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High dissolved oxygen selection against *Nitrospira* sublineage I in full-scale activated sludge

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ABSTRACT

A single *Nitrospira* sublineage I OTU was found to perform nitrite oxidation in full-scale domestic wastewater treatment plants (WWTPs) in the tropics. This taxon had an apparent oxygen affinity constant lower than that of the full-scale domestic activated sludge cohabitating ammonium oxidizing bacteria (AOB) (0.09 ± 0.02 g O₂ m⁻³ versus 0.3 ± 0.03 g O₂ m⁻³). Thus, nitrite oxidizing bacteria (NOB) may in fact thrive under conditions of low oxygen supply. Low dissolved oxygen (DO) conditions selected for and high aeration inhibited the NOB in a long-term lab-scale reactor. The relative abundance of *Nitrospira* sublineage I gradually decreased with increasing DO until it was washed out. Nitrification was sustained even after the DO was lowered subsequently. The morphologies of AOB and NOB microcolonies responded to DO levels in accordance with their oxygen affinities. NOB formed densely packed spherical clusters with a low surface area-to-volume ratio compared to the *Nitrosomonas*-like AOB clusters, which maintained a porous and non-spherical morphology. In conclusion, the effect of oxygen on AOB/NOB population dynamics depends on which OTU predominates given that oxygen affinities are species-specific, and this should be elucidated when devising operating strategies to achieve mainstream partial nitrification.

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1. INTRODUCTION

Understanding interactions between ammonia oxidizing bacteria (AOB) and nitrite oxidizing bacteria (NOB) is crucial for optimizing biological nitrogen removal (BNR) wastewater treatment design and operation. AOB and NOB typically cooperate to oxidise ammonium to nitrate via nitrite. This is then followed by heterotrophic nitrate reduction to dinitrogen gas ¹. If nitrite accumulates, anaerobic ammonium oxidation (Anammox) to dinitrogen gas can occur to completely remove nitrogen with less energy ^{2, 3}. This requires stable partial nitrification, which can only be achieved by eliminating NOB. Partial nitrification-Anammox can be accomplished relatively simply in full-scale systems treating high strength wastewaters. High ammonium concentrations coupled with varying nitrogen loadings and aeration regimes, and the right dissolved oxygen (DO) and pH set points, selectively promote AOB growth and inhibit NOB ^{4, 5}. Achieving partial nitrification is more challenging with domestic wastewaters, however, due to significantly lower ammonium loads ⁶ where the same controls for inhibiting NOB may not be so effective. Further work is therefore required to elucidate inhibition strategies for NOB at lower ammonium concentrations.

Implementing NOB inhibition strategies is confounded by phylogenetic variability among AOB and NOB. Members of the genus *Nitrosomonas* are among the most frequently detected AOB in wastewater treatment plants (WWTPs) ⁷, but different treatment plants can be dominated by single or multiple *Nitrosomonas*-like AOB populations ^{8, 9}. For NOB, *Nitrospira* and *Nitrobacter* have been reported to co-exist in WWTPs where nitrite concentrations fluctuate within operational cycles, which allows for niche differentiation ⁹⁻¹¹. While most *Nitrospira* in domestic WWTPs are associated with sub-lineages I and II, functional differences have been reported for even closely related sub-lineages with regards to nitrite and DO concentration preferences and

their spatial co-aggregation with AOB¹²⁻¹⁴. In addition, differences in the AOB to NOB ratio can significantly impact process performance¹⁵. Optimum operational strategies for achieving partial nitritation may in fact depend on the identities of the AOB and NOB populations present, and could therefore be system specific.

Elucidating NOB control strategies is further confounded by uncertainty over the relative half saturation constants (K_s) and affinities for oxygen (maximum specific growth rate per half-saturation constant, $\mu_{\max}/K_s$), which are key factors driving competition dynamics between AOB and NOB¹⁶. In most full-scale partial-nitritation installations treating high-strength wastewater, low oxygen set points of less than 0.35 mg O₂/L are used to curb NOB activity⁴, which is consistent with the prevailing belief that AOB have a higher oxygen affinity (i.e. lower K_s for oxygen) than NOB^{17, 18}. However, while low oxygen selection has also been demonstrated in a lab-scale system treating domestic wastewater¹⁹, several studies based on activity tests suggest that the K_s of AOB may not always be lower than that of NOB^{5, 20, 21}.

One possible explanation for these inconsistencies regarding the relative oxygen affinities of NOB and AOB is that it depends on which NOB are dominant. The genus *Nitrospira* that predominates under mainstream conditions generally has a higher affinity for oxygen and nitrite than does *Nitrobacter*, which prevails in high-strength systems^{5, 22}. This explanation was questioned by Picioroanu *et al.*²³, however, who showed by 3D modelling that the reversal of K_s (i.e. NOB lower than AOB) is an artifact of the generally larger average cluster sizes of AOB compared to NOB coupled with the higher oxygen consumption per unit of biomass produced for AOB relative to NOB. DO is consumed more quickly in AOB clusters, resulting in DO limitation and lower measured activity. Thus, colony morphology needs to be considered when resolving the factors that contribute to oxygen competition between AOB and NOB.

The aim of this study was, therefore, to assess the extent to which phylogenetic variability and colony morphology contribute to AOB/NOB oxygen competition, under tropical conditions with operating temperatures between 29 and 31 °C. Deconvoluting the contributions of genus type and microcolony morphology to K_s was achieved by combining (1) AOB and NOB microbial community characterisation in activated sludge with (2) determining their respective apparent oxygen half saturation constant, $K_{s(app)}$ and (3) describing the physical properties of AOB and NOB clusters using 3D-fluorescence *in situ* hybridization (FISH) and image analysis. In addition, the effect of varying oxygenation levels on AOB and NOB activity was also investigated in a long term lab-scale reactor. The findings suggest that cluster shape and size are a consequence of rather than the cause of different $K_{s(app)}$. This informs competition dynamics between two key players in nitrification and can lead to improved DO control strategies for achieving nitrification for more cost effective wastewater treatment.

2. MATERIALS AND METHODS

2.1 Sampling of activated sludge from major WWTPs treating domestic wastewater.

Activated sludge was sampled from three main WWTPs (Plants 1, 2 and 3) collectively treating 90% of the total domestic wastewater loading in Singapore. Three sampling events were carried out for each plant between December 2016 and February 2017. On each sampling event, liquid samples were collected from the influent and effluent of the activated sludge treatment unit for nutrient analyses. Samples for DNA extraction and FISH were collected from the mid aerobic zone. Samples for DNA extraction were snap-frozen in liquid nitrogen and stored at -80 °C until extraction. Samples for FISH were immediately fixed in 4% paraformaldehyde and were washed twice with 1% phosphate-buffered saline solution after 2 h and stored at -20 °C in a 50:50 mixture of 1% phosphate-buffered saline solution and 100% ethanol.

2.2 Long-term lab-scale reactor start-up and operation. A sequencing batch reactor (SBR) with a working volume of 4 L was seeded in June 2016 with returned activated sludge (RAS) from Plant 3 that receives domestic wastewater at a rate of 800,000 m³/day. The SBR operation mode was chosen to ensure uniform mixing at each tested DO concentration. Wastewater was collected from the plant once a week and stored in a chiller at 4 °C as feed for the SBR. The nutrient content of every wastewater batch was analysed and is summarised in Table S1 (Supporting Information, SI). The SBR was operated to achieve partial nitrification mode (50% conversion of ammonium to nitrite, with the remaining ammonium unconverted) for the first 150 days of operation and switched to nitrification (complete conversion of ammonium to nitrite) mode thereafter. Anoxic phases for denitrification, whereby aeration was switched off, were also included in the SBR operation to exhaust the organics in the wastewater and to restore the alkalinity in the reactor. Therefore, pH was not specifically controlled in the reactor but stabilised between 6.3 and 7.0. The anoxic phase precedes the aerobic phase followed by settling and decant. Therefore, effluent nutrient content captures the performance of the nitrification activity in the reactor. The effect of DO concentration on nitrite accumulation was investigated by varying the DO setpoint between 0.5 and 5.5 mg O₂/L. In addition, the effect on the length of aeration period (between 14 and 60 min) on the AOB and NOB activities were also investigated while maintaining the overall wastewater loading rate with a hydraulic retention time (HRT) of 10.7 h. The SBR temperature was maintained at 31.0 ± 0.5 °C, typical temperature of domestic wastewater in Singapore. DO and pH were continuously monitored using Mettler Toledo-InPro 3250i pH and DO sensors, respectively. The solid retention time (SRT) was not controlled for the first 160 days of reactor operation and was estimated to be 14.4 ± 2.0 days due to the inevitable sludge loss with the effluent but subsequently controlled at approximately 8 days. When stable partial nitrification was achieved,

the SBR was switched to nitrification mode from day 151 onwards by increasing the length of the aerobic phase to investigate whether NOB will recur with extended aeration. Each operational change in DO set point, aeration cycle length and solid retention time was implemented individually and separated as different experimental phases (I to X), summarised in Table 2.

2.3 Batch activity experiments & analytical procedures. Three types of activity batch tests were conducted using Plant 3 RAS: (1) AOB activity test, (2) NOB activity test and (3) simultaneous AOB and NOB activity tests. Given that AOB and NOB microcolonies are found to cluster in close proximity to one another the simultaneous AOB and NOB activity tests are important to elucidate how oxygen consumption would impact oxygen availability and hence each other's activity²³. All three activity tests were conducted at DO concentrations of 0.5 ± 0.1 , 1.5 ± 0.1 , 2.5 ± 0.1 , 5.5 ± 0.1 and 6.5 ± 0.1 mg O₂/L. Additional DO concentrations of 0.2 ± 0.1 and 0.3 ± 0.1 mg O₂/L were used for type (2) and (3) batch tests, respectively, since maximum NOB activity was attained at a lower DO concentration compared to AOB. In type (3) batch tests, nitrite oxidation rates were determined based on the total consumption of nitrite externally supplied and of that produced from ammonium oxidation. DO concentration was manually controlled using a gas mixture of N₂ and air. Fresh sludge was collected every 48 h and diluted with effluent from the lab-scale SBR at a 1:1 ratio before each batch experiment. When steady nitrification activity had been achieved in the lab-scale SBR, sludge was also collected to perform AOB activity batch tests at the aforementioned DO concentrations. Given that NOB was washed out from the system, NOB activity was not detected. Ammonium and/or nitrite was added at the start of each activity test to initial concentrations of 25 mg N/L and 15 mg N/L, respectively. All activity tests were conducted in triplicate and the pH controlled at 7.0 ± 0.1 . Nutrient samples were collected every 10-20 min and filtered immediately with 0.2 µm filters.

Ammonium and COD were measured using Hach® kits whereas nitrate, nitrite and orthophosphate were analysed using ion chromatography (Prominence, Shimadzu). Total Kjeldahl Nitrogen (TKN) was measured using a total nitrogen measuring unit (Shimadzu). Mixed liquor samples were collected for suspended solids (MLSS) and volatile suspended solids (MLVSS) analyses, and analyzed according to the standard methods ²⁴.

2.4 Estimation of AOB and NOB associated kinetics. A Monod-based model with well-established biokinetics of AOB and NOB was applied ¹⁸ particularly to evaluate the maximum growth rate of AOB (μ_{AOB}) and NOB (μ_{NOB}) and the apparent oxygen half saturation coefficient for AOB ($K_{O_2}^{AOB}$) and NOB ($K_{O_2}^{NOB}$). The literature values for affinity constants of ammonium and nitrite were used due to the excessive supply of ammonium or nitrite in the activity batch tests in comparison to the reported relatively low affinity constants of ammonium and nitrite as shown in Table S2 in the SI. These values have been well applied to describing mixed cultures of AOB and NOB, irrespective of their detailed species. Table S2 summarizes the stoichiometrics and kinetics of the model, while Table S3 lists the definitions, values, units and sources of all parameters used in the model.

Specifically, the NOB biokinetics (i.e., μ_{NOB} and $K_{O_2}^{NOB}$) were first calibrated and validated using the NOB activity batch tests conducted on the Plant 3 sludge at all tested DO levels. On top of the NOB biokinetics obtained, the calibration and validation of the AOB biokinetics (i.e., μ_{AOB} and $K_{O_2}^{AOB}$) was then carried out using AOB activity batch tests conducted on Plant 3 sludge at the aforementioned DO levels. The NOB and AOB biokinetics were also validated with simultaneous AOB and NOB activity batch tests data and further applied to verify the dominance of AOB over NOB after enrichment using batch tests conducted on the lab reactor sludge at the aforementioned DO levels. MLVSS measurements were used in the model evaluation process in combination with

the microbial abundance analysis detailed in the above section. The model was implemented on the software AQUASIM 2.1d²⁵ and configured according to the specific conditions of the batch tests.

2.5 Microbial Community Characterisation. DNA from biomass samples collected from full-scale plants and the lab-scale nitrification reactor was extracted based on the FastDNATM 2 mL SPIN Kit for Soil (MP Biomedicals, USA) optimised for DNA extraction from activated sludge²⁶. 16S rRNA gene amplicon sequencing was conducted by DNA Sense (<http://dnasense.com/>) to determine the microbial community structure of the activated sludge. Amplicon library preparation, sequencing and data processing are described in SI, Section S1.1. The heatmap of the community compositions was plotted using the R package pheatmap Version 1.0.8 (<https://cran.r-project.org/web/packages/pheatmap>) with cluster analysis of the square root transformed community composition. The absolute abundance of AOB and NOB in full-scale plants and the lab-scale reactor was further determined with quantitative PCR (SI, Section S1.2). In addition, qPCR was used to detect the potential presence of Comammox *Nitrospira* according to Pjevac et al.²⁷.

2.6 Fluorescence in situ Hybridisation (FISH) and image processing. The method described by Daims et al.²⁸ was used to prepare biomass samples for FISH analysis. The following probes were used: NSO1225 and NSO190, specific for ammonia oxidizing betaproteobacteria; Ntspa662 targeting all *Nitrospira*; and EUB-mix (EUB338, EUB338-II, and EUB338-III), covering most bacteria. All probes were either labeled with indocarbocyanine (Cy3 or Cy5) or 6-carboxyfluorescein (6-FAM). Z-stacks of FISH-probed samples were acquired using a Zeiss LSM 780 inverted confocal microscope equipped with 100x/NA1.4 oil immersion objective (Carl Zeiss, Jena, Germany) and pre-processed using FIJI (background subtraction, 3D median)²⁹. DAIME

software was used for 3D visualization, segmentation and quantification of bacterial clusters³⁰. Surface, volume, surface-to-volume ratio and shape were measured for every recognized cluster in respective AOB and NOB channels for 20 distinct regions-of-interest (ROIs). Unpaired nonparametric Mann-Whitney U test was conducted to assess differences between AOB and NOB clusters.

Accession number for 16S rRNA Amplicon Sequencing. The Illumina MiSeq reads of the 16S rRNA amplicon sequencing have been deposited under the accession number SRP137036.

3. RESULTS

3.1 Diversity of nitrifying communities in major WWTPs. A single *Nitrospira* sublineage I OTU was the dominant NOB in all three treatment plants sampled (Figure 1), despite differences in process design and operational conditions. In contrast, up to 18 OTUs in the family of Nitrosomonadaceae were detected by 16S rRNA gene amplicon sequencing, of which only eight were assigned to the genus *Nitrosomonas* (Figure 1). Other known ammonia oxidizing and nitrite oxidizing taxa were not detected. The total relative abundance in OTUs of AOB was lower than that of NOB in Plants 2 and 3, with ratios of NOB to AOB of 2.6 and 5.5, respectively (Figure 1 and Table 1). The opposite was observed for Plant 1 where AOB were present at a higher abundance relative to NOB with a ratio of NOB to AOB of 0.5. The lower AOB-to-*Nitrospira* NOB ratio observed for Plant 1 compared to Plant 2 and 3 was confirmed by qPCR (Table S4, SI). Apart from differences in plant design, Plant 1 had the highest DO concentration in the aerobic zone and the longest SRT compared to Plants 2 and 3 (Table 1). The lower relative abundance of AOB in these plants is also reflected in the ammonium removal efficiency with a higher residual ammonium concentration compared to Plant 1. This increase in AOB:NOB ratio under higher DO

conditions in a full-scale WWTP is consistent with the observation that NOB have higher relative oxygen affinities.

Table 1. The key operational condition, effluent quality and corresponding relative abundance of AOB and NOB in three major wastewater treatment plants treating domestic waste water (values are averages and standard error of the mean from three sampling events).

	Plant 1	Plant 2	Plant 3
Process configuration	Pre-denitrification/Anaerobic/Aerobic/ MBR	Modified Ludzack Ettinger	Anoxic/Oxic Step Feeding
Dissolved Oxygen (mg O ₂ /L)	1.6±0.6	0.8±0.2	1.2±0.2
pH	7.7-8.3	6.9-7.7	6.4-7.0
Solid retention time (day)	15-18	4-6	4-6
Effluent ammonium (mg N/L)	0.2±0.2	3.1±1.5	2.4±1.0
Effluent nitrate (mg N/L)	8.1±0.8	7.5±0.9	10.6±8.0
Effluent nitrite (mg N/L)	0	0.7±0.2	0.3
Effluent phosphate (mg P/L)	0.8±0.8	0	1.6±0.7
Effluent TCOD (mg/L)	17±2	17.9±1.5	11.3±0.9
Mixed liquor suspended solids (g/L)	3.16±0.07	2.06±0.14	2.44±0.01
Relative abundance of <i>g_Nitrospira</i> (%)	0.83±0.05	1.67±0.38	1.81±0.30
Relative abundance of <i>f_Nitrosomonadaceae</i> (%)	1.79±0.12	0.64±0.05	0.33±0.09

Figure 1. Ammonia oxidizing and nitrite oxidizing bacteria detectable by 16S rRNA gene amplicon sequencing in three major wastewater treatment plants in Singapore and the lab-scale partial nitrification/ nitrification-denitrification reactor during different experimental phases (see Table 2 and Figure 2). The scale on the heat map denotes relative abundance of each taxon in %.

3.2 Dynamics of microbial community structure in response to varying DO set points.

determine whether DO can be used to select against NOB, sludge from Plant 3 (with a high NOB:AOB ratio) was transferred to a lab-scale reactor, exposed to a range of DO conditions, and the AOB to NOB competition dynamics were observed. The DO concentration and length of aeration phase were systematically varied in each experimental phase to investigate DO selection against NOB in a lab-scale reactor (Table 2). Consistent with what was observed in the full-scale

plant, only a single *Nitrospira* sublineage I OTU was detected whereas multiple OTUs were annotated to Nitrosomonadaceae throughout the experiment. In experimental phases I, II and III, all of the ammonium oxidised by AOB was converted to nitrate by NOB when DO set points < 1.5 mg O₂/L were applied (Table 2 and Figure 2A). *Nitrospira* sublineage I had a much higher relative abundance than AOB during these initial experimental phases (Figure 2B). The increase in DO set point to 2.0 – 2.5 mg O₂/L resulted in a slight increase in the nitrite:NO_x (sum of nitrite and nitrate in the effluent) ratio in experimental phase IV and a corresponding reduction in the relative abundance of *Nitrospira* from 3.7% to 2.1%, albeit it was still more abundant than AOB (Figure 2B).

When the aeration phase was shortened from 60 min to 20 – 35 min while maintaining the DO set point at 2.0 – 2.5 mg O₂/L (Table 2), no apparent change was observed in both the relative abundance of *Nitrospira* and nitrite production. However, *Nitrospira* gradually decreased to below the detection limit in experimental phase VI, when the DO set point was further increased to 4.5 – 5.5 mg O₂/L. Partial nitrification was accordingly achieved with a concomitant increase in final effluent nitrite concentration and overall nitrite:NO_x ratio (Figure 2A and B). Nitrite accumulation was sustained with subsequent changes in operational conditions, including 1) extension of the aerobic period to achieve complete conversion of ammonium to nitrite (nitrification) in experimental phase VII; 2) decrease in SRT in experimental phase VIII; and 3) return of the DO concentration back to 1.0 – 1.5 mg O₂/L in experimental phase VIII and 0.5 – 1.0 mg O₂/L in experimental phase X (Figure 2A and B, Table 2). The susceptibility of *Nitrospira* to high DO concentrations was further confirmed using qPCR, whereby the abundance of *nxrB* genes targeting nitrite-oxidising *Nitrospira* decreased with increasing DO concentration (Figure S1, SI).

In addition to *Nitrospira*, two OTUs annotated to the genera *Candidatus Amarilinum* and *Blastocatella* decreased in relative abundance with increasing DO concentration. The disappearance of *Nitrospira* also coincided with the proliferation of multiple heterotrophic taxa belonging to the families Chitinophagaceae, Saprospiraceae, Comamonadaceae and PHOS-HE51 (Figure 2C), which could be the consequence of increased availability of nitrite or organic carbon sources or both. These taxa displayed cyclical dynamics whereby the increase in relative abundance of one OTU was at the expense of another, indicating competition for a limiting resource, likely organic carbon since the effluent total COD was consistently below 20 mg/L throughout the experiment (Figure 2C). The changes in relative abundances were particularly apparent when the SRT was decreased to 8 days in experimental phase VIII, with a *Comamonadaceae* OTU increasing periodically in relative abundance up to approximately 34%. However, most other heterotrophic bacteria were present at relative abundances of <1% throughout all experimental phases.

In contrast to the effect on the NOB population in the activated sludge, we observed that ammonium oxidation was not compromised across a wide range of oxygen concentrations in the lab-scale reactor. In contrast to *nxrB* genes, the abundance of *amoA* genes targeting AOB was relatively stable across the various operational phases (Figure S1, SI). While multiple AOB OTUs were detectable during the various experimental phases, different OTUs seemed to predominate at different DO set points. Thus, increasing the oxygen set point in a sludge dominated by *Nitrospira* sublineage I resulted in inhibition of nitrite oxidation and eventual wash out of nitrite oxidizers from the system, consistent with the observation in the full-scale treatment plants that *Nitrospira* has a preference for lower oxygen concentrations compared to the coexisting AOB.

Table 2. Operational parameters at different experimental stages of the lab-scale partial nitrification (PN)/ nitrification (N)-denitrification (DN) sequencing batch reactor.

Experimental Phase	Period (day)	pH	Dissolved Oxygen Set point (mg O ₂ /L)	Solid retention time (day)	Aerobic period (min)	Mode of operation	Temperature (°C)
I	0-16	6.6-7.1	0.5-1.5	>12 (Not controlled)	60	PN-DN	30.5 ± 0.5
II	17-36	6.6-7.1	0.4-0.8	>12 (Not controlled)	60	PN-DN	30.5 ± 0.5
III	36-45	6.6-7.1	1.0-1.5	>12 (Not controlled)	60	PN-DN	30.5 ± 0.5
IV	46-66	6.4-6.9	2.0-2.5	>12 (Not controlled)	60	PN-DN	30.5 ± 0.5
V	67-77	6.5-7.0	2.0-2.5	>12 (Not controlled)	20-25	PN-DN	30.5 ± 0.5
VI	78-149	6.5-7.0	4.5-5.5	>12 (Not controlled)	16-18	PN-DN	30.5 ± 0.5
VII	150-160	6.1-7.1	4.5-5.5	>12 (Not controlled)	35-40	N-DN	30.5 ± 0.5
VIII	161-244	6.1-7.1	4.5-5.5	8	35-40	N-DN	30.5 ± 0.5
VIII	245-309	6.1-7.1	1.0-1.5	8	45-50	N-DN	30.5 ± 0.5
X	309-402	6.1-7.1	0.5-1.0	8	60	N-DN	30.5 ± 0.5

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Figure 2. Effluent ammonium, nitrate and nitrite concentrations, (B) relative abundance of detectable ammonia oxidizing bacteria (*f_Nitrosomonadaceae*) and nitrite oxidizing bacteria (*g_Nitrospira*-sublineage I) and the corresponding nitrite:NO_x ratio, and (C) relative abundance of all other detectable taxa in the sludge of the lab-scale partial nitrification(PN)/ nitrification(N)-denitrification(DN) sequencing batch reactor as a function of time. In (C) all detected taxa are displayed but only the top ten most abundant annotated taxa in all three samples are listed. Dashed lines indicate changes made to operational conditions summarized in Table 2.

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3.3 Nitrifying activity in response to varying DO set points. To further understand the competitive dynamics between AOB and NOB in Plant 3 sludge for oxygen, the ammonia and nitrite oxidizing activities were characterized across a wide range of DO concentrations, raw data presented in Figure S2, SI and summarized in Figure 3. Comparable nitrite oxidizing activities were observed in batch experiments in the presence of nitrite only (i.e. NOB activity test) and when both nitrite and ammonia were supplied (i.e. simultaneous AOB and NOB activity tests) at DO concentrations from 0.2 to 6.5 mg O₂/L (Figure 3). In both NOB activity test and simultaneous AOB and NOB activity tests on Plant 3 sludge, the maximum specific nitrite oxidizing activity of approximately 6.0 mg N/h/g VSS was attained at a DO concentration of 1.5 mg O₂/L. In contrast, a lower maximum specific ammonium oxidizing activity of 7.1 mg N/h/g VSS was observed in the simultaneous AOB and NOB batch activity tests compared to 9.3 mg N/h/g VSS in the AOB-only batch activity tests (i.e. ammonium only) (Figure 3). In the lab-scale reactor, following the wash out of the NOB, a much higher maximum ammonium oxidation rate of approximately 12.2 mg N/h/g VSS was achieved, suggesting that AOB activity in the sludge was significantly impacted when NOB activity was simultaneously occurring, whereas the NOB activity was not affected by concurrent oxygen consumption by AOB. The maximum AOB activities in all cases were higher than those of NOB and were attained at a DO concentration of 2.5 mg O₂/L (Figure 3). However, in the simultaneous AOB and NOB batch activity tests at DO concentrations <1.5 mg O₂/L, the nitrite oxidation rates were higher than the ammonia oxidation rates (Figure 3), further supporting that *Nitrospira* taxa in Plant 3 sludge have lower relative *K_s* for oxygen compared to *Nitrosomonas*-like AOB. The comparable nitrite oxidizing activity beyond DO concentration of 1.5 mg O₂/L (up to 6.5 mg O₂/L) also suggests that NOB are able to cope with the relatively high oxygen concentration and that the wash out of NOB observed in the long-term

lab-scale study was from the imbalance in their ability to compete for oxygen with AOB when DO concentration was increased.

Figure 3. The effect of dissolved oxygen concentration on the average specific nitrite or ammonia oxidation rate (NOR or AOR) of activated sludge from Plant 3 in the nitrite oxidising bacteria (NOB) batch tests, ammonia oxidising bacteria (AOB) batch tests and the concurrent AOB and NOB batch tests and of sludge from lab-scale reactor in the AOB batch tests.

3.4 Microbial kinetics of AOB and NOB. The various batch experiments were then modelled according to a Monod-based model for AOB and NOB ¹⁸ to determine the apparent $K_{S(app)}$ and μ_{max} of the AOB and NOB from the batch tests (Figure S2, SI). The good agreement between model predictions and measured results supported the validity of the estimated kinetic parameters to describe the competitive dynamics between AOB and NOB at varying DO levels. The oxygen $K_{S(app)}$ for AOB in Plant 3 sludge was estimated to be 0.30 ± 0.03 mg O₂/L, higher than that of

NOB with an estimated value of 0.09 ± 0.02 mg O₂/L (Table S3, SI). In addition, the estimated $\mu_{\max}$ of 0.126 ± 0.003 h⁻¹ of AOB was almost ten times that of NOB, at 0.0128 ± 0.0003 h⁻¹. The estimated kinetic parameters suggest that *Nitrosomonas*-like AOB will outgrow *Nitrospira* in Plant 3 sludge when oxygen supply is high, whereas *Nitrospira* will predominate under oxygen limitation conditions; this could explain their high relative abundance in Plants 2 and 3. In addition, using the estimated kinetic parameters, the model was able to reproduce the batch experiment results with the lab-scale sludge (Figure S1, SI), which further supports that the washout of *Nitrospira* was a result of operation at high DO.

3.5 Physical properties of AOB and NOB microcolonies. To determine whether the lower $K_{S(app)}$ of the NOB compared to AOB was an artifact of microcolony size, three dimensional FISH imaging was carried out on Plant 3 sludge to visualize the AOB and NOB colonies followed by image processing (Figure 4). Both AOB and NOB form cell clusters and reside in close proximity to each other. Image analysis revealed high variability in the volume and surface area of both AOB and NOB microcolonies (Figure S2, SI). Cluster sizes are therefore not uniformly distributed in the sludge. However, while the total surface area of the AOB clusters were significantly larger compared to that of the NOB clusters, the NOB clusters consistently occupied a higher volume than the AOB clusters (Figure 5). Thus, despite inconsistent microcolony sizes, the AOB microcolonies had significantly higher surface area to volume ratios than did NOB microcolonies (p value < 0.0001) and displayed far higher morphological variability. AOB microcolonies were less regular in appearance compared to those of NOB, which tended to be more spherical (Figures 4 and 5). The irregularity in shape resulted in conflicting maximum and minimum diameter of the microcolonies, indicating that microcolony description based on size is not sufficient when describing substrate diffusion (Figure S3, SI).

Figure 4. Ammonia oxidising bacteria (probes NSO1225 & NSO190 in green) and nitrite oxidising bacteria (probe Ntspa662 in red) colonies detected by fluorescence in situ hybridisation (FISH) in Plant 3 sludge. Boxes indicate colonies identified based on a threshold described in materials and methods under further analyses. Ticks on all axes are uniformly distributed at 5 µm. All other detected bacteria are shown in blue (probes EUB338, EUB338-II and EUB338-III).

Figure 5. Physical properties of ammonia oxidising bacteria (AOB) and nitrite oxidising bacteria (NOB) clusters identified by fluorescence in situ hybridisation (FISH) with an example shown in Figure 4. Data represent the median with 95% confidence interval. P values are listed for T-test comparisons of means. The value for shape denotes the sphericity of the clusters with the value 1 being round.

4. DISCUSSION

Understanding the competition dynamics between AOB and NOB for oxygen is confounded by a combination of physical and biological factors acting at different scales, all the way from micro- to macro-scale^{23, 31}. In this study, we resolved physicochemical and biological aspects of

AOB/NOB interactions in full-scale activated sludge systems by integrating kinetic, microbial community and microcolony structure characterization along with field sampling and short term and long term lab-scale experimentation. Contrary to the paradigm that lower DO concentrations eliminate NOB in nitrogen rich side stream systems⁴, we observed in this study into activated sludge systems receiving relatively low nitrogen loadings that the opposite was true.

Based on 16S rRNA gene amplicon sequencing, the relative abundance of the NOB community was significantly higher compared to that of AOB within the same system, with a single *Nitrospira* sublineage I OTU dominating across different treatment plants in Singapore. Due to the great diversity of *Nitrospira* species detected in soil³² and in activated sludge¹² with highly similar 16S rRNA sequences, species or strain level identification is challenging. Using available cluster specific FISH probes¹², only cluster Id was detected in all three treatment plants (Figure S4). Other clusters may have been present but were not detectable with the methods used in this study. Phylogenetic analysis based on 16S rRNA amplicon sequence revealed that the observed *Nitrospira* OTU is only 89% similar to the comammox *Candidatus Nitrospira inopinata* (e value: $5e^{-85}$), but most similar (94% similarity) to the non-comammox *Candidatus Nitrospira defluvii* (e value: $4e^{-103}$). In addition, comammox *Nitrospira* could not be detected by qPCR targeting *amoA*²⁷ in any of the three plants suggesting that the relevant NOB communities were unable to perform complete nitrification. When subjected to perturbations in oxygenation levels (Figure 2), *Nitrospira* numbers decreased in relative abundance with increasing DO concentration and were eventually washed out from the lab-scale system altogether. The nitrite oxidizing activity did not recover and sustained nitrite accumulation was observed even at low DO concentrations. The inability to reverse the nitrite oxidizing activity at lower DO concentration demonstrates that high DO may only be required for a short period of time to suppress NOB activity, after which oxygen

levels can be adjusted depending on process requirements. This provides operational flexibility when combining partial nitrification with Anammox to achieve nitrogen removal, with single-stage systems requiring low oxygen levels to prevent inhibition of Anammox bacteria, whereas higher oxygen levels can be applied for two-stage systems to maximize conversion rates.

NOB are a highly diverse functional group that possesses fundamental ecophysiological diversity mainly stemming from differences in affinity to nitrite and energetic yield depending on the localization of the enzyme nitrite oxidoreductase (NXR)²². Environmental conditions such as DO concentration¹⁴, temperature^{9, 33, 34} and pH³⁵ have also been shown to drive niche differentiation of NOB. The absence of taxa from the other NOB genera in this study is not unexpected given the lack of temporal fluctuations in the plants and the year round stable wastewater temperature of 30 °C, which favours *Nitrospira* over *Nitrotoga*³⁴, and the relatively diluted ammonium content with a continuous flow configuration that limits the nitrite production rate by AOB. Such conditions favour the selection of *Nitrospira* taxa that possess a periplasmic NXR with a high nitrite affinity, allowing them to adapt to low nitrite concentrations^{36, 37} as opposed to *Nitrobacter* that have higher nitrite conversion rates but lower nitrite affinity¹⁰. On the contrary, excess ammonium availability will result in dynamic changes in ammonium profiles that potentially provide ecological niches for the co-occurrence of multiple AOB strains^{9, 38}. Mass balance analysis revealed a significantly higher proportion of NOB compared to AOB in Plants 2 and 3, suggesting that the growth of *Nitrospira* was not exclusively dependant on autotrophic nitrite oxidation. Indeed, mixotrophic growth has been shown for *Ca. N. defluvii*³⁹ and *Nitrospira marina*⁴⁰. The *Ca. N. defluvii* genome also contains genes that encode pathways for the transport, oxidation, and assimilation of simple organic compounds³⁷. In the lab-scale reactor, the increase in a number of heterotrophic taxa after *Nitrospira* was washed out from the system suggests that

they occupy the same ecological niche. The antagonistic relationships between OTUs annotated to Chitinophagaceae, Saprospiraceae and Comamonadaceae (Figure 2C) are more likely to be due to competition for organic carbon than nitrite given that nitrite was always in excess when *Nitrospira* had been inhibited. Identifying complete metabolic function of the detected *Nitrospira* in this study would require genome-wide analysis. Given the relatively low abundance of *Nitrospira* in activated sludge, genome recovery could be possible with single-cell genomics⁴¹ otherwise prior enrichment with synthetic medium will be required to increase the relative abundance of *Nitrospira*³⁷.

Our study also shows that AOB and NOB exhibited microcolonies of distinct morphology when subjected to the same level of oxygenation in Plant 3 of 1.2 ± 0.2 mg O₂/L (Figures 4 and 5). *Nitrospira*-like NOB were previously reported to form denser microcolonies than *Nitrosomonas*-like AOB in a biofilm system⁴². In suspension, *Nitrospira* also formed tightly packed and dense clusters that limits mass transfer of oxygen in agreement with kinetic properties determined in this study and their preference for lower oxygen concentrations³⁷. In contrast, *Nitrosomonas*-like AOB formed open porous aggregates that maximises diffusional mass transfer. Porous colonies are expected to yield lower $K_{S(app)}$ values (i.e., higher affinity), whereas dense microcolonies would lead to the reverse⁴³. Yet, the estimated $K_{S(app)}$ for NOB is still significantly lower than that of AOB, suggesting that the intrinsic oxygen affinity constant (i.e., not affected by diffusion) of NOB could be even lower than the estimated value in this study. While AOB are generally thought to form larger colonies than NOB based on the measurement of colony diameter^{23, 44, 45}, we show that colony characterization solely based on diameter may lead to skewed conclusions given the inconsistency between the maximum and minimum diameter of non-spherical colonies (Figure S3, SI). Therefore, our results are contrary to the contention of

Piciooreanu *et al.*²³ that observation of a lower oxygen affinity for NOB could be the consequence of the difference in microcolony size.

Although the same *Nitrospira* taxon is prevalent across the different treatment plants indicating comparable oxygen dependent NOB activity, however, the relative *K*s may still differ between treatment plants given the high diversity of AOB in the different plants. In addition, given that temperature has a significant impact on the NOB community structure³⁴ and activity⁴⁶, the oxygen competition dynamics between AOB and NOB may differ in temperate activated sludge plants. Further investigation is required to understand oxygen preference for closely related AOB and NOB sub-lineages for better oxygen control in engineered systems.

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Supporting Information Available

Method description of the 16S rRNA amplicon library preparation, sequencing and data processing, quantitative PCR analysis for absolute quantification of ammonia oxidizing bacteria and all nitrite-oxidizing *Nitrospira* and the screening for the presence of specific *Nitrospira* clusters. A table showing the composition of the primary effluent augmented with nitrite, two tables showing the stoichiometric matrix and associated stoichiometric and kinetic parameters for

the metabolic model, respectively, a figure showing the experimentally observed and model-based ammonia oxidising and/or nitrite oxidising activity at varying dissolved oxygen concentrations in the various batch experiments. A figure and a table showing the abundance of *amoA* genes and *nxrB* genes in the long-term lab-scale experiment and in the investigated full-scale plants, respectively. Pre-print of the manuscript is available on bioRxiv⁴⁷

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