

RESEARCH ARTICLE

Ammonium oxidizing bacterial populations in South African activated sludge wastewater treatment plants

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Abstract

This is the first study that describes ammonium oxidizing bacterial populations and correlations of these populations with a range of criteria in activated sludge wastewater treatment plants in South Africa. In this study, not only the influent but also the activated sludge chemistry was comprehensively characterized. Multivariate statistical analyses were used to determine the relative significances of the geographical location (factor: site), wastewater treatment plant process (factor: configuration), seasonality (factor: season), and environmental parameters on the ammonium oxidizing bacterial genera in six municipal activated sludge wastewater treatments plants from two sites (the cities of Cape Town and Ekurhuleni). The geographical location (site) was significant for selection of the ammonium oxidizing genera (Global ANOSIM R value = 0.538, $p = 0.001$). It was established that the inter-site differences were not climatic in origin, nor related to the composition of the influent, but were rather driven by the activated sludge chemistry. It was found using BEST analysis that the activated sludge ammonia, activated sludge total phosphate, and activated sludge total chemical oxygen demand were the most significant ($p < 0.001$) drivers for ammonium oxidizing bacterial selection (ANOSIM Global R value 0.419) and were significantly higher in the activated sludge from the City of Cape Town wastewater treatment plants. *Nitrosospira* was the most abundant ammonium oxidizing bacterial genus, with notably higher relative and estimated actual abundances in the City of Cape Town wastewater treatment plants than the City of Ekurhuleni wastewater treatment plants. The strong selection of *Nitrosospira* in the City of Cape Town wastewater treatment plants with higher nutrient concentrations strongly suggests that high concentrations of activated sludge ammonia, activated sludge total phosphate, and activated sludge total chemical oxygen demand are key selective drivers for this genus.

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Practitioner Points

- First comprehensive study describing ammonium oxidizing bacterial populations in Southern African domestic activated sludge wastewater treatment plants.
- The geographical location (site) was significant for selection of different ammonium oxidizing genera (Global ANOSIM R value = 0.538, $p = 0.001$).
- Inter-site differences driven by the activated sludge chemistry, not climate or influent wastewater composition.
- Selection of *Nitrosospira* driven by high concentrations of activated sludge ammonia, total phosphate and total chemical oxygen demand.

KEYWORDS

configuration, nitrification, *Nitrosospira*, process, season

INTRODUCTION

High but variable levels of ammonia are present in domestic wastewater/municipal sewage. For example, in monthly influent samples taken from 16 wastewater treatment plants (WWTPs) located in three cities in South Africa (SA), inter-city averages of 58.7 ± 19.0 mg/L (range: 38.1–102.0 mg/L, $n = 82$), 37.5 ± 19.2 mg/L (range: 6.5–94.9 mg/L, $n = 76$), and 23.8 ± 18.8 mg/L (range: 0.7–82.8 mg/L, $n = 48$) were measured (Welz et al., 2022). Both unionized ammonia (NH_3) and ammonium ions (NH_4^+) are present in wastewater, with the unionized form being particularly toxic to aquatic biota (Alonso et al., 2022; Lin et al., 2022). The proportion of NH_3 increases with temperature and/or pH ($\text{pK}_a = 9.25$ at 25°C) (Alonso et al., 2022). At temperatures ranging from 0 to 30°C , and within pH values typical of domestic wastewater (7–8), the proportion of NH_3 is low, ranging from 0.082% to 7.46%. However, this increases markedly as the pH increases and NH_3 concentrations as low as 1 mg/L can have adverse effects on biotic species (Alonso et al., 2022; Lin et al., 2022). It is therefore desirable to reduce the amount of $\text{NH}_3/\text{NH}_4^+$ in wastewater before discharge (Wade et al., 2010).

Nitrification refers to the biologically mediated conversion of NH_3 to nitrite (NO_2^-) and nitrate (NO_3^-) by autotrophic nitrifying bacteria and archaea. The primary function of conventional activated sludge (AS) WWTPs treating domestic sewage is to reduce the biological/chemical oxygen demand (BOD/COD) and $\text{NH}_3/\text{NH}_4^+$ concentrations to protect the receiving environment (Wade et al., 2010). Aeration of the AS promotes organic biodegradation by chemoorganotrophic (heterotrophic) microbial species as well as nitrification by specialized

autotrophic species (Chen et al., 2022; Li et al., 2021; Soliman & Eldyasti, 2018; Zhou et al., 2020). In biological nutrient removal (BNR) and extended biological phosphorus removal (EBPR) WWTPs, additional total nitrogen (TN) is removed by promoting both nitrification as well as subsequent denitrification of the formed NO_2^- and NO_3^- to nitrogen (N_2) and nitrous oxide (N_2O) gas in aerated and non-aerated reactors, respectively.

In terms of ammonium oxidation (AO), ammonium oxidizing bacteria (AOB), ammonium oxidizing archaea (AOA), and the more recently described complete ammonium oxidizing bacteria (CAOB) are involved in the oxidation of $\text{NH}_3/\text{NH}_4^+$ to NO_2^- or in the case of CAOB, directly to NO_3^- (Stein, 2019; Zhao et al., 2019). Nitrite is converted to NO_3^- by nitrite oxidizing bacteria (NOB), but oxidation of $\text{NH}_3/\text{NH}_4^+$ is usually the rate-limiting step. The AO microbes are therefore critically important in AS WWTPs. However, these autotrophic species grow more slowly, are metabolically less active, and are more sensitive to environmental perturbations than their heterotrophic counterparts, which includes the denitrifiers (Soliman & Eldyasti, 2018). Conditions therefore need to be created to promote the growth of AO species. Physicochemical parameters reported to impact on AO growth and activity are as follows: (i) Temperature—growth is severely retarded at low temperatures; (ii) aeration—sufficient dissolved oxygen (DO) is required for nitrification; (iii) pH—a stable pH range is required; (iv) sludge retention time (SRT)—sludge needs to be retained for long enough to prevent washout of the slow-growing nitrifiers; (v) toxic metals—nitrifiers are particularly sensitive to toxic metals and a range of other substances; (vi) NO_2^- concentration—excessive NO_2^- inhibits nitrifiers; (vii) carbon to nitrogen (C/N) ratio—excessive

organic substrate promotes growth of heterotrophs (Cui et al., 2019; Chen et al., 2022; Gholami-Shiri et al., 2021; Johnson et al., 2019; Li et al., 2021; Novotnik et al., 2014; Soliman & Eldyasti, 2018; You et al., 2009; Yu & Zhang, 2012; Zhou et al., 2020). Loss of nitrification through washout of nitrifiers, nitrifier toxicity, or competitive exclusion is common in AS WWTPs (Johnson et al., 2019). After “upsets,” it can take weeks to reestablish functional nitrifying populations (Bhattacharya & Mazumder, 2020; Johnson et al., 2019). Studies that focus specifically on AOB can provide critical insight into the fundamental aspects of biological nitrification in AS WWTPs.

The microbial community composition in AS WWTPs varies from one geographical location to another (Dueholm et al., 2022; Kim et al., 2019; Kim et al., 2021a; Thobejane et al., 2023; Valentin-Vargas et al., 2012; Wu et al., 2018; Zhang et al., 2012; Zhao et al., 2014). Variations have been ascribed to seasonal and/or climatic differences and variations, (Cai et al., 2020; Kang et al., 2020; Kim et al., 2021b; Sun et al., 2021) and/or WWTP process configurations and operations (Begmatov et al., 2022; Dueholm et al., 2022; Hu et al., 2012; Jiang et al., 2018; Kim et al., 2021b; Nierychlo et al., 2020; Wu et al., 2018), and/or sociocultural aspects, such as dietary habits, on the influent composition (Begmatov et al., 2022; Zhao et al., 2014). In SA, monthly sampling over 18 months from nine WWTPs from two geographical locations (the City of Cape Town [CPT, $n = 3$] and the City of Ekurhuleni [COE, $n = 6$]) revealed highly significant differences in the overall bacterial community structures from each site, with only negligible effects being found for the factor “season” and “WWTP process configuration” (Thobejane et al., 2023).

Literature describing comprehensive studies on microbial communities in AS WWTP are skewed toward those conducted in Europe, Asia and the United States, with Africa and South America being particularly

under-represented (Dueholm et al., 2022). To date, there are no studies describing the AOB populations in AS WWTPs in SA, and, to the best of our knowledge, in sub-Saharan Africa. In addition, from a global perspective, there are no studies that have linked AOB populations in AS WWTP to physicochemical parameters in AS. In this study, the AOB genera in six WWTPs from the CPT and the COE have been elucidated, and the AOB composition has been analyzed for significant correlations with site, season, WWTP process configuration and environmental parameters, which includes analysis of both influent and AS.

MATERIALS AND METHODS

Sampling and sampling sites

Six WWTPs, three each from COE and CPT, were included in the study. Despite the fact that no University of Cape Town (UCT)-configured WWTPs were present in COE and there is only one three-stage Bardenpho (3SB)-configured WWTP in CPT, in terms of wastewater type (mainly domestic), influent type (no primary settling), and process (configuration), the closest matches were selected from the two cities (Table 1). Extended biological phosphorus removal (EBPR) WWTPs such as five-stage Bardenpho-configured WWTPs were excluded from the study.

For the larger study, monthly samples were taken wherever possible over a period of 20 months from each WWTP (Thobejane et al., 2023; Welz et al., 2022). Of a total of 141 AS and influent samples, 50 samples were selected for high-throughput sequencing (HTS) and nitrification studies (Thobejane et al., 2023). Samples were transported on ice and physicochemical analyses were performed within 24 h. Aliquots of the AS samples for DNA extractions were immediately washed in equal

TABLE 1 Basic design details of study wastewater treatment plants.

Site	Configuration	Capacity (ML/day)	Industrial (%)
CPT	3SB	14.0	5
CPT	MLE	12.0	0
CPT	UCT	17.5	5
COE	MLE	1.0	0
COE	3SB (A)	4.7	0
COE	3SB (B)	20.0	0

Abbreviations: 3SB, 3-stage Bardenpho; five-stage Bardenpho; COE, City of Ekurhuleni; CPT, City of Cape Town; ML, megaliter; MLE, modified Ludzack–Ettinger; UCT, University of Cape Town.

volumes of $1\times$ phosphate-buffered saline (PBS) and stored at -20°C until required.

Analysis of environmental and operational parameters

The AS dissolved oxygen (DO) concentration was measured using a YSI 550A DO meter (Xylem Analytics, New York, USA). Both the temperature and DO readings were taken manually by submerging probes into the AS at a minimum of three locations in the aerobic reactors and taking an average of the readings. The diluted sludge volume index (DSVI) and mixed liquor suspended solids (MLSS) were determined according to standard methods (APHA, 2005).

Chemical analyses were performed on influent and AS samples as described by Thobejane et al. (2023) and included measurements of pH, BOD, total COD (COD_t), total phosphate (TP), ortho phosphate ($\sigma\text{-P}$), sulfate as sulfur ($\text{SO}_4^{2-}\text{-S}$), NH_3 as N ($\text{NH}_3\text{-N}$), NO_3^- and NO_2 as N ($\text{NO}_3^-/\text{NO}_2\text{-N}$), total alkalinity (T.alk), and volatile fatty acids (VFA).

Microbial analyses

DNA extraction

Metagenomic DNA was extracted using the PowerSoil[®] DNA extraction kit (MoBio Qiagen, Hilden, Germany) according to the manufacturers' instructions with the following modifications: The amounts of AS used for the extraction step and buffer used for the final elution step were increased from 0.5 to 1 g and 50 to 100 μL , respectively. The quantity and quality of the DNA was assessed using a NanoDrop[™] 2000 Spectrophotometer (Thermo Scientific, Waltham, MA, USA). In order to reduce PCR bias, the DNA extractions were conducted in duplicate and the extracts were pooled in equimolar amounts. The eluted DNA aliquots were stored at -20°C until required for analysis.

High-throughput amplicon sequencing

An Illumina MiSeq (San Diego, CA, USA) instrument was used for high-throughput amplicon sequencing (HTAS) of the V4 region of the small subunit (SSU) of the 16S rRNA gene using the primer pairs 515F-Y (5'-GTG YCA GCM GCC GCG GTA A-3') (Parada et al., 2016) and revised 806-R (5'-GGA CTA CNV GGG TWT CTA AT-3') (Apprill et al., 2015) at MR DNA

(Shallowater, TX, USA) according to the in-house protocol as described by Thobejane et al. (2023). Zero radius operational taxonomic units (zOTUs) were generated from processing of the DNA libraries using the MR DNA analysis pipeline as previously described (Thobejane et al., 2023). The zOTUs were assigned taxonomic classification using BLASTn against a curated database derived from the Ribosomal Database Project II (RDP II) and the National Centre for Biotechnology Information (NCBI) databases (<http://rdp.cme.mus.edu> and <http://www.ncbi.nlm.nih.gov>).

Quantitative polymerase chain reaction and estimated absolute abundance

The quantity and quality of the DNA was determined at MR DNA using a NanoDrop[™] 2000 instrument (Thermo Scientific). The DNA samples were then amplified by quantitative PCR (qPCR). Sequences of three genes were amplified: (i) a sequence in the V4 region of the small subunit (SSU) of the 16S rRNA as per the HTAS, (ii) a 491-bp sequence of the bacterial ammonium oxidizing (*amoA*) gene using the primer pair *amoA*-1F: 5'-GGG GTT TCT ACT GGT GGT-3' and *amoA*-2R: 5'-CCC CTC KGS AAA GCC TTC TTC-3' (Rotthauwe et al., 1997), and (ii) a 436-bp region of the newly described CAOB *amoA* gene using the primer pair *comamoA*-F: 5'-AGG NGA YTG GGA YTT CTG G-3' and *comamoA*-R: 5'-CGG ACA WAB RTG AAB CCC AT-3' (Zhao et al., 2019).

For both *amoA* amplicon qPCRs, the DNA was normalized, and 10 ng DNA from each sample was used to perform the reactions using a StepOnePlus[™] Real-Time PCR System (Applied Biosystems, Waltham, MA, USA) with PowerUp SYBER[®] Green Master Mix (Applied Biosystems). The reactions were carried out with an initial holding stage of 50°C for 2 min followed by 95°C for 2 min. The cycling stage consisted of 40 cycles of 95°C for 15 s, 53°C for 40 s, and 72°C for 1 min. Each reaction was conducted in duplicate. *Nitrosomonas europaea* ATCC 19718 DNA was used as a standard for *amoA*-1F/2R, but no standard DNA was available for *comamoA*-F/R.

For the 16S rRNA gene amplicon qPCR, 1 μL of the template DNA and 2X Universal Taqman[™] PCR Mastermix (Applied Biosystems) was used. The reactions were performed with an initial holding stage of 50°C for 2 min followed by 95°C for 10 min. The cycling stage consisted of 40 cycles of 95°C for 15 seconds, followed by 60°C for 1 minute. The estimated actual abundance (EAA) of the number of bacterial nitrifying genera per gram of AS was calculated using 10-fold dilutions of *Escherichia coli* strain K12 as linear standards to

extrapolate cell number from the qPCR cycle thresholds (Ct) and applying Equation 1.

$$EAA = (RA \times n \times 100) \quad (1)$$

where RA = relative abundance of taxa determined from amplicon sequencing, n = number of bacterial cells in 1 μL template DNA, and 100 = elution volume from 1.0 g AS, which was standardized for each sample extraction.

Statistical analyses

The RA of the nitrifying bacteria genera obtained from Illumina MiSeq data (Section 2.3.3) as well as the environmental data were analyzed using Primer 7[®] software (Primer-e, Auckland, New Zealand).

Square root transformed data was used to create Bray–Curtis similarity matrices that were then used for nested and one-way unordered analysis of similarity (ANOSIM) based on Spearman rank correlations and to create non-metric multidimensional scaling (nMDS) plots to assess the influence of the geographical location (site) and process (WWTP configuration) on the overall bacterial and nitrifying bacterial community compositions.

Student's t -tests (Microsoft Excel[®]) were performed on the environmental data to determine whether there were significant differences between the results obtained from the two sites ($T > T_{\text{crit}}$, $p < 0.05$). The data were then analyzed using Primer 7[®] software. Fourth root transformed and normalized data were used for principal component analyses (PCA) and ANOSIM. Significant correlations were determined by regression analyses using Microsoft Excel[®]. RELATE and BEST analyses of

Spearman rank correlations were performed on the Bray–Curtis similarity data of nitrifying bacterial community compositions and the Euclidean distance of the environmental data. The presence or absence of significant correlations between *amoA* qPCR (per ng DNA and per gram AS) and nitrifier abundance (%RA and EAA) at genus level was determined using regression analysis (Microsoft Excel[®]).

RESULTS AND DISCUSSION

Composition of ammonium oxidizing bacterial populations in activated sludge samples

There was a highly significant correlation between the RA of the identified bacterial genera and the geographical location (factor “site”) determined by unordered one-way ANOSIM (Spearman global $R = 0.538$, $p = 0.001$). There was also a significant but lower correlation between RA of the AOB genera subset and “site” (unordered one-way ANOSIM, Spearman global $R = 0.396$, $p = 0.001$). These relationships can be visualized on the nMDS plots that show (i) distinct separation of the data points representing the RA of all the identified bacterial genera from each site on either side of the plot (Figure 1a) and (ii) less distinct clustering of most data points representing the RA of the AOB genera from each site on either side of the plot (Figure 1b).

Significant inter-site differences were previously found in the core bacterial compositions in the WWTPs from the COE and CPT. The distinctions were mainly driven by different nutrient concentrations, the most important being the AS TP, the AS NH_3 , and the influent COD_t

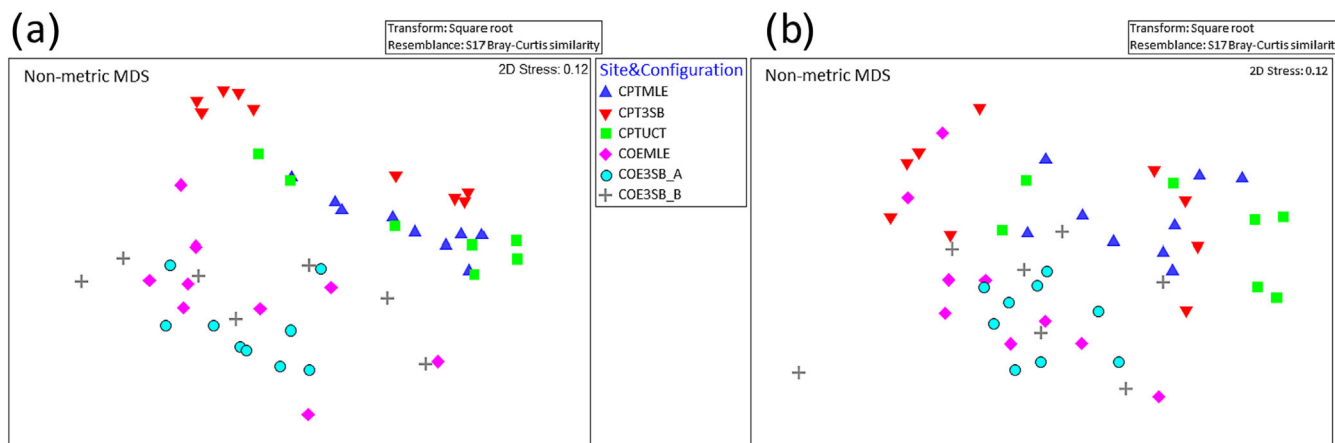


FIGURE 1 nMDS plots of the relative abundance of all bacterial genera (a) and ammonium oxidizing bacteria (b) from all study samples ($n = 50$).

concentrations that were significantly higher in the CPT WWTPs (Thobejane et al., 2023). In that study, climatic factors were discounted from being the primary drivers of bacterial community selection, as the temperature differences between each city were minimal (personal communication South African Weather Services [SAWS]) (Figure 2a), and there was no correlation between the AS temperature and the bacterial community composition (Thobejane et al., 2023). Although the COE is a summer rainfall area and CPT is a winter rainfall area (Figure 2b), dilution effects were deemed as negligible because none of the study WWTPs receive stormwater in the sewage networks (Thobejane et al., 2023).

Low temperatures are known to retard de-ammonification of wastewater (Gholami-Shiri et al., 2021; Johnson et al., 2019; Pan et al., 2018), but in this study, the factor “season” had no significant influence on the AOB community composition (ANOSIM $p > 0.05$). There was also no significant

correlation of AS temperature with AOB community composition (Section 3.2). Historically, nitrification rates do not decrease in winter in AS WWTPs in the CPT (Welz, 2009; Welz et al., 2014). Even in the COE where slightly colder average minimum temperature are experienced, nitrification rates determined from historical influent and effluent NH_3 measurements from 2013 to 2021 remained high throughout the year in all three study WWTPs (Figure 3). There were low but significant positive correlations between temperature and nitrification rates at the COEMLE (Pearson's $R = 0.228$, $0.05 < p > 0.01$) and COE3SB_A WWTP (Pearson's $R = 0.258$, $0.01 < p > 0.005$), but not at the COE3SB_B WWTP ($p > 0.05$). Overall, the results indicated that winter temperatures in SA are not low enough have deleterious effects on the AO microbial function that cannot be overcome by slight adjustments to the operational parameters as suggested anecdotally (personal communication) (Johnson et al., 2019; Pan et al., 2018).

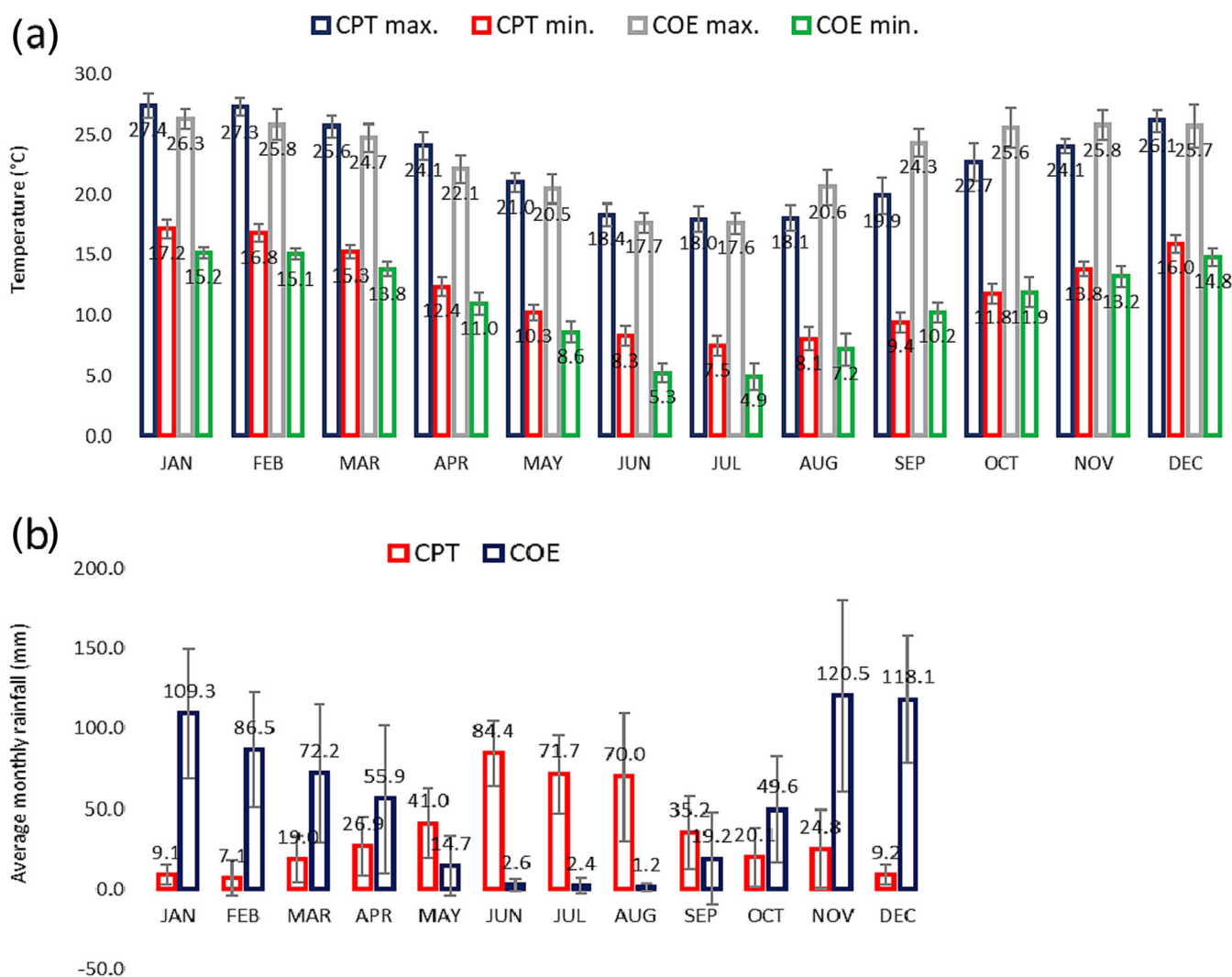


FIGURE 2 Average monthly temperature (a) and rainfall (b) data measured at the City of Cape Town and the City of Ekurhuleni.

FIGURE 3 Average monthly ammonia removal efficiency from 2013 to 2021 measured at the study wastewater treatment plants in the City of Ekurhuleni. Error bars represent the standard deviation from the mean. Four outliers removed (three from COE3SB_B during plant upgrading).

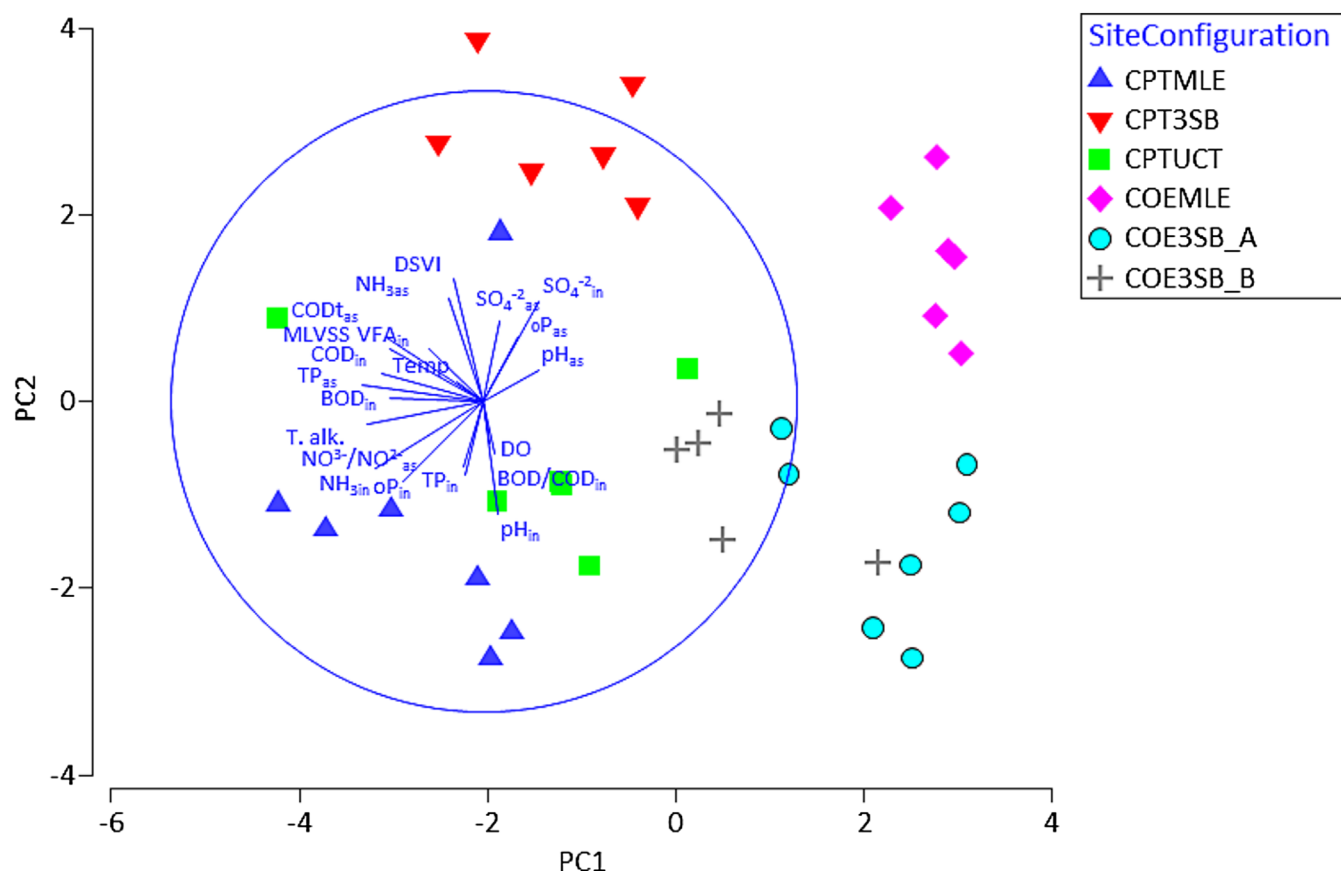
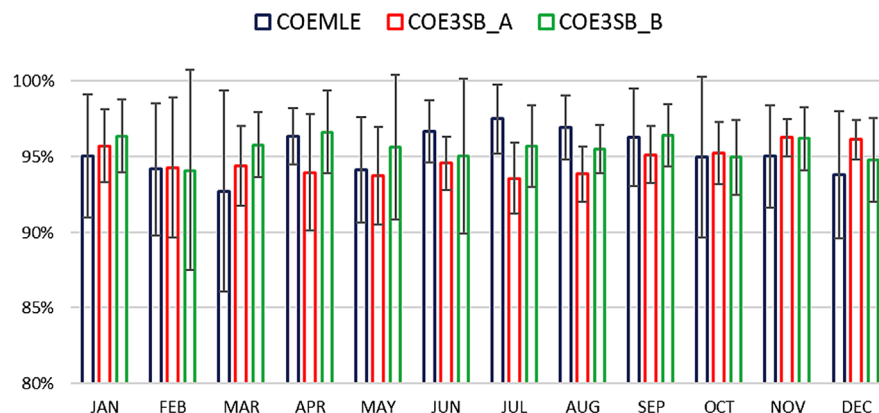


FIGURE 4 PCA plot of the Euclidean distance of all measured environmental parameters in the samples taken from the study wastewater treatment plants.

Correlation of environmental parameters with AOB community composition

Of the 50 AS samples that were analyzed using HTAS, comprehensive environmental data were available for 37 samples. The physicochemical profiles from each WWTP were mostly unique, as demonstrated on the PCA plot where data points denoting each sample from each WWTP tend to cluster together (Figure 4).

Analysis of all the environmental data revealed significant differences between some parameters from the two geographical locations (Table 2). Possible correlations between environmental data and the AOB composition (based on RA) were determined using RELATE analyses, with the data being analyzed concurrently (all) as well as in three separate categories, namely, influent, AS, and “other” (Table 2). There were no significant correlations between “all,” “influent,” and “other” ($p > 0.5$).

TABLE 2 Physicochemical parameters measured in the study samples.

Parameter	CPT (ave $\pm$ SD)	COE (ave $\pm$ SD)	T	T _{crit}
Influent chemistry ($n = 37$)				
BOD (mg/L)	407 $\pm$ 106	243 $\pm$ 146	3.886***	1.700
COD _t (mg/L)	1034 $\pm$ 217	598 $\pm$ 303	5.004***	1.700
BOD/COD (ratio)	0.40 $\pm$ 0.12	0.44 $\pm$ 0.21	0.836	1.703
pH	7.6 $\pm$ 0.3	7.8 $\pm$ 0.4	1.706*	1.690
T.Alk (mg/L as CaCO ₃)	406 $\pm$ 69	273 $\pm$ 94	4.867***	1.700
NH ₃ (mg/L as N)	73 $\pm$ 21	42 $\pm$ 27	3.907***	1.692
TP (mg/L as P)	13 $\pm$ 6	12 $\pm$ 17	0.197	1.717
σP (mg/L)	9.0 $\pm$ 3.1	7.4 $\pm$ 5.5	2.616**	1.700
SO ₄ ²⁻ (mg/as S)	70 $\pm$ 38	88 $\pm$ 33	1.569	1.689
VFA (mg/L)	89 $\pm$ 75	47 $\pm$ 28	2.263*	1.714
Activated sludge chemistry ($n = 37$)				
COD _t (mg/L)	6315 $\pm$ 1592	4152 $\pm$ 870	5.163***	1.701
pH	6.8 $\pm$ 0.3	7.2 $\pm$ 0.5	3.404**	1.703
NH ₄ ⁺ (mg/L as N)	16 $\pm$ 21	4.6 $\pm$ 1.9	2.298*	1.734
NO ₃ ⁻ /NO ₂ ⁻ (mg/L as N)	1.9 $\pm$ 3.2	1.0 $\pm$ 1.9	0.968	1.699
TP (mg/L)	126 $\pm$ 40	15 $\pm$ 12	11.51***	1.72
σP (mg/L)	5.4 $\pm$ 8.5	8.6 $\pm$ 7.3	1.231	1.689
SO ₄ ²⁻ (mg/L as S)	63 $\pm$ 19	63 $\pm$ 17	0.003	1.689
Other ($n = 37$)				
MLVSS (mg/L)	4432 $\pm$ 1119	2650 $\pm$ 1299	4.459***	1.691
DSVI (mL/g)	202 $\pm$ 86	165 $\pm$ 77	1.364	1.690
DO (mg/L)	1.3 $\pm$ 0.9	0.9 $\pm$ 1.4	0.291	1.692
Temp (°C)	19.6 $\pm$ 2.0	19.5 $\pm$ 3.1	1.013	1.699

Note: P (T_{crit} < =T) two-tail *** significance $p < 0.001$ **significance ($0.001 \geq p < 0.01$) *significance ($0.01 \geq p < 0.05$).

Abbreviations: BOD, biological oxygen demand; COD_t, total chemical oxygen demand; DO, dissolved oxygen; DSVI, diluted sludge volume index; MLVSS, mixed liquor volatile suspended solids; N, nitrogen; NH₃, ammonia; NO₃⁻/NO₂⁻, nitrates/nitrites; SO₄²⁻, sulfate; TP, total phosphorus; VFA, volatile fatty acids; σP, ortho phosphate.

However, there was a significant correlation between the AS environmental data and the AOB composition (Spearman rank 0.368, $p = 0.001$). Similarly, the BEST analyses showed only low Spearman correlations between the chemical parameters measured in the influent (maximum 0.138 for a combination of pH, T.alk, SO₄²⁻, and VFAs) and “other” parameters (maximum 0.144 for a combination of DSVI, DO and temperature). Conversely, notably higher correlations were found for the AS chemical parameters (maximum 0.419 for a combination of AS COD_t, AS NH₃, and AS TP).

A recent comprehensive literature review yielded 18 studies focused on microbial community composition in AS WWTPs (Thobejane et al., 2023). None of the studies measured chemical parameters in the AS, only in the influent and in some cases, effluent. Correlations between removal rates and/or influent values are

commonly reported. However, this study on the AOB populations and our previous study on the overall bacterial community composition show that the AS nutrient concentrations, not the influent nutrient concentrations, are the major drivers of bacterial community composition. It is contended that this is because the composition of the AS in each individual WWTP is a holistic reflection of the influent composition, the operational performance, and the WWTP operation (including the sludge recycle (SR) ratio and sludge wasting rate [SWR]). This is a highly noteworthy finding, and it is proposed that all future microbial studies on AS WWTPs, and possibly all biological reactors, should include measurements of a range of parameters in the AS and/or mixed liquor.

The links between the most important drivers of AOB community composition (AS COD_t, AS TP, AS NH₃) can clearly be seen in Figure 5. In the nMDS plot showing

the RA of the AOB in the different WWTPS (Figure 5a), the data point from the COE group on the upper half of the plot, while those from the CPT WWTPs group on the lower half of the plot (indicated by a line). The bubble overlays on the nMDS plot that depict the concentrations of AS COD_t, AS TP, and AS NH₃ clearly show that in most instances, the concentrations were notably higher in the CPT WWTPs.

Ammonium oxidizing bacterial composition in the study wastewater treatment plants

The average RA of the *Nitrosomonadaceae* in the core bacterial population (*Nitrosomonas*, *Nitrosococcus*, *Nitrosospira*, and *Nitrosovibrio*) for each WWTP was calculated (Figure 6a). As expected, the RA was higher in the CPT WWTPs that contained higher nutrient and NH₃/NH₄⁺ concentrations in the AS. However, when the abundance

was normalized for the number of AOB cells present in 1 g AS, there was less variability (Figure 6b). The same was true for the individual genera (Figure 6). While quantification using RA provides a measure of microbial taxonomic distribution, it does not account for differing amounts of microbial biomass in the samples. There are also inherent biases in quantification methods based on 16S rRNA HTAS as amplicon copy numbers vary from taxon to taxon (Větrovský & Baldrain, 2013; Williamson et al., 2021). Nevertheless, the normalized EAA provided more credible data in this study.

No seasonal trends were noted in the distribution of the AOB genera in any of the study WWTPs. *Nitrosospira* were typically more abundant in the CPT WWTPs than the COE WWTPs when measured as RA and EAA, while the converse was true for *Nitrosomonas*, and to a lesser extent, the EAA of *Nitrosococcus* (Figure 7). The strong selection of *Nitrosospira* in the CPT WWTPs strongly suggests that high AS nutrient concentrations (AS COD_t, AS NH₃, and AS TP) are key selective drivers for this genus.

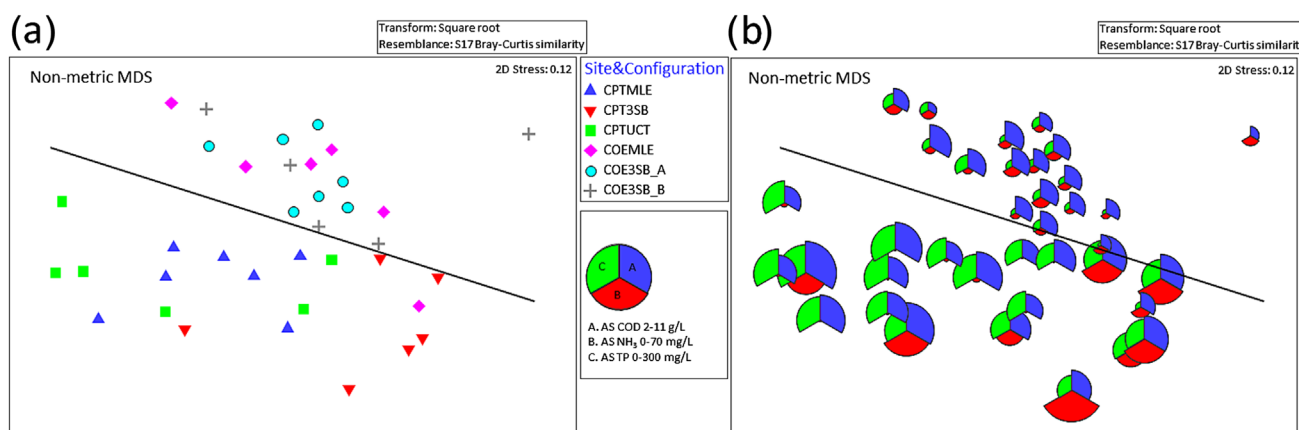


FIGURE 5 nMDS plot of the relative abundance of the ammonium oxidizing genera (a) the same nMDS plot with bubble overlays of the three most significant drivers of ammonium oxidizing bacterial composition (b) ($n = 37$).

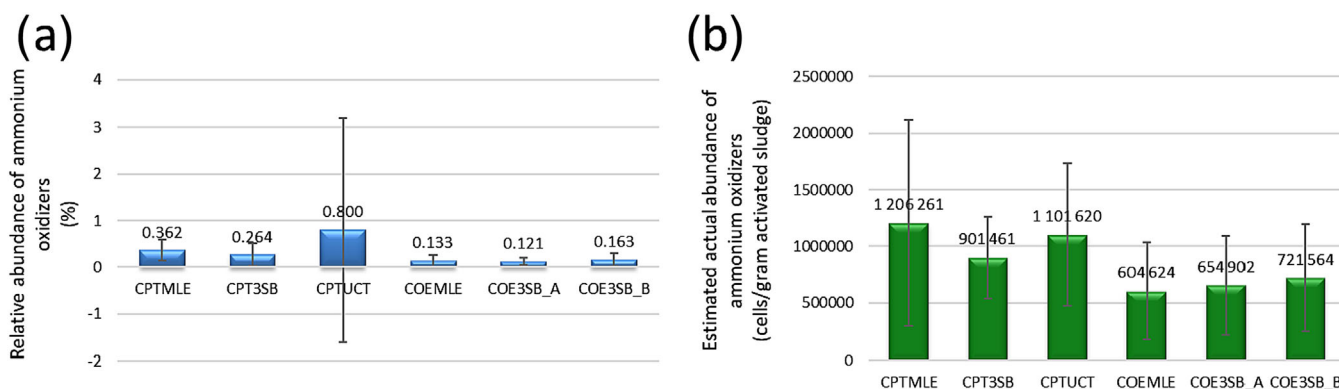


FIGURE 6 Average relative abundance (a) and estimated actual abundance (b) of ammonium oxidizing bacterial genera. Error bars represent standard deviation from the mean.

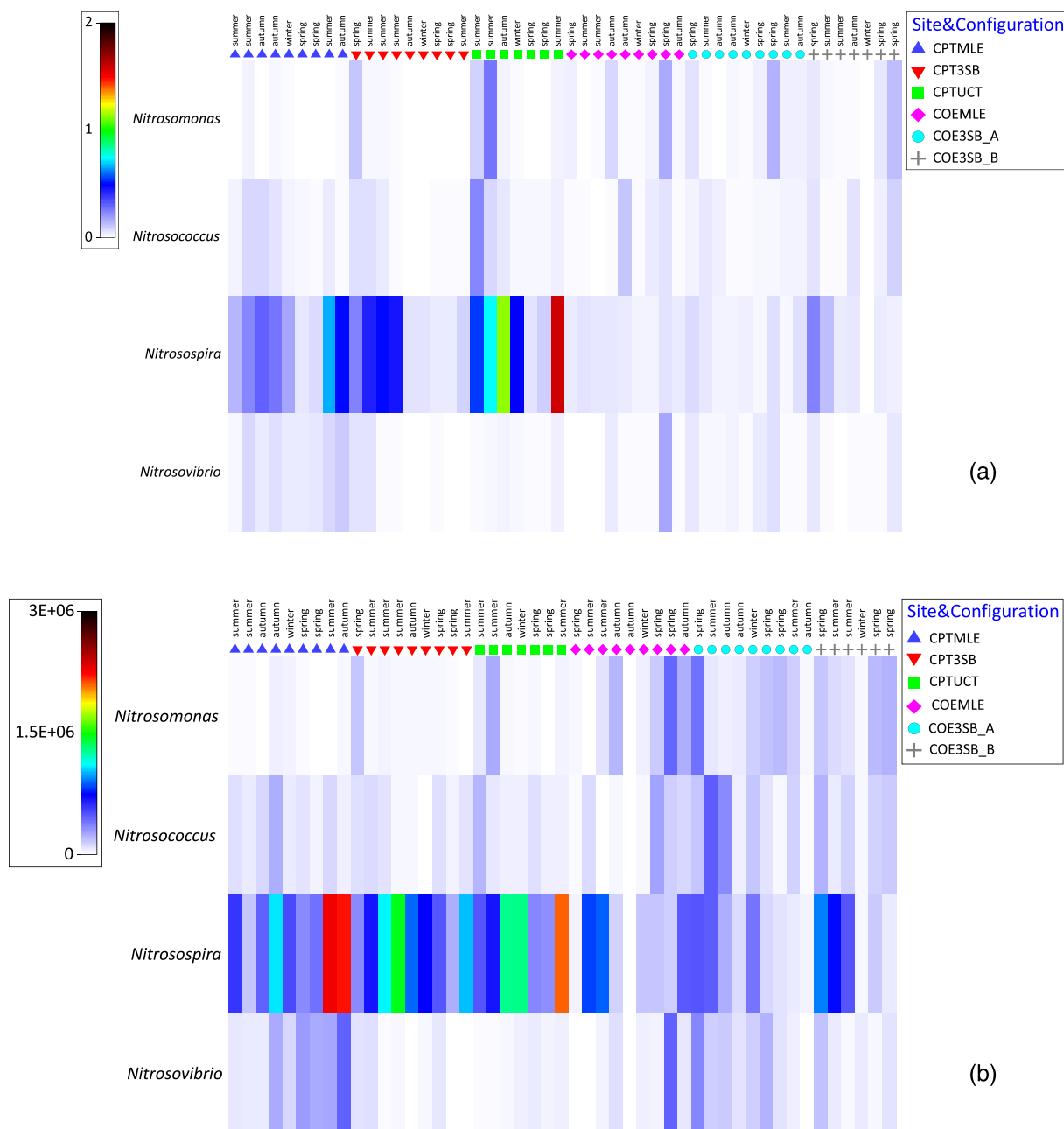


FIGURE 7 Shadeplots of (a) the % relative abundance and (b) estimated actual abundance of ammonium oxidizing bacterial genera.

In a similar study, He et al. (2022) found that *Nitrosomonas* usually dominated over the second most prevalent genus, *Nitrosospira*, in eight AS WWTPs in China treating domestic effluent. *Nitrosomonas oligotropha* was the most abundant species, ostensibly because of its high affinity for $\text{NH}_3/\text{NH}_4^+$. In Minnesota, USA, only two AOB genera (*Nitrosomonas* and *Nitrosospira*) comprising 0.2%–0.5% of the core community were found in samples collected over 55 consecutive weeks from three parallel full-scale SBRs that historically experience loss of nitrification in winter (Johnson et al., 2019). *Nitrosospira* and *Nitrosomonas* were also the most abundant AOB genera

found in a WWTP in Hong Kong (Yu & Zhang, 2012). These two genera correspond to the most abundant AOB genera in the SA study, where the RA of the *Nitrosomonadaceae* also fell within a similar range (0.06%–1.21% of the core bacterial population). In the US study, distinct shifts were seen in the overall bacterial community composition, but these could not be definitively defined as seasonal because no year-on-year samples were taken. None of the known nitrifiers showed significant correlations between RA and temperature or effluent $\text{NH}_3/\text{NH}_4^+$, but unfortunately, as per convention, no AS chemical analyses were included (Johnson et al., 2019). To

validate the SA study findings, it would be interesting to determine if correlations exist between AS parameters and AO communities in other countries.

The CAOB fall within the genus *Nitrospira* that are traditionally known to oxidize NO_2^- to NO_3^- , with only a few strains known to oxidize $\text{NH}_3/\text{NH}_4^+$ directly to NO_3^- . Although CAOB have been shown to dominate the AO community in low DO laboratory studies, their role in AS WWTPs is still being defined (Beach & Noguera, 2019). For example, *Nitrospira* spp. dominated in a low-DO sequencing batch reactor that was operated using wastewater from a well-aerated full-scale system where no *Nitrospira* spp. were detected (Roots et al., 2019). From the results, the authors hypothesized that low DO is a selective driver for CAOB (Roots et al., 2019). The rationale is supported by a study conducted by Gonzalez-Martinez et al. (2016) in the Netherlands where no CAOB were detected in six full-scale AS systems and AOB dominated the AO population. In addition, no CAOB (*Nitrospira amoA* species) were detected in samples from an SBR in the United States (Johnson et al., 2019). In contrast, CAOB were found to be more abundant than AOB or AOA in eight WWTPs in China treating municipal wastewater (He et al., 2022). However, Beach and Noguera (2019) demonstrated that universal CAOB *amoA* primers significantly overestimate the abundance of this functional gene. By applying species specific primer sets for *Nitrospira nitrosa*, *Ca. Nitrospira inopinata* and *Ca. Nitrospira nitrificans*, the authors found that *Ca. N. nitrificans* was the only species that was enriched in low-DO AS reactors (He et al., 2022). In the SA study, *Nitrospira* spp. were present in higher RA than the AOB (data not shown), but, because only a few strains of *Nitrospira* are CAOB, it was impossible to speculate on the RA of CAOB within the genus from the HTAS results. However, there was no significant difference ($p > 0.5$) in the qPCR CAOB *amoA* Ct numbers obtained from the samples from the two geographical locations (CPT: 29.06 ± 3.57 , range 24.42–37.42, COE: 31.75 ± 4.04 , range 25.14–38.86) determined using universal CAOB *amoA* primers (Zhao et al., 2019).

A comprehensive study was conducted by Pan et al. (2018) to determine the “low-temperature” distribution of AOB, AOA, and CAOB *Nitrospira* spp. in 23 municipal WWTPs in China. Once-off sampling was conducted in

winter, when the ambient temperatures ranged from 5 to 20°C, but no further temperature data were provided. Copy numbers of CAOB *Nitrospira* gene amplicons were one to two orders of magnitude lower than the AOA and AOB gene amplicons, and, with the exception of one WWTP sample, the ratios of AOA to AOB gene amplicons ranged from 1:1 to ≥ 10.1 (Pan et al., 2018). Pan et al. (2018) also explored structure–function relationships by DNA-stable isotope probing (DNA-SIP) experiments using AS from three selected AS WWTPs for microcosm experiments. The AOA (*Nitrososphaera* spp.) were functionally dominant in the microcosms, *Nitrosomonas* was the most dominant functional AOB genus, and CAOB were only active at lower $\text{NH}_3/\text{NH}_4^+$ concentrations. The authors postulated that temperature may be a key driver in terms of the functional contribution of AOB to AOA in WWTPs, although temperature effects were not validated at different temperatures in their study (Pan et al., 2018). In addition, the authors found a lack of correlation between functional gene copy numbers and numerical abundance of the AO community, leading them to postulate that there is a disconnect between functional and structural dominance of AO (Pan et al., 2018). Similar results were found in the SA study, where no significant correlations were found between *amoA* qPCR results and AOB RA/EAA (Table 3). However, Johnson et al. (2019) found a highly significant correlation (linear regression $R^2 = 0.64$, $p < 0.001$) between the EAA of the AOB (*Nitrosomonadaceae*) and *amoA* gene copy numbers, but the study was limited to one SBR WWTP in the United States. Interestingly, they found no seasonal shifts in AOB EAA or *amoA* abundance, despite loss of nitrification in winter. They hypothesized that the AOB were somehow able to lower energy generation activity via AO but still maintain the same growth rates during winter (Johnson et al., 2019).

In this study, AOA were not investigated. Studies conducted in AS WWTPs in other countries have reported low abundance or absence of AOA in AS WWTPs treating domestic wastewater. Yu and Zhang (2012) failed to detect AOA from an AS WWTP in Hong Kong using metagenomic and metatranscriptomic approaches to evaluate the microbial community structure and gene expression. In eight WWTPs in China treating municipal wastewater, He et al. (2022) found that AOB were

TABLE 3 Analysis of similarity of *amoA* cells determined by qPCR with ammonium oxidizing bacterial abundance determined using high-throughput amplicon sequencing.

	<i>amoA</i> (cells/ngDNA)	<i>amoA</i> (cells/g _{AS})	<i>amoA</i> (Ct number)
RA (%)	0.113*	0.209*	0.106*
EAA (cells/g _{AS})	0.157*	0.004*	0.012*

*Not significant: $p > 0.05$.

typically around an order of magnitude in higher abundance than AOA. Studies have demonstrated a clear inverse relationship between AOA abundance and NH_4^+ concentration. Kurt et al. (2017) showed decreases in AOA to AOB ratios in full-scale AS WWTPs in Turkey, a country with similar climatic conditions to SA. Pan et al. (2016) validated a model based on the hypothesis that AOA have higher NH_4^+ affinities (0.06 g N/m^{-3}) than AOB (2.4 g N/m^{-3}) so that AOA are only competitive with AOB in low NH_4^+ environments. This link between NH_4^+ concentration and AOA abundance is also supported by results obtained by He et al. (2022) with refinery wastewater and Sun et al. (2022) with “high influent strength” swine wastewater ($\text{COD}, 516.94 \pm 4.18 \text{ mg/L}$; $\text{NH}_4^+, 100.65 \pm 2.65$) in a constructed wetland, where the calculated AOB to AOA abundance ratio was 51.6. Based on the results of previous studies, it was hypothesized that AOA would not be abundant in the SA WWTPs as the influent NH_4^+ concentrations was high in the WWTPs from both geographical locations (Table 2). However, this hypothesis requires experimental validation.

CONCLUSIONS

The results of this study showed that the AS nutrient concentrations were the primary drivers of AOB selection in WWTPs from the COE and CPT, with higher values being found in the CPT WWTPs where *Nitrosospora* spp. dominated the AOB population.

In contrast to countries with colder winters, severe reductions in nitrification in winter are not expected in SA, and no significant seasonal variations in either the structure or function of the AOB populations were seen in samples from the study sites. These results are meaningful to WWTP operators in SA when troubleshooting loss of nitrification efficiency in winter, as temperature effects can be discounted.

AUTHOR CONTRIBUTIONS

Pamela J. Welz: Conceptualization; visualization; data curation; funding acquisition; formal analysis; investigation; methodology; project administration; student supervision; validation; writing original draft. **Mfundisi P. Thobejane:** Methodology; reviewing original draft. **Gerhardus N. van Blerk:** Student supervision; methodology; reviewing and editing original draft.

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CONFLICT OF INTEREST STATEMENT

The authors have no relevant financial or non-financial competing interests to declare.

DATA AVAILABILITY STATEMENT

Raw sequence data obtained in this study has been deposited in the National Center for Biotechnology Information (NCBI) SRA database under BioProject PRJNA907162 with accession numbers SAMN31953178-31953227. All other raw data are available on request and will be freely available on the CPUT Institutional eSango repository at <https://esango.cput.ac.za>.

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