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Nitrification Efficiency and Community Structure of Municipal Activated Sewage Sludge

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***Abstract*—**Nitrification is essential to biological processes designed to remove ammonia and/or total nitrogen. It removes excess nitrogenous compound in wastewater which could be very toxic to the aquatic fauna or cause serious imbalance of such aquatic ecosystem. Efficient nitrification is linked to an in-depth knowledge of the structure and dynamics of the nitrifying community structure within the wastewater treatment systems. In this study, molecular technique was employed for characterizing the microbial structure of activated sludge [ammonia oxidizing bacteria (AOB) and nitrite oxidizing bacteria (NOB)] in a municipal wastewater treatment with intention of linking it to the plant efficiency. PCR based phylogenetic analysis was also carried out. The average operating and environmental parameters as well as specific nitrification rate of plant was investigated during the study.

During the investigation the average temperature was 23±1.5^{o}C. Other operational parameters such as mixed liquor suspended solids and chemical oxygen demand inversely correlated with ammonia removal. The dissolved oxygen level in the plant was constantly lower than the optimum (between 0.24 and 1.267 mg/l) during this study. The plant was treating wastewater with influent ammonia concentration of 31.69 and 24.47 mg/L. The influent flow rates (ML/Day) was 96.81 during period. The dominant nitrifiers include: *Nitrosomonas* spp. *Nitrobacter* spp. and *Nitrospira* spp. The AOB had correlation with nitrification efficiency and temperature. This study shows that the specific ammonia oxidizing rate and the specific nitrate formation rates can serve as good indicator of the plant overall nitrification performance.

***Keywords*—**Ammonia monooxygenase α-subunit (amoA) gene, ammonia-oxidizing bacteria (AOB), nitrite-oxidizing bacteria (NOB), specific nitrification rate, PCR.

I. Introduction

BIOREACTOR treating wastewater is a complex ecosystem, having different microbial interactions resulting in breakdown of pollutants and removal of nutrients. The nitrifying bacterial communities form an important part of this ecological system and are responsible for ammonia removal through nitrification [1]. Nitrification involves the bioconversion of ammonia into nitrite by either ammonia-oxidizing bacteria (AOB) or ammonia-oxidizing archaea (AOA) and subsequent conversion of nitrite to nitrate by nitrite-oxidizing bacteria (NOB) [2]. Nitrifiers are "fragile," with characteristic slow specific growth rate [3] and are usually sensitive to toxic compounds, operational and environmental shocks which predispose biological wastewater treatment (WWT) systems to nitrification depletion or failure [4]. Monitoring the nitrifiers, especially AOB which carry out the rate-limiting step of nitrification in wastewater treatment systems, is key to prevent nitrification failure [5].

Seasonal fluctuations and failures in nutrient removal due to operational and environmental changes have been widely reported in engineered wastewater treatment systems [6]. Among these, seasonal temperature variation has been implicated as a major cause of breakdown in ammonia removal efficiency and nitrification [7], [8]. However, research efforts have focused on laboratory and pilot scale plant which do not fully represent the complex ecological relationships that occur in the full scale plant. In furtherance majority of the studies were conducted over short period of time. Developments with regards to organisms involved in nitrification have progressed with some novel findings [9], [10]. There have been reports by different authors [11]–[13] on the probable involvement of ammonia oxidizing archaea (AOA) in wastewater nitrification. However, there is still need to establish if AOA are ubiquitous in all engineered systems in order to establish a holistic knowledge of nitrogen biotransformation and AOA contribution in wastewater treatment. *Nitrolancetus hollandicus,* a nitrite oxidizing bacteria which belongs to the phylum *Chloroflexi*, was also recently isolated and cultured from a nitrifying reactor. All of these novel discoveries indicate our inconclusive knowledge of wastewater microbiology and suggests the need for more research drive in that direction.

This study examines the nitrifying community in a full-scale municipal wastewater treatment plant over long term of 237 days. The Phase 1 of the study was from the first day to the 78th day which represents the winter whilst the remaining days constitute the Phase 2 (summer). The plant's nitrification efficiency (ammonia removal) was investigated in correlation to abundance of the different nitrifying populations. A combination polymerase chain reaction (PCR) and quantitative polymerase chain reaction (qPCR) was employed in understanding the microbial community structure of the plant. According to [14], it is important to understand how microbial community structure correlates to system stability in a bid to design functionally reliable wastewater treatment systems. The nitrifiers can also serve as indicator parameter for preventing washout of important functional groups in wastewater treatment facility. Various studies have reported nitrification depletion or loss due to seasonal temperature variations especially during winter [15]–[17]. It is therefore important to understand the role of nitrifiers' diversity and abundance in such functional fluctuations or failure. Also,

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majority of the studies on nitrification and seasonal variations have been carried out in regions with extremely low winter average temperatures (temperate regions) contrary to the sub-tropical temperature regime experienced in some parts of the Southern hemisphere.

II. Methods

A. Full Scale Plant and Sample Collection

The full-scale WWTP under study is situated in the Midlands of KwaZulu-Natal province, South Africa. The plant receives a discharge of 82,880 ± 20,832 m^3/d (average dry weather flow), including 90% domestic and 10% industrial wastewaters. The plant was designed based on the criteria of a modified Johannesburg configuration, which offered anaerobic, anoxic, and aerobic biological processes. As shown in Fig. 1, the anaerobic zone receives primary treated wastewater, while the pre-anoxic basin is enriched with return activated sludge from the bottom of a final settler. The effluent from pre-anoxic/anaerobic tank is discharged to an aerobic unit. An internal recycle is pumped from the aerobic units to the anoxic zone. The mixed liquor, containing activated sludge, flows from the aerobic zone to a secondary settler, where it is separated under the action of a quiescent condition into treated wastewater and return activated sludge. Composite wastewater samples (from the aerobic chamber), influent and effluent water samples were collected twice a month for 237 days. The first phase of the sampling which represents the winter was from May to July 2012 whilst the second phase (summer) was between November 2012 and March 2013.

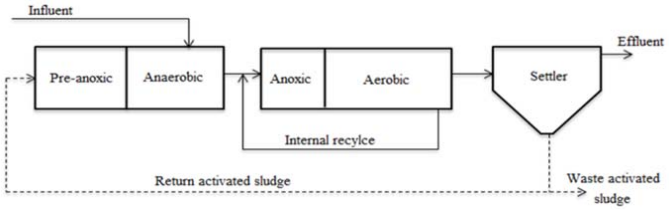


Fig. 1 Schematic representation of wastewater treatment plant in this study

B. Analytical Methods

The nitrogen species (NH_4^+ −N, NO_2^- −N, NO_3^- −N) and chemical oxygen demand (after digestion), were measured using Gallery™ Automated Photometric Analyzer. Temperature, dissolved oxygen (DO) concentrations and pH measurements were done using the YSI 556 MPS (Multiprobe System). The mixed liquor suspended solids (MLSS) was also measured using standard methods [18].

C. Modification Genomic DNA Extraction and PCR Amplification

Genomic DNA were extracted from sludge samples (aerobic samples) taken over the winter and summer seasons. Two milliliter volume of each sample was centrifuged at 5000x g and the solids used for DNA extraction. Genomic DNA was extracted using [19] procedure. The nucleic acid was precipitated with 0.6 volume isopropanol and wash with 70% cold ethanol. The quality of the extracted DNA was ascertained with NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, USA). The extracted genomic DNA was stored at –20°C until when needed.

The DNA was amplified using Veriti TM 96-well Thermal Cycler (Applied Biosystems) and the optimized tempetures for the primers are as shown in Table I. PCR was performed in a total volume of 50 µL containing 10 ng of DNA template. The final concentrations of the different components in the reaction mix were according to modified protocols from [20]. The resulting amplicons were electrophoresed in 1.2% (wt/vol) agarose gel for the presence the expected gene product sizes.

D. Cloning, Sequencing and Phylogenetic Analysis

The amplification products were purified using QIAquick PCR purification kit (Qiagen) and cloning of the purified PCR products was performed using InsTAclone PCR Cloning Kit (Thermo Scientific) according to the manufactures instructions. Colony PCR was carried out in order to confirm the plasmid insert using the appropriate primer sets as listed in Table I. The sequencing of the selected clones was carried out at Inqaba Biotechnical Industries (Pty), South Africa. The sequences obtained were edited using Finch TV software. The sequences obtained were checked against the National Center for the Biotechnology Information (NCBI) GenBank database. The GenBank accession numbers for the nucleotide sequences of the clones isolated in this study are KP337415-KP337452.

E. Quantitative PCR (qPCR)

Individual standard curves were prepared for the different nitrifiers using purified 16S rRNA gene fragments (target DNA) obtained from PCR-amplified *Nitrobacter* spp. (FGPS872f and FGPS1269r), *Nitrospira* spp. (NSR1113F and NSR1264R) and AOB (amoA-1F and amoA-2R) respectively as described by [21]. The concentrations (µg/µL) of these templates were determined using NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, USA). These concentrations were used in calculating their copy by considering their molecular weight and Avogadro's number. The Ten-fold serial dilutions of the target DNA were prepared from 10^8 to 10^1 copy numbers. The real time PCR quantification was carried out according to the modification of method described by [22] using Bio-Rad C1000 Touch Thermal Cycler-CFX96 Real-Time System (BIO-RAD, USA).

TABLE I
PRIMERS AND THEIR OPTIMIZED ANNEALING TEMPERATURES

Target	Primer	Annealing (°C)	References
AOB amoA	amoA-1F/ amoA-2R	55	[2]
Nitrospira 16S rDNA	NSR1113F/NSR1264R	65	[4]
Nitrobacter 16S rDNA	FGPS872/FGPS1269	50	[20]

F. Laboratory Batch Experiment for Specific Nitrification Rate Determination Laboratory Batch Experiment for Specific Nitrification Rate Determination

Specific nitrification rate which includes specific ammonium oxidization rate (SAOR) and specific nitrate formation rate (SNFR) were measured on sampling days according to the method previously described [23]. Batch tests were performed to evaluate the variations of nitrification activities of activated sludge (AOB, and NOB) in the bioreactor. A 100 ml sludge sample from the aerobic tank was centrifuged at 8,000 rpm for 10 min and then washed with buffer medium to remove the background concentrations of nitrogen. Then, the biomass was transferred to a 250-ml Erlenmeyer flask and suspended with 100 ml of mineral medium. The batch experiment was performed in a shaking incubator at 30°C. Samples were taken at an interval of 30 min to analyse the concentrations of NH_4^+-N and NO_2^--N. The SAOR and SNFR was determined by monitoring the decreased rate of NH_4^+-N concentration and NO_2^--N concentration versus time, respectively. The SAOR and SNFR were carried out between the 35th and 193rd day of the study. The 35th to 83rd day represent the phase 1 (winter) whilst the 96th to 193rd represents the Phase 2 (summer) of the study.

G. Statistical Analysis

GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego California USA) was used in carrying out Pearson correlation, one-way analysis of variance and unpaired t-test.

III. RESULTS

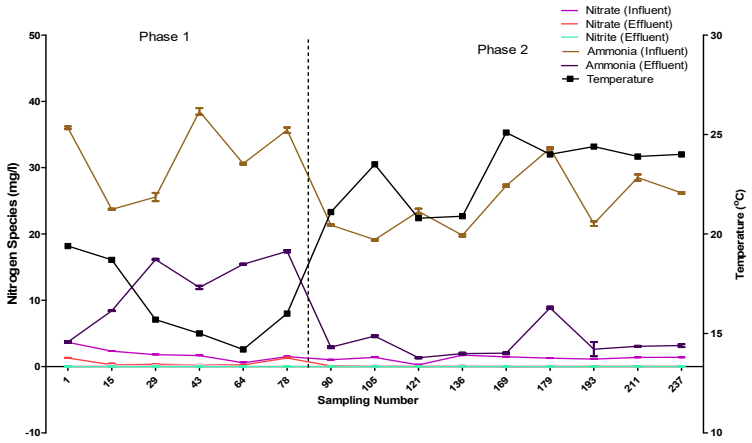
A. Process Performance

The temperature observed in the aeration basins ranged from 14.2°C to 25.1°C. The average temperature during first (day 1 – 78) and second (day 79 – 237) phases were 16.5 ± 2.0 and 22.86 ± 1.5°C (Fig. 2 (a)) respectively. The DO range observed during the study was between 0.24 and 1.267 mg/l. The influent chemical oxygen demand varied during the sampling period. Over the study period, the average influent ammonia concentrations was 24.47 ± 4.58 mg/l during first 78th days that corresponds to the Phase 1 (winter) whilst it was 31.69±6.04 mg/l during the rest of the sampling period which was the Phase 2 (winter). The effluent NH_4^+–N concentrations were 3.403 and 13.24 mg/l summer and winter respectively (Fig. 2 (a)). The influent and effluent nitrite concentrations were always lower than 1 mg/L during winter and summer in the plant. The nitrogen removal performance of the plant in terms of ammonia is shown in Fig. 2 (b). There was a significant variation in ammonia removal efficiency across the two seasons. The average ammonia removal during winter was 61.99±2.2% whereas it was 96.81 ± 14.5% during summer and this was found to correlate with temperature ($r = 0.7671$; $P = 0.0008$). The ammonia removal also demonstrated significant correlation with the ammonia oxidizing bacteria population (copies/l) ($r = 0.55$; $P = 0.03$). There was however a significant variation in ammonia removal efficiency throughout the study.

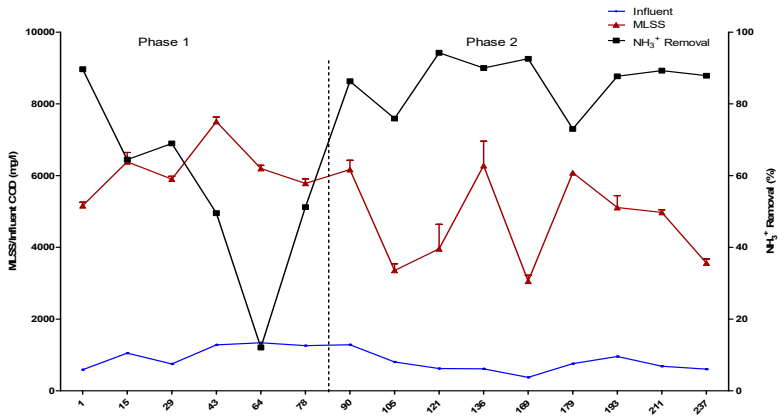
The lowest influent COD (377 mg/L) was recorded during the second phase, whereas the highest COD of 1340 mg/L (Fig. 2 (b)) was observed during the second phase. The experimental site usually has its highest rainfall during its Phase 2 as observed during this study. It was observed that the rainfall dilution effect contributed to lower concentration of influent chemical constituents as shown in the parameters monitored, since they were generally diluted during Phase 2 and this corresponds with the season that had the highest rainfall. Generally, the plant had an efficient COD removal rate during the two phases (95.44 ± 2.8%). The ammonia removal rate of the plant was found to have a significant positive correlation with the ammonia oxidizing bacteria population (copies/L) ($r = 0.553$; $P = 0.033$) and temperature ($r = 0.767$; $P = 0.001$) whilst it exhibited a negative correlation ($r = -0.555$; $P = 0.032$) with influent COD concentration of the plant. It was also observed that the organic loading rate (Fig. 2 (c)) impacted on the AOB population density showing a weak negative correlation ($r = -0.614$; $P = 0.015$). The pH in the reactor over the entire sampling period was relatively stable with a mean of 7.3±0.2 pH units. The reactors had average mixed liquor suspended solids (MLSS) of 5300±1297 mg/L (3057±291 – 7506±220) which showed a negative correlation with the NH_4 removal ($r = -0.530$; $P = 0.040$). The organic loading rate had a negative correlation ($r = -0.732$; $P = 0.002$) with the ammonia removal rate.

B. Detection of Nitrifiers Using PCR

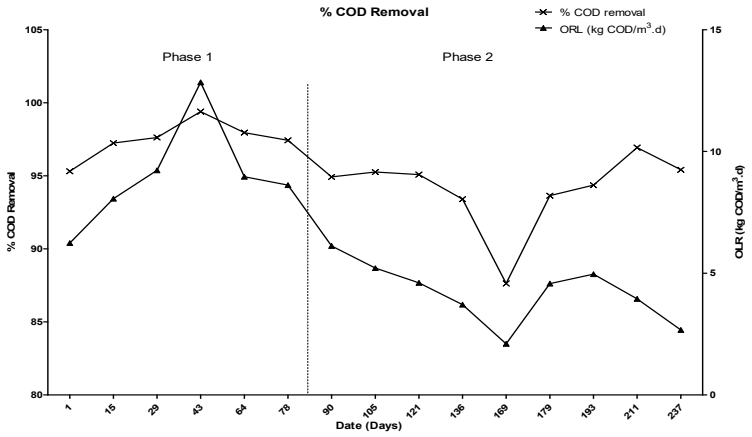
Detection of the nitrifiers was carried out using the primers listed in Table I, the optimized PCR conditions resulted in specific amplicons at the expected base pair length (AOB: 490 bp; *Nitrospira* spp.: 151 bp; *Nitrobacter* spp.: 386 bp). The PCR amplification further confirmed that AOB, *Nitrospira* spp. and *Nitrobacter* spp. were present all through the sampling period. The uncultured AOB and *Nitrosomonas* sp. were the AOB identified during this study with the uncultured ammonia bacteria being the dominant group based on the PCR and phylogenetic analysis. However, AOA the new player identified in ammonia oxidation was not detected in this wastewater treatment plant throughout the study. The partial sequences obtained were used in constructing the phylogenetic tree and also deposited with GenBank to obtain accession numbers (KP337415-KP337452).



(a)



(b)



(c)

Fig. 2 (a) Changes in nitrogen species concentrations in the plant; (b) Changes in MLSS, influent COD, and percentage ammonia removal of the plant; (c) COD removal rate and organic loading rate of the plant

C. Quantification of AOB and NOB

The efficiencies for all the qPCR were between 90 and 110% and the standard curves were linear over six orders of magnitude (R2 > 0.99). The AOB population abundance was quantified using the primer set targeting the amoA (ammonia monooxygenase) gene locus whereas *Nitrospira* and *Nitrobacter* 16S rDNA were targeted for the NOB. The AOB abundance was within the range of 1.6 x 10^7 – 1.7 x 10^9

copies/L. *Nitrospira* and *Nitrobacter* spp. were found to be 2.4 x 10^8 – 3.8 x 10^9 copies/L and 9.3 x 10^9 – 1.4 x 10^{11} copies/L respectively. The qPCR standard curve parameters used for the analysis is shown in Table II. Throughout the study period we observed *Nitrobacter* spp. population abundance was highest with about 2 orders of magnitude above the AOB. *Nitrobacter* spp. was also found to be the dominant NOB throughout the study period. The changes in AOB, *Nitrospira* spp. and *Nitrobacter* spp. during this study is shown in Fig. 3. A significant correlation was found between the AOB (amoA gene) copy numbers and temperature in the reactors (α= 0.05; P=0.0498). The lowest AOB abundance was recorded during the winter, whereas there was no correlation observed between the NOB and temperature. The NOB population remained almost stable with slight shifts throughout the sampling period.

Peak values for both SAOR and SNFR were observed during the summer; 0.1488 and 0.6310 g N g^{-1} MLSS d^{-1} respectively. The SNFR was observed to be higher than the SAOR all through the experiment.

A significant correlation was observed between the plant's nitrification rate and the specific ammonium oxidization rate determined in the lab (r = 0.6524; P = 0.04).

D.Specific Nitrification Rate Determination

The specific nitrification rate of the biomass in terms of specific ammonium oxidization rate (SAOR) and specific nitrate formation rate (SNFR) were determined in a laboratory batch experiment (Fig. 4).

TABLE II
DESCRIPTION OF qPCR STANDARD CURVES PARAMETERS

Parameter	Target		
	AOB	*Nitrobacter* spp.	*Nitrospira* spp.
Efficiency	102.5±2.1	92.75±1.63	107.3±1.9
Slope	-3.3±0.05	-3.5±0.05	-3.2±0.04
R^2 of Slope	0.998±0.001	0.99±0.01	0.998 ± 0.04
Intercept	39.8±2.2	35.0±0.59	37.2±0.14

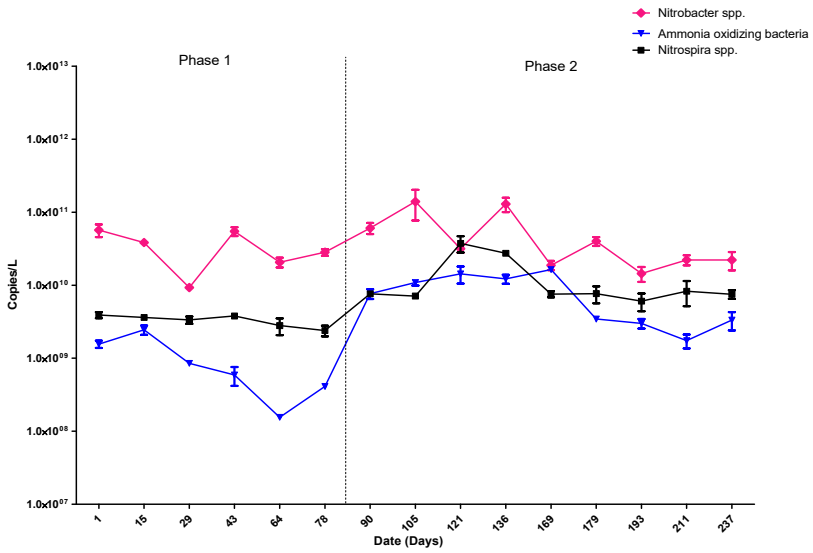


Fig. 3 Changes in copy number variations of nitrifiers in the plant

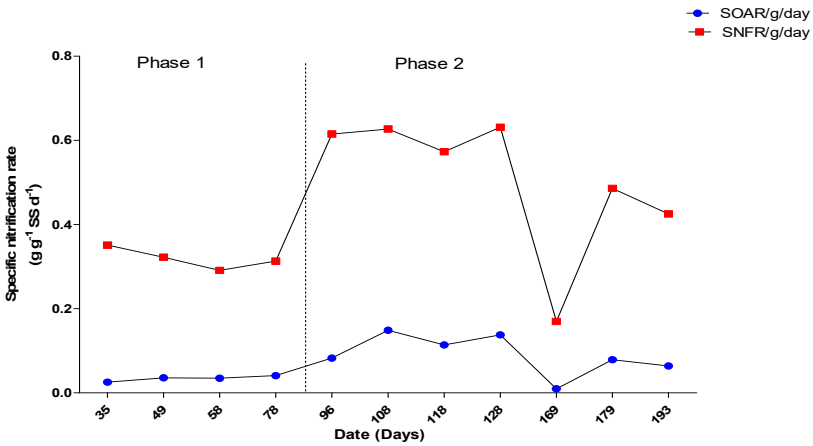


Fig 4 The specific nitrification rate of the biomass in terms of specific ammonium oxidization rate

IV. Discussion

Seasonal temperature variation has been implicated as one of the environmental factors [24], [25] affecting total nitrogen and ammonia oxidizing rates biotransformation in wastewater treatment facilities. In this study, a significant seasonal variations in temperature ($\alpha = 0.05$; $P = < 0.0001$) was observed in the reactor. The maximum temperature amplitude was 10.2°C. The lowest ammonia removal of 12.1% and AOB abundance ($1.6 \times 10^8 \pm 1.3 \times 10^7$) were observed when the lowest temperature of 14.2°C was recorded (Fig. 2 (b)). Reference [26] earlier has reported a similar high correlation of temperature with ammonia oxidation. In this study, it was observed that the NH_3 removal efficiency was reduced from 86.34±7% during summer when the organic loading rate (OLR) was 4.212 kg COD/m^3.d (Fig. 2 (c)), to 56.01±26% during winter when the OLR 8.993 kg COD/m^3.d. This is in line with the report by [27] that also noted a poor nitrification rate at high organic loading rate. This was also found to correlate significantly with seasonal temperature fluctuation ($r = 0.767$; $P = 0.001$). However, neither temperature nor NOB populations was found to correlate with nitrite oxidation rate.

The average copy number of AOB to NOB ratio varied from 0.11 (summer) to 0.02 (winter) during the period investigated (Fig. 3), which was lower than the theoretical ratio 2.0 reported for good nitrification by [28]. Seasonal temperature was observed to have positive correlation with the AOB gene copy number; whereas NOB was not affected by the temperature shifts. The average influent parameters of the plant showed great variation comparing the summer to the winter period during this study and the COD concentration was perpetually higher during the winter compare to summer. According to various studies high COD concentration usually favours the proliferation of the more competitive heterotrophic bacteria population over the autotrophic nitrifiers [29], [30]. In this study the combination of the generally low DO and the higher COD during the winter impacted on the ammonia removal rate of the plant due to probable increased heterotrophic competition elicited by the increased COD concentration observed during the period.

Quantitative PCR results revealed the dominance of NOB over AOB throughout the study period. Due to the limiting oxygen levels observed during this study (Table I), there is a possibility of nitrite-loop. Under low DO level, denitrifiers can carry out incomplete nitrate reduction which can serve as additional nitrite source for NOB and partially uncouple their growth from AOB, thereby resulting in their elevated population density [28], [31] noted that NOB exhibited a significant O_2 affinity under prolonged low DO concentrations (0.16 - 0.37 mg/l) which in turn made them a better competitor for O_2 as their abundance increased comparably to AOB. Another possible reason for the higher population load of NOB observed in this study is suggested by [32]. According to them, under extended period of low dissolved oxygen concentrations ($\leq$ 0.5 mg/l) as observed in this study, the endogenous decay of both ammonia/nitrite oxidizing bacteria was retarded. This resulted in increased biomass density which nullified some low DO impact on nitrification. They also reported near complete nitrification with 0.16-0.37 mg/l DO range. Furthermore, they noted that under extended low DO period NOB demonstrated significant increase in O_2 affinity which in turn made them a better O_2 competitor than AOB.

The dominance of *Nitrospira* (K-strategist) over Nitrobacter (r-Strategist) in activated sludge has been reported [33], [34]; however in this study a contrary observation was noted. This could possibly be explained based on the earlier observations of [35] and [36] where they have noted an irreversible prevalence of *Nitrobacter* spp. over *Nitrospira* spp. in WWTP after spike in nitrite concentration and even after subsequent reduction in nitrite concentration. Nitrobacter usually exhibit inhibitory effect on the growth of *Nitrospira* once it dominates. Reference [37] also reported that *Nitrobacter* spp. though a weak competitor compared to *Nitrospira* under low nitrite concentration can be selected over *Nitrospira* spp. in plants with low inorganic carbon in addition to low nitrite concentration. The plant studied exhibited low AOB population density which could result in lowered rate of ammonia conversion to nitrite. This could probably give a selective advantage to *Nitrobacter* spp. as observed in this study. Although various authors [11]–[13] have reported the possible involvement of AOA in nitrification in WWTP, this group of nitrifier was not detected in this study which indicates that AOA may not be ubiquitous in all engineered wastewater treatment systems.

The SAOR was found to be lower than the SNFR throughout the study with the average values as 0.07036 and 0.4368 g N g^{-1} MLSS d^{-1} SAOR and SNFR respectively. This result indicates a similar trend with earlier study by [38] that reported a lower SAOR compared to SNFR in their aerobic batch experiment. They [38] also found out that the SAOR and SNFR obtained did not reflect any proportional relationship between both ammonia and nitrite-oxidation measured in their experiment. On the contrary, the specific nitrification rates determined in this present study (higher SNFR as compared to SAOR) reflects the population densities (higher NOB copy numbers compared to AOB) of the nitrifiers obtained using qPCR. This result indicates that specific nitrification rate determined in the laboratory batch experiment, can serve as indicator of the plant's nitrifying community and performance. Reference [39] in an earlier batch experiment reported a similar trend, where the SAOR and SNFR obtained were related to the AOB: NOB ratio in the plant.

V. Conclusion

The seasonal dynamics of nitrifying community in full-scale municipal bioreactor was investigated and variation in nitrification efficiency was noted with the lowest rate observed during winter. Seasonal temperature variation correlates positively with AOB population and ammonia removal rate but not with NOB (*Nitrobacter* spp. and *Nitrospira* spp.). Significant correlation could not be established between nitrification and DO levels in this study as the microbial community have adapted to the low DO concentrations. Contrary to previous reports, our findings showed that AOA is not ubiquitous in all wastewater treatment

plants as we could not amplify this group through this study. Seasonal temperature variation and organic loading ratio were major factor impacting the microbial population abundance which in turn modifies nitrification in wastewater treatment. The specific nitrification rate batch experiment could be a speedy way of deducing the plant's nitrification performance in a full scale plant. The findings of the current study have set the foundation for future research on using seasonal impact on nitrification performance of full scale activated sludge in sub-tropical temperature.

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