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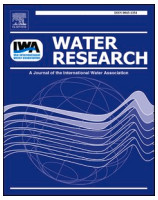

Low diversity and microdiversity of comammox bacteria in wastewater systems suggest specific adaptations within the *Ca.* Nitrospira nitrosa cluster

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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 the species-level diversity of comammox bacteria with that of strict nitrifiers in full-scale wastewater treatment systems and assess whether this comparison is consistent or diverged at the strain-level. Full-length 16S rRNA gene sequencing indicated that *Nitrosomonas*-like bacteria exhibited higher species-level diversity in comparison with other nitrifying bacteria, while the strain-level diversity (also called microdiversity) of most *Nitrospira*-like bacteria were higher than *Nitrosomonas*-like bacteria with few exceptions (one *Nitrospira* lineage II population). Comammox bacterial metagenome assembled genomes (MAGs) were associated with *Ca.* Nitrospira nitrosa. The average amino acid identity between principal comammox bacterial MAGs (93% ± 3) across systems was significantly higher than that of the *Nitrosomonas*-like ammonia oxidizers (73% ± 8), the *Nitrospira*_A-like nitrite oxidizer (85% ± 4), and the *Nitrospira*_D-like nitrite oxidizer (83% ± 1). This demonstrated the low species-level diversity of comammox bacteria compared with strict nitrifiers and further suggests that the same comammox population was detected in all systems. Comammox bacteria (*Nitrospira* lineage II), *Nitrosomonas* and, *Nitrospira*_D (*Nitrospira* lineage II) MAGs were significantly less microdiverse than the *Nitrospira*_A (lineage I) MAGs. 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.

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1. 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) (Klotz and Stein, 2008). Since their discovery (Daims et al., 2015; Pinto et al., 2015; van Kessel et al., 2015), several studies have detected comammox bacteria in a wide range of environmental and engineered systems (Palomo et al., 2016; Pjevac et al., 2017), including laboratory and full-scale wastewater treatment bioreactors (Annavajhala et al., 2018; Camejo et al., 2017; Roots et al., 2019). In contrast to oligotrophic engineered systems with low nitrogen concentrations, such as drinking water (Palomo et al., 2019; Pjevac et al., 2017) and even tertiary treatment systems at full-scale wastewater treatment plants (WWTPs) (Spasov et al., 2020) where several comammox species co-exist, most studies have identified *Candidatus* Nitrospira nitrosa-like comammox as the principal or sole comammox bacteria in lab- and full-scale wastewater secondary treatment systems (Camejo et al., 2017; Xia et al., 2018b; Zhao et al., 2019). This apparent lack of diversity (at the species-level) of comammox bacteria in secondary treatment processes is not only surprising but has the potential to impact treatment strategies centered around comammox bacteria (Gottshall et al., 2021; D. Li et al., 2021) as functional outcomes reliant on low diversity communities may be less resilient to environmental fluctuations and perturbations.

Studies often report the coexistence of multiple *Nitrosomonas*-like AOB and *Nitrospira*-like NOB populations (J.-F. Gao et al., 2013; Ofiţeru et al., 2010; Siripong and Rittmann, 2007) likely occupying different ecological niches (i.e., adapted to different conditions) within a complex community. The functional redundancy of multiple co-exiting populations can confer stability to microbial ecosystems ensuring the long-term persistence (Konopka et al., 2015; Schloter et al., 2000). Despite the apparent low species-level diversity of comammox bacteria compared with strict nitrifiers, they have exhibited remarkable stability in secondary treatment systems (Camejo et al., 2017; Cotto et al., 2020; Roots et al., 2019). One plausible reason for their temporal persistence could be that comammox bacteria may harbor intra-population (i.e., strain) level diversity (also known as microdiversity), compared with population (i.e., species) level diversity. In this manuscript, the words "diversity" and "microdiversity" will refer to the species-level diversity and strain-level diversity, respectively. Previous studies have demonstrated that microdiversity within populations can not only allow them to adapt to rapidly changing conditions, but also contribute to their persistence (Meziti et al., 2019; Orellana et al., 2019). Further, small variations in metabolic capacity may underpin metabolic diversity and thus enable the co-existence of multiple closely related strains within a single population. *Nitrospira*-like bacteria have been shown to harbor high levels of microdiversity in drinking water (Gülay et al., 2016; Palomo et al., 2022) and wastewater (Gruber-Dorninger et al., 2015) systems. Moreover, studies have reported that closely related co-existing *Nitrospira* lineage I strains can exhibit differences in substrate affinities, and utilization rates (Gruber-Dorninger et al., 2015). Since, both levels of diversity (i.e., species and strain-level) have been associated with microbial persistence and ecosystem stability, it is plausible that the persistence of comammox bacteria may be associated with higher levels of microdiversity. This would be consistent with observations for *Nitrospira*-like bacteria (Gruber-Dorninger et al., 2015).

To test this hypothesis, we used a combination of full-length 16S rRNA gene sequencing and hybrid assembly and binning approaches to systematically explore the species and strain-level diversity of nitrifiers in three full-scale nitrogen removal systems with different process configurations. These systems were selected to investigate the species- and strain-level diversity of nitrifiers due to the high abundance and temporal persistence of similar comammox bacterial species despite the differences in process configuration (Cotto et al., 2020). While 16S rRNA gene sequencing cannot provide information on genome content or metabolic potential, the single nucleotide resolution across the full length 16S rRNA gene can help determine differences in (micro)diversity among nitrifying populations (García-García et al., 2019; Gruber-Dorninger et al., 2015). Nevertheless, 16S rRNA gene sequences cannot be used to reliably distinguish comammox bacteria from strict *Nitrospira*-NOB. One approach to differentiate between the different guilds within *Nitrospira* bacteria and to analyze their diversity at high resolution is to leverage a genome resolved approach to obtain high quality metagenome assembled genomes (MAGs). Various methods exist to quantify diversity (e.g., average nucleotide identity [ANI] and average amino acid identity [AAI]) and microdiversity (e.g., average nucleotide identity from reads and nucleotide diversity from single nucleotide polymorphisms [SNPs]) within populations. Nevertheless, their applicability depends on the quality of MAGs which can be impacted when relying on short read sequencing (e.g., Illumina) in the presence of closely related strains (Bertrand et al., 2019). While long-read sequencing on the Nanopore platform can help mitigate this constraint (Mantere et al., 2019; Singleton et al., 2021), this requires high levels of coverage to obtain polished consensus sequences with lower error rates compared with the raw data (Bertrand et al., 2019). Here, we utilize a hybrid metagenomic assembly approach, including long- and short-read data in an effort to assemble high quality MAGs for subsequent microdiversity analyses (Bertrand et al., 2019).

Thus, the overall objective of this study was to test the hypothesis that the widespread distribution and persistence of nearly identical comammox bacterial populations in multiple secondary wastewater treatment systems was due to the co-existence of multiple strains (i.e., high microdiversity). To accomplish this, we used a combination of full-length 16S rRNA gene sequencing and hybrid metagenome assembly and binning approach to systematically explore the species- and strain-level diversity of nitrifiers in three full-scale nitrogen removal systems with different process configurations. In doing so, we also aimed to assess factors that may influence the inter-population (i.e., strain) and intra-population (i.e., species) level diversity of co-existing nitrifiers.

2. Materials and methods

2.1. Samples selection and processing

Samples were taken from the nitrifying bioreactors of three municipal wastewater treatment plants with different process configurations (i.e., sequencing batch reactor (SBR), Integrated Fixed Film Activated Sludge (IFAS), and Bardenpho 5-stages activated sludge system) and high abundances of comammox bacteria reported in our previous study (Cotto et al., 2020). The process parameters of these systems are discussed elsewhere (Cotto et al., 2020). Biomass samples were collected in 2017–2018 and stored at −80 °C until subsequent analysis. Post DNA extraction, a total of thirty-three, twenty, and six samples were selected for full-length 16S rRNA gene sequencing on the PacBio Sequel IIe platform, short read metagenomic sequencing on the Illumina NovaSeq platform and long read metagenomic sequencing on the Nanopore MinION platform, respectively (Table 1). Sample collection and processing (including DNA extraction) and process data collection were described previously (Cotto et al., 2020) and are outlined in the supplementary text.

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

Sample DNA extracts (Table 1) were sent for full-length 16S rRNA gene sequencing at the Roy J. Carver Biotechnology Center (Sequencing Core, University of Illinois Urbana-Champaign). The full-length 16S rRNA gene amplicons were generated with universal barcoded primers (27F and 1492R)(Frank et al., 2008), PCR products were subject to library preparation and sequenced on the PacBio Sequel IIe using the circular consensus sequencing (CCS) mode. Raw reads were demultiplexed and CCS analyses were performed to obtain consensus reads. Further details on PCR, library preparation, and sequencing are

Table 1
Overview of system process type and sub-type, operational scale and samples included in 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 Bardenpho	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[+]

[1] Nitrification-denitrification,.
[2] Sequencing batch reactor,.
[3] Integrated Fixed-film Activated Sludge.
[*] Samples selected for Illumina NovaSeq sequencing in August 2019.
[+] Samples selected for PacBio full-length 16S rRNA gene sequencing in April 2021.
[†] Samples selected for nanopore sequencing on the MinION platform in July 2020.

provided in the supplementary text. Downstream data processing was performed on a system-by-system basis using the DADA2 v1.19.2 (Callahan et al., 2016) in R v4.0.4. Specifically, we used the DADA2 sample inference method for full-length 16S rRNA gene with single-nucleotide resolution. The DADA2 workflow included primer removal, quality filtering, dereplication, learning the dataset-specific error model, amplicon sequence variants (ASVs) inference, chimera removal, and taxonomic assignment with the SILVA SSU 138 Ref NR database using default settings. Further details on the downstream data processing are provided in the supplementary text. Table S1a summarizes the reads per samples at different stages of data processing in DADA2. ASVs with $\geq$100 reads in any of the three systems ($\sim$>0.01% relative abundance) were clustered into operational taxonomy units (OTUs) using the function 'IdClusters' in DECIPHER v2.16.1. (Wright, 2020) at 98.7% sequence identity, a previously recommended threshold for clustering full-length 16S rRNA gene sequences at the species level (Stackebrandt, 2006; Yarza et al., 2014). The most abundant ASV within each OTU was used as representative sequence and its taxonomic affiliation, determined using the SILVA SSU 138.1 database, was used as the consensus taxonomy for the OTU. Additional details on ASV to OTU data processing are provided in the supplementary text. Representative ASV sequence from all OTUs were aligned with MUSCLE v3.8.1551 (Edgar, 2004), and the maximum likelihood phylogenetic tree was constructed and visualized using IQ-TREE v2.0.3 (Nguyen et al., 2015) and iTOL v2.1.7 (Price et al., 2010), respectively. Principal Coordinates Analysis (PCoA) was performed with the weighted and unweighted UniFrac distance metric (Lozupone and Knight, 2005) using the 'ordinate' function of phyloseq v1.32.0 and the 'plot_ordination' function of ggplot2 v3.3.5 (Wickham, 2016).

ASVs classified at the genus level as *Nitrospira* and *Nitrosomonas* (the only two nitrifying genera detected in this study) were extracted from the rarefied ASV table (rarefied to the sample with lowest read count) and reference 16S rRNA gene sequences for the two genera were obtained from the SILVA SSU 138.1 database (Tables S2 and S3). Comammox bacterial 16S rRNA genes were extracted from references genomes downloaded from NCBI (Table S4). MUSCLE v3.8.1551 was used to align ASVs sequences from each genus with their respective references, and a maximum likelihood phylogenetic tree of each species was generated using IQ-TREE v2.0.3 and visualized in iTOL v2.1.7. To assess the species (OTUs)-level diversity the Shannon diversity was calculated per sample from the relative abundance of the OTUs classified as the same genus/lineage as the exponential of the Shannon index. To estimate the strain (ASV)-level diversity, the effective microdiversity of each OTU was calculated on a per sample basis from the relative abundance of its ASVs as the exponential of the Shannon index, which is analogous to the effective number of strains (i.e., ASVs) within a species (i.e., OTU) (García-García et al., 2019).

2.3. Illumina and Oxford Nanopore sequencing, co-assembly, and hybrid assembly

Seven samples from GRE and NEU and six samples from JAMMSM were sent for sequencing on the Illumina NovaSeq 6000 platform, and two samples from each system on the Oxford Nanopore Technologies GridIONx5 (Table 1) to the Roy J. Carver Biotechnology Center at the University of Illinois Urbana-Champaign Sequencing Core. Library preparation and sequencing details are provided in the supplementary text. These runs resulted in 1.78 billion paired-end short reads (2 $\times$ 150 nt reads) and 45.3 Gbps of long read data (Table S1b and c). Raw short reads were filtered using fastp v0.20.0 (Chen et al., 2018) and the Univec database was used to remove contamination from the filtered reads as previously described (Cotto et al., 2020). The resulting bam files were sorted using SAMtools v1.9 (Danecek et al., 2021) and converted into fastq files with bedtools v2.29.0 (Quinlan and Hall, 2010). All reads from the same system were co-assembled into contigs using metaSpades v3.13.0 (Nurk et al., 2017). Co-assemblies were performed with kmer sizes of 21, 33, 55 and 77. Contigs smaller than 500 bp were removed from the co-assemblies using the Anvi'o v6.1 command 'anvi-script-reformat-fasta' (Eren et al., 2015). The reformatted assembly fasta files were indexed with bwa index v0.7.17 (H. Li and Durbin, 2009) and the paired end reads from each metagenome were mapped to the respective co-assembly using bwa mem v0.7.17. The resulting sam files were converted to bam files using 'samtools view -F 4 -bhS' to retain only mapped reads. Hybrid metagenomic assemblies were performed using OPERA-MS v0.9.0., which combines the advantages of short and long-read technologies to improve genome assemblies (Bertrand et al., 2019). The OPERA-MS inputs were short-read metagenomic assemblies to provide a good representation of the sequences in the metagenome, and long and short reads to identify connections between the contigs and obtain contiguous assemblies with low base-pair error. OPERA-MS was executed with the flags –no-ref-clustering and –long-read-mapper minimap2. Both sets of co-assemblies were evaluated using QUAST v5.0.2 (Gurevich et al., 2013) (Table S5).

2.4. Recovery, annotation, refining and dereplication of metagenome assembled genomes (MAGs)

Binning was performed separately, with co-assemblies from Illumina short reads only and hybrid assemblies from each system using MetaBAT2 v2.12.1 (Kang et al., 2019), CONCOCT v1.1.0 (Alneberg et al., 2014), and MaxBin2 v2.2.7 (Wu et al., 2016) using contigs greater than 2000 bp. Pilon v1.23 (Warren et al., 2019) was used for polishing to improve the draft bins obtained from the hybrid assemblies. The quality and taxonomy of the resulting bins were determined with CheckM v1.1.2 (D. H. Parks et al., 2015) and the Genome Taxonomy Database Toolkit (GTDB-Tk 1.1.1, database release r86 v3) (D. H. Parks et al., 2018), respectively. Bins were subject to gene calling using Prodigal v2.6.3 (Hyatt et al., 2010) and gene annotation against the KEGG database (Kanehisa et al., 2016) using kofamscan v1.2.0 (Aramaki et al., 2020). Only bins that were taxonomically assigned to known nitrifying genera or those containing genes associated with nitrification (i.e., *amoA* [KO number K10944], *amoB* [K10945], *amoC* [K10946], *hao* [K10535], *nxrA* [K00370], *nxrB* [K00371]) were retained for manual refinement

with Anvi'o v6.1. DAStool v1.1.2 (Sieber et al., 2018) was used to combine and curate the refined bins from the three binning methods and generate a non-redundant set of bins from each co-assembly (i.e., one set of bins per system for the short-read only and for the hybrid assemblies). In total, 43 nitrifiers bins from the short-read co-assemblies and 30 from the hybrid assemblies were obtained. These bins were de-replicated using drep v2.5.4 at 95% ANI with completeness and contamination thresholds set to 50% and 10%, respectively. This resulted in 44 nitrifier MAGs with completeness and redundancy estimates higher than 50% and lower than 10%, respectively, of which 21 contained genes associated with nitrification (i.e., *amoA, amoB, amoC, hao, nxrA*, and/or *nxrB*).

2.5. Taxonomy aware re-assembly using nitrifying bacterial MAGs

We performed re-assemblies from reads mapped to the 44 nitrifying bacterial MAGs to improve MAG quality. First, contigs associated with *Nitrospira* (including comammox) and *Nitrosomonas* MAGs from each system (i.e., GRE, JAMMSM, NEU) were collated into a single fasta file per system. The resulting fasta files were indexed using bwa index v0.7.17 and short reads from the respective system were mapped using bwa mem v0.7.17. The resultant bam file was split into *Nitrospira* and *Nitrosomonas* specific bam files per system and respective fastq files were generated per using samtools fastq. Long reads were also recruited by mapping the reads from Nanopore sequencing to the fasta files with 'bwa mem -x ont2d'. Short reads mapping to the contigs of the MAGs classified as *Nitrospira* and *Nitrosomonas* were re-assembled using metaSpades v3.13.0 with kmers 21, 33, 55 and 77 on a system-by-system basis. The new genus-specific assemblies and fastq files of mapped long reads were used as input for OPERA-MS to perform hybrid metagenomic re-assemblies (n = 9). Quality assessment, binning, taxonomy annotation, manual refining and gene calling were performed as described before. This resulted in 36 nitrifier MAGs with completeness and redundancy estimates higher than 50% and lower than 10%, respectively, of which 24 contained genes associated with nitrification (*amoA, amoB, amoC, hao, nxrA*, and/or *nxrB*). The abundance of each MAG per sample (reads per kilobase million, RPKM) and proportion of genome covered was calculated with coverM (Woodcroft and Newell, 2021). Four low abundance MAGs (3 AOB and 1 *Nitrospira*-NOB) with less than 50% genome coverage in any of the samples were removed from subsequent analyses. The entire workflow for assembly and re-assembly of MAGs is outlined in Figure S1. The nitrifier MAGs were subsequently phylogenetically placed in the context of reference *Nitrospira* (Table S4) and *Nitrosomonas* (Table S6) genomes (supplementary text).

2.6. Diversity and microdiversity analysis of nitrifiers

FastANI v1.3 (Jain et al., 2018) was used to calculate the pairwise average nucleotide identity (ANI) between MAGs within each functional group (i.e., AOB, *Nitrospira*_A-NOB, *Nitrospira*_D-NOB and *Nitrospira*-comammox). ANI represents the mean nucleotide identity of the orthologous genes shared between two genomes offering a robust resolution between similar or identical species (i.e., ~80–100% ANI) (Jain et al., 2018). Since ANI values lower than 80% are not reported, we also calculated the average amino acid identity (AAI) to estimate similarity between two genomes at the amino acid level using compareM (D. Parks et al., 2020). To determine strain-level diversity (i.e., microdiversity), we calculated the average nucleotide identity from reads (ANIr) (Meziti et al., 2019) with 90% read identity threshold, as recommended for intra-population comparisons. inStrain v1.3.9 (Olm et al., 2021) was used to determine the nucleotide diversity for each MAG in each sample and their population average nucleotide identity (popANI) between the samples where they were detected. Nucleotide diversity is a measurement of genetic microdiversity at every position along the genome using mapped reads, while popANI is a unique ANI calculation performed by inStrain that considers both major and minor alleles. This is different from the traditional ANI (called consensus ANI [conANI] in inStrain), which only considers major alleles to call (or not) a substitution. Details on parameters and procedures associated with the implementation of ANIr and inStrain are presented in the supplementary text.

2.7. Statistical analyses

Statistical tests were performed using R v4.0.4. All correlations were performed using a linear regression model. The Spearman's rank correlation coefficients between MAG abundances were performed with the 'rcorr' function of the R package Hmisc v4.5.0. Pair-wise significances were calculated with the Kruskal-Wallis test, a nonparametric approach to the one-ANOVA. Principal coordinates analysis (PCoA) with the weighted and unweighted UniFrac distance metrics was used to compare the community composition among systems.

3. Results

3.1. Community composition in nitrogen removal systems

Three nitrification-denitrification systems with high absolute abundance of comammox bacteria were selected for this time-series study on the basis of our previous findings (Cotto et al., 2020) since high sequencing coverage is critical for evaluation of strain-level diversity. Specifically, over the sampling period (June 2017 to June 2018) comammox bacterial *amoB* genes constituted approximately 0.77 (± 0.32), 4.7 (± 4.21), and 0.45 (± 0.24)% of total bacterial 16S rRNA gene abundances at GRE, JAMMSM, and NEU, respectively.

The PacBio full-length 16S rRNA gene sequencing resulted in 2317,019 total reads (Table S1a) with 7501, 5783, and 8260 ASVs at GRE, JAMMSM and NEU, respectively. Each ASV set was rarefied to the sample with the smallest library size per system resulting in 7040, 4349, and 5749 ASVs from GRE, JAMMSM, and NEU, respectively, and a total of 16,651 unique ASVs across all three systems. ASVs with a total of 100 reads in each system were clustered into OTUs at 98.7% identity, resulting in 846 OTUs (Figure 1A; Table S7). The most abundant OTUs at GRE and NEU were from the classes *Gammaproteobacteria* and *Alphaproteobacteria* and the phylum *Bacteroidota* while the class *Nitrospiria* had the highest relative abundance in JAMMSM. PCoA using weighted (WUF) and unweighted UniFrac (UUF) distance metrics (Fig. 1B) demonstrated that samples clustered by system and the community structure between GRE and NEU (WUF: 0.18 ± 0.03; UUF: 0.46 ± 0.03) were significantly more similar than GRE and JAMMSM (WUF: 0.28 ± 0.02; UUF: 0.56 ± 0.05) and NEU and JAMMSM (WUF: 0.29 ± 0.03; UUF: 0.52 ± 0.04) ($p<0.05$; Figure S2A and B). The higher similarity between GRE and NEU is likely due to the fact that these were suspended phase communities as compared with attached phase (i.e., biofilm samples) communities collected from the IFAS system at JAMMSM.

Nitrosomonas- (31, 25, and 14 ASVs at GRE, JAMMSM, and NEU, respectively) and *Nitrospira*-like bacteria (37, 36, and 29 ASVs at GRE, JAMMSM, and NEU, respectively) were the only nitrifiers present in the systems. *Nitrospira* lineage I ASVs were the most abundant group across all systems with average relative abundances of 2.91, 25.13, and 2.88%, followed by *Nitrospira* lineage II with average relative abundances 1.41, 7.07, and 0.64% in GRE, JAMMSM, and NEU, respectively (Fig. 1C). *N. oligotropha* (Cluster 6a) and *N. communis* (Cluster 8) -like ASVs were present in all systems with relative abundances of 0.5 and 0.33, 1.68 and 0.93, and 0.58 and 0.19% in GRE, JAMMSM, and NEU, respectively. *N. europaea/mobilis* (Cluster 7)-like ASVs were also detected in GRE and JAMMSM at very low abundances compared with the other two *Nitrosomonas* linages (average ~0.032 and 0.002%, respectively).

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

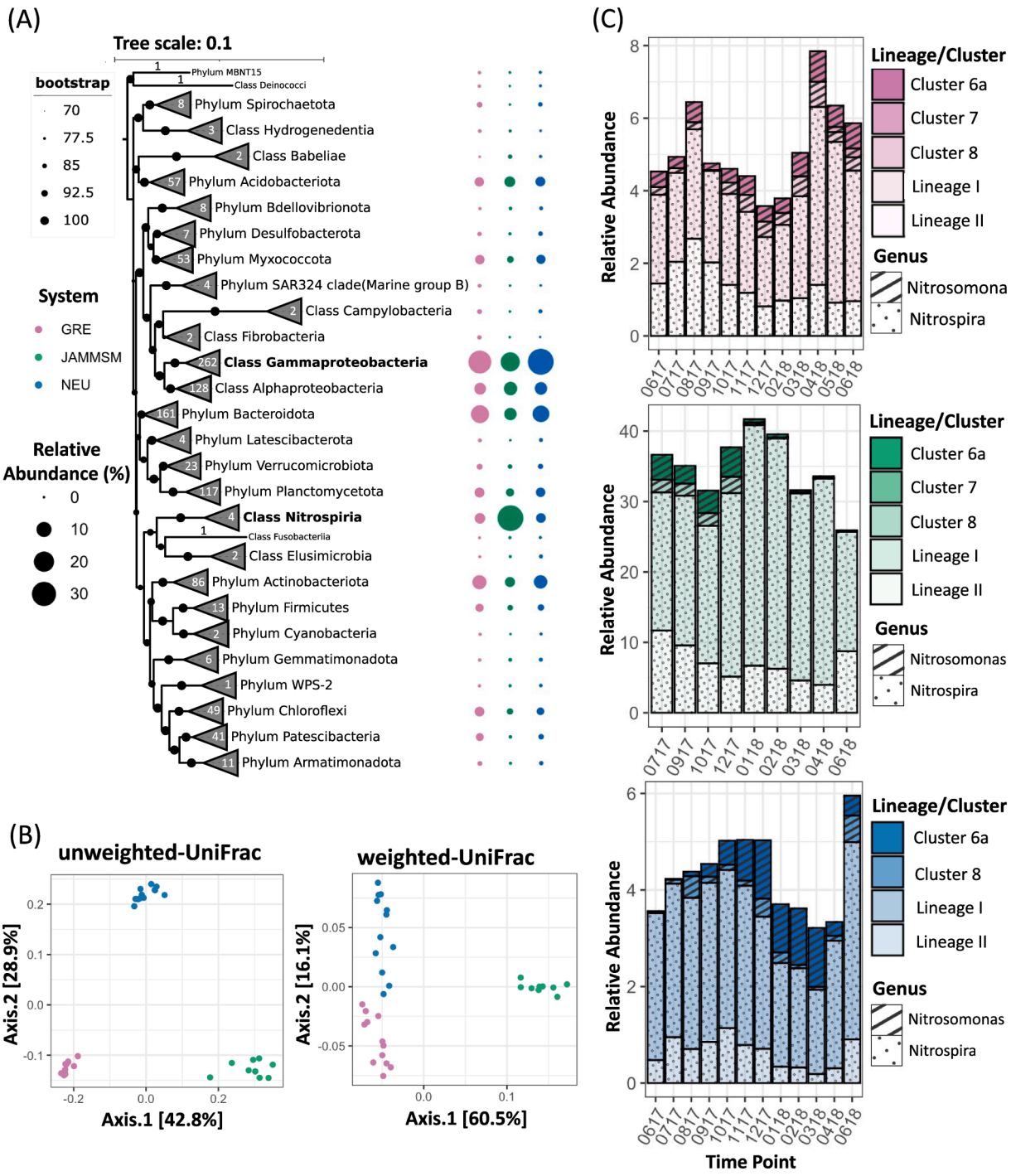

Fig. 1. Community composition of the nitrogen removal systems. (A) Phylogenetic tree constructed using full-length 16S rRNA gene sequences of the representative ASV in each OTU (98.7% sequence similarity cluster), with their corresponding relative abundances shown at the right. Branches are collapsed 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).

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). There are five *Nitrospira* related genera (e.g., *Nitrospira*_A, *Nitrospira*_C, *Nitrospira*_D, *Nitrospira*_E, and *Nitrospira*_F) according to the Genome Taxonomy Database (D. H. Parks et al., 2018) with high metabolic flexibility and physiological diversity (Sampara et al., 2022). However, the taxonomy of the 16S rRNA ASVs was evaluated with the SILVA SSU 138.1 database which classified all *Nitrospira*-like bacteria as the same genus. Since lineage I appears to be represented by the GTDB *Nitrospira*_A, and lineage II by the other GTDB genera (including *Nitrospira*_C-F) (Sampara et al., 2022) and taking into consideration that *Nitrospira*_A, *Nitrospira*_D,

and *Nitrospira*_F where the only *Nitrospira*-like bacteria found in the systems (see Section 3.4) the (micro)diversity analysis was performed separately by *Nitrospira* lineages (i.e., lineage I and II). The *Nitrospira* lineage I, *Nitrospira* lineage II and *Nitrosomonas*-like ASVs clustered into 1, 2 and 21 OTUs for GRE; 1, 2 and 14 OTUs for JAMMSM; and 1, 1 and 10 OTUs for NEU, respectively. The Shannon diversity (a species-level diversity parameter) of *Nitrosomonas*-like OTUs was significantly higher than *Nitrospira*-like OTUs from both lineages, while the effective microdiversity (a measurement of strain-level diversity) of *Nitrospira* lineage I OTUs was significantly higher than *Nitrosomonas*-like OTUs in all systems ($p<0.05$; Fig. 2A,). *Nitrospira* lineage I OTUs consistently showed the highest microdiversity (Fig. 2B) while two *Nitrospira* lineage II OTUs from GRE and JAMMSM had a lower effective microdiversity than most nitrifier OTUs. These results suggest high diversity (more OTUs) and low microdiversity (less ASVs per OTUs) for *Nitrosomonas*-like bacteria, while the opposite was observed for *Nitrospira*-like bacteria in lineage I.

3.3. Improving the recovery of metagenome assembled genomes of nitrifying bacteria

We pursued taxonomy aware re-assembly (Uritskiy et al., 2018) of 44 non-redundant nitrifer MAGs after dereplicating bins from short-read and hybrid assembly approach (see supplemental information for discussion of multiple assembly and bin approaches used). The resultant 36 nitrifier MAGs had completeness greater than 70% and redundancy lower than 10% (Table S8), with nitrification genes present in 24 MAGs. Although the number of retrieved nitrifier MAGs was a little lower after the reassembly process, there was significant improvement in quality (i.e., higher completeness, lower redundancy, and less fragmentation)

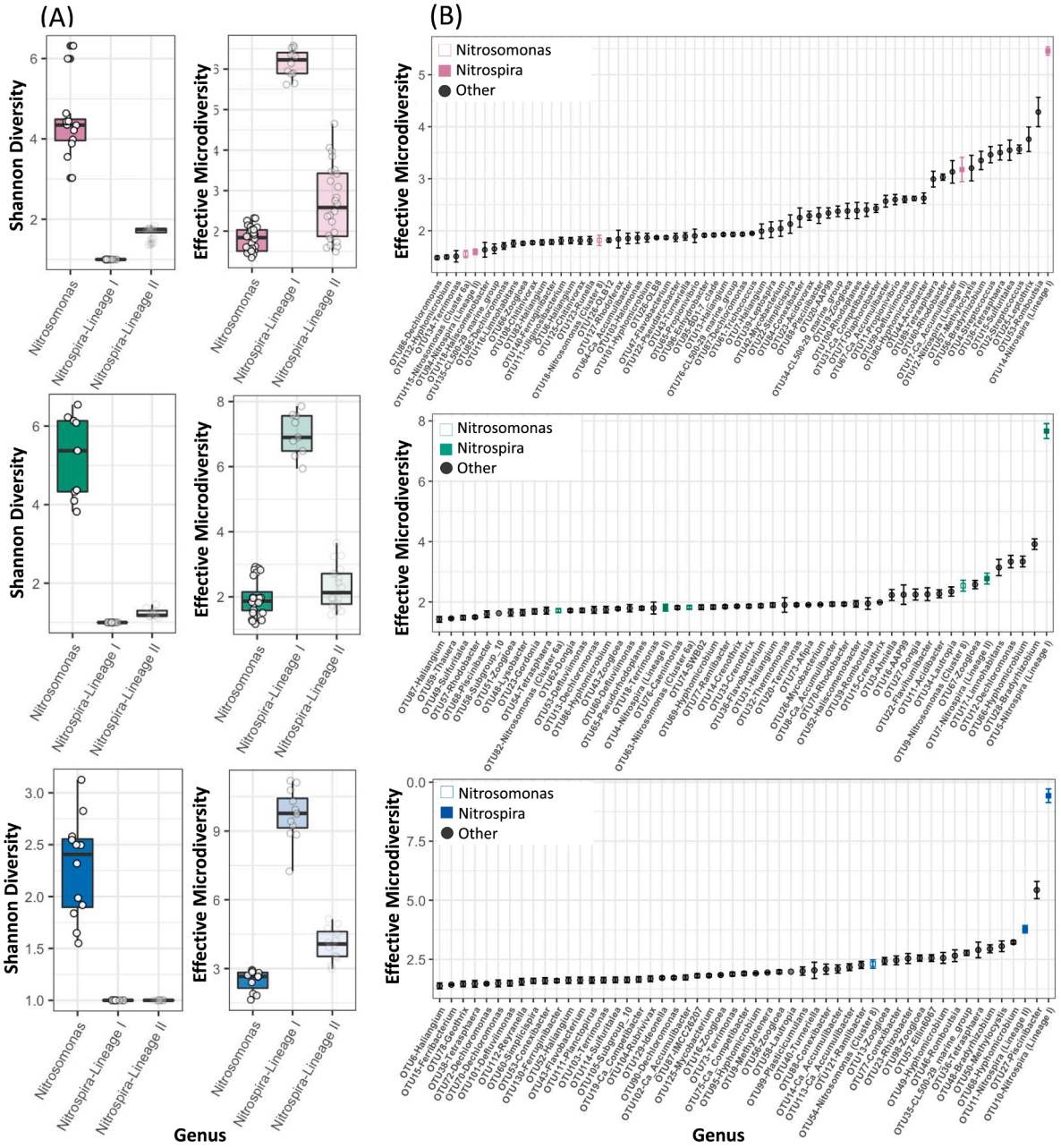

Fig. 2. Diversity and microdiversity based on ASV analyses. (A) Boxplot of Shannon diversity and effective microdiversity of *Nitrospira* lineage I and II, and *Nitrosomonas* at each system. (B) Average effective microdiversity of major OTUs per system. *Nitrospira* and *Nitrosomonas* OTUs are colored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

(Table S8), especially for comammox and AOB genomes. For example, both analyses (before and after reassembly) resulted in 4 *Nitrospira*-comammox MAGs. However, 2 of the 4 comammox MAGs recovered after reassembly contained the entire genetic repertoire required for nitrification, and the other two lack only one gene. In contrast, all pre-reassembly comammox MAGs lacked at least one nitrification gene. These improvements resulted from longer contigs created during the reassembly process compared with previous co-assemblies. In contrast to comammox and AOB, despite becoming less fragmented post-reassembly, fewer *Nitrospira*-NOB MAGs contained all genes associated with nitrite oxidation.

3.4. Phylogenomic placement and abundance of nitrifier MAGs

The 11 canonical NOB and 4 comammox were associated with *Nitrospira* lineage I (8 *Nitrospira*_A-NOB) and *Nitrospira* lineage II (3

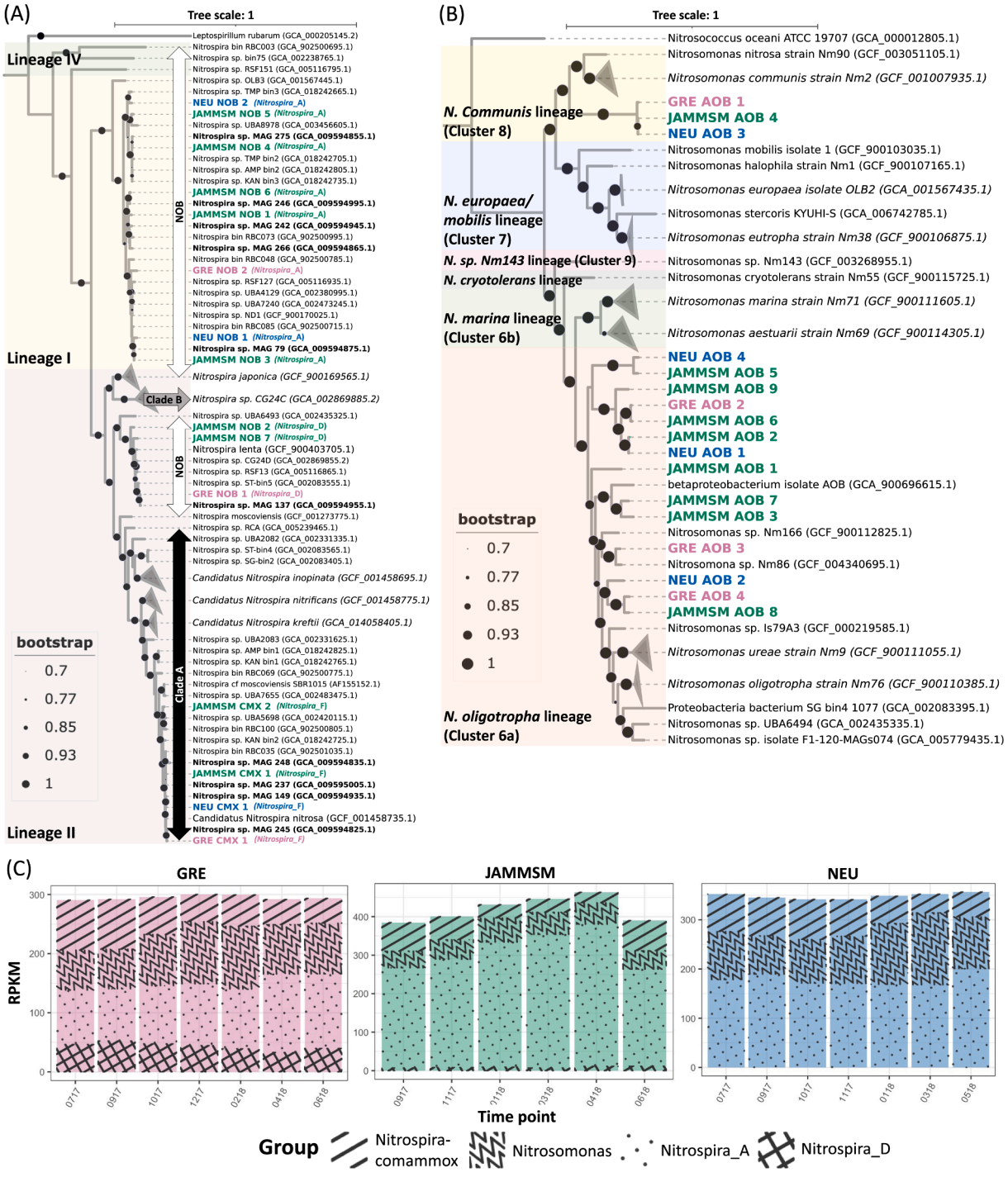

Fig. 3. Phylogenetic placement of (A) *Nitrospira* and (B) *Nitrosomonas* MAGs (GRE, pink; JAMMSM, green; NEU, blue) with 78 and 48 reference genomes (black), respectively. Comammox clade A (black arrow), clade B (gray arrow) and canonical NOB (white arrow) are indicated by arrows and phylogenetic lineages by colored boxes. The *Nitrospira* genus (e.g., *Nitrospira*_A) are presented next to the MAG label in parenthesis. 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*_F-comammox, *Nitrospira*_A-NOB, *Nitrospira*_D-NOB, and *Nitrosomonas* MAGs at each time point per system.

*Nitrospira*_D-NOB and 4 *Nitrospira*-comammox), with all comammox MAGs closely related with *Ca.* Nitrospira nitrosa (Fig. 3A) and belonged to *Nitrospira*-comammox clade A.1 based on the *hao* gene phylogenetic inference (Figure S4). 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) (Palomo et al., 2019). While non-WWTP settings (e.g., soils, sediments, lakes, rivers, DWTPs) harbor diverse comammox bacteria (Palomo et al., 2019; Pjevac et al., 2017; Shi et al., 2020; Xia et al., 2018a; Y. Xu et al., 2020), our findings suggest very low diversity of comammox bacteria in wastewater systems which is consistent with other wastewater studies (Beach and Noguera, 2019; M. Wang et al., 2018; Zhao et al., 2019; Zhou et al., 2021). All AOB MAGs ($n = 20$) were associated with the *N. oligotropha* ($n = 14$) and *N. communis* ($n = 3$) lineages (Fig. 3B). Although several members of the *N. europaea/mobilis* lineage have been detected in sewage treatment plants (H.-P. Koops et al., 2005), 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 (Pommerening-Röser et al., 1996), with *N. communis* species being urease negative and detected primarily in agricultural soils, and isolates of *N. nitrosa* being urease positive,

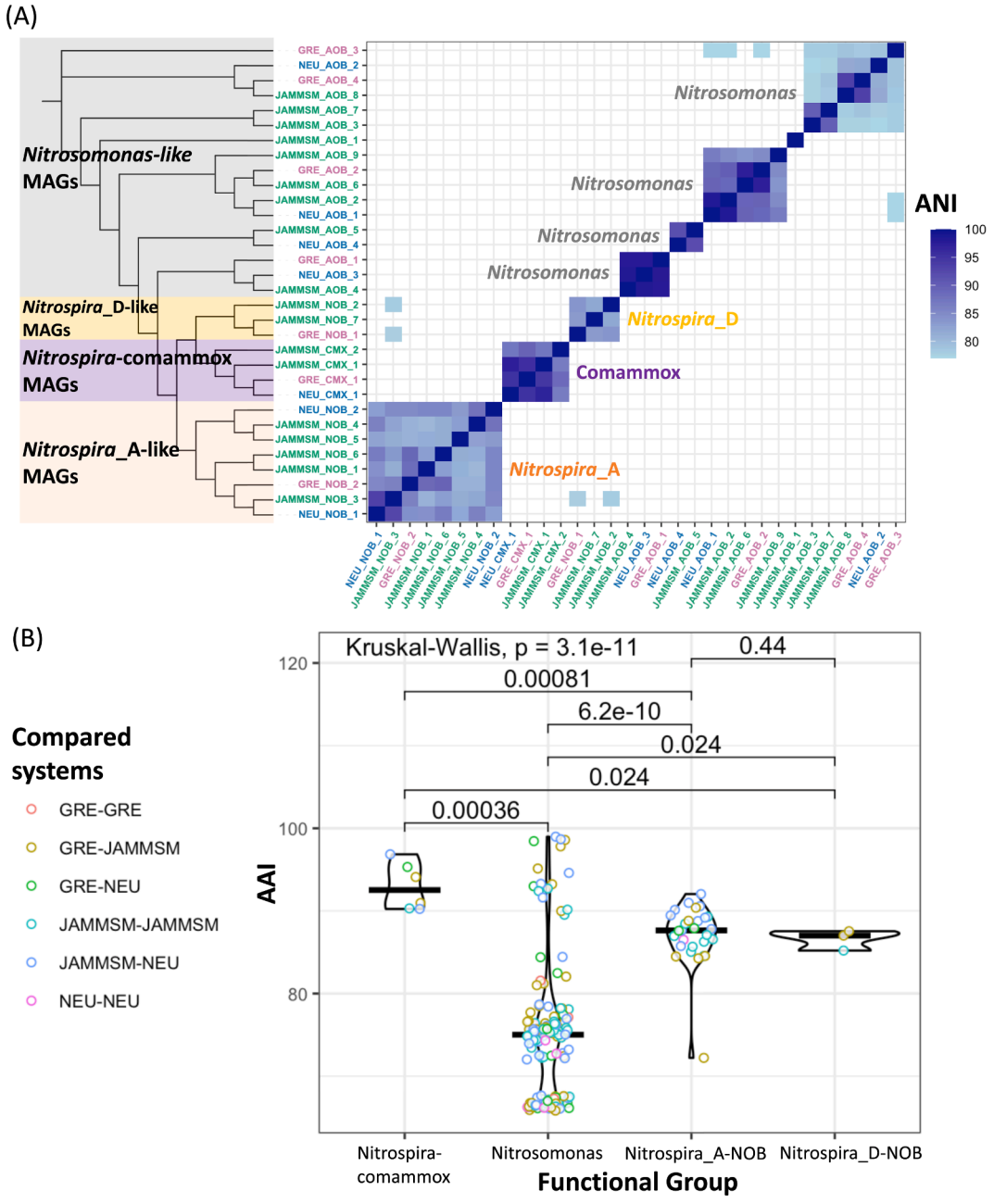

Fig. 4. (A) ANI values calculated between *Nitrosomonas, Nitrospira*_A-NOB, *Nitrospira*_D-NOB, and *Nitrospira*-comammox MAGs along with the phylogenetic placement of the MAGs. MAG labels are colored by system (GRE, pink; JAMMSM, green and NEU, blue). (B) AAI values per functional group. Black lines 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.

preferring aquatic habitats and often found in wastewater treatment plants (H. P. Koops et al., 1991). Our three MAGs contain urease-encoding genes but were still distant to *N. nitrosa* in the phylogenomic analysis (Fig. 3B), suggesting the presence of a novel phylogenetic lineage. Lastly, most species of the *N. oligotropha* lineage have been recovered from oligotrophic freshwaters and almost all are urease positive (H. P. Koops et al., 1991; H.-P. Koops et al., 2005). Accordingly, 12 of the 14 AOB MAGs placed within this *Nitrosomonas* lineage contain urease-encoding genes.

*Nitrospira*_A-NOB was the most abundant functional group in all three systems for the duration of the study with higher abundances in JAMMSM (305.2 ± 48.2 RPKM) as compared with NEU (178.2 ± 12.5 RPKM) and GRE (106.0 ± 16.8 RPKM). This suggests that *Nitrospira*_A bacteria may prefer biofilm-based growth over suspended phase (Navada et al., 2020). *Nitrospira*_D-NOB were only present in GRE (43.3 ± 6.4 RPKM) and JAMMS (9.3 ± 2.1 RPKM) at lower concentrations compared with *Nitrospira*_A-NOB. *Nitrosomonas*-like MAGs were more abundant in NEU (105.2 ± 23 RPKM) and GRE (86 ± 15.8 RPKM) as compared with JAMMSM (53.2 ± 27.4). *Nitrospira*-comammox relative abundances were very similar in all systems, ranging from 55.5 to 64.9 RPKM in JAMMSM and NEU, respectively (Fig. 3C). The abundances of nitrifying MAGs were weakly correlated with each other, with only 37 of 195 correlations significant (p-value <0.05). Most of the significant correlations between *Nitrosomonas*-AOB and *Nitrospira*-NOB were positive (Figure S5), likely arising as consequence of metabolic interactions of these groups within the nitrification process. Comammox bacteria had very few correlations with other nitrifiers, with primarily negative correlations with MAGs from *N. oligotropha*-like bacteria (cluster 6a).

3.5. Species-level diversity of nitrifying bacteria within and between systems

Post dereplication at 95% ANI (Jain et al., 2018; Konstantinidis et al., 2022), pairwise ANI and AAI were calculated between all MAGs within the same functional group (i.e., *Nitrosomonas, Nitrospira*_A-NOB, *Nitrospira*_D-NOB, and *Nitrospira*-comammox) (Fig. 4A-B, Tables S9a-b). Of the 136 pairwise comparisons between the 17 *Nitrosomonas* MAGs, only 18 (13.2%) had ANI values higher than 80% (ANI values lower than 80% are not reported) suggesting high species-level diversity within and between nitrogen removal systems for the *Nitrosomonas*-like AOB (Fig. 4A). Pairwise AAIs for *Nitrospira*-NOB MAGs within the same genus (i.e., *Nitrospira*_A) within or across systems were usually >85% (Table S9b) which, typically, represent members of the same species (Luo et al., 2014). These results suggest that while multiple *Nitrosomonas*-AOB and *Nitrospira*-NOB coexist in each system, *Nitrosomonas*-AOB were substantially more diverse than all *Nitrospira*-NOB genus. In contrast, three of the four comammox MAGs (one from each system) share ANI values between 95 and 97% and are closely related with *Ca.* Nitrospira nitrosa (Cotto et al., 2020). 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 (Fig. 4B) with them and also falls within the *Ca.* Nitrospira nitrosa cluster (Fig. 3A) (Luo et al., 2014). Pairwise AAI comparisons (which are highly correlated with ANI – Figure S6) 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 ($p < 0.05$; Fig. 4B). Moreover, similar to the ASV analysis, *Nitrospira*_A-NOB (lineage I) and *Nitrospira*_D-NOB (lineage II) MAGs have highly similar populations within each genus/lineage (Fig. 4B) being detected across systems. These findings further confirm that in contrast to AOB that demonstrate high diversity, comammox bacteria show very low diversity and, in fact, may all be associated with the same population across multiple wastewater systems.

3.6. Microdiversity of nitrifying bacteria

The ANIr distributions for *Nitrospira*-comammox, *Nitrosomonas*-AOB and Nitrospira_D-NOB MAGs were not significantly different from each other, and significantly higher than for *Nitrospira*_D-NOB MAGs (Fig. 5A). While *Nitrospira*_A-NOB overall demonstrated lower ANIr indicating higher microdiversity compared with the other three groups, there was some variability within each system (Figure S7). These observed trends were similar between ANIr and inStrain estimated nucleotide diversity (Fig. 5B), with *Nitrosomonas*-AOB showing on average lowest microdiversity and *Nitrospira*_A-NOB the highest. Specifically, the average nucleotide diversity of *Nitrosomonas*-AOB was significantly lower than *Nitrospira*_*A*-NOB and *Nitrospira*-comammox ($p < 0.05$), while with ANIr only *Nitrospira*_A-NOB were significantly more microdiverse than the three other functional groups. This difference arises from the fact that the two measures of microdiversity (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*_A-NOB exhibit significantly higher microdiversity as compared with the other groups. These results are consistent with the 16S rRNA gene analysis where *Nitrospira* lineage I and some 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 the lower effective microdiversity likely correspond to comammox bacteria (Fig. 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). All popANI values from GRE_CMX_1, JAMMSM_CMX_1, and NEU_CMX_1 were above or very close to the 99.999% threshold, suggesting low strain-level diversity of comammox bacteria (Fig. 5C). Only one comammox genome retrieved from JAMMSM (JAMMSM_CMX_2) had popANI values lower than the recommended threshold. However, the average relative abundance of this comammox (RPKM = 7 ± 2) was particularly low as compared with the more abundant comammox in JAMMSM (RPKM = 48 ± 21) and those retrieved from GRE (RPKM = 60 ± 19) and NEU (RPKM = 65 ± 17). Therefore, the principal or sole comammox bacteria in each system is not only a single population but likely also a specific strain. The ANIr for each comammox MAG estimated by mapping reads from a different system (e.g., mapping GRE or NEU reads to MAGs assembled from JAMMSM) were lower than when mapping of reads from the system from where the MAG was obtained (e.g., mapping JAMMSM reads to MAGs assembled from JAMMSM; Figure S8). This suggests that on the one hand the comammox bacterial population within a single system is restricted to a specific *Ca.* Nitrospira nitrosa-like strain, but on the other hand that different systems contain different strains.

4. Discussion

4.1. Comammox bacteria exhibit lower diversity in secondary wastewater treatment as compare with strict AOB and NOB and comammox species in other environments

Structural diversity and functional redundancy are inherently linked to process stability when environmental and process conditions vary (Siripong and Rittmann, 2007). Both, metagenomics and 16S rRNA gene sequencing, indicated that *Nitrosomonas* and *Nitrospira*-like bacteria were the only known nitrifiers present in the systems investigated. This is consistent with numerous studies (Dionisi et al., 2002; J.-F. Gao et al., 2013; Gruber-Dorninger et al., 2015; Zhang et al., 2011) suggesting the specific adaptation of these genera to the wastewater environment. The majority of the *Nitrosomonas*-like AOB MAGs, within and between systems, had AAI values below 85% (Fig. 4) indicating that this functional

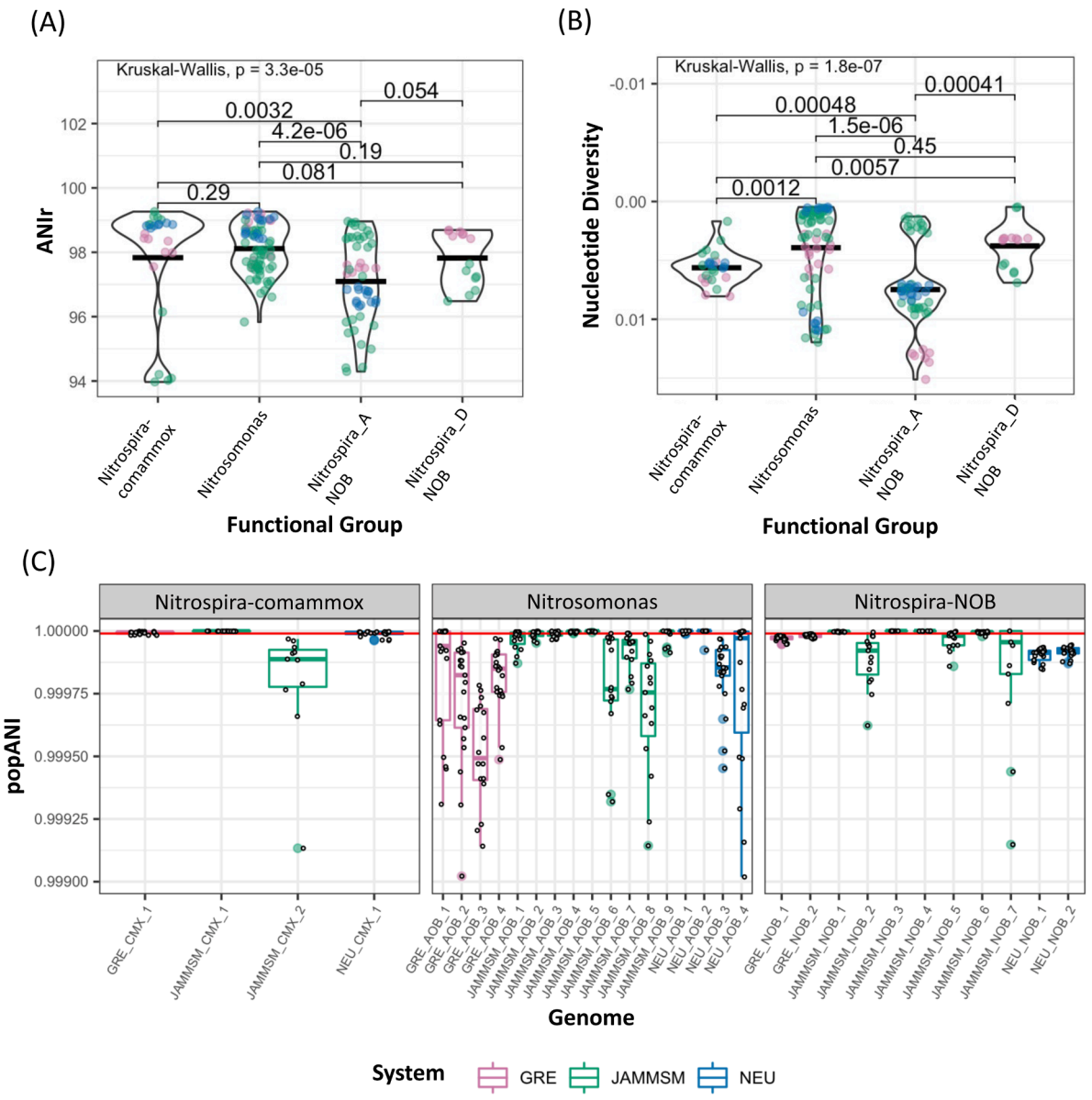

Fig. 5. (A) ANIr and (B) nucleotide diversity values from each functional group. Points represent the average value of each MAG and are colored according to the systems (GRE, pink; JAMMSM, green; NEU, blue) from which the MAGs were recovered. (C) popANI values from each MAG. MAGs are colored by system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

group exhibited high species-level diversity. Meanwhile, *Nitrospira*-like MAGs within the same genus (e.g., *Nitrospira*_A or *Nitrospira*_D) reported AAI values above 85% in most cases. The high diversity of the *Nitrosomonas*-like bacteria was also confirmed with full length 16S rRNA gene sequencing (Figure S3B) and corroborates earlier studies on their diversity in wastewater (J. Gao et al., 2014; J.-F. Gao et al., 2013; Zhang et al., 2011). Although *N. europaea, N. oligotropha* and the *Nitrosomonas*-like cluster closely associated with *N. communis/nitrosa* are often the most abundant groups in wastewater systems, different *Nitrosomonas* populations dominate different processes configuration (J.-F. Gao et al., 2013). For example, *N. europaea* are often the major AOB in high ammonia concentration environments (e.g., anammox reactors) due to their low ammonia affinity (J. Gao et al., 2014). The observed dominance of *N. oligotropha* and *N. communis* in this study are consistent with previous studies of WWTPs with relatively lower ammonia concentrations (J. Gao et al., 2014; J.-F. Gao et al., 2013; Wells et al., 2009).

Comammox bacteria exhibited significantly lower diversity as compared with strict AOB and most NOB (Fig. 4). All comammox *Nitrospira* MAGs, independent of the system they were recovered from, belong to *Nitrospira* lineage II and were closely associated with *Ca.* Nitrospira nitrosa. These findings are consistent with several studies that have found *Ca.* Nitrospira nitrosa-like bacteria as the unique or principal comammox bacteria present in their lab- and full-scale wastewater treatment systems (Beach and Noguera, 2019; Camejo et al., 2017; Roots et al., 2019), using metagenomics and gene targeted analyses. In contrast, other engineered and natural habitats (e.g., soils, sediments, lakes, drinking water, groundwater, and estuaries) typically harbor multiple co-existing species across the two primary clades (i.e., comammox clade A and B) (Palomo et al., 2022; Pjevac et al., 2017; Vilardi et al., 2022; X. Wang et al., 2021; Y. Xu et al., 2020). However, this co-existence of comammox populations across clades does not seem to be prevalent in wastewater treatment systems (Beach and Noguera, 2019; M. Wang et al., 2018). These findings suggest that the diversity of comammox bacteria in WWTPs is lower than in other habitats where several comammox species co-exist.

In contrast, Spasov et al. (2020) reported the detection of multiple comammox MAGs in samples collected from rotating biological contactors (RBCs) used for tertiary treatment at a municipal WWTP in Ontario, Canada. The difference in diversity between that study and other WWTP investigations, including this one, may be associated with ammonia availability. Specifically, tertiary treatment systems are typically employed as a polishing step, designed and operated to remove low residual amounts of nitrogen. The influent ammonium concentrations reported for the RBC tertiary treatment system range from 0.2 to 16.3 μM (Spasov et al., 2020), while the three systems in this study have influent ammonia concentrations between 2 and 3 mM. The comammox *Nitrospira* MAGs with overall higher abundances in the tertiary plant (RBC001 and RBC083) were less abundant at the beginning of the train (higher ammonium concentrations) than at the end of the train (lower ammonium concertation). In contrast, the abundance of RBC035, the only MAG phylogenetically associated with *Ca.* Nitrospira nitrosa (ANI >95%), decreased in abundance with decreasing ammonium concentrations. The high comammox diversity in the RBCs and other ammonium-limited systems suggest that ammonium concentration and/or flux through the system may be a key factor driving comammox bacteria diversity in nitrification systems. In fact, Palomo et al. (2022) reported that the influent ammonium concentration was the key explanatory variable associated with comammox bacterial diversity in 12 groundwater-fed sand filters when higher comammox population diversity was detected in systems receiving lower ammonium concentrations. *Ca.* Nitrospira inopinata and *Ca.* Nitrospira kreftii, two comammox species with known kinetic parameters, have high apparent ammonia affinities (Kits et al., 2017; Sakoula et al., 2021). Assuming this is a conserved trait in comammox bacteria, this explains their prevalence and diversity in ammonium-limited systems. However, the kinetic parameters (i.e., affinity constant, growth rate) of other comammox species especially of the *Ca.* Nitrospira nitrosa clade have not yet been reported, and our findings suggest that at least this comammox clade may have substantially different kinetic traits that allow it to thrive in systems with higher ammonium concentrations. Low DO has also been associated with the prevalence of comammox bacteria over canonical nitrifiers in wastewater systems (Beach and Noguera, 2019; Roots et al., 2019). However, the high DO concentrations (>2 mg/L) in this and other studies (M. Wang et al., 2018; S. Xu et al., 2022) suggest DO is not a crucial factor for the proliferation of *Ca.* Nitrospira nitrosa in nitrogen removal systems.

4.2. *Comammox bacteria not only exhibit low diversity at the species-level but also at the strain-level across wastewater systems*

The high abundance and microdiversity of *Nitrospira*_A-NOB have been previously reported in wastewater and other engineered and natural environments (Gruber-Dorninger et al., 2015; Gülay et al., 2016; Palomo et al., 2016). However, it is unclear whether the high microdiversity of *Nitrospira* is associated with high functional diversity or is indicative of the coexistence of functionally identical *Nitrospira* with allelic diversity. Gruber-Dorninger et al. (Gruber-Dorninger et al., 2015) reported variable responses of closely related bacteria within *Nitrospira* lineage I (sequence identities ranging from 95.8 to 99.6%) to different nitrite availabilities. Similarly, ecological niche partitioning was also identified as potential mechanisms for co-existence of three *Nitrospira* lineage I strains that used formate under different conditions: two used formate when incubated with nitrite and ammonia, respectively, while the third used formate efficiently as the sole substrate (Gruber-Dorninger et al., 2015). Although these results shed light on the possible niche partitioning of *Nitrospira* sub-species, this does not shed light on whether the microdiversity in our study represents coexisting *Nitrospira* strains with slight functional differences. Although functional implications of such high microdiversity are not clear, the persistence of high microdiversity may suggest it plays an important role in the distribution and success of *Nitrospira* populations in wastewater systems. Few studies in other environments have shown that persistent populations exhibit increased intra-population sequence diversity (Meziti et al., 2019; Orellana et al., 2019). For example, a study in a saltern pond suggested that the ecologically important genes of the major archaeal population were carried by distinct sub-populations (strains), indicating that the adaptation to different salinity concentrations had led to sub-population differentiation and speciation (niche partitioning) (Konstantinidis et al., 2022). Moreover, a study in eight different temperate bog lakes concluded that high microdiversity is associated with the maintenance of functional microbial communities during changes in environmental conditions (García-García et al., 2019).

In contrast, comammox bacteria not only exhibited an unexpected low diversity within and between systems but also showed significantly lower microdiversity as compared with strict NOB within the genus *Nitrospira*_A. These results may indicate specific adaptations of comammox bacteria within the *Ca.* Nitrospira nitrosa cluster to the wastewater environment and particularly secondary treatment systems. We confirmed that the principal or sole comammox bacteria in each system was not only a single population but likely also a specific *Ca.* Nitrospira nitrosa-like strain. However, each system was dominated by a different strain. This is in accordance with Wang et al. (2018) who found in their study that most comammox OTUs were associated with the *Ca.* Nitrospira nitrosa cluster (94.34% of all comammox *amoA* sequences) with different OTUs representing the dominant comammox species in different systems.

Palomo et al. (2022) observed a negative correlation between the species-level diversity of comammox *Nitrospira* and ammonium concentrations in groundwater sand filters. However, that was not the case for microdiversity, suggesting that different mechanisms may shape inter- versus intra-population diversities, or that the range of ammonium concentrations in the investigated drinking water systems was too narrow to capture any underlying associations. For instance, the comammox nucleotide diversity in our study ranged from 0.002 to 0.008, while for the MAGs obtained from these drinking water systems was from ~0.005 to ~0.013, with only three of the twelve comammox MAGs presenting nucleotide diversities lower than 0.008 (Palomo et al., 2022). This may suggest that comammox microdiversity is also associated with ammonium concentrations and/or flux through the system, with the observed low microdiversity in wastewater systems associated with the higher prevailing ammonium concentrations.

The low microdiversity of comammox bacteria compared with other nitrifier groups rejected the hypothesis that the persistence of comammox bacteria in wastewater secondary treatment is associated with high levels of microdiversity, suggesting other possible reasons. The reasons for their persistence could include higher growth yield as compared with AOB and preference for biofilm-based growth (Costa et al., 2006; Kits et al., 2017). Further, their metabolic versatility could help them adapt to changes in environmental conditions and thus, contribute to their persistence. Finally, several metabolic differences between comammox bacteria and canonical nitrifiers (e.g., ammonia and nitrite affinity, CO_2 fixation mechanisms, etc.) (Koch et al., 2015) can potentially create a unique niche which may contribute to their persistence. Nevertheless, further research is required to clearly identify factors that explain the presence and temporal stability of comammox bacterial strains in WWTPs.

Despite the broad detection of comammox bacteria in WWTPs, their role and process relevance are as yet unclear. In contrast to other environments, comammox *Nitrospira* present very low species and strain-level diversity in wastewater treatment systems for nitrogen removal. This observed lack of diversity and, consequently, lack of functional redundancy may influence the feasibility of potential design and operational strategies relying primarily on comammox *Nitrospira* for nitrogen removal. Although ammonium concentration and/or availability apparently influences the diversity of comammox bacteria, further studies are necessary to determine other factors driving the success of their clonal community in wastewater. Moreover, studies are required to

estimate the activity of comammox bacteria, specifically *Ca.* Nitrospira nitrosa, and to assess their contribution to nitrification in full-scale nitrogen removal systems. It is also important to note that the strain-level diversity cannot be solely studied with shotgun metagenomic methods since high microdiversity can prevent robust assembly of individual genomes (Konstantinidis et al., 2022). Therefore, more accurate techniques are needed to obtain high-quality strain-level genome assemblies for *Nitrospira*_A and other highly microdiverse bacteria.

5. Conclusion

- Full-length 16S rRNA and metagenomic analysis demonstrated high diversity and low microdiversity for *Nitrosomonas*-like bacteria while the opposite was observed for *Nitrospira* (lineage I) bacteria. Meanwhile, *Nitrospira* lineage II (which include comammox bacteria) had lower microdiversity than *Nitrospira* (lineage I).
- Hybrid assembly was not completely suitable for the reconstruction of *Nitrospira*-NOB genomes. This is likely caused by the high levels of microdiversity within *Nitrospira*_A-NOB (lineage I), which may impact the read mapping and *de novo* assembly process.
- Comammox bacteria exhibited significantly lower diversity compared with strict AOB (i.e., *Nitrosomonas*-like bacteria) and comammox bacteria present in other environments and lower microdiversity compared with strict *Nitrospira*_A-NOB. These results may indicate specific adaptations of comammox bacteria within the *Ca.* Nitrospira nitrosa cluster to the wastewater environment and particularly secondary treatment systems.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

All raw sequencing data from Illumina and Nanopore platforms and nitrifiers MAGs are available on NCBI under bioproject number PRJNA846349.

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All raw sequencing data from Illumina and Nanopore platforms and nitrifiers MAGs are available on NCBI under bioproject number PRJNA846349.

Supplementary materials

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