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Exploring Community and Kinetic Shifts in Nitrifying Microbial Communities in Low Dissolved Oxygen Activated Sludge Facilities for Energy-Efficient Biological Nitrogen Removal

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Cite This: *ACS EST Water* 2024, 4, 707−718

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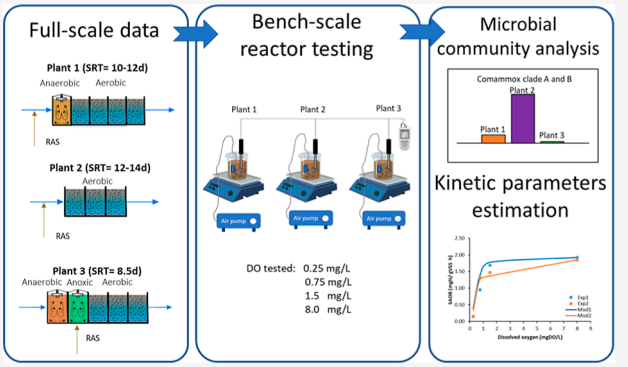
ABSTRACT: The discovery of the complete ammonia oxidation process (comammox) has challenged conventional nitrification theory, showing microbial adaption to very low dissolved oxygen (DO) concentrations. This study aimed at investigating the effects of different DO concentrations using a series of bioreactors inoculated with biomass from three operationally diverse water resource recovery facilities. Results show that microbial populations adapted to low DO environments can maintain high rates across a range of DO concentrations, indicating their ability to function well even at high DO concentrations. Additionally, long solids retention times (>10 days) can encourage the persistence of comammox populations adapted to different DO concentrations. Molecular analyses revealed that the low DO-facility had a nitrifying population with similar ratios of comammox clades A and B, while the high DO facility was dominated by clade A. Modeling results suggest that the nitrifying population including comammox bacteria from the low DO facility has a different half-saturation coefficient for DO (e.g., 0.05 mg L^{-1}) and possible intrapopulation diversity within clades A and B. This study highlights that a changing nitrification community can enable the activated sludge process to operate effectively at low DO concentrations, leading to low-energy biological nitrogen removal.



KEYWORDS: *ammonia removal, aeration, SRT, nitrospira, comammox*

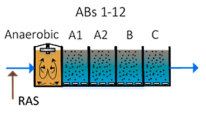
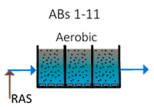
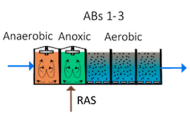
1. INTRODUCTION

Wastewater treatment plants (WTTPs) are currently implementing a variety of strategies to achieve energy-neutral goals, among those which are reducing their carbon footprint and providing cost savings.[1,2] As a result, in recent years, there has been a shift in how WTTPs are viewed, from traditional "flow-through and treat" systems to water resource recovery facilities (WRRFs) that recover resources such as energy, carbon, and nutrients.[3] Aeration is one of the most energy-intensive processes in WRRFs and optimizing its requirements in the activated sludge process is essential to address this issue.[4,5] Nitrification is a critical microbial process during biological wastewater treatment that involves the oxidation of ammonia (NH_4^+) to nitrite (NO_2^-) and NO_2^- to nitrate (NO_3^-) and is carried out by two groups of bacteria: ammonia oxidizing bacteria (AOB) and nitrite oxidizing bacteria (NOB). AOB and ammonia oxidizing archaea (AOA) are responsible for the first step of nitrification, but they are slow growers and not known to be strong competitors for oxygen.[6−8] In contrast, NOB have a higher affinity for dissolved oxygen (DO) and are less sensitive to its depletion.[6]

Recent advances in wastewater treatment processes have focused on how to capitalize on the physiological differences among nitrifying bacteria to reduce aeration energy and chemical addition while enhancing effluent quality.[9] For example, partial nitrification and low DO nitrification have gained attention as ways to reduce energy and aeration demand, as well as operational costs of aeration during secondary treatment.[10,11] Other processes that require less oxygen to remove NH_4^+ have also been implemented as alternatives, such as partial nitritation (PNA) coupled with anaerobic ammonium oxidation (anammox), which combines two different nitrogen removal processes: PNA and anammox.[12−14] The PNA process has been successfully implemented to sidestream treatments, including biomass digesters'

Received: November 14, 2023
Revised: January 5, 2024
Accepted: January 8, 2024
Published: January 19, 2024

Table 1. Key Operational Parameters and Comparison of the Secondary Treatments at Each Plant in CRWS, TMCRWS, and DCRWS

Parameter	CRWS plant	TMCRWS plant	DCRWS plant
SRT	Total SRT= 10-12 d	Total SRT= 12-14 d	Total SRT= 8.5 d
MLSS	Avg ABs= 6,000 mg L^{-1}	Avg ABs= 5,800 mg L^{-1}	Avg ABs= 3,800 mg L^{-1}
DO	Based on DO setpoints	Avg DO= 5.0 mg L^{-1}	Avg DO= 2.33 mg L^{-1}
Aeration strategy	ABAC System	N/A	N/A
Bio-P	Yes	No	Yes
Settleability	Avg SVI= 55 mL g^{-1}	Avg SVI= 85 mL g^{-1}	Avg SVI= 85 mL g^{-1}
BNR configuration	A/O Process	CAS	A2/O Process
			

effluent and landfill leachate, and has proven to be more energy-efficient.[15] Recently partial denitrification has been coupled with anammox (PdNA) as alternative nitrogen removal strategy providing opportunities to achieve a more sustainable wastewater treatment.[16,17]

In 2015, our conventional knowledge of the two-step nitrification process was overturned by the newly discovered process called complete ammonia oxidation or "comammox". Studies by Daims et al.[18] and van Kessel et al.[19] reported the enrichment of *Nitrospira* species capable of complete NH_4^+ oxidation to NO_3^- via NO_2^- (without accumulation). These species were characterized by high growth yield and a low growth rate.[20,21] While different comammox species, such as *Nitrospira inopinata*, *Candidatus Nitrospira nitrificans*, and *Candidatus Nitrospira kreftii*,[18,19,22] have been identified, Xia et al.[23] found that *Nitrospira nitrosa*-like comammox could be a key nitrifier in secondary wastewater systems. Additionally, comammox bacteria generally release less nitrous oxide (N_2O) but rather may contribute to emission reduction as they tend to accumulate less or no NO_2^-.[24]

Since their discovery, comammox bacteria have been detected at both lab- and full-scale and in natural and engineered systems, including WRRFs.[25−28] How et al.[29] found that low DO (~0.9 mg L^{-1}) reactors with dominant *Nitrospira* spp. had putative comammox bacteria accounting for over 50% of *Nitrospira* spp. Law et al.[30] also reported a decrease in the relative abundance of *Nitrospira* spp. with an increase in DO concentration, suggesting an inverse correlation between the competitiveness of comammox bacteria and DO concentration. WRRFs are starting to utilize low DO operation and achieve optimal nitrogen effluent water quality while using less energy than conventional DO-operated WRRFs.[31] However, there is a lack of documentation systematically comparing the microbial population compositions in operational WRRFs with varying configurations and DO concentrations. Therefore, in this study, three distinct WRRFs with diverse nutrient removal and aeration control strategies were selected to achieve the following objectives:

(1) Understand the impacts of different DO concentrations on the behavior of various nitrifying communities, including comammox, and determine their nitrogen removal pathways and rates.
(2) Use bench scale batch reactor data to estimate kinetic rates across the operationally diverse WRRFs and evaluate the impact of full-scale operational strategies on nitrifying community selection.
(3) Identify key microbial players through MinION 16S rRNA amplicon sequencing and quantitative polymerase chain reaction (qPCR).

The findings of this study will help optimize operational strategies for WRRFs and enhance our understanding of the behavior of nitrifying communities at different DO concentrations.

2. METHODS AND MATERIALS

2.1. Description of Tested WRRFs. The Trinity River Authority (TRA) of Texas owns and operates five WRRFs in its Northern Region. This study focuses on three of the five WRRFs: Central Regional Wastewater System (CRWS), Ten Mile Creek Regional Wastewater System (TMCRWS), and Denton Creek Regional Wastewater System (DCRWS). Key operational parameters for each plant are shown in Table 1 while typical wastewater chemistry is shown in Table S1 in the Supporting Information.

2.1.1. Central Regional Wastewater System. The CRWS treatment plant is rated for an annual average flow of 859,000 m^3 d^{-1}. Wastewater is treated with headworks, primary treatment, advanced secondary activated sludge, and tertiary treatment prior to disinfection and discharge. The secondary setup of CRWS features an anaerobic/oxic (A/O) layout with low DO operation (Figure S1 in the Supporting Information). Beginning in 2012, the CRWS staff began operating their activated sludge process at progressively lower DO set points. For the purposes of this study, CRWS will represent the low-DO operation facility with biological nitrogen and phosphorus removal. During the study, CRWS maintained a total solids retention time (SRT) of 10−12 days and mixed liquor suspended solids (MLSS) of 6000 mg L^{-1}. Sludge volume index (SVI) values for the plant are historically low, averaging 55 mL g^{-1}.

The CRWS treatment plant is equipped with advanced aeration control capabilities. The aeration controls were programmed to provide ammonium-based airflow control (ABAC) in addition to the existing DO control mode. The DO concentrations within each zone are controlled to maintain an effluent ammonium (NH_4^+) target set point. These set points are optimized to reduce energy, optimize treatment performance, and provide a low amount of residual NH_4^+. When the effluent NH_4^+ exceeds the set point for a predetermined length of time, the DO set points automatically shift from the low to high DO set points (more details on the DO set point system can be found in Table S2 in the Supporting Information). This

https://doi.org/10.1021/acsestwater.3c00715
ACS EST Water 2024, 4, 707−718

setup provides CRWS with the flexibility to optimize nitrification and energy input into the system while also allowing operations staff to mitigate the risk of NH_4^+ exceedance with the ABAC control logic. Before the implementation of ABAC controls, the activated sludge system had transitioned to these lower DO set points, which resulted in selective pressures for a low DO nitrifying bacterial population in the CRWS activated sludge system.

2.1.2. Ten Mile Creek Regional Wastewater System. The TMCRWS treatment plant is rated for an annual average flow of 109,000 m^3 d^{-1}. Wastewater is treated with headworks, primary treatment, secondary treatment, and tertiary treatment prior to disinfection. The secondary treatment in TMCRWS is a conventional nitrification-only activated sludge process (Figure S2 in the Supporting Information). TMCRWS has conventional aeration controls with a target DO concentration of 2.0 mg L^{-1}. For this study, TMCRWS will represent the conventional DO facility with traditional nitrification and no biological P removal. During the time of the study, TMCRWS maintained an average SRT of 12−14 days and MLSS values of 5800 mg L^{-1}. The facility's SVIs averaged 85 mL g^{-1}. The plant meets effluent NH_4^+ concentrations of <0.5 mg N L^{-1} with no other nutrient reduction requirements.

2.1.3. Denton Creek Regional Wastewater System. The DCRWS treatment plant is rated for an annual average flow of 52,000 m^3 d^{-1}. Wastewater is treated with headworks, secondary treatment, and tertiary treatment prior to disinfection. The secondary treatment in DCRWS follows an anaerobic/anoxic/oxic (A2O) layout (Figure S3 in the Supporting Information) with the flexibility to be converted to Modified University of Cape Town (MUCT) configuration. The process at DCRWS has a conventional aeration control and meets DO targets of 2.0 mg L^{-1}. For the purposes of this study, DCRWS represents the conventional DO facility with biological N and P removal. At the time of this study, DCRWS had an effluent phosphorus limit of 0.5 mg P L^{-1} and meets an effluent TP of <0.25 mg P L^{-1}. The facility also maintained an average SRT of 8.5 days and MLSS values of 3800 mg L^{-1}. The typical SVIs for DCRWS are 85 mL g^{-1}. The DCRWS treatment plant is currently undergoing an expansion with liquid stream process changes.

2.2. Lab-Scale Reactors for Batch Experiments. Biomass samples were collected from each facility (CRWS, TMCRWS, and DCRWS) and used to seed bench scale experiments at the CRWS' lab facility. The samples were collected in 4 L Nalgene bottles at TMCRWS and DCRWS and then shipped in coolers and used the same day for further processing. The samples were then allowed to acclimate at room temperature (T = 20 °C) before the experiments were performed. The experimental setup was like the one used in Sabba et al.[33] Bench scale experiments were carried out in three 4 L glass cylindrical beakers with an active volume of 3 L. Three parallel nitrification tests (Figure S4 in the Supporting Information) were performed for the same DO concentration, from low DO (0.25 mg L^{-1}) to nonrate limiting DO (8 mg L^{-1}).

The samples were aerated with diffusers and compressed air; DO concentration was continuously monitored, and flow rates were carefully adjusted to achieve target DO concentrations. The tests were performed with an initial NH_4−N concentration of 15 mg N L^{-1} and lasted 2 h. Each test was run in duplicate. Samples were collected at 20 min intervals, resulting in a total of seven samples, including time zero (t_0). Key parameters were tracked using standard methods and procedures outlined in Table S3 in the Supporting Information. NH_4^+, NO_2^-, NO_3^-, orthophosphate (OP), and alkalinity were measured at specific time points, as indicated in Table S3 in the Supporting Information. Samples of total and volatile suspended solids (TSS and VSS) were collected at t_0 only since the concentration of solids was not expected to change within 2 h of testing. DO and pH were constantly monitored via their specific probes (Table S3 in the Supporting Information). Specific NH_4^+ oxidation rates, SAOR (mg N^{-1} g VSS^{-1} h^{-1}), were used to estimate the oxidation of NH_4^+ based on Zhou et al.[34]

2.3. Determination of Apparent Maximum Substrate Utilization Rates and Half-Saturation Coefficients for DO. Equation 1 was used to predict the oxidation of NH_4^+ by the composite community and to fit the experimental data to a biokinetic model. In these tests, the apparent maximum substrate utilization rate, $\hat{q}_{app}$, was determined at a nonlimiting DO concentration of 8.0 mg L^{-1} while the both parameters were determined using parameter fitting[35,36] solely based on NH_4^+ oxidation rates. Since the experiments were conducted over a short time (e.g., 2 h), growth and decay were not considered. The values of the parameters $\hat{q}_{app}$ and $\hat{K}_{DOapp}$ were varied by +10 and −10% to determine their sensitivity (Supporting Information Figures S7−S9), and the root-mean-square error analysis was used to explore how the observed data clustered around the predicted values.

$$\text{Ammonia oxidation } (r_{amm\_ox}) = \hat{q}_{max} \cdot \left(\frac{NH_4^+}{NH_4^+ + K_{NH_4}}\right) \cdot \left(\frac{O_2}{O_2 + \hat{K}_{DOapp}}\right) \cdot X \quad (1)$$

where $\hat{q}_{max}$ is the apparent maximum substrate utilization rate, mg N g VSS^{-1} h^{-1}; NH_4^+ is the concentration of ammonia during the experiment, mg N L^{-1}; K_{NH_4} is the ammonia half-saturation coefficient, mg N L^{-1}; O_2 is the concentration of oxygen during the experiment, mg O_2 L^{-1}; $\hat{K}_{DOapp}$ is the apparent oxygen half-saturation coefficient, mg O_2 L^{-1}; and X, the biomass concentration during the experiment, g VSS L^{-1}.

2.4. DNA Extraction and Quantitative PCR. Biomass samples of 50 mL were collected and preserved in dimethyl sulfoxide, disodium EDTA, and saturated NaCl (DESS) until further processing.[37] Prior to DNA extraction, samples containing DESS were centrifuged for 1 min at 10,000 rpm. The supernatant was removed, and the pellet was resuspended in 750 μL of a buffer for DNA extraction. Genomic DNA was extracted from the biomass samples using the Quick-DNA Fecal/Soil Microbe Miniprep Kit, cat. no. D6010 (Zymo Research, Irvine, CA) following the manufacturer's instructions and was eluted in 100 μL of manufacturer buffer solution. Isolated genomic DNA concentration was estimated using an AccuGreen Broad Range dsDNA Quantitation Kit, cat. no. 31069, (Biotium, Fremont, CA), and 5 μL of eluted DNA was estimated following the manufacturer's directions using an Invitrogen Qubit 4 fluorometer (Thermo Fisher Scientific, Waltham, MA). The fluorometer was calibrated using kit-supplied DNA standards.

qPCR analyses were employed to quantify total AOB, total NOB, and comammox clade A and clade B. Total AOB qPCR chemistry and proprietary primers targeting *Nitrosomonas marina* and *Nitrosomonas nitrosa amoA* genes were developed by Aster Bio, Inc. (Houston, TX). Each assay was performed

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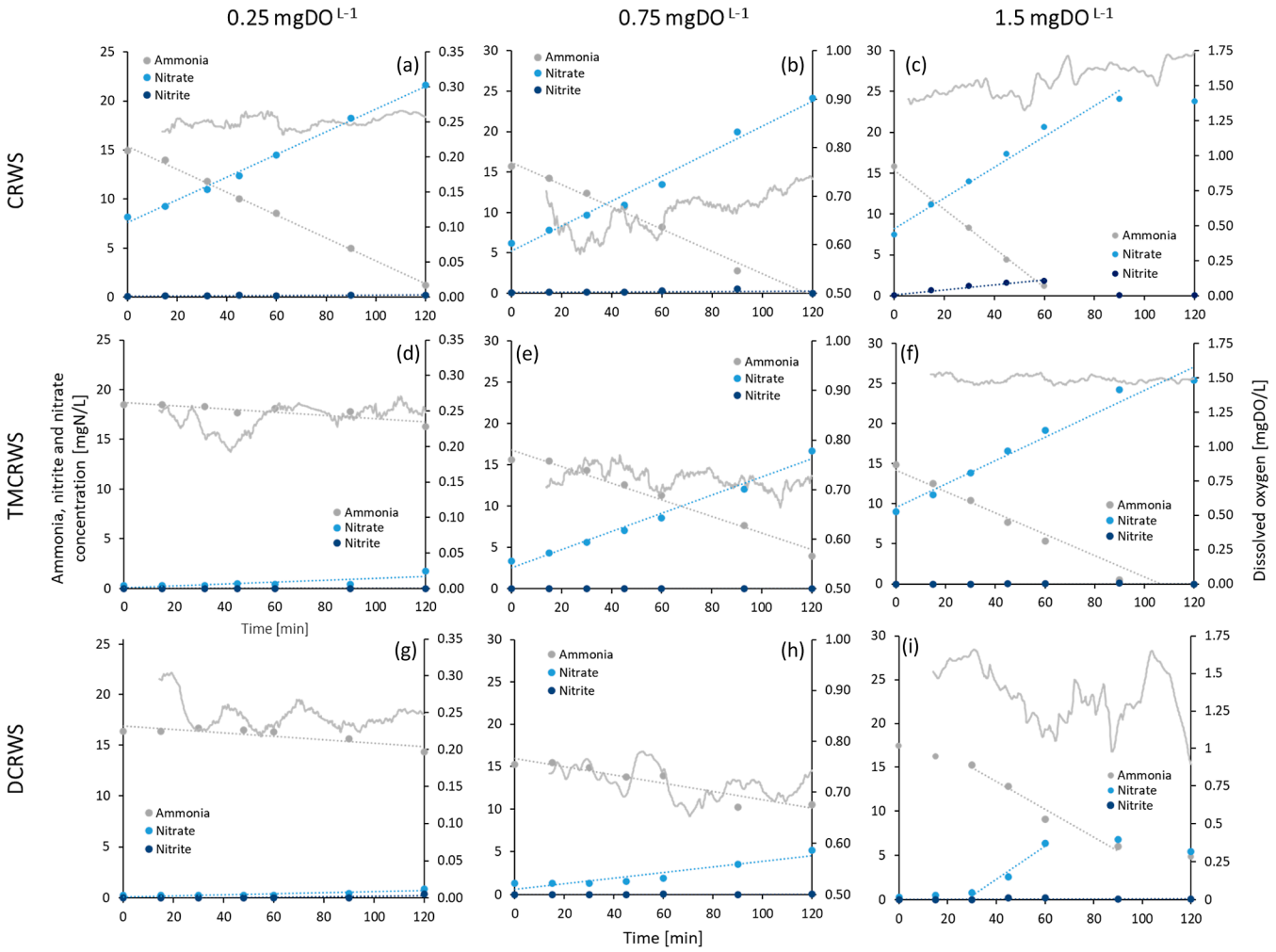

Figure 1. Profiles of nitrification batch tests with biomass from CRWS, TMCRWS, and DCRWS at 0.25, 0.75, and 1.5 mg L^{-1} DO concentrations. The DO, 0.25, 0.75, and 1.5 mg L^{-1} are organized vertically, while the WRRFs, CRWS, TMCRWS, and DCRWS are organized horizontally.

on a Bio-Rad C1000 CFX96 Real-Time PCR System (Bio-Rad, Hercules, CA). To normalize the *amoA* copy numbers by the total bacterial population, eubacterial 16S rRNA genes were also quantified using the method recommended by López-Gutiérrez et al.[38] Samples and standards were analyzed in duplicate, and melt curve analysis and agarose gel electrophoresis were used to verify the specificity of amplification. Further information about qPCR and qPCR thermocycling conditions can be found in Tables S4 and S5 in the Supporting Information.

2.5. MinION 16S Amplicon Sequencing and Data Analysis. For high-resolution microbial community profiling, 16S rRNA genes were sequenced using Oxford MinION (Oxford Nanopore Technologies, Oxford, UK). The 16S rRNA genes were amplified using the primers 27F 5′-AGAGTTTGATCCTGGCTCAG-3′ and 1492R 5′-GGTTACCTTGTTACGACTT-3′, and the amplicons were cleaned up using the DNA clean & concentrator-5 kit (Zymo Research, Irvine, CA) and barcoded using a 16S barcoding kit (Oxford Nanopore Technologies, Oxford, UK) following the manufacturer's protocol. Barcoded PCR products were pooled and cleaned up using Mag-Bind TotalPure NGS beads (Omega Bio-Tek, Norcross, GA) and sequenced using the Oxford MinION.

The MinION software (Release 22.05.5) performed base calling and demultiplexed the barcoded library into individual sample folders of .fastq-formatted files. The .fastq files were trimmed with quality and length cutoffs of 7 and 400 bp, respectively. Then, the .fastq files were converted to the fasta format. The sequencing reads in the .fasta file were annotated using blastn against the MiDAs 4 database.[39,40] The NCBI taxonomy identification number (TaxID) of the top BLAST hit (BLAST+ 2.9.0) was assigned to each sequencing read, and the frequencies of TaxIDs were used to compute microbial community profiles. Sequencing data are available at NCBI accession PRJNA1049977.

2.6. Identification of the Terminal Oxidases with High-Affinity Oxygen Using Public Database. The inventories of the genes encoding cytochrome oxidases (cbb_3-type and bd_1-type), putatively involved in high-affinity oxygen reduction, was examined in comammox *Nitrospira* genome and metagenome-assembled genomes (MAGs) available in the database. Genomes and MAGs assigned to *Nitrospira* spp. were downloaded from the NCBI GenBank database (accessed October 7th, 2023). The quality of each MAG was assessed using CheckM v1.2.2 with default option, and those with completeness >90% and contamination (<5%) were retained for downstream analyses.[41] For each qualified *Nitrospira* genome, gene calling was performed using Prodigal

https://doi.org/10.1021/acsestwater.3c00715
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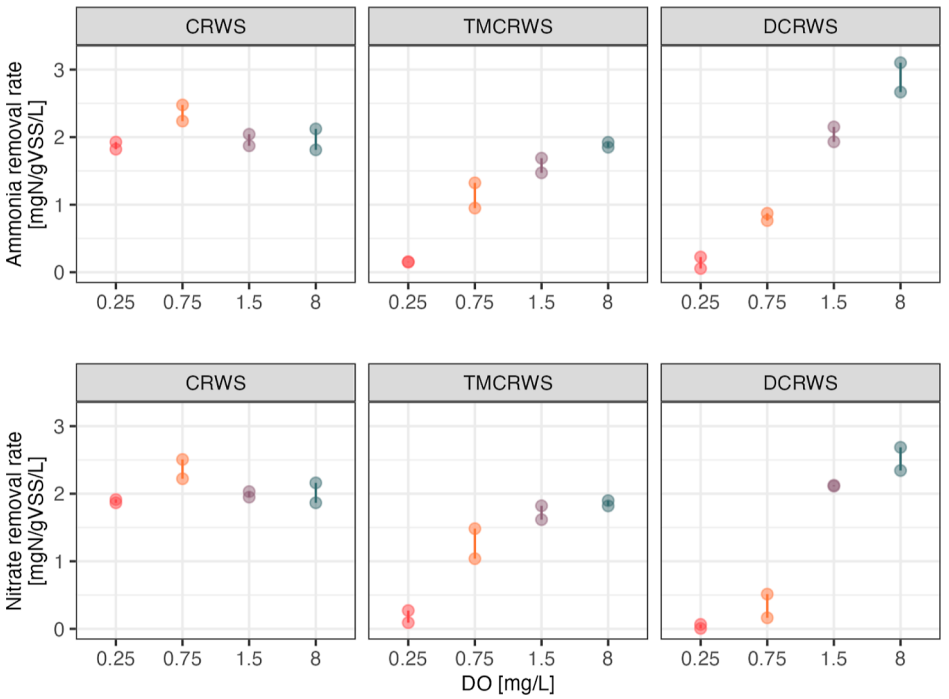

Figure 2. Comparison of CRWS, TMCRWS, and DCRWS, (a) NH_4^+ removal rates and (b) NO_3^- production rates at the different DO concentrations (0.25, 0.75, 1.5, and 8.0 mg L^{-1}). Duplicate points for each DO condition represent the test replicate.

v2.6.3[42], and the predicted gene-coding sequences were submitted to GhostKOALA server for assignment to KEGG orthology groups.[43] The genomes with a complete inventory of the KEGG module M00804 were considered as those of comammox *Nitrospira*. The inventories of high-oxygen-affinity terminal oxidase genes *ccoN* (K00404), *ccoO* (K00405), *ccoNO* (K15862), *cydA* (K00425), and *cydB* (K00426) were examined in these genomes. The comammox genomes were classified into clade A and B based on the phylogeny of their translated AmoA sequences.[32] Sequences were aligned using L-INS-I algorithm implemented in MAFFT v7.520[44], and the alignment was refined using trimAl v1.4.1.[45] A maximum-likelihood tree (LG + G4 + F model) was constructed by using RaxML-NG v1.2.0. The strains that clustered with *N. inopinata* were classified as clade A and those that clustered with *Nitrospira* sp. CG24A as clade B.[46]

3. RESULTS AND DISCUSSION

3.1. Nitrogen Transformation Rates at the Three WRRFs. Duplicate bench scale tests were conducted in parallel for each facility. These tests were designed to evaluate the removal of NH_4^+, formation of NO_3^-, and accumulation of NO_2^-. Nitrogen transformation was impacted by different DO concentrations in all WRRFs; a typical profile of NH_4^+ oxidation can be found in Figure 1. Upon incubation at 0.25 mg DO L^{-1}, the CRWS biomass was capable of fully oxidizing 15 mg NH_4–N L^{-1} within 2 h, while the TMCRWS and DCRWS biomass removed only 1–2 mg NH_4–N L^{-1} in the first 2 h (Figure 1a,d,h). The absence of NH_4^+ oxidation activity in the DCRWS biomass was also supported by the near absence of the NO_3^- accumulation. The trends were similar to the 0.75 mg DO L^{-1} condition (Figure 1b,e,i). CRWS biomass removed complete NH_4^+ removal within 2 h, with stoichiometric NO_3^- production, while TMCRWS and DCRWS biomass showed an improvement for NH_4^+ removal as compared to the 0.25 mg DO L^{-1} condition but did not achieve full removal within the 2 h testing. Interestingly, the TMCRWS sample showed a more significant drop in NH_4^+ with over 10 NH_4–N L^{-1} removed, while the DCRWS sample showed only a 5 NH_4–N L^{-1} drop.

To capture the nitrogen transformation rates occurring in all tests, the biomass-normalized NH_4^+ removal and NO_3^- production rates were calculated as discussed in Section 2.2; these rates are shown in Figure 2 (volumetric nitrification rates are shown in Figure S6 in the Supporting Information). In addition to the 0.25, 0.75, and 1.5 mg DO L^{-1} conditions, a nonrate limiting condition of 8 mg DO L^{-1} was used to confirm the maximum NH_4^+ removal rate (batch tests profiles for nonrate limiting condition can be found in Figure S5 in the Supporting Information). The NH_4^+ removal rates for TMCRWS and DCRWS showed similar trends with increasing removal at increased DO concentrations. One discrepancy of note between the two samples is that while TMCRWS biomass NH_4^+ oxidation peaked at 1.5 mg of DO L^{-1}, DCRWS was still able to achieve a higher rate at a higher DO concentration (e.g., 8 mg of DO L^{-1}). In contrast, the CRWS sample reached a near-maximum NH_4^+ oxidation rate at the lowest DO concentration (e.g., 0.25 mg DO L^{-1}) and did not change significantly at higher DO concentrations. Similar trends can be observed for the NO_3^- production, as shown in Figure 2, where both TMCRWS and DCRWS showed a clear correlation between increasing DO and NO_3^- production, while for CRWS, the maximum NO_3^- production rate was already achieved at the lowest DO concentration (e.g., 0.25 mg DO L^{-1}).

Important operational outcomes can be deduced from the comparison of the NH_4^+ removal rates in all three WRRFs. The results suggest that the increase of DO would not exert a similar outcome in all the WRRFs. The unique response of CRWS activated sludge probably owes to its unique properties of the microbial community, which will be further discussed in

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ACS EST Water 2024, 4, 707–718

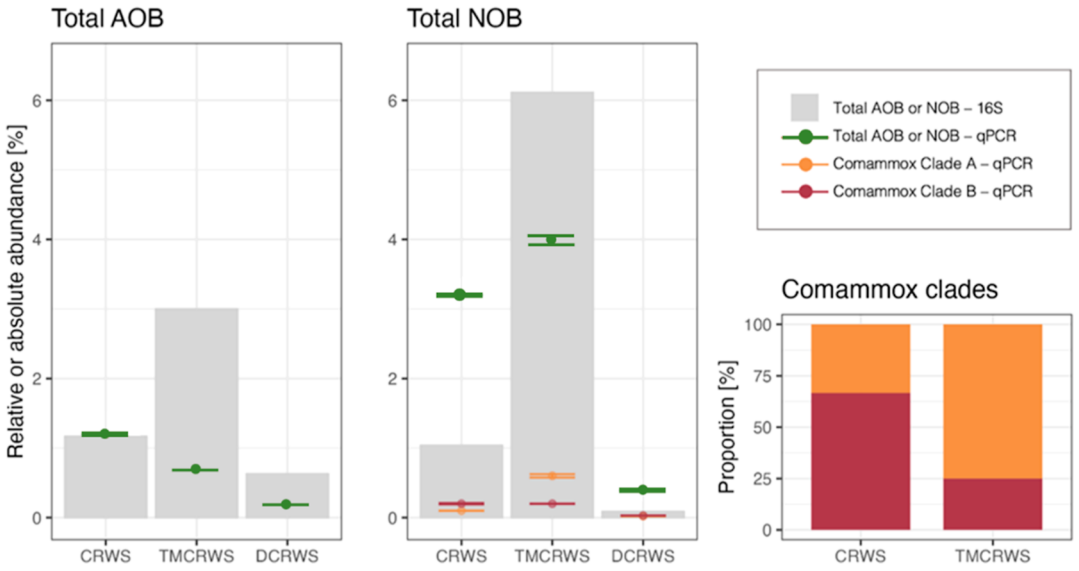


Figure 3. MinION 16S total AOB and total NOB percentage of reads (gray shade); total AOB and total NOB absolute abundance, normalized to the total bacterial community (green); and comammox clade A and clade B absolute abundance, normalized to the total bacterial community (orange and red, respectively) for biomass from the CRWS, TMCRWS, and DCRWS facility.

Section 3.2. Increasing the DO in CRWS would not result in an increased NH_4^+ removal rate, while it shows that the current average DO concentration for TMCRWS (5.0 mg L^{-1}) is higher than what is required to achieve a near-maximum NH_4^+ removal rate. Figure 2a shows that 1.5 mg DO L^{-1} assures 85% of the NH_4^+ removal rate achieved at nonrate limiting DO (e.g., 8.0 mg DO L^{-1}). DCRWS, at 1.5 mg DO L^{-1}, instead achieves 70% of the NH_4^+ removal rate achieved at nonrate limiting DO. While not a distinct removal difference, it is possible that different aeration strategies (Table 1 in Section 2.1) may have led to the development of nitrifier populations with different affinity to oxygen (Section 3.2).

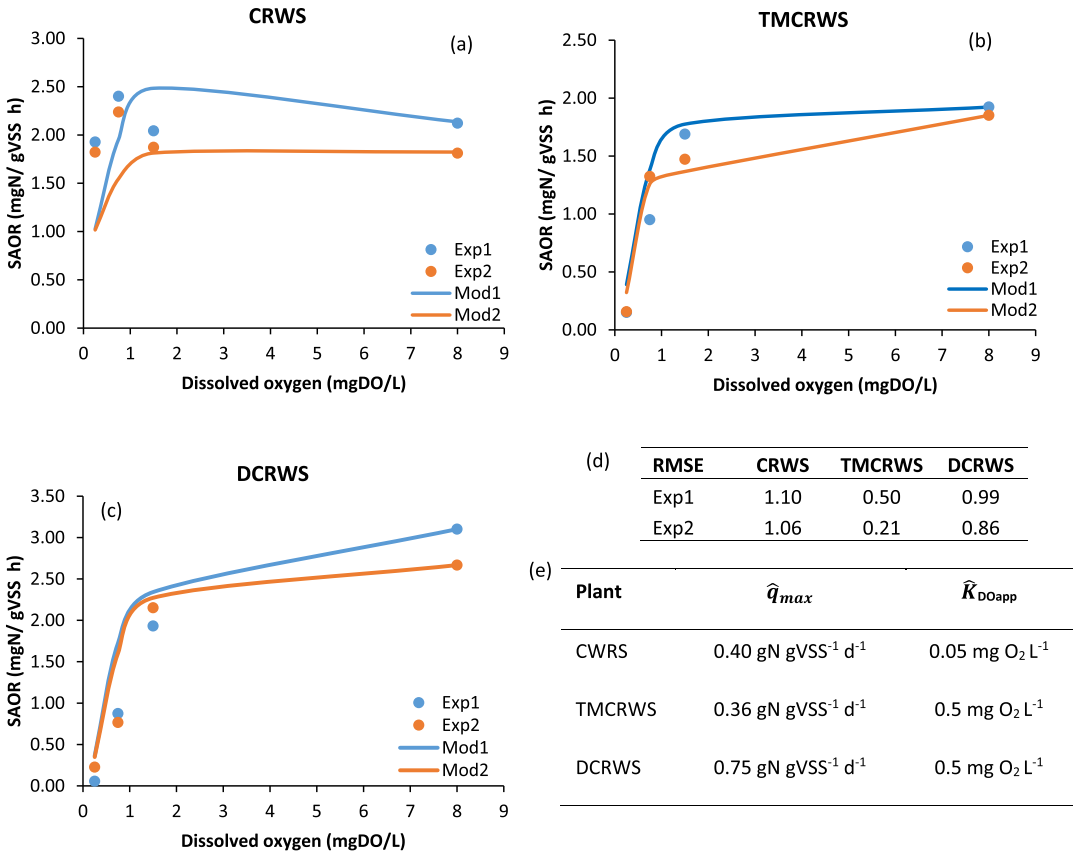
3.2. Overall Microbial Community Trends. The microbial communities of the three different biomasses used for the bench scale testing were characterized via long-read sequencing of 16S rRNA amplicons using a Nanopore sequencer (Figure S10 in the Supporting Information, Figure 3a,b). The MinION technology was used here solely to identify large trends and potential dominant bacterial guilds. The trends were further confirmed through qPCR for quantitative analysis (Figure 3a,b).

Overall, the microbial community in each facility was dominated by the following taxa: *Candidatus Accumulibacter* (4.3%), *Candidatus Competibacter* (11.9−18.7%), *Defluviicoccus* (4.2%), *Nitrospira* (6.1%), *Comamonadaceae* (3.2−4.8%), and *Streptococcus* (2.3%). The community composition was influenced by some of the operational parameters presented in Table 1 (Section 2.1). For instance, CRWS and DCRWS both conduct biological phosphorus removal, and their communities contain players involved in phosphorus and glycogen accumulation (Figure S10 in the Supporting Information). *Candidatus Accumulibacter* (4.3% of total reads in CRWS) is a polyphosphate-accumulating organism (PAO) that plays a crucial role in enhanced biological phosphorus removal (EBPR) in WRRFs that use carbon, commonly in the form of volatile fatty acids (VFAs) to perform phosphorus cycling.[47] *Candidatus Competibacter* (18.7% of total reads in CRWS) is a glycogen-accumulating organism (GAO) and is considered a competitor for carbon during EBPR because it consumes VFAs but does not take up phosphorus. However, recent findings have shown that *Candidatus Accumulibacter* and *Candidatus Competibacter* can coexist without impacts on overall process performance.[48] The presence of *Denitratisoma* (1% of total reads in CRWS) also confirms the presence of bacteria involved in denitrification or partial denitrification; these heterotrophic bacteria were previously confirmed to directly convert NO_2^- to N_2.[49] The occurrence of denitrifying bacteria could be explained by the low DO operation of CRWS and by the possibility of the activated sludge flocs performing simultaneous nitrification and denitrification (SND). Low DO and the presence of sludge flocs can allow the formation of anoxic inner portions where denitrification can occur.[50]

EBPR also influenced the composition of the DCRWS biomass, as shown in Figure S10 in the Supporting Information. The GAO population included *Candidatus Competibacter* (11.9% of total reads in DCRWS) and *Defluviicoccus* (1.2% of total reads), and the PAO population included also *Terrimonas* spp. (1.2% of total reads in DCRWS) and *Tetrasphaera* (0.95% of total reads in DCRWS). Although the role of *Terrimonas* in phosphorus cycling is still being studied, the presence of *Tetrasphaera* is significant. These bacteria are capable of fermentation to produce VFAs and assimilation of complex organics as intracellular storage anaerobically rather than only relying on VFAs as carbon sources.[51]

As TMCRWS does not perform EBPR, the community composition of the TMCRWS facility did not include major phosphorus cycling microorganisms. However, microorganisms that play a role in carbon cycling, such as *Candidatus Competibacter* (1.7% of total reads in TMCRWS) and *Defluviicoccus* (4.2% of total reads in TMCRWS), were found in TMCRWS biomass. Additionally, it is noteworthy that there were heterotrophic microorganisms present in the biomass that are involved in the nitrogen cycle. For example, *Haliangium* and *Comamonas* (2.4 and 2.5% of the total reads in TCMCRWS, respectively) are aerobic heterotrophic capable of NO_2^- reduction,[52,53] while members of the family

https://doi.org/10.1021/acsestwater.3c00715
ACS EST Water 2024, 4, 707−718



(d)

RMSE	CRWS	TMCRWS	DCRWS
Exp1	1.10	0.50	0.99
Exp2	1.06	0.21	0.86

(e)

Plant	$\hat{q}_{max}$	$\hat{K}_{DOapp}$
CWRS	0.40 gN $gVSS^{-1}$ d^{-1}	0.05 mg O_2 L^{-1}
TMCRWS	0.36 gN $gVSS^{-1}$ d^{-1}	0.5 mg O_2 L^{-1}
DCRWS	0.75 gN $gVSS^{-1}$ d^{-1}	0.5 mg O_2 L^{-1}

Figure 4. Batch and modeling (data fitting) results of SAOR for biomass from (a) CRWS, (b) TMCRWS, and (C) DCRWS at different DO concentrations and (d) a table with the RMSE of each model vs their experimental data points. A lower RMSE value indicates a better model prediction of the observed experimental data. The table shows a summary of the apparent parameters for the composite samples from the three WRRFs.

Gemmataceae (1.25% of the total reads in TCMCRWS) can be N_2O-reducing organisms.[54]

The sum of the relative abundances of the putative AOB (*Nitrosomonas* and *Nitrosospira*, etc.) and NOB taxa (*Nitrospira* and *Nitrotoga*) were estimated from the microbial composition profiles (Figure 3a,b). Of the three activated sludge microbiomes, TMCRWS had the highest relative abundances of putative AOB and NOB taxa (3 and 6% of total reads in TMCRWS, respectively). Interestingly, the putative NOB taxa were nearly absent in DCRWS. To further validate these trends, qPCR targeting total AOB and total NOB was performed on the biomass samples, and the results are shown in Figure 3a,b. The results of qPCR analysis were, in general, consistent with the trends observed with the 16S rRNA-based community profiling. Particularly, NOB had 3−4-fold higher absolute abundance than AOB, and the highest abundance of NOB was found in the CRWS and TMCRWS samples.

For quantification of the commamox *Nitrospira* population, the activated sludge samples were also subjected to qPCR assays specifically targeting the *amoA* genes in comammox clade A and clade B (Figure 3b). The total comammox *amoA* copy number was at least 3-fold larger in the TMCRWS sample than the CRWS and DCRWS samples, and interestingly, the relative abundance of clade A comammox *amoA* (0.6% of 16S rRNA gene counts) was higher than clade B comammox *amoA* (0.2%). The CRWS sample contained a more balanced comammox *amoA* population, with clade B being more abundant than clade A (0.2% of the total bacteria). Comammox clades were found near detection limits in the DCRWS activated sludge; this may relate to the poor NH_4^+ oxidation performance under low DO conditions. The coexistence of clade A and clade B is not surprising as this has also been shown in rapid gravity sand filter, WRRFs, sequencing batch reactors (SBR), and soil.[25,32,55,56]

In this study, it was found that comammox *Nitrospira* coexisted with AOB. Koch et al.[57] suggested that comammox *Nitrospira* are rarely the only nitrifying guild present in a habitat and that the co-occurrence of comammox and canonical ammonia oxidizers indicates a potential function-based differentiation between the two microbial groups. However, while niche separation factors for AOA and AOB are known (e.g., mixotrophy, ammonia affinities, and different pH), those for comammox and canonical ammonia oxidizers are not.[58−61]

3.3. Kinetic Parameter Estimation Via Experimental Data Fitting. The experimental data obtained during the bench scale experiments were used for parameter estimation via AQUASIM modeling and the parameter estimation function[35,36] (Figure 4a−e). Kinetic parameters were determined for the biomass samples from each facility (CRWS, TMCRWS and DCRWS).

The apparent half-saturation constant for DO, $\hat{K}_{DOapp}$, and the maximum substrate utilization rate, $\hat{q}_{max}$, were estimated for CRWS, TMCRWS, and DCRWS (Figure 4a−c) biomass samples (table in Figure 4e). CWRS and TMCRWS (Figure 4a,b, respectively) both exhibited a higher abundance of comammox bacteria, and a lower maximum utilization rate was

https://doi.org/10.1021/acsestwater.3c00715
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estimated. This is an important consideration when developing a minimum SRT for systems with nitrifying populations with high comammox abundance. A second important observation worth noting is the low $\hat{K}_{DOapp}$ for the CRWS biomass (table in Figure 4e). This low DO facility has shown an adaptation to the lower DO concentrations, and a very low $\hat{K}_{DOapp}$ of 0.05 mg L^{-1} was estimated. As shown in Section 3.2 this adaptation is also confirmed by the enrichment in comammox *Nitrospira*. Additionally, research by Camejo et al.[26] showed a high degree of comammox *Nitrospira* enrichment in a bioreactor system inoculated with activated sludge and operated under low DO concentrations, indicating a competitive advantage of comammox *Nitrospira* over canonical ammonia oxidizers under microaerophilic conditions.

When the modeling fit is examined more closely, the actual $\hat{K}_{DOapp}$ may even be substantially lower than this calculated value, as the nitrification rate did not show a significant decrease at the DO concentration of 0.25 mg L^{-1} from that measured at the saturating DO concentration ($p < 0.05$). Practically speaking, measuring the DO concentration below 0.25 mg L^{-1} in a full-scale activated sludge system is not achievable with current sensor technology, and therefore, no decrease in the nitrification rate would be anticipated in an activated sludge system controlled with DO measurement if the ecology is given sufficient time to adapt.

3.4. Insights into Low Dissolved Oxygen Operation Through Experimental, Molecular, and Modeling Findings. The batch tests in Section 3.1 showed a distinct behavior of the three WRRFs with the different DO concentrations. It has been clearly shown that DO is a controlling factor in nitrogen removal systems, particularly secondary treatment processes involving nitrification.[25,62] The CRWS facility was characterized by an effective low-DO operation, while the other two WRRFs (e.g., TMCRWS and DCRWS) required higher DO concentrations to perform properly and carry out partial or complete NH_4^+ removal. Possibly, the key to the effectiveness of low-DO operation may lie with the comammox population found in the CRWS activated sludge. In both CRWS and TMCRWS plants, NOB (including comammox *Nitrospira*) abundance was, in fact, found to outnumber that of AOB. Such comammox abundance is not surprising, as Roots et al.[25] and Shao and Wu[63] showed that comammox *Nitrospira* can be relatively active and can become the dominant ammonia oxidizers in biological nitrogen removal processes, especially those operated at low DO concentrations. An interesting point to note, however, was the clade A-versus-clade B composition of the comammox *Nitrospira amoA* in these plants (Section 3.2). A survey of 43 genomes (36 clade A and 7 clade B) of comammox *Nitrospira* identified a consistent genomic feature regarding the genes involved in high-affinity oxygen utilization. The gene encoding one of the cytochrome bd_1 oxidase subunits, *cydB*, was found in the genomes of clade A comammox *Nitrospira* but not in those of clade B (Figure S11 in the Supporting Information). As both CydA and CydB subunits are required for the insertion of heme-d_{631} and thus formation of a functional cytochrome bd_1 oxidase, the clade A and clade B comammox *Nitrospira* may differ in their capability to grow under low-DO environments.[64] The qPCR-based observation was somewhat contradictory to these genome survey results, in that the clade A abundance was more conspicuous in TMCRWS than in CRWS. This discrepancy may suggest that the DO concentration at CRWS (~0.25 mg of DO L^{-1}) was not sufficiently low to select for the comammox *Nitrospira* equipped with a functional cytochrome bd_1. Therefore, it may be hasty to conclude that only comammox *Nitrospira* was responsible for the distinguished ability of the CRWS biomass to oxidize NH_4^+ under the low-DO concentrations.

One additional factor shaping the community in the WRRFs studies is SRT. Recent studies have shown that SRT is a key component in the selection for comammox *Nitrospira*-based populations. Cotto et al.[28] showed that, regardless of the process configuration, comammox was prevalent in high SRT systems, including attached growth systems. In the case of CRWS, the relatively long SRT of 10−12 days and the low DO operation achieved through the ABAC system facilitated the selection of comammox *Nitrospira*-based populations. Sensor-based control strategies, such as ABAC and ammonia vs NOx (AvN) control, are gaining popularity in the wastewater treatment field. These strategies regulate airflows to achieve an effluent ammonia set point (ABAC) or an ammonia/(nitrate + nitrite) or (nitrate + nitrite)/ammonia set point (AvN).[65] Using ABAC offers two additional benefits: first, it has the potential to restrict aeration during periods with low NH_4^+ effluent, which may limit complete nitrification and second, it allows to increase aeration intensity to limit NH_4^+ peaks during peak load. Despite efforts to implement advanced control strategies in WRRFs, their global application remains limited. This is due to the high complexity of the process and the substantial capital investment required.[66] Additionally, there is a certain level of skepticism toward optimization of aeration in activated sludge processes; this stems from the sensitivity of effluent quality to aeration and the need to protect against permit violations. In the absence of a clear understanding of the relationship between process conditions and the required aeration rate, to avoid the effects of upstream disturbances (e.g., weather events, increased loads, etc.), it is often preferred to maintain total aeration rates higher than necessary.[67] Finally, CRWS demonstrated healthy sludge volume indices with an average of 55 mL g^{-1}. The gradual reduction of DO over time allowed the nitrifying population to adapt to lowered DO conditions and increase their affinity for DO, contributing to the establishment of the low-DO community.[68]

While TMCRWS instead was operated at a rather high DO (5.0 mg L^{-1}), its high SRT (12−14 days) might have had an impact on the selection of comammox *Nitrospira*-based populations. This is also supported by a recent study from Zhao et al.[69] that reported the dominance of comammox *Nitrospira* in a moving bed biofilm reactor (MBBR) fed with synthetic mainstream wastewater and operated at a DO concentration above 6 mg L^{-1}. Additionally, like in our study, different bacteria from clade A comammox *Nitrospira* were identified as key players in the biofilm of the MBBR. Finally, DCRWS was not select for comammox *Nitrospira*-populations; this was likely due to a short SRT operation of the WRRF (8.5 days).

The $\hat{K}_{DOapp}$ found at TMCRWS was estimated to be around 0.5 mg O_2 L^{-1}, which is higher than the ones reported in literature.[70] In the same study Park et al.[70] suggested that a combination of NO_2^--limited environments and long SRTs can serve as favorable conditions for the growth of comammox *Nitrospira*. One hypothesis is that the potential selective pressure for the nitrifying population with comammox at TMCRWS can be also due to a high SRT e.g., 12−14 days, but the comammox species found might not be adapted to low DO operation or might not have the metabolic capability to switch

https://doi.org/10.1021/acsestwater.3c00715
ACS EST Water 2024, 4, 707−718

to low-DO nitrification (as shown in the bench scale tests at lower DO). Furthermore, as suggested by Cotto et al.,[71] one additional reason for the temporal persistence of comammox in some WRRFs might be due to intrapopulation level diversity (at the strain level rather than species level). Other studies have confirmed that microdiversity within populations allow them to adapt to changing conditions.[72] This could possibly favor the persistence of comammox strains with higher $\hat{K}_{Doapp}$ values. While DCRWS showed the same $\hat{K}_{Doapp}$ found at TMCRWS, the plant was characterized by a rather short SRT (8.5 days) and the community comprised mainly AOB and NOB with very little comammox. Therefore, DCRWS $\hat{K}_{Doapp}$ might rather be attributed to conventional AOB. The study of the comammox presence along with activity at higher DO concentrations warrants further research.

4. CONCLUSIONS

In this study, the effects of different DO concentrations were tested on the microbial communities of three operationally diverse WRRFs through bench scale testing. One of the key takeaways from this work is the apparent resiliency of microbial populations adapted to low DO environments and their ability to maintain high nitrification rates across a range of DO concentrations. These results show that not only does the facility operating low DO full scale select for a population adapted to these conditions but the microbial population also has the ability to exert high nitrification rates at high DO concentrations. Additionally, long SRT (>10 days) operation can favor the persistence of comammox population adapted to different DO concentrations.

Sequencing of 16S showed that NOB were prevalent in the low DO-operated facility, while an unexpected higher presence of NOB (6% of the total reads) was found at the higher DO-operated facility. Further molecular analyses via qPCR showed that comammox clades A and B (0.1 and 0.2%, respectively) were enriched in the low DO facility, while clade A (0.6%) was the dominant one in the higher DO facility. Data from the bench scale tests were utilized for modeling and parameter estimation. The analysis revealed that two WRRFs had a "low DO" and a "higher DO" nitrifying population with comammox, with $\hat{K}_{Doapp}$ of 0.05 and 0.5 mg L^{-1}, respectively. These findings provide evidence that comammox could potentially have intrapopulation diversity within clade A and clade B leading to diverse kinetic behavior and affinity for oxygen. Future research would help expand our understanding of low-DO-adapted nitrifying communities. For instance, long-term continuously fed batch studies can help track changes in microbial communities over time and gain a better understanding of their response to varying DO concentrations.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsestwater.3c00715.

Flow diagram of liquid and solid phases for CRWS, TMCRWS, and DCRWS at Trinity River Authority; experimental setup for testing; profiles of nitrification batch tests; volumetric nitrification rates; sensitivity analysis on the parameters $\hat{q}_{app}$ and $\hat{K}_{DOapp}$ for biomass from CRWS, TMCRWS, and DCRWS; top 25 genera in the CRWS, TMCRWS, and DCRWS biomass; maximum-likelihood phylogenetic tree; CRWS-optimized ABAC DO set points; list of qPCR primers; and list of thermocycling conditions for the qPCR primers (PDF)

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CRediT: **Fabrizio Sabba** conceptualization, data curation, formal analysis, investigation, validation, visualization, writing-original draft, writing-review & editing; **Eric Redmond** conceptualization, data curation, formal analysis, supervision; **Caitlin Ruff** data curation, investigation, methodology; **Monica Ramirez** data curation, methodology, validation; **Mike Young** funding acquisition, investigation, project administration; **Min Joon Song** formal analysis, investigation, methodology; **Sukhwan Yoon** formal analysis, investigation, writing-review & editing; **Leon Downing** project administration, supervision, visualization, writing-original draft, writing-review & editing.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We would like to thank Theo Chan, Raudel Juarez, and operations with the Trinity River Authority for onsite testing facilities and assistance. Additionally, we extend our thanks to McKenna Farmer (Northwestern University) for the fruitful discussion and help with data analysis, as well as to Paul Campbell from Aster Bio, Inc. (Houston, TX) for his help with MinION and qPCR sequencing analyses.

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