a total of 16651 unique ASVs across all

282 three systems. ASVs with a total of 100 reads in each system were clustered into OTUs at 97%

283 identity, resulting in 846 OTUs (Figure 1A; Table S7). The most abundant OTUs at GRE and NEU

12

284   were from the classes *Gammaproteobacteria* and *Alphaproteobacteria* and the phylum

285   *Bacteroidota* while the class *Nitrospiria* had the highest relative abundance in JAMMSM. PCoA

286   using weighted (WUF) and unweighted UniFrac (UUF) distance metrics (Figure 1B) demonstrated

287   that samples clustered by system and the community structure between GRE and NEU (WUF:

288   0.18±0.03; UUF: 0.46±0.03) were significantly more similar than GRE and JAMMSM (WUF:

289   0.28±0.02; UUF: 0.56±0.05) and NEU and JAMMSM (WUF: 0.29±0.03; UUF: 0.52±0.04)

290   (p<0.05; Figure S2A and B). The greater similarity between GRE and NEU is likely due to the

291   fact that these were suspended phase communities as compared to attached phase (i.e., biofilm

292   samples) communities collected from the IFAS system at JAMMSM.

293

294   *Nitrosomonas-* (31, 25, and 14 ASVs at GRE, JAMMSM, and NEU, respectively) and *Nitrospira-*

295   like bacteria (37, 36, and 29 ASVs at GRE, JAMMSM, and NEU, respectively) were the only

296   nitrifiers present in the systems. *Nitrospira* lineage I ASVs were the most abundant group across

297   all systems with average relative abundances of 2.91, 25.13, and 2.88%, followed by *Nitrospira*

298   lineage II with average relative abundances 1.41, 7.07, and 0.64% in GRE, JAMMSM, and NEU,

299   respectively (Figure 1C). *N. oligotropha* (Cluster 6a) and *N. communis* (Cluster 8) -like ASV's

300   were present in all systems with relative abundances of 0.5 and 0.33, 1.68 and 0.93, and 0.58 and

301   0.19% in GRE, JAMMSM, and NEU, respectively. *N. europaea/mobilis* (Cluster 7)-like ASVs

302   were also detected in GRE and JAMMSM at very low abundances compared to the other two

303   *Nitrosomonas* linages (average ~0.032 and 0.002%, respectively).
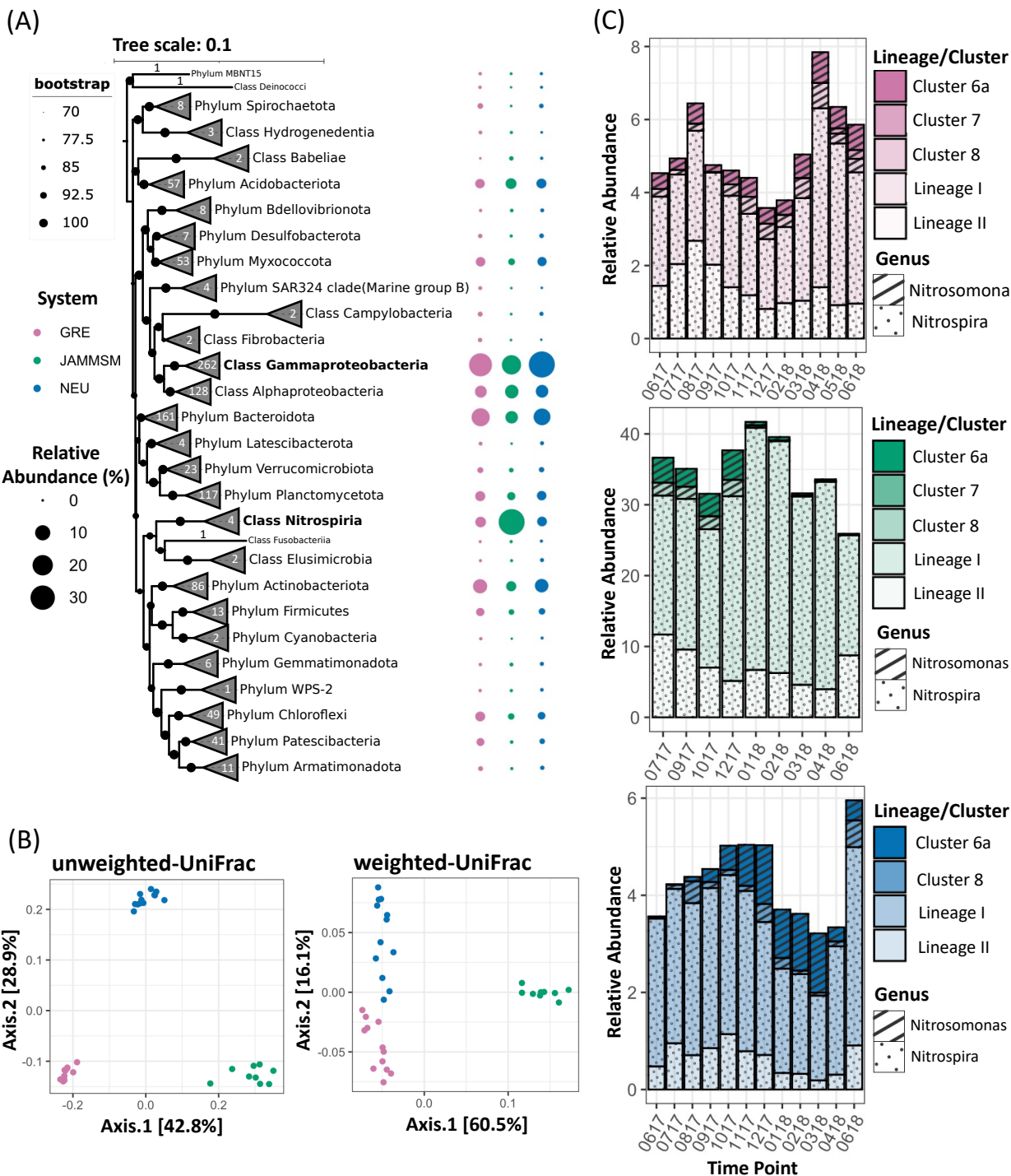
Figure 1: Community composition of the nitrogen removal systems. (A) Phylogenetic tree constructed using full-length 16S rRNA gene sequences of the representative ASV in each OTU (98.7% sequence similarity cluster), with their corresponding relative abundances shown at the right. Branches are collapsed at the phylum/class level and corresponding number of OTUs within each phylum/class across all three systems are indicated. Black circles on branches designate bootstrap support. Bubbles represent cumulative relative abundances of all OTUs within each phylum/class per system. (B) Principal Coordinate Analysis of community composition with unweighted and weighted UniFrac distance metrics using OTU data. Points represent the samples. (C) Cumulative relative abundances of OTUs corresponding to *Nitrospira* Lineages and *Nitrosomonas* clusters per system at each time point analyzed. Colors correspond to each nitrogen removal system with (GRE, pink; JAMMSM, green; NEU, blue).

3.2 16S rRNA gene sequence-based diversity and microdiversity of nitrifiers

The *Nitrospira*-like ASVs belonged to lineage I (20, 24, and 22 ASVs at GRE, JAMMSM, and NEU, respectively) or lineage II (17, 12, and 7 at GRE, JAMMSM, and NEU, respectively) (Figure S3A), while *Nitrosomonas*-like ASVs were associated with *N. oligotropha* (23, 21, and 10 at GRE, JAMMSM, and NEU, respectively), *N. europaea*/*mobilis* (5 and 1 at GRE and JAMMSM, respectively), and *N. communis* (3, 3, and 4 at GRE, JAMMSM, and NEU, respectively) lineages (Figure S3B). The *Nitrospira*- and *Nitrosomonas*-like ASVs clustered into 3, 3 and 2, and 21, 14 and 10 OTUs for GRE, JAMMSM and NEU, respectively. The Shannon diversity (i.e., population diversity) of *Nitrosomonas*-like OTUs was significantly higher than *Nitrospira*-like OTUs, while the effective microdiversity of *Nitrospira*-like OTUs was significantly higher than *Nitrosomonas*-like OTUs in all systems (p<0.05; Figure 2A,). *Nitrospira* lineage I OTUs consistently showed the highest microdiversity (Figure 2B) while two *Nitrospira* lineage II OTUs from GRE and JAMMSM had a lower effective microdiversity than most nitrifier OTUs. These results suggest high population level diversity and low microdiversity for *Nitrosomonas*-like bacteria, while the opposite was observed for *Nitrospira*-like bacteria in Lineage I (more ASVs per OTU). The effective microdiversity of *Nitrospira*-like OTUs was positively correlated with their relative abundance (Figure S4A). However, this was neither the case for *Nitrosomonas*-like OTUs (Figure S4B) and the overall bacterial community (Figure S4C). Although the relative abundances of some other highly microdiverse OTUs were positively correlated with effective microdiversity (e.g., *Hyphomicrobium*), this correlation was not statistically significant for most (e.g., Figure S5). This suggests that the increase in effective microdiversity of *Nitrospira*-like OTUs with increasing relative abundance is more likely to be an ecological phenomenon rather than a sampling artifact.

15
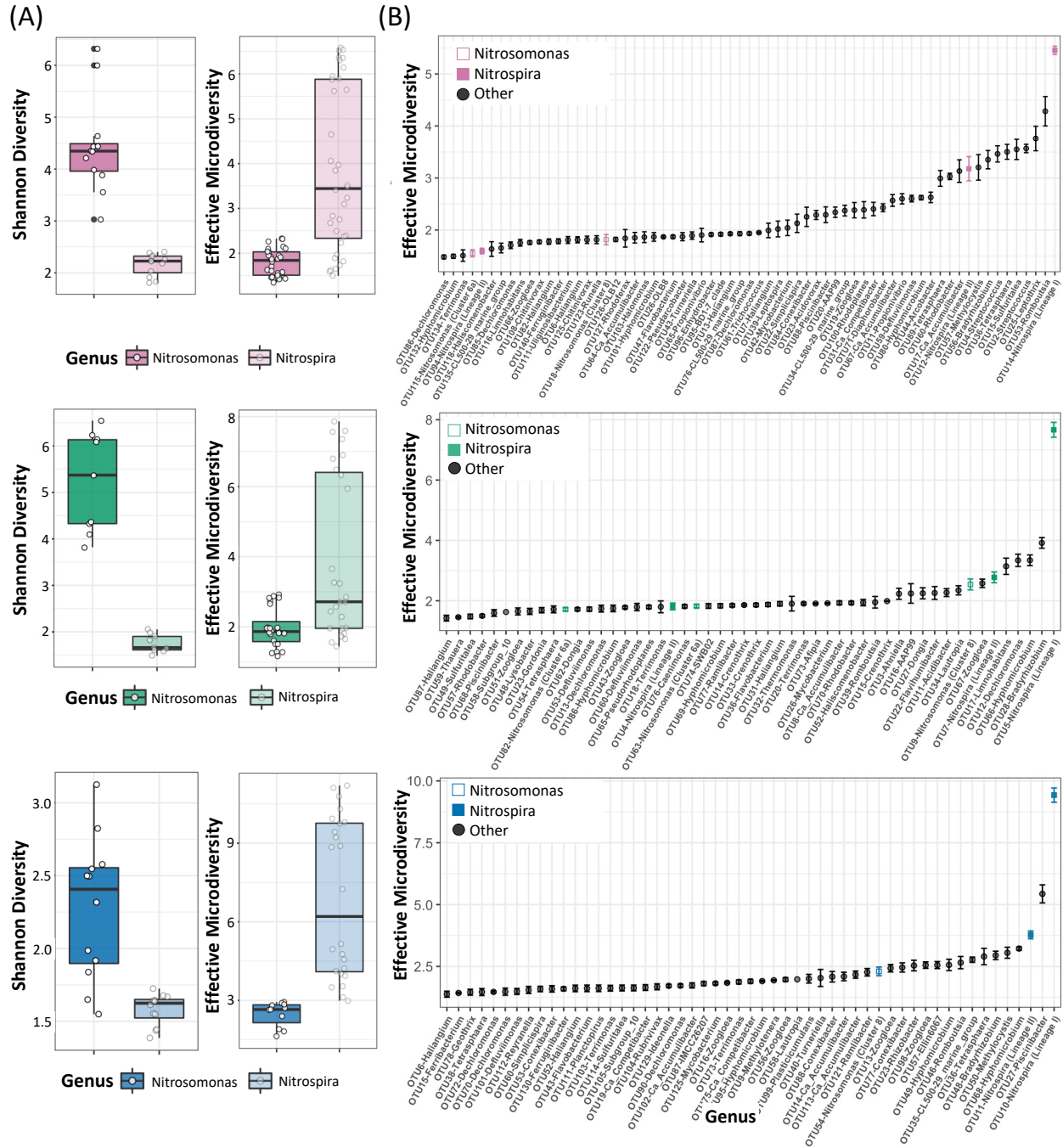
Figure 2: Diversity and microdiversity based on ASV analyses. (A) Boxplot of Shannon diversity and effective microdiversity of *Nitrospira* (lighter shade) and *Nitrosomonas* (darker shade) at each system. (B) Average effective microdiversity of major OTUs per system. *Nitrospira* and *Nitrosomonas* OTUs are colored by system (GRE, pink; JAMMSM, green; NEU, blue), represented by closed and open symbols, respectively.

345   **3.3   Improving the recovery of metagenome assembled genomes of nitrifying bacteria**

346   A total of 43 dereplicated nitrifiers MAGS were recovered from short-read assemblies. However,

347   several MAGs lacked key genes associated with nitrification (i.e., *amoA, amoB, amoC, hao, nxrA,*

348   *nxrB*). For example, only 2 of the 7 *Nitrosomonas* MAGs (from JAMMSM and NEU) contained

349   the complete *amoCAB* operon and only one had the *hao* gene. To improve MAG quality, we

350   incorporated long reads from Nanopore sequencing and a hybrid assembly using OPERA-MS (37).

351   The hybrid approach increased the contiguity of the assembly and resulted in 30 nitrifier MAGs,

352   with nitrification genes present in 19 MAGs (Table S8). It resulted in significant improvement of

353   MAGs retrieved from JAMMSM but not for GRE or NEU. Thus, we pursued taxonomy aware re-

354   assembly (72) of the 44 non-redundant nitrifer MAGs after dereplicating bins from short-read and

355   hybrid assembly approach. The resultant 36 nitrifier MAGs had completeness greater than 70%

356   and redundancy lower than 10% (Table S8), with nitrification genes present in 24 MAGs.

357   Although the number of retrieved nitrifier MAGs was lower after the reassembly process, there

358   was significant improvement in quality, especially for comammox and AOB genomes. For

359   example, both analyses (before and after reassembly) resulted in 4 *Nitrospira*-comammox MAGs.

360   However, 2 of the 4 comammox MAGs recovered after reassembly contained the entire genetic

361   repertoire required for nitrification, and the other two lack only one gene. In contrast, all pre-

362   reassembly comammox MAGs lacked at least one nitrification gene. These improvements were

363   also observed for AOB including significant improvement in MAG quality (i.e., higher

364   completeness, lower redundancy, and less fragmentation) (Table S8). These improvements

365   resulted from longer contigs created during the reassembly process compared with previous co-

366   assemblies. In contrast to comammox and AOB, despite becoming less fragmented post-

367   reassembly, fewer *Nitrospira*-NOB MAGs contained all genes associated with nitrite oxidation.

17

3.4 Phylogenomic placement and abundance of nitrifier MAGs

The 11 canonical NOB and 4 comammox were associated with *Nitrospira* lineage I (8 *Nitrospira*-NOB) and *Nitrospira* lineage II (3 *Nitrospira*-NOB and 4 *Nitrospira*-comammox), with all comammox MAGs closely related with *Ca.* Nitrospira nitrosa (Figure 3A) and belonged to *Nitrospira*-comammox clade A.1 based on *hao* gene phylogenetic inference (Figure S6). This is in agreement with a previous study that suggests comammox clade A.1 usually co-occur with the *Nitrospira* lineage I populations in wastewater treatment plants (WWTPs) while clade A.2 and most clade B are typically found in drinking water treatment plants (DWTPs) (21). While non-WWTP settings (e.g., soils, sediments, lakes, rivers, DWTPs) harbor diverse comammox bacteria (9,18,21,73,74), our findings suggest very low diversity of comammox bacteria in wastewater systems which is consistent with other wastewater studies (12,15,17,22,75–77). All AOB MAGs (n=20) were associated with the *N. oligotropha* (n=14) and *N. communis* (n=3) lineages (Figure 3B). Although several members of the *N. europaea*/*mobilis* lineage have been detected in sewage treatment plants (78), only three AOB MAGs related to this lineage were recovered from JAMMSM at very low abundances and less than 50% genome coverage. Thus, these MAGs were excluded from subsequent analyses. The three *N. communis* cluster MAGs (GRE AOB 1, JAMMSM AOB 4, NEU AOB 3) were nearly 100% identical but shared less than 75% ANI with all reference genomes. This lineage can be divided into the *N. communis* and the *N. nitrosa* clusters (79), with *N. communis* species urease negative and detected primarily in agricultural soils, and isolates of *N. nitrosa* urease positive, preferring aquatic habitats and often found in wastewater treatment plants (80). Our three MAGs were urease positive but still distant to *N. nitrosa* in the phylogenomic analysis (Figure 3B), suggesting the presence of a novel phylogenetic lineage. Lastly, most species of the *N. oligotropha* lineage have been recovered from oligotrophic

18

391   freshwaters and almost all are urease positive (78,80). Accordingly, 12 of the 14 AOB MAGs

392   placed within this *Nitrosomonas* lineage were urease positive.

393

394   *Nitrospira*-NOB was the most abundant functional group in all three systems for the duration of

395   the study with higher abundances in JAMMSM (314.5 ±70.6 RPKM) as compared to NEU (178.2

396   ±12.5 RPKM) and GRE (149.3 ±11.7 RPKM). *Nitrosomonas*-like MAGs were more abundant in

397   NEU (105.2 ±23 RPKM) and GRE (86 ±15.8 RPKM) as compared to JAMMSM (53.2 ±27.4).

398   This suggests that *Nitrospira*-like bacteria may prefer biofilm-based growth over suspended phase

399   (81). *Nitrospira*-comammox relative abundances were very similar in all systems, ranging from

400   55.5 to 64.9 RPKM in JAMMSM and NEU, respectively. The abundances of nitrifying MAGs

401   were weakly correlated with each other, with only 37 of 195 correlations significant (p-value

402   <0.05). Most of the significant correlations between AOB and *Nitrospira*-NOB were positive

403   (Figure S7), likely arising as consequence of metabolic interactions of these groups within the

404   nitrification process. Comammox bacteria had very few correlations with other nitrifiers, with

405   primarily negative correlations with MAGs from *N. oligotropha*-like bacteria (cluster 6a).

19
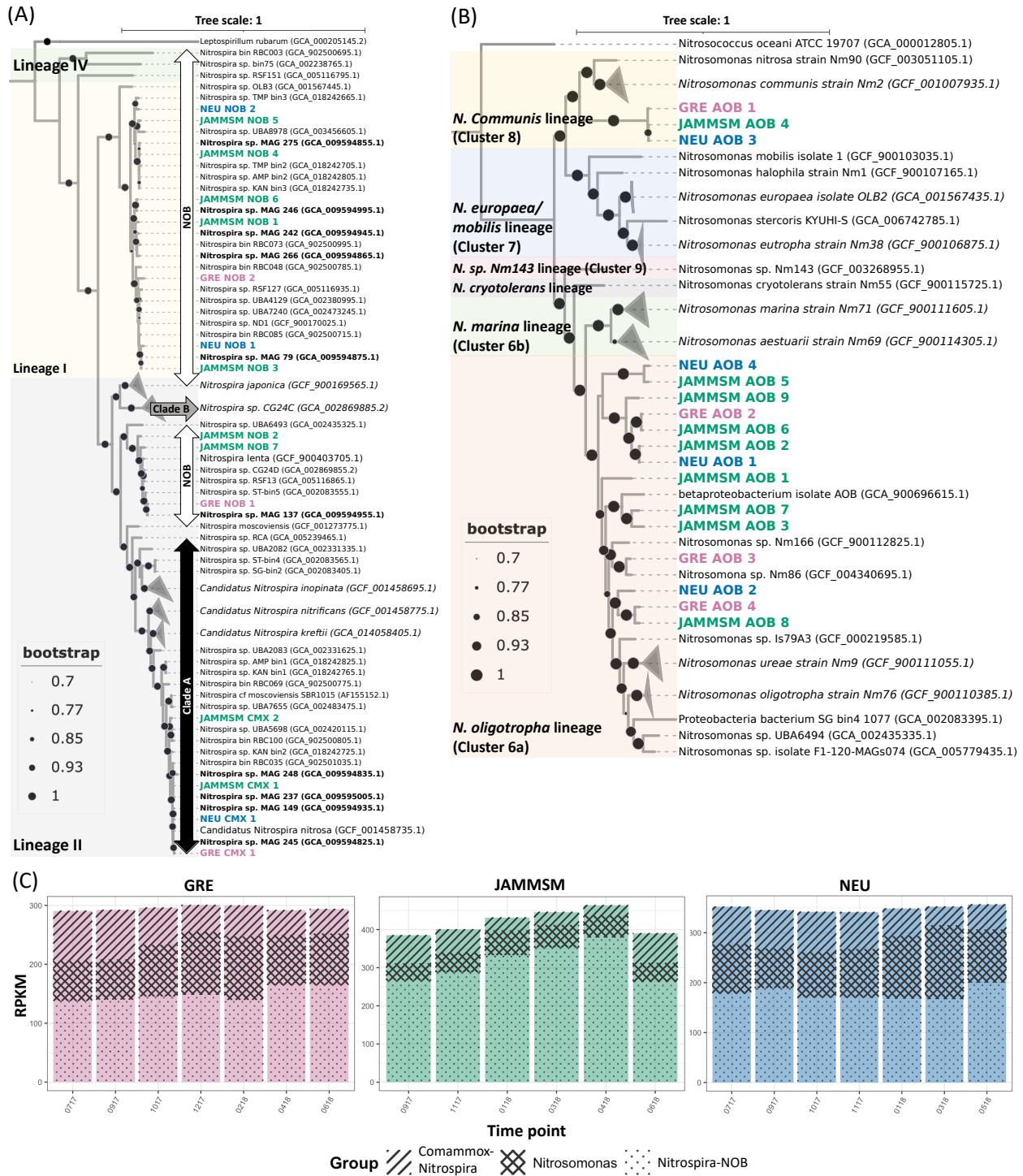
Figure 3: Phylogenetic placement of (A) *Nitrospira* and (B) *Nitrosomonas* MAGs (GRE, pink; JAMMSM, green and NEU, blue) with 78 and 48 reference genomes (black), respectively. Comammox clade A (black arrow), clade B (grey arrow) and canonical NOB (white arrow) are indicated by arrows and phylogenetic lineages by colored boxes. Branches with reference genomes that did not include MAGs from this study were collapsed and labeled with a representative reference genome. All reference genomes used for the reconstruction of the trees are listed in Tables S4 and S6. (C) Cumulative abundances (RPKM) of *Nitrospira*-comammox, *Nitrospira*-NOB and *Nitrosomonas* MAGs at each time point per system.

406

407
408
409
410
411
412
413

20

3.5 Population-level diversity of nitrifying bacteria within and between systems

Post dereplication at 95% ANI (69,82), pairwise ANI and AAI were calculated between all MAGs within the same functional group (i.e., AOB, *Nitrospira*-NOB, and *Nitrospira*-comammox) (Figure 4A-B, Tables S9a-b). Of the 136 pairwise comparisons between the 17 AOB MAGs, only 18 (13.2%) had ANI values higher than 80% suggesting high population-level diversity within and between nitrogen removal systems for the *Nitrosomonas*-like AOB (Figure 4A). The pairwise AAI for *Nitrospira*-NOB MAGs showed a bimodal distribution (Figure 4B) reflective of the two lineages detected in each system (i.e., Lineage I and II). Pairwise AAIs for *Nitrospira*-NOB MAGs of the same lineage within or across systems were typically >85% (Table S9b). These results suggest that while multiple AOB and *Nitrospira*-NOB coexist in each system, AOB were substantially more diverse than *Nitrospira*-NOB. In contrast, three of the four comammox MAGs (one from each system) share ANI values between 95-97% and are closely related with *Ca.* Nitrospira nitrosa (31). Although a comammox MAG from JAMMSM (JAMMSM CMX 2) has lower than 95% with the other three MAGs, it still exhibited greater than 85% AAI (Figure 4B) with them and also falls within the *Ca*. Nitrospira nitrosa cluster (Figure 3A)(83). Pairwise AAI comparisons (which are highly correlated with ANI – Figure S8) indicated that *Nitrospira*-comammox MAGs have significantly higher AAI compared with AOB and NOB, while AOB were the most diverse functional group at the population level (p <0.05; Figure 4B). Moreover, similar to ASV analysis, *Nitrospira*-NOB MAGs were associated with one of two clusters (*Nitrospira* lineage I or II), with highly similar populations within each cluster (Figure 4B) being detected across systems. These findings further confirm that in contrast to AOB that demonstrate high population diversity, comammox bacteria show very low population diversity and, in fact, may all be associated with the same population across multiple wastewater systems

21
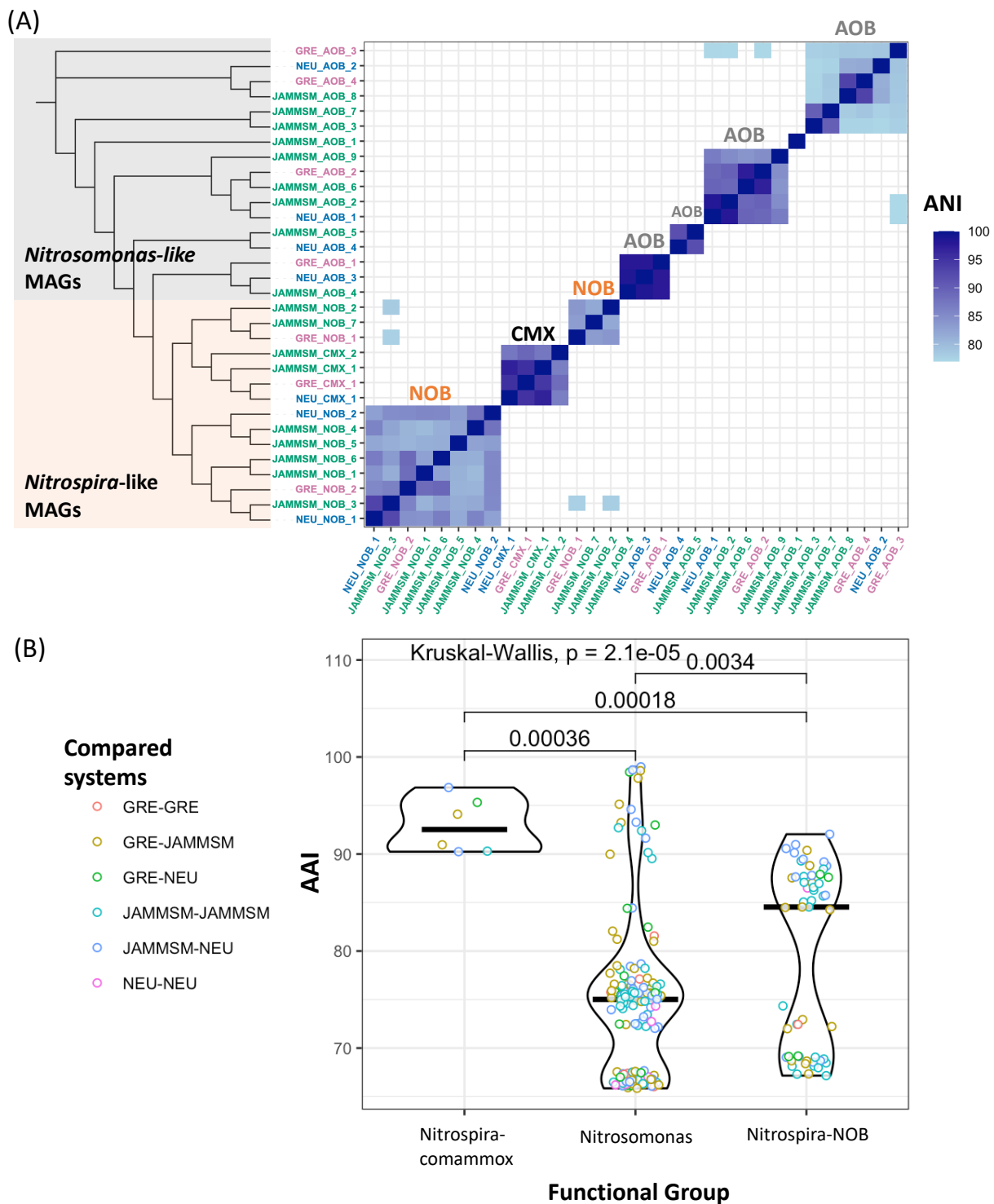
Figure 4: (A) ANI values calculated between *Nitrosomonas* (AOB), *Nitrospira*-NOB and *Nitrospira*-comammox (CMX) MAGs along with the phylogenetic placement of the MAGs. MAG labels are colored by system (GRE, pink; JAMMSM, green and NEU, blue). (B) AAI values per functional group. Black lines represent the average, points represent the AAI value between two MAGs and colors decode the systems that are compared. Pairwise statistical comparisons were performed with the Kruskal-Wallis test.

3.6 . Microdiversity of nitrifying bacteria

The ANIr distributions for comammox and AOB MAGs were not significantly different from each other, and significantly higher than for *Nitrospira*-NOB MAGs (Figure 5A). While *Nitrospira*-NOB overall demonstrated lower ANIr indicating higher microdiversity compared to AOB and comammox bacteria, there was some variability within each system (Figure S9). These observed trends were similar between ANIr and inStrain estimated nucleotide diversity (Figure 5B), with AOB showing on average lowest microdiversity and *Nitrospira*-NOB the highest. Specifically, the average nucleotide diversity of *Nitrosomonas*-like AOB was significantly lower than of both *Nitrospira* groups, i.e., NOB and comammox ($p < 0.05$), while with ANIr only *Nitrospira*-NOB were significantly more microdiverse than the two other functional groups. This difference arises from the fact that the two measures of intra-population diversity (i.e., ANIr and nucleotide diversity) are calculated differently. For instance, ANIr considers only the major alleles in the consensus sequence to call a substitution, while nucleotide diversity is calculated using base pair frequencies at each position. Nonetheless, both approaches indicate that *Nitrospira*-NOB exhibit significantly higher microdiversity as compared to *Nitrosomonas*-like AOB and *Nitrospira*-comammox bacteria. These results are consistent with the 16S rRNA gene analysis where *Nitrospira* lineage I and most lineage II OTUs had significantly higher effective microdiversity than the *Nitrosomonas*-like OTUs and the remaining *Nitrospira* lineage II OTUs. Based on these results, we speculate that the *Nitrospira* lineage II OTUs with low effective microdiversity likely correspond to comammox bacteria (Figure 2B).

Finally, popANI, a microdiversity-aware ANI calculation, was used to discriminate between strains across samples using the recommended popANI threshold of 99.999% (Olm et al., 2021).

23

467   All popANI values from GRE_CMX_1, JAMMSM_CMX_1, and NEU_CMX_1 were above or

468   very close to the 99.999% threshold, suggesting low strain-level diversity of comammox bacteria

469   (Figure 5C). Only one comammox genome retrieved from JAMMSM (JAMMSM_CMX_2) had

470   popANI values lower than the recommended threshold. However, the average relative abundance

471   of this comammox (RPKM = 7 ±2) was particularly low as compared to the more abundant

472   comammox in JAMMSM (RPKM = 48 ±21) and those retrieved from GRE (RPKM = 60 ±19) and

473   NEU (RPKM = 65 ±17). Therefore, the principal or sole comammox bacteria in each system is

474   not only a single population but likely also a specific strain. The ANIr for each comammox MAG

475   estimated by mapping reads from a different system (e.g., mapping GRE or NEU reads to MAGs

476   assembled from JAMMSM) were lower than when mapping of reads from the system from where

477   the MAG was obtained (e.g., mapping JAMMSM reads to MAGs assembled from JAMMSM;

478   Figure S10). This suggests that on the one hand the comammox bacterial population within a single

479   system is restricted to a specific *Ca.* Nitrospira nitrosa-like strain, but on the other hand that

480   different systems contain different strains.

24

481
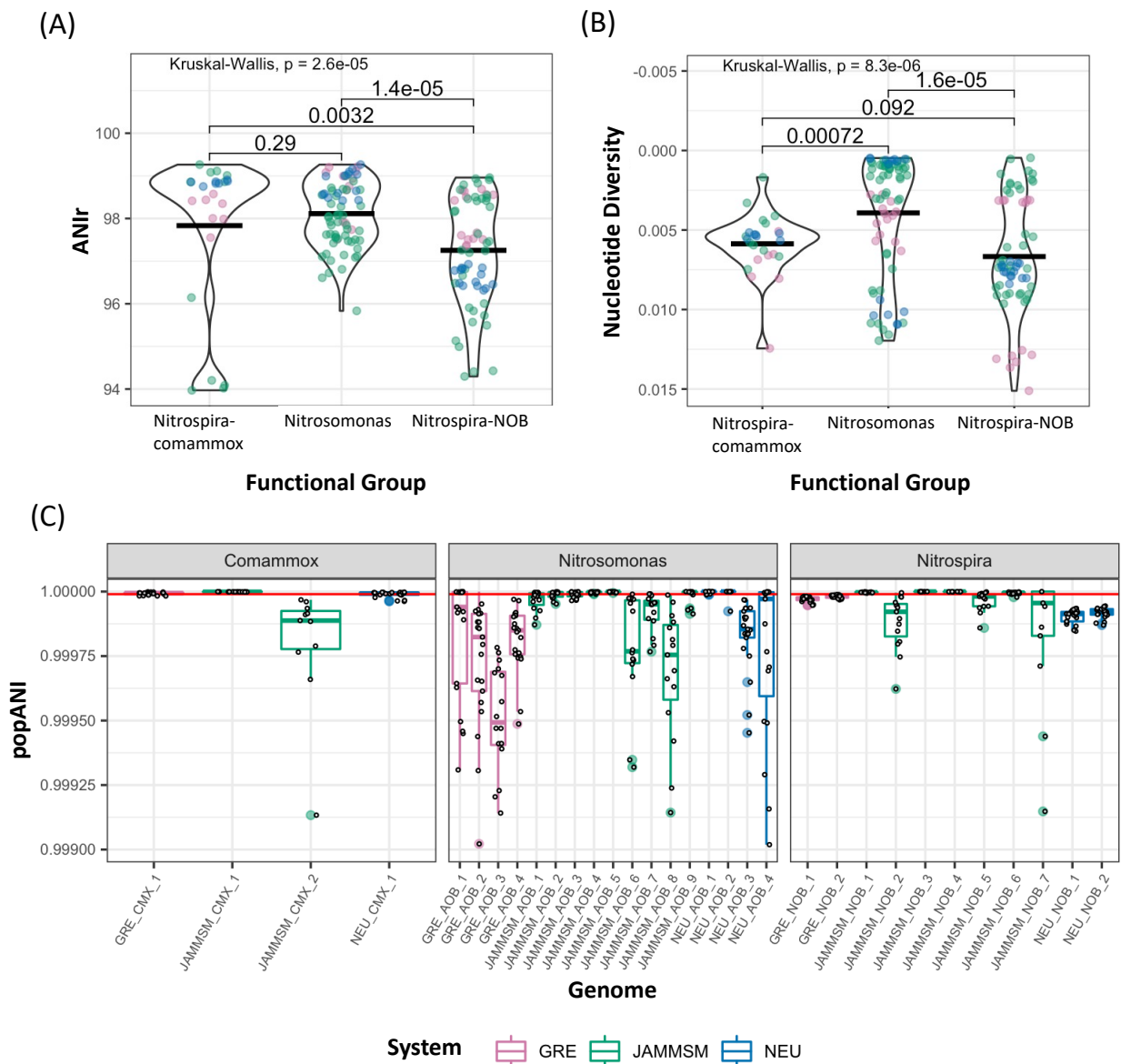
Figure 5: (A) ANIr and (B) nucleotide diversity values from each functional group. Points represent the average value of each MAG and are colored according to the systems (GRE, pink; JAMMSM, green and NEU, blue) from which the MAGs were recovered. (C) popANI values from each MAG. MAGs are colored by system and points represent the popANI value of the MAG between two samples.

486
487
488
489
490
491
492
493
494

495   **4   Discussion**

496   **4.1   Hybrid assembly and re-assembly processes improve the quality of metagenome**

497   **   assembled genomes**

498   Reliance on short reads for genome assembly from complex metagenomes can lead to incomplete

499   and fragmented MAGs especially in systems containing closely related populations (84). Indeed,

500   short read analysis resulted in highly fragmented nitrifier MAGs lacking several nitrification

501   genes. To improve the short read assemblies, we used long reads generated from Nanopore

502   sequencing with a hybrid assembly approach (38). Recent studies have demonstrated the ability of

503   hybrid assemblies to significantly improve the recovery of complex bacterial genomes (85,86) by

504   increasing the contiguity and accuracy of metagenome assembly (87). Combining the two

505   sequencing techniques (i.e., short and long reads) with the hybrid assembler OPERA-MS resulted

506   in modest assembly and binning improvements regarding genome contiguity and completeness.

507   However, the bead beating based DNA extraction protocol may have resulted in shearing of

508   genomic DNA into smaller fragments which is not ideal for long-read sequencing (88) and resulted

509   in lower read lengths. In an attempt to further improve MAG quality, we performed taxonomy

510   aware reassemblies (72) as described in the materials and methods section. Since a hybrid approach

511   resulted in some improvements in MAG quality, we also adopted a hybrid strategy with OPERA-

512   MS for taxonomy aware reassembly. This significantly improved the completeness and contiguity

513   of MAGs and number of annotated nitrifier genes (especially for *Nitrosomonas* and comammox

514   *Nitrospira*) binned within them. While the *Nitrospira*-NOB MAGs also became less fragmented,

515   they showed fewer improvements with respect to the presence of nitrite oxidation genes. This is

516   likely caused by the high levels of microdiversity within *Nitrospira*-NOB (Figures 2 and 5), which

517   likely impacts both the read mapping and de novo assembly process. In the future, this issue might

26

518   be improved by incorporating more long-read data into the re-assemblies, especially once the

519   accuracy of long-read sequencing technologies further increases.

520

521   **4.2 *Ca.* Nitrospira nitrosa-like bacteria are the primary comammox bacteria in secondary**

522   **wastewater treatment for nitrogen removal**

523   Structural diversity and functional redundancy are inherently linked to process stability when

524   environmental and process conditions vary (28). Both, metagenomics and 16S rRNA gene

525   sequencing, indicated that *Nitrosomonas* and *Nitrospira*-like bacteria were the only known

526   nitrifiers present in the systems investigated. This is consistent with numerous studies

527   (26,35,89,90) suggesting the specific adaptation of these genera to the wastewater environment.

528   The majority of the *Nitrosomonas*-like AOB MAGs, within and between systems, had AAI values

529   below 85% (Figure 4) indicating that this functional group exhibited high species level diversity.

530   Meanwhile, *Nitrospira*-like MAGs within the same lineage reported AAI values above 85% in

531   most cases. The high population level diversity of the *Nitrosomonas*-like bacteria was also

532   confirmed with full length 16S rRNA gene sequencing (Figure S3B) and corroborates earlier

533   studies on their diversity in wastewater (26,90,91). Although *N. europaea*, *N. oligotropha* and the

534   *Nitrosomonas*-like clusters closely associated with *N. communis*/*nitrosa* are frequently the most

535   abundant groups in wastewater, different *Nitrosomonas* populations dominate different processes

536   and the extent of their diversity is system dependent (26). For example, *N. europaea* are often the

537   major AOB in high ammonia concentration environments (e.g., anammox reactors) due to low

538   ammonia affinity and low DO level adaptation (91). The observed dominance of *N. oligotropha*

539   and *N. communis* in this study are consistent with previous studies of WWTPs with relatively

540   lower ammonia concentrations (26,91,92).

541

542   Comammox bacteria exhibited significantly lower population level diversity as compared to strict

543   AOB and NOB (Figure 4). All comammox *Nitrospira* MAGs, independent of the system they were

544   recovered from, belong to *Nitrospira* lineage II and were closely associated with *Ca.* Nitrospira

545   nitrosa. These findings are consistent with several other studies that have found *Ca.* Nitrospira

546   nitrosa-like bacteria as the unique or principal comammox bacteria present in their laboratory and

547   full-scale wastewater treatment systems (11,15,17,20,22,75,76), both using metagenomics and

548   gene targeted analyses. In contrast, other engineered and natural habitats typically harbor multiple

549   co-existing species across the two primary clades (i.e., comammox clade A and B). For example,

550   a study of multiple groundwater-fed sand filters recovered 12 comammox MAGs (5 clade A and

551   7 clade B) and revealed the co-occurrence of at least five comammox species (10 on average) per

552   system (34). Other studies in soils, sediments, lakes, drinking water, groundwater, and estuaries

553   have also reported the co-existence of multiple comammox species (9,73,93,94). However, this

554   co-existence of comammox populations across clades does not seem to be prevalent in wastewater

555   treatment systems. For instance, a study of 8 WWTPs found 9 of 14 comammox OTUs were

556   associated with the *Ca*. Nitrospira nitrosa cluster and accounted for 94.34% of all comammox

557   *amoA* sequences (17) with different OTUs representing the dominant comammox species in

558   different systems. Moreover, Beach and Noguera (75) reported the dominance of *Ca*. Nitrospira

559   nitrosa in five low DO nitrogen removal bioreactors using qPCR with species-specific primers.

560   These findings suggest that the diversity of comammox bacteria in WWTPs is lower than in other

561   habitats where several comammox species co-exist.

562

28

563   In contrast, Spasov et al. (16) reported the detection of multiple comammox MAGs in samples

564   collected from rotating biological contactors (RBCs) used for tertiary treatment at a municipal

565   WWTP in Ontario, Canada. The difference in population diversity between that study and other

566   WWTP investigations, including this one, may be associated with ammonia availability.

567   Specifically, tertiary treatment systems are typically employed as a polishing step; they are

568   designed and operated to remove low residual amounts of nitrogen. The influent ammonium

569   concentrations reported for the RBC tertiary treatment system range from 0.2 to 16.3 µM (16),

570   while the three systems in this study have influent ammonia concentrations between 2 and 3 mM.

571   The comammox *Nitrospira* MAGs with overall higher abundances in the tertiary plant (RBC001

572   and RBC083) were less abundant at the beginning of the train (higher ammonium concentrations)

573   than at the end of the train (lower ammonium concertation). In contrast, the abundance of

574   RBC035, the only MAG phylogenetically associated with *Ca.* Nitrospira nitrosa (ANI >95%),

575   decreased in abundance with decreasing ammonium concentrations. The high comammox

576   diversity in the RBCs and other ammonium-limited systems suggest that ammonium concentration

577   and/or flux through the system may be a key factor driving comammox bacteria diversity in

578   nitrification systems. In fact, Palomo et al. (34) reported that the influent ammonium concentration

579   was the key explanatory variable associated with comammox bacterial diversity in 12

580   groundwater-fed sand filters when higher comammox population diversity was detected in systems

581   receiving lower ammonium concentrations. *Ca*. Nitrospira inopinata and *Ca.* Nitrospira kreftii,

582   two comammox species with known kinetic parameters, have high apparent ammonia affinities

583   (95,96); assuming this is a conserved trait in comammox bacteria, this explains their prevalence

584   and diversity in ammonium-limited systems. However, the kinetic parameters (i.e., affinity

585   constant, growth rate) of other comammox species especially of the *Ca*. Nitrospira nitrosa clade

586 have not yet been reported, and our findings suggest that at least this comammox clade may have

587 substantially different kinetic traits that allow it to thrive in systems with higher ammonium

588 concentrations. Low DO has also been associated with the prevalence of comammox bacteria over

589 canonical nitrifiers in wastewater systems (15,75). However, the high DO concentrations (>2

590 mg/L) in this and other studies (17,20) suggest DO is not a crucial factor for the proliferation of

591 *Ca.* Nitrospira nitrosa in nitrogen removal systems.

592

593 **4.3 Comammox bacteria not only exhibit low diversity at the species/population level but**

594 **also at the strain/intra-population level across wastewater systems**

595 The high abundance and microdiversity of *Nitrospira*-NOB have been previously reported in

596 wastewater and other engineered and natural environments (4,33,35). However, it is unclear

597 whether the high microdiversity of *Nitrospira* is associated with high functional diversity or is

598 indicative of the coexistence of functionally identical *Nitrospira* with allelic diversity. Gruber-

599 Dorninger et al. (35) reported variable responses of closely related bacteria within *Nitrospira*

600 lineage I (sequence identities ranging from 95.8 to 99.6%) to different nitrite availabilities.

601 Similarly, ecological niche partitioning was also identified as potential mechanisms for co-

602 existence of three *Nitrospira* lineage I strains that used formate under different conditions: two

603 used formate when incubated with nitrite and ammonia, respectively, while the third used formate

604 efficiently as the sole substrate (35). Although these results shed light on the possible niche

605 partitioning of *Nitrospira* sub-species, this does not shed light on whether the microdiversity in

606 our study represents coexisting *Nitrospira* strains with slight functional differences. Although

607 functional implications of such high microdiversity are not clear, the persistence of high intra-

608 population diversity may suggest it plays an important role in the distribution and success of

30

609   *Nitrospira* populations in wastewater systems. A few studies in other environments have shown

610   that persistent populations exhibit increased intra-population sequence diversity (7,32). A study in

611   a saltern pond suggested that the ecologically important genes of the major archaeal sequence-

612   discrete population were carried by distinct sub-populations (strains), indicating that the adaptation

613   to different salinity concentrations had led to sub-population differentiation and speciation (niche

614   partitioning) (82). Moreover, a study in eight different temperate bog lakes concluded that high

615   microdiversity is associated with the maintenance of functional microbial communities during

616   changes in environmental conditions (36).

617

618   Contrastingly, comammox bacteria not only exhibited an unexpected low population-level

619   diversity within and between systems but also showed significantly lower microdiversity as

620   compared to strict NOB within the genus *Nitrospira*. These results may indicate specific

621   adaptations of comammox bacteria within the *Ca.* Nitrospira nitrosa cluster to the wastewater

622   environment and particularly secondary treatment systems. Palomo et al. (34) observed a negative

623   correlation between the species-level diversity of comammox *Nitrospira* and ammonium

624   concentrations. However, that was not the case for microdiversity, suggesting that different

625   mechanisms may shape inter- versus intra-population diversities, or that the range of ammonium

626   concentrations in the investigated drinking water systems was too narrow to capture any

627   underlying associations. For instance, the comammox nucleotide diversity in our study ranged

628   from 0.002 to 0.008, while this was from ~0.005 to ~0.013 for the MAGs obtained from these

629   drinking water systems, with only three of the twelve comammox MAGs presenting nucleotide

630   diversities lower than 0.008 (34). This may suggest that comammox microdiversity is also

631   associated with ammonium concentrations and/or flux through the system, with the observed low

31

632 microdiversity in wastewater systems associated with the higher prevailing ammonium

633 concentrations.

634 Despite the broad detection of comammox bacteria in WWTPs, their role and process relevance

635 are as yet unclear. In contrast to other environments, comammox *Nitrospira* present very low

636 population and intra-population diversity levels in wastewater treatment systems for nitrogen

637 removal. This observed lack of diversity and, consequently, lack of functional redundancy may

638 influence the feasibility of potential design and operational strategies relying primarily on

639 comammox *Nitrospira* for nitrogen removal. Although ammonium concentration and/or

640 availability apparently influences the diversity of comammox bacteria, further studies are

641 necessary to determine other factors driving the success of their clonal community in wastewater.

642 Moreover, studies are required to estimate the activity of comammox bacteria, specifically *Ca.*

643 Nitrospira nitrosa, and to assess their contribution to nitrification in full-scale nitrogen removal

644 systems. It is also important to note that the intra-population diversity cannot be solely studied

645 with shotgun metagenomic methods since high microdiversity can prevent robust assembly of

646 individual genomes (82). Therefore, more accurate techniques are needed to obtain high-quality

647 strain-level genome assemblies for *Nitrospira* and other highly microdiverse bacteria.

648

649 **4   Acknowledgements**

654

655   **5   Data availability**

656   All raw sequencing data from Illumina and Nanopore platforms and nitrifiers MAGs are

657   available on NCBI under bioproject number PRJNA846349.

6 References

1. Klotz MG, Stein LY. Nitrifier genomics and evolution of the nitrogen cycle. FEMS Microbiol Lett. 2008;278(2):146–56.

2. Daims H, Lebedeva E v, Pjevac P, Han P, Herbold C, Albertsen M, et al. Complete nitrification by Nitrospira bacteria. Nature. 2015;528(7583):504–9.

3. Pinto AJ, Marcus DN, Ijaz UZ, Bautista-de Lose Santos QM, Dick GJ, Raskin L. Metagenomic evidence for the presence of comammox Nitrospira-like bacteria in a drinking water system. Msphere. 2016;1(1): e00054–15.

4. Palomo A, Jane Fowler S, Gülay A, Rasmussen S, Sicheritz-Ponten T, Smets BF. Metagenomic analysis of rapid gravity sand filter microbial communities suggests novel physiology of Nitrospira spp. ISME J. 2016;10(11):2569–81.

5. van Kessel MAHJ, Speth DR, Albertsen M, Nielsen PH, den Camp HJM, Kartal B, et al. Complete nitrification by a single microorganism. Nature. 2015;528(7583):555–9.

6. Hu HW, He JZ. Comammox—a newly discovered nitrification process in the terrestrial nitrogen cycle. Journal of Soils and Sediments. 2017;17(12):2709–17.

7. Orellana LH, ben Francis T, Krüger K, Teeling H, Müller MC, Fuchs BM, et al. Niche differentiation among annually recurrent coastal Marine Group II Euryarchaeota. ISME J. 2019;13(12):3024–36.

8. Wang Y, Ma L, Mao Y, Jiang X, Xia Y, Yu K, et al. Comammox in drinking water systems. Water Res. 2017; 116:332–41.

9. Pjevac P, Schauberger C, Poghosyan L, Herbold CW, van Kessel MAHJ, Daebeler A, et al. AmoA-targeted polymerase chain reaction primers for the specific detection and quantification of comammox Nitrospira in the environment. Front Microbiol. 2017; 8:1508.

10. Annavajhala MK, Kapoor V, Santo-Domingo J, Chandran K. Comammox functionality identified in diverse engineered biological wastewater treatment systems. Environ Sci Technol Lett. 2018;5(2):110–6.

11. Camejo PY, Santo Domingo J, McMahon KD, Noguera DR. Genome-enabled insights into the ecophysiology of the comammox bacterium "Candidatus Nitrospira nitrosa." Msystems. 2017;2(5): e00059–17.

12. Chao Y, Mao Y, Yu K, Zhang T. Novel nitrifiers and comammox in a full-scale hybrid biofilm and activated sludge reactor revealed by metagenomic approach. Appl Microbiol Biotechnol. 2016;100(18):8225–37.

13. Fan XY, Gao JF, Pan KL, Li DC, Dai HH. Temporal dynamics of bacterial communities and predicted nitrogen metabolism genes in a full-scale wastewater treatment plant. Rsc Advances. 2017;7(89):56317–27.

14. Gonzalez-Martinez A, Rodriguez-Sanchez A, van Loosdrecht MC, Gonzalez-Lopez J, Vahala R. Detection of comammox bacteria in full-scale wastewater treatment bioreactors using tag-454-pyrosequencing. Environmental Science and Pollution Research. 2016;23(24):25501–11.

15. Roots P, Wang Y, Rosenthal AF, Griffin JS, Sabba F, Petrovich M, et al. Comammox Nitrospira are the dominant ammonia oxidizers in a mainstream low dissolved oxygen nitrification reactor. Water Res. 2019; 157:396–405.

16. Spasov E, Tsuji JM, Hug LA, Doxey AC, Sauder LA, Parker WJ, et al. High functional diversity among Nitrospira populations that dominate rotating biological contactor

703   microbial communities in a municipal wastewater treatment plant. ISME J.
704   2020;14(7):1857–72.

705   17.   Wang M, Huang G, Zhao Z, Dang C, Liu W, Zheng M. Newly designed primer pair
706   revealed dominant and diverse comammox amoA gene in full-scale wastewater treatment
707   plants. Bioresour Technol. 2018; 270:580–7.

708   18.   Xia F, Wang JG, Zhu T, Zou B, Rhee SK, Quan ZX. Ubiquity and diversity of complete
709   ammonia oxidizers (comammox). Appl Environ Microbiol. 2018;84(24): e01390–18.

710   19.   Yang Y, Daims H, Liu Y, Herbold CW, Pjevac P, Lin JG, et al. Activity and metabolic
711   versatility of complete ammonia oxidizers in full-scale wastewater treatment systems.
712   Mbio. 2020;11(2): e03175–19.

713   20.   Xu S, Chai W, Xiao R, Smets BF, Palomo A, Lu H. Survival strategy of comammox
714   bacteria in a wastewater nutrient removal system with sludge fermentation liquid as
715   additional carbon source. Science of The Total Environment. 2022; 802:149862.

716   21.   Palomo A, Dechesne A, Smets BF. Genomic profiling of Nitrospira species reveals
717   ecological success of comammox Nitrospira. BioRxiv. 2019;612226.

718   22.   Zhao Z, Huang G, He S, Zhou N, Wang M, Dang C, et al. Abundance and community
719   composition of comammox bacteria in different ecosystems by a universal primer set.
720   Science of The Total Environment. 2019; 691:146–55.

721   23.   Xia F, Wang JG, Zhu T, Zou B, Rhee SK, Quan ZX. Ubiquity and diversity of complete
722   ammonia oxidizers (comammox). Appl Environ Microbiol. 2018;84(24): e01390–18.

723   24.   Gottshall EY, Bryson SJ, Cogert KI, Landreau M, Sedlacek CJ, Stahl DA, et al. Sustained
724   nitrogen loss in a symbiotic association of Comammox Nitrospira and Anammox bacteria.
725   Water Research. 2021; 202:117426.

726   25.   Li D, Fang F, Liu G. Efficient nitrification and low-level N2O emission in a weakly acidic
727   bioreactor at low dissolved-oxygen levels are due to comammox. Appl Environ Microbiol.
728   2021;87(11): e00154–21.

729   26.   Gao JF, Luo X, Wu GX, Li T, Peng YZ. Quantitative analyses of the composition and
730   abundance of ammonia-oxidizing archaea and ammonia-oxidizing bacteria in eight full-
731   scale biological wastewater treatment plants. Bioresour Technol. 2013; 138:285–96.

732   27.   Ofi\c{t}eru ID, Lunn M, Curtis TP, Wells GF, Criddle CS, Francis CA, et al. Combined
733   niche and neutral effects in a microbial wastewater treatment community. Proceedings of
734   the National Academy of Sciences. 2010;107(35):15345–50.

735   28.   Siripong S, Rittmann BE. Diversity study of nitrifying bacteria in full-scale municipal
736   wastewater treatment plants. Water Res. 2007;41(5):1110–20.

737   29.   Konopka A, Lindemann S, Fredrickson J. Dynamics in microbial communities: unraveling
738   mechanisms to identify principles. ISME J. 2015;9(7):1488–95.

739   30.   Schloter M, Lebuhn M, Heulin T, Hartmann A. Ecology and evolution of bacterial
740   microdiversity. FEMS Microbiol Rev. 2000;24(5):647–60.

741   31.   Cotto I, Dai Z, Huo L, Anderson CL, Vilardi KJ, Ijaz U, et al. Long solids retention times
742   and attached growth phase favor prevalence of comammox bacteria in nitrogen removal
743   systems. Water Res. 2020; 169:115268.

744   32.   Meziti A, Tsementzi D, Rodriguez-R LM, Hatt JK, Karayanni H, Kormas KA, et al.
745   Quantifying the changes in genetic diversity within sequence-discrete bacterial
746   populations across a spatial and temporal riverine gradient. ISME J. 2019;13(3):767–79.

747 33. Gülay A, Musovic S, Albrechtsen HJ, Al-Soud WA, Sørensen SJ, Smets BF. Ecological
748   patterns, diversity and core taxa of microbial communities in groundwater-fed rapid
749   gravity filters. ISME J. 2016;10(9):2209–22.
750 34. Palomo A, Dechesne A, Cordero OX, Smets BF. Evolutionary ecology of natural
751   comammox Nitrospira populations. Msystems. 2022;7(1): e01139–21.
752 35. Gruber-Dorninger C, Pester M, Kitzinger K, Savio DF, Loy A, Rattei T, et al.
753   Functionally relevant diversity of closely related Nitrospira in activated sludge. ISME J.
754   2015;9(3):643–55.
755 36. Garc\'\ia-Garc\'\ia N, Tamames J, Linz AM, Pedrós-Alió C, Puente-Sánchez F.
756   Microdiversity ensures the maintenance of functional microbial communities under
757   changing environmental conditions. ISME J. 2019;13(12):2969–83.
758 37. Bertrand D, Shaw J, Kalathiyappan M, Ng AHQ, Kumar MS, Li C, et al. Hybrid
759   metagenomic assembly enables high-resolution analysis of resistance determinants and
760   mobile elements in human microbiomes. Nat Biotechnol. 2019;37(8):937–44.
761 38. Mantere T, Kersten S, Hoischen A. Long-read sequencing emerging in medical genetics.
762   Front Genet. 2019; 10:426.
763 39. Singleton CM, Petriglieri F, Kristensen JM, Kirkegaard RH, Michaelsen TY, Andersen
764   MH, et al. Connecting structure to function with the recovery of over 1000 high-quality
765   metagenome-assembled genomes from activated sludge using long-read sequencing. Nat
766   Commun. 2021;12(1):1–13.
767 40. Frank JA, Reich CI, Sharma S, Weisbaum JS, Wilson BA, Olsen GJ. Critical evaluation of
768   two primers commonly used for amplification of bacterial 16S rRNA genes. Applied and
769   Environmental Microbiology. 2008 Apr;74(8):2461–70.
770 41. Callahan BJ, McMurdie PJ, Rosen MJ, Han AW, Johnson AJA, Holmes SP. DADA2:
771   High-resolution sample inference from Illumina amplicon data. Nat Methods.
772   2016;13(7):581–3.
773 42. Callahan BJ, Wong J, Heiner C, Oh S, Theriot CM, Gulati AS, et al. High-throughput
774   amplicon sequencing of the full-length 16S rRNA gene with single-nucleotide resolution.
775   Nucleic Acids Res. 2019;47(18): e103–e103.
776 43. Wright ES. RNAconTest: comparing tools for noncoding RNA multiple sequence
777   alignment based on structural consistency. RNA. 2020;26(5):531–40.
778 44. Stackebrandt E. Taxonomic parameters revisited: tarnished gold standards. Microbiol
779   Today. 2006; 33:152–5.
780 45. Yarza P, Yilmaz P, Pruesse E, Glöckner FO, Ludwig W, Schleifer KH, et al. Uniting the
781   classification of cultured and uncultured bacteria and archaea using 16S rRNA gene
782   sequences. Nature Reviews Microbiology. 2014;12(9):635–45.
783 46. Edgar RC. MUSCLE: multiple sequence alignment with high accuracy and high
784   throughput. Nucleic Acids Res. 2004;32(5):1792–7.
785 47. Nguyen LT, Schmidt HA, von Haeseler A, Minh BQ. IQ-TREE: a fast and effective
786   stochastic algorithm for estimating maximum-likelihood phylogenies. Mol Biol Evol.
787   2015;32(1):268–74.
788 48. Price MN, Dehal PS, Arkin AP. FastTree 2–approximately maximum-likelihood trees for
789   large alignments. PLoS One. 2010;5(3): e9490.
790 49. Lozupone C, Knight R. UniFrac: a new phylogenetic method for comparing microbial
791   communities. Appl Environ Microbiol. 2005;71(12):8228–35.
792 50. Wickham H. ggplot2: elegant graphics for data analysis. springer; 2016.

793   51.   Chen S, Zhou Y, Chen Y, Gu J. fastp: an ultra-fast all-in-one FASTQ preprocessor.
794   Bioinformatics. 2018;34(17): i884–i890.
795   52.   Danecek P, Bonfield JK, Liddle J, Marshall J, Ohan V, Pollard MO, et al. Twelve years of
796   SAMtools and BCFtools. Gigascience. 2021;10(2): giab008.
797   53.   Quinlan AR, Hall IM. BEDTools: a flexible suite of utilities for comparing genomic
798   features. Bioinformatics. 2010;26(6):841–2.
799   54.   Nurk S, Meleshko D, Korobeynikov A, Pevzner PA. metaSPAdes: a new versatile
800   metagenomic assembler. Genome Res. 2017;27(5):824–34.
801   55.   Eren AM, Esen ÖC, Quince C, Vineis JH, Morrison HG, Sogin ML, et al. Anvi'o: an
802   advanced analysis and visualization platform for 'omics data. PeerJ. 2015;3: e1319.
803   56.   Li H, Durbin R. Fast and accurate short read alignment with Burrows–Wheeler transform.
804   bioinformatics. 2009;25(14):1754–60.
805   57.   Gurevich A, Saveliev V, Vyahhi N, Tesler G. QUAST: quality assessment tool for
806   genome assemblies. Bioinformatics. 2013;29(8):1072–5.
807   58.   Kang DD, Li F, Kirton E, Thomas A, Egan R, An H, et al. MetaBAT 2: an adaptive
808   binning algorithm for robust and efficient genome reconstruction from metagenome
809   assemblies. PeerJ. 2019;7: e7359.
810   59.   Alneberg J, Bjarnason BS, Bruijn I de, Schirmer M, Quick J, Ijaz UZ, et al. Binning
811   metagenomic contigs by coverage and composition. Nature Methods. 2014
812   Apr;11(11):1144–6.
813   60.   Wu YW, Simmons BA, Singer SW. MaxBin 2.0: an automated binning algorithm to
814   recover genomes from multiple metagenomic datasets. Bioinformatics. 2016;32(4):605–7.
815   61.   Warren RL, Coombe L, Mohamadi H, Zhang J, Jaquish B, Isabel N, et al. ntEdit: scalable
816   genome sequence polishing. Bioinformatics. 2019;35(21):4430–2.
817   62.   Parks DH, Imelfort M, Skennerton CT, Hugenholtz P, Tyson GW. CheckM: assessing the
818   quality of microbial genomes recovered from isolates, single cells, and metagenomes.
819   Genome Res. 2015;25(7):1043–55.
820   63.   Parks DH, Chuvochina M, Waite DW, Rinke C, Skarshewski A, Chaumeil PA, et al. A
821   standardized bacterial taxonomy based on genome phylogeny substantially revises the tree
822   of life. Nat Biotechnol. 2018;36(10):996–1004.
823   64.   Hyatt D, Chen GL, LoCascio PF, Land ML, Larimer FW, Hauser LJ. Prodigal:
824   prokaryotic gene recognition and translation initiation site identification. BMC
825   Bioinformatics. 2010;11(1):1–11.
826   65.   Kanehisa M, Sato Y, Kawashima M, Furumichi M, Tanabe M. KEGG as a reference
827   resource for gene and protein annotation. Nucleic Acids Res. 2016;44(D1): D457–D462.
828   66.   Aramaki T, Blanc-Mathieu R, Endo H, Ohkubo K, Kanehisa M, Goto S, et al.
829   KofamKOALA: KEGG ortholog assignment based on profile HMM and adaptive score
830   threshold. Bioinformatics. 2020;36(7):2251–2.
831   67.   Sieber CMK, Probst AJ, Sharrar A, Thomas BC, Hess M, Tringe SG, et al. Recovery of
832   genomes from metagenomes via a dereplication, aggregation and scoring strategy. Nat
833   Microbiol. 2018;3(7):836–43.
834   68.   Woodcroft B, Newell R. CoverM (Version 0.6.1). 2021.
835   69.   Jain C, Rodriguez-R LM, Phillippy AM, Konstantinidis KT, Aluru S. High throughput
836   ANI analysis of 90K prokaryotic genomes reveals clear species boundaries. Nat Commun.
837   2018;9(1):1–8.

838 70. Parks D, Skennerton C, Brown C. CompareM. https://github.com/dparks1134/CompareM;
839   2020.
840 71. Olm MR, Crits-Christoph A, Bouma-Gregson K, Firek BA, Morowitz MJ, Banfield JF.
841   inStrain profiles population microdiversity from metagenomic data and sensitively detects
842   shared microbial strains. Nature Biotechnology. 2021;39(6):727–36.
843 72. Uritskiy G v, DiRuggiero J, Taylor J. MetaWRAP—a flexible pipeline for genome-
844   resolved metagenomic data analysis. Microbiome. 2018;6(1):1–13.
845 73. Xu Y, Lu J, Wang Y, Liu G, Wan X, Hua Y, et al. Diversity and abundance of
846   comammox bacteria in the sediments of an urban lake. Journal of Applied Microbiology.
847   2020;128(6):1647–57.
848 74. Shi Y, Jiang Y, Wang S, Wang X, Zhu G. Biogeographic distribution of comammox
849   bacteria in diverse terrestrial habitats. Science of the Total Environment. 2020;
850   717:137257.
851 75. Beach NK, Noguera DR. Design and assessment of species-level qPCR primers targeting
852   comammox. Front Microbiol. 2019; 10:36.
853 76. Zheng M, Wang M, Zhao Z, Zhou N, He S, Liu S, et al. Transcriptional activity and
854   diversity of comammox bacteria as a previously overlooked ammonia oxidizing
855   prokaryote in full-scale wastewater treatment plants. Science of the Total Environment.
856   2019; 656:717–22.
857 77. Zhou X, Li B, Wei J, Ye Y, Xu J, Chen L, et al. Temperature Influenced the Comammox
858   Community Composition in Drinking Water and Wastewater Treatment Plants. Microbial
859   Ecology. 2021;82(4):870–84.
860 78. Koops HP, Purkhold U, Pommerening-Röser A, Timmermann G, Wagner M. The
861   lithoautotrophic ammonia-oxidizing bacteria. Bergey's manual of systematic bacteriology,
862   volume two: the proteobacteria, part A introductory essays, Springer. 2005;141–7.
863 79. Pommerening-Röser A, Rath G, Koops HP. Phylogenetic diversity within the genus
864   Nitrosomonas. Systematic and Applied Microbiology. 1996;19(3):344–51.
865 80. Koops HP, Stehr G, others. Classification of eight new species of ammonia-oxidizing
866   bacteria: Nitrosomonas communis sp. nov., Nitrosomonas ureae sp. nov., Nitrosomonas
867   aestuarii sp. nov., Nitrosomonas marina sp. nov., Nitrosomonas nitrosa sp. nov.,
868   Nitrosomonas eutropha sp. nov., Nitrosomonas oligotropha sp. nov. and Nitrosomonas
869   halophila sp. nov. Microbiology (N Y). 1991;137(7):1689–99.
870 81. Navada S, Knutsen MF, Bakke I, Vadstein O. Nitrifying biofilms deprived of organic
871   carbon show higher functional resilience to increases in carbon supply. Sci Rep.
872   2020;10(1):1–11.
873 82. Konstantinidis KT, Viver T, Conrad RE, Venter SN, Rossello-Mora R. Solar salterns as
874   model systems to study the units of bacterial diversity that matter for ecosystem
875   functioning. Curr Opin Biotechnol. 2022; 73:151–7.
876 83. Luo C, Rodriguez-r LM, Konstantinidis KT. MyTaxa: an advanced taxonomic classifier
877   for genomic and metagenomic sequences. Nucleic Acids Res. 2014;42(8): e73–e73.
878 84. Sczyrba A, Hofmann P, Belmann P, Koslicki D, Janssen S, Dröge J, et al. Critical
879   assessment of metagenome interpretation—a benchmark of metagenomics software. Nat
880   Methods. 2017;14(11):1063–71.
881 85. de Maio N, Shaw LP, Hubbard A, George S, Sanderson ND, Swann J, et al. Comparison
882   of long-read sequencing technologies in the hybrid assembly of complex bacterial
883   genomes. Microb Genom. 2019;5(9).

884   86.   Xie H, Yang C, Sun Y, Igarashi Y, Jin T, Luo F. PacBio long reads improve metagenomic
885   assemblies, gene catalogs, and genome binning. Front Genet. 2020;1077.
886   87.   Brown CL, Keenum IM, Dai D, Zhang L, Vikesland PJ, Pruden A. Critical evaluation of
887   short, long, and hybrid assembly for contextual analysis of antibiotic resistance genes in
888   complex environmental metagenomes. Sci Rep. 2021;11(1):1–12.
889   88.   Dilhari A, Sampath A, Gunasekara C, Fernando N, Weerasekara D, Sissons C, et al.
890   Evaluation of the impact of six different DNA extraction methods for the representation of
891   the microbial community associated with human chronic wound infections using a gel-
892   based DNA profiling method. Amb Express. 2017;7(1):1–11.
893   89.   Dionisi HM, Layton AC, Harms G, Gregory IR, Robinson KG, Sayler GS. Quantification
894   of Nitrosomonas oligotropha-like ammonia-oxidizing bacteria and Nitrospira spp. from
895   full-scale wastewater treatment plants by competitive PCR. Appl Environ Microbiol.
896   2002;68(1):245–53.
897   90.   Zhang T, Ye L, Tong AHY, Shao MF, Lok S. Ammonia-oxidizing archaea and ammonia-
898   oxidizing bacteria in six full-scale wastewater treatment bioreactors. Appl Microbiol
899   Biotechnol. 2011;91(4):1215–25.
900   91.   Gao J, Luo X, Wu G, Li T, Peng Y. Abundance and diversity based on amoA genes of
901   ammonia-oxidizing archaea and bacteria in ten wastewater treatment systems. Appl
902   Microbiol Biotechnol. 2014;98(7):3339–54.
903   92.   Wells GF, Park HD, Yeung CH, Eggleston B, Francis CA, Criddle CS. Ammonia-
904   oxidizing communities in a highly aerated full-scale activated sludge bioreactor:
905   betaproteobacterial dynamics and low relative abundance of Crenarchaea. Environ
906   Microbiol. 2009;11(9):2310–28.
907   93.   Vilardi KJ, Cotto I, Rivera MS, Dai Z, Anderson CL, Pinto A. Comammox Nitrospira
908   bacteria outnumber canonical nitrifiers irrespective of electron donor mode and
909   availability. FEMS Microbiol Ecol. 2022;98(4): fiac032.
910   94.   Wang X, Lu L, Zhou X, Tang X, Kuang L, Chen J, et al. Niche differentiation of
911   comammox Nitrospira in the mudflat and reclaimed agricultural soils along the north
912   branch of Yangtze River estuary. Front Microbiol. 2021;3482.
913   95.   Sakoula D, Koch H, Frank J, Jetten MSM, van Kessel MAHJ, Lücker S. Enrichment and
914   physiological characterization of a novel comammox Nitrospira indicates ammonium
915   inhibition of complete nitrification. ISME Journal. 2021 Apr 1;15(4):1010–24.
916   96.   Kits KD, Sedlacek CJ, Lebedeva E v, Han P, Bulaev A, Pjevac P, et al. Kinetic analysis of
917   a complete nitrifier reveals an oligotrophic lifestyle. Nature. 2017;549(7671):269–72.
918