Environmental Research 222 (2023) 115394

Contents lists available at ScienceDirect

Environmental Research

journal homepage: www.elsevier.com/locate/envres

Influence of seasonality, wastewater treatment plant process, geographical location and environmental parameters on bacterial community selection in activated sludge wastewater treatment plants treating municipal sewage in South Africa

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ARTICLE INFO

Handling Editor: Aijie Wang

Keywords:
Bacterial community composition
Chemistry
Climate
Configuration
Influent
Mixed liquor

ABSTRACT

This is the first comprehensive study that focusses on the correlation between the bacterial community composition and a range of previously identified selective criteria in activated sludge wastewater treatment plants on the African continent. Multivariate statistical analyses were used to determine the relative significance of the geographical location (factor: site), wastewater treatment plant process (factor: configuration), seasonality (factor: season), and environmental parameters on the bacterial communities in nine wastewater treatments plants from two sites in South Africa using terminal restriction fragment length polymorphism as a screening tool to rationalize the number of samples (to 50 samples) for high throughput (Illumina MiSeq) sequencing.

Site was the most significant factor (Global ANOSIM R value = 0.91, $p = 0.001$), and it was established that the inter-site differences were not climatic in origin but related to differences in the composition of the influent and activated sludge. Previous studies that have reported associations between microbial community structure and environmental parameters have measured influent chemistry, and this is the first time, to our knowledge, that the comprehensive chemical character of activated sludge itself has been included in this type of study. It was found using BEST analysis that the activated sludge ammonia, activated sludge total phosphate and influent chemical oxygen demand were the most significant ($p < 0.001$) drivers for inter-site bacterial community selection (ANOSIM Global R values of 0.862, 0.782 and 0.428, respectively). This link would not have been established with only influent chemical analyses as there was no significant difference (*t*-test, $p > 0.05$) in the average influent phosphate concentrations between the 2 sites, but there was a highly significant difference ($p < 0.001$, t (15.5)>t-crit (2.01)) in the activated sludge total phosphate concentrations (20.8 ± 17.0 and 127.8 ± 40.2 mg/L). This is notable for all future studies on a global level aimed at identifying factors for selection of microbial communities in activated sludge.

1. Introduction

Microbial communities are responsible for the bioremediation of wastewater (WW) in activated sludge (AS) wastewater treatment plants (WWTPs). Process performance is reliant on the metabolic activities of the microbial species present in the AS, which include, amongst other functional groups, chemoorganotrophic (heterotrophic) bacteria responsible for mineralization of organic carbon and denitrification, and nitrifying autotrophs and heterotrophs (Gupta et al., 2021; Sam et al., 2022; Qin et al., 2018). Factors that play pivotal roles in microbial community selection are both climatic and non-climatic (Luo et al., 2020; Gu et al., 2022; Maza-Márquez et al., 2022), and the AS microbial community compositions in WWTPs are in variable states of flux (Gao et al., 2016; Kim et al., 2021a).

South Africa (SA) is a large country (1.22 million km^2) with different microclimates ranging from sub-tropical to Mediterranean. There is a need to collect more data on the influence of different factors on microbial community selection in AS WWTPs in countries like SA which

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https://doi.org/10.1016/j.envres.2023.115394
Received 13 December 2022; Received in revised form 25 January 2023; Accepted 29 January 2023

have been under-represented in earlier studies. Previous publications reporting on results from long-term sampling from multiple WWTPs in Africa are limited to filamentous bacterial populations without the use of high throughput sequencing (HTS) (Blackbeard et al., 1988; Lacko et al., 1999; Welz et al., 2014, 2022). The only reports from Africa based on HTS are limited to those emanating from the Microbial Database for Activated Sludge (MiDAS) project, where results from once-off sampling from 6 WWTPs in 3 cities (Wu et al., 2018) and 25 WWTPs in 10 cities (Dueholm et al., 2022) were used to compile the global database. Although the methodologies employed and the bioinformatical information obtained in these studies was of the highest order, WWTP and location-specific temporal variations could not be accounted for as long-term sampling was (understandably) not conducted.

In this study, monthly samples were taken over 20 months from AS WWTPs in 2 geographical locations in SA with different climates, namely the City of Ekurhuleni (COE) and the City of Cape Town (CPT). After screening using terminal restriction fragment length polymorphism (TRFLP), amplicon sequencing using a universal primer set targeting the V4 region of the 16S rRNA gene was performed on a rationalized dataset of 50 samples. Multivariate statistical tools were used to analyze the data and assess the influence of seasonality, WWTP process (configuration), geographical location (site) and a comprehensive set of environmental variables on bacterial community selection. Furthermore, the primary environmental drivers impacting on bacterial selection were determined and results obtained with TRFLP were compared with those obtained using the Illumina MiSeq® platform. To our knowledge, this is the first study that has (i) comprehensively characterized the composition of the AS and correlated both the influent and AS physicochemical data with bacterial community composition, and (ii) assessed the use of TRFLP as a cost-effective screening tool to rationalize datasets for HTS for AS microbial studies.

2. Methods

2.1. Sampling and sampling sites details

Nine WWTPs from 2 locations approximately 1200 km apart and treating primarily municipal WW were included in the study (Table 1). The COE is a summer rainfall area in the northern interior (central GPS co-ordinates 26.1511°S, 28.3696°E), while CPT is a winter rainfall area on the south-west coast (central GPS co-ordinates 33.9249°S, 18.4241°E) of SA.

Both cities experience relatively mild winters and similar temperature trends. The daily maximum (max) and minimum (min) temperatures recorded in the hottest month (January) were 27.4 ± 1.0 °C (max) and 17.2 ± 0.8 °C (min) and 26.3 ± 0.8 °C (max) and 15.2 ± 0.4 °C (min) in CPT and COE, respectively. In the coldest month (July) the recorded temperatures were: 18.0.4 ± 1.1 °C (max) and 7.5 ± 0.9 °C (min) and 17.6 ± 0.9 °C (max) and 4.9 ± 1.1 °C (min) in CPT and COE, respectively (Fig. S1). The slightly higher winter minima in CPT are due to the stabilizing effect of the ocean on the climate of this city.

Table 1
Basic Information on study wastewater treatment plants.

	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-3SB1	19.0	0
COE-3SB2	4.7	0
COE-3SB3	20.0	0
COE-3SB4	15.0	0
COE-5SB	10.0	0

CoCT: City of Cape Town; CoE: City of Ekurhuleni.
3SB: 3-stage Bardenpho; 5SB: 5-stage Bardenpho.
MLE: Modified Ludzack-Ettinger.
UCT: University of Cape Town.

The WWTPs from the 2 locations were matched as far as possible in terms of wastewater type (mainly domestic), influent type (no primary settling), and process (configuration). This presented some challenges as there are no University of Cape Town (UCT)-configured WWTPs in COE and there is only one three-stage Bardenpho (3SB)-configured WWTP in CPT. One representative of Modified Ludzack Ettinger (MLE)-configured WWTP was included from each site. As the 3SB process is widely used in COE, three WWTPs with this configuration were included to increase the statistical validity of the results obtained with ‘process’ as a factor during the initial screening.

Monthly samples were taken over a period of 20 months except for a few instances where sampling was not possible (Table S1). A total of 141 AS and influent samples were taken for microbial and physiochemical analyses using the grab technique and 24-h composite sampling, respectively. The AS samples were taken from the exits from the final aerobic zones of the reactors. Samples were transported on ice and physicochemical analyses were performed within 24 h. On arrival at the laboratories, aliquots of the AS samples for DNA extractions were immediately washed in equal volumes of 1X phosphate buffered saline (PBS) and stored at −20 °C until required. Permission to obtain final effluent samples was not granted for this study and WWTP performance is excluded from the discussion.

2.2. Analysis of environmental and operational parameters

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

Physicochemical (environmental) analyses were performed on influent and AS samples as described by Welz et al. (2022) and included measurements of pH, biological oxygen demand (BOD), chemical oxygen demand (COD), total phosphate (TP), ortho phosphate (σ-P), sulfate as sulfur (SO_4^{2-}–S), ammonia as nitrogen (NH_3–N), nitrates and nitrites as nitrogen (NO_3^-/NO_2–N), total alkalinity (T.alk.) and volatile fatty acids (VFA). Details of equipment and methods are provided in the Supplementary Material (Table S2).

2.3. Microbial analyses

2.3.1. DNA extraction

The AS samples were defrosted at ambient temperature and thoroughly mixed by vortexing. Metagenomic DNA was extracted using the PowerSoil® DNA extraction kit (MoBio Qiagen, Hilden, Germany) according to the manufacturers' instructions with the following modification: instead of 0.5 g, 1 g of AS was used for the extraction and the final elution step was conducted using 100 μl eluent (buffer). A blank control was included during the processing to ensure that no contaminants were introduced during extraction. A NanoDrop 2000 Spectrophotometer (Thermo Scientific, Waltham, USA) was used to quantify and assess the quality of the extracted DNA. Where required, the eluted DNA was aliquoted and stored at −20 °C until further analysis. All DNA extractions were conducted in duplicate and then pooled in equimolar amounts to reduce PCR bias.

2.3.2. Polymerase chain reaction and terminal-restriction fragment length polymorphism

The 16S rRNA gene sequences were amplified using the universal bacterial primers E9F (5′-GAGTTTGATCCTGGCTCAG-3′) and U1510R (5′-GGTTACCTTGTTACGACTT-3′) (Marchesi et al., 1998). The primers were synthesized by Integrated DNA Technologies (Coralville, USA) and the forward primer E9F was FAM (fluorescein amidite dye) labelled. The reactions were carried out in 50 μl volumes, each containing 1X DreamTaq PCR Mastermix (Fermentas, Burlington, Canada), 0.5 μM of each primer, 0.1% (w/v) bovine serum albumin (BSA) and between 10 and 20 ng of metagenomic DNA. The amplification was carried out using a Qiagen Rotor Gene 6000 2-plex thermal cycler with HRM capability using the following PCR program: 4 min at 94 °C for denaturation; 30 cycles of 30 s denaturation at 94 °C, 30 s annealing at 56 °C and 105 s elongation at 72 °C, and a final elongation step of 10 min at 72 °C. All reactions were conducted in duplicate.

The PCR amplicons were visualized at 254 nm on 1% (w/v) agarose gel containing ethidium bromide. The PCRBIO Ladder II (PCR Biosystems Ltd., Aztec House, London) Universal Ladder Kit was used to determine the amplicons' approximate size on the agarose gel. Amplicons were purified using the Nucleospin® gel and PCR clean-up kit (Macherey-Nagel, Durën, Germany) and quantified using a NanoDrop 2000 Spectrophotometer.

The purified amplicons were digested for 3 h at 37 °C using the restriction enzyme *Hae*III (Sigma-Aldrich, St. Louis, USA cat no 10693944001) and then inactivated at 80 °C for 10 min. The digestion products were purified using the NucleoSpin gel and PCR clean up kit (Machery-Nagel). Restriction fragment lengths and quality was determined via capillary electrophoresis at the Central Analytical Facility (CAF), Stellenbosch University, South Africa, and the data was processed as previously described using the freeware PeakScanner™ (version 1.0) ((Applied Biosystems (Waltham, USA) https://products.appliedbiosystems.com/)) and T-REX (http://trex.biohpc.org/) (Welz et al., 2018).

2.3.3. Amplicon sequencing

The TRFLP results were used to rationalize the number of AS samples from 141 to 50 bearing in mind the objective to assess the influence of 3 factors on the microbial community structure (process, seasonality, geographic location). Samples taken at the same times from each WWTP, as well as year-on year (same month, different years) samples were included (Table S1). Amplicon sequencing of the V4 region of the small subunit (SSU) of the 16S rRNA gene using the primer pairs 515F–Y (5′-GTGYCAGCMGCCGCGGTAA) (Parada et al., 2016) and revised 806-R (5′-GGA CTA CNV GGG TWT CTA AT-3′) (Apprill et al., 2015) was conducted using an Illumina MiSeq instrument according to the MR DNA (Shallowater, TX, USA) in-house protocol.

The forward primers were barcoded, and the PCR program was: initial denaturation at 95 °C for 5 min, 30 cycles of denaturation (95 °C for 30 s), annealing (53 °C for 40 s) and extension (72 °C for 1 min) and final elongation (72 °C for 10 min) using the Qiagen HotStarTaq Plus Master Mix kit. Each reaction was conducted in duplicate. The PCR products were quality checked by visualized in 2% agarose gel. Aliquots of samples were multiplexed using unique dual indices. Based on their DNA concentrations and molecular weights, samples were pooled in equal ratios, purified using calibrated Ampure XP beads (Beckman Coulter, Brea, USA) and used to prepare a DNA library according to the Illumina TruSeq manufacturers' protocol.

Sequencing was performed using an Illumina MiSeq instrument according to the manufacturers' instructions. The data was analyzed using the MR DNA analysis pipeline. The sequences were joined together, the barcodes were removed, and those with <150 bp and/or ambiguous base calls were removed. Quality filtration was performed by applying a maximum expected error threshold of 1.0, after which the sequences were dereplicated and denoised by removing unique sequences identified by PCR error points and chimeras in order to generate zero radius operational taxonomic units (zOTUs). 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/). Advanced bioinformatic analyses were also performed at MR DNA. Processed data outputs consisted of Excel spreadsheets containing the relative abundance (RA) of zero radius operational taxonomic units (zOTUs) as well as the RA of different taxa from phyla to species.

2.3.4. Quantitative polymerase chain reaction and estimated absolute abundance

The quantity and quality of the submitted DNA was determined at MR DNA (Shallowater, USA) using a NanoDrop2000 instrument (Thermo Scientific). Samples were then amplified by quantitative PCR (qPCR) using the same primer pair as per the amplicon sequencing protocol at MR DNA. Briefly, 1 μl of the template DNA was used to perform the qPCR reaction using 2XUniversal Taqman PCR Mastermix (Applied Biosystems) in StepOnePlus Real-Time PCR System (Applied Biosystems). Three replications were used for each sample. The qPCR reaction was carried out 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 s, followed by 60 °C for 1 min. In order to estimate the abundance of taxa, numbers of *Escherichia coli* strain K12 in different liquid concentrations were determined using multiple plate counts. Assuming 80% extraction efficiency, the DNA extracts from 10-fold dilutions of *E. coli* strain K12 were used as linear standards to extrapolate bacterial cell numbers from the qPCR cycle thresholds (Ct). The Ct values obtained from the sample templates were then used to estimate the number of bacterial cells in 1 μl of the sample templates. Each reaction was conducted in triplicate.

The estimated absolute abundances (EAA) of different bacterial taxa per ng DNA in 1.0 g AS were calculated using Equation (1).

$$EAA = (RA\ x\ n) / DNAconc.) / AS \tag{1}$$

where: RA = relative abundance of taxa determined from amplicon sequencing, n = number of bacterial cells in 1 μl template DNA, $DNAconc.$ = DNA concentration of template DNA, AS = 1.0 g AS which was standardized for each sample extraction.

2.4. Statistical analyses

The operational taxonomic units (OTUs) from processing of the TRFLP data (Section 2.3.2) and zOTUs 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).

The operational taxonomic units (OTUs) from processing of the TRFLP data (Section 2.3.2) and zOTUs from Illumina MiSeq data were analyzed using Primer 7® software (Primer-e, Auckland, New Zealand) as detailed in the Supplementary material (M1.3). Square root transformed data was used to create Bray-Curtis similarity matrices which 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 geographical location (site), season and process (WWTP configuration) on the bacterial community compositions (site(configuration(season))). Shade-plots of the most abundant bacterial taxa determined using HTS were constructed using processed Illumina MiSeq data. Rarefaction curves of the sequencing depth plotted against (i) the numbers of zOTUs ((also known as amplicon sequence variants (ASVs)) and (ii) the Faith diversity index were constructed using the Qiime2 microbiome pipeline (https://qime2.org/).

2.4.1. Environmental and combined microbial and environmental data

Student's t-tests (Microsoft Excel) were performed on the environmental data to determine whether there were significant differences between the results obtained from different sites, seasons or WWTP processes. The data was then analyzed using Primer 7® software. Fourth root transformed and normalized data was used for Principal Component Analyses (PCA) and ANOSIM.

BEST analyses of Spearman rank correlations were performed on the

Bray-Curtis similarity data of the functional microbial community structures and the Euclidian distance of the transformed and normalized environmental data. To limit the environmental variables, co-linearity checks were performed to establish whether physicochemical variable could be used as proxies for others, with R values of 0.95 being used as the cut-off. The results obtained using BEST were verified for the environmental variables by performing 3-way ANOSIM (site(configuration (season))) on similarity plots of the environmental data based on Euclidian distance. In addition, the 'best' correlated parameters were used to construct LINKTREE plots to visualize and assess the ranges of parameters that explained clustering of the functional microbial communities.

3. Results

3.1. *Influence of geographical location (site), process (configuration) and season on the overall bacterial community structure*

Distinct separation of the data points representing each geographical location (factor 'site') was noted on the nMDS plots obtained using the TRFLP OTU data, irrespective of configuration (Fig. 1a) or season (Fig. 1b). The same trends were noted on the nMDS plots obtained using the HTS zOTU data (Fig. 1c and d). The results were validated by 3-way fully nested ANOSIM, where highly significant differences ($p = 0.001$) were calculated for the factor 'site' (Global ANOSIM R value $= 0.91$). Differences in the bacterial community structures for the factors 'season' and 'configuration' were not significant ($p < 0.05$) with Global ANOSIM R values of 0.069 and 0.088 (season) and 0.385 and 0.281 (configuration), for the TRFLP OTU and HTS zOTU data, respectively. The pairwise ANOSIM tests also showed no significant differences for 'season' or 'configuration'.

As the geographical location played such a dominant role on bacterial community selection, the factors 'season' and 'configuration' were analyzed separately for each site to interrogate whether these factors played lesser roles in bacterial community selection that were masked by the inclusion of 'site' as a confounding variable.

In the nMDS plots constructed from the TRFLP data (Fig. 2a) and HTS data (Fig. 2cd), some loose clustering of data points representing samples from the 3SB-configured WWTP in CPT was noted (encircled in Fig. 2a and c). The 2D stress value of 0.1 for the nMDS indicated that the plot was a good representation of the results, but they should ideally be validated with another statistical analysis (Clark et al., 2014). No significant differences ($p > 0.05$) for the factor 'season' (Global R $= 0.125$ and 0.160, respectively) or 'configuration' (Global R $= 0.042$ and 0.302, respectively) were obtained from 2-way nested ANOSIM of the COE TRFLP and HTS WWTP data, respectively. While no significant differences for 'season' were noted for the CPT WWTP data (Global R $= 0.115$ and 0.237 for TRFLP and HTS, respectively), a significant difference ($0.05 > p < 0.01$) for the factor 'configuration' was noted (Global R $=$ 0.271 and 0.634, respectively), validating the nMDS.

One-way unordered ANOSIM was performed to establish whether 'season' or 'configuration' had any effects on the bacterial community structures independent of one another. The 'configuration' results (Table 2) confirmed the nMDS and nested ANOSIM results, with significant differences being found only in the CPT WWTPs. In contrast to the nested ANOSIM, significant differences were shown for 'season' in both the COE and CPT WWTPs, with some minor differences between the TRFLP and HTS data being noted.

3.2. *Environmental parameters measured in influent and activated sludge*

The highly significant differences in the bacterial compositions in the AS from the two geographical locations could not be ascribed to climatic temperature differences (Section 2.1) and AS temperature was not significantly correlated ($p < 0.05$) with the TRFLP data. However, there were highly significant differences in some of the physicochemical parameters from each 'site' (*t*-test Table 3), with the influent to the WWTPs at the COE being more dilute in nature.

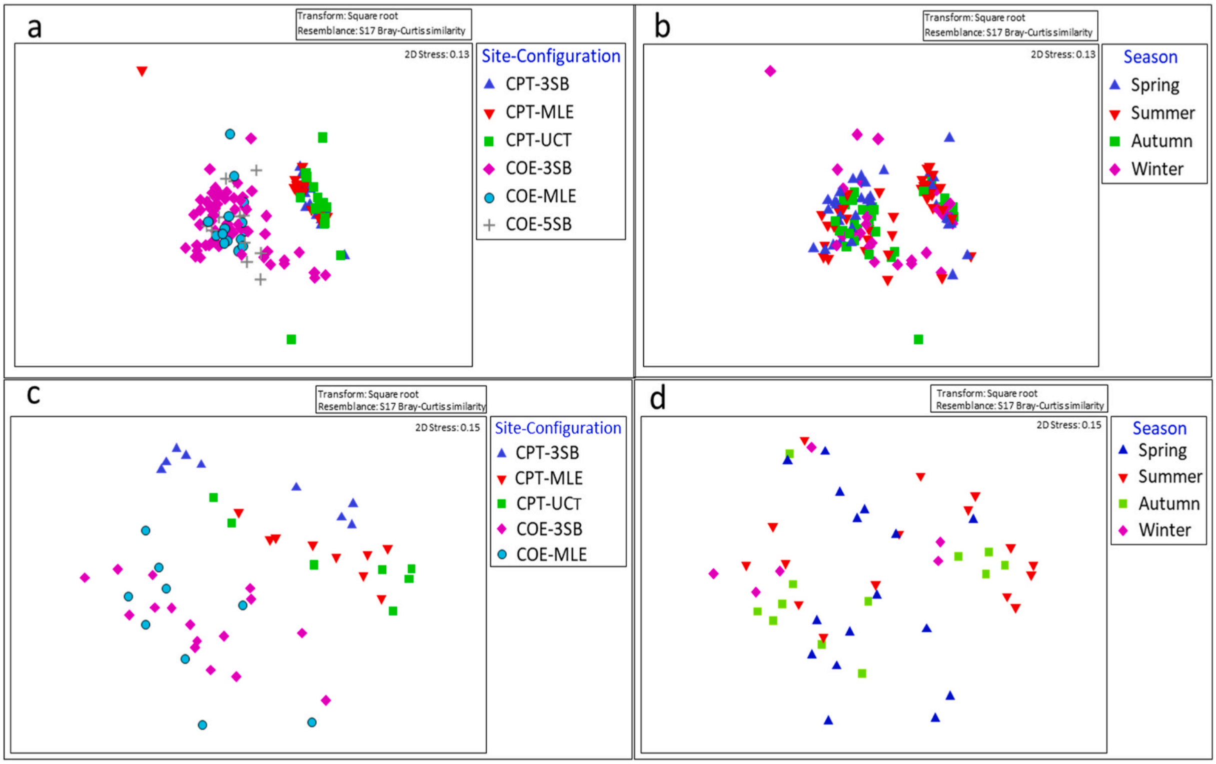

Fig. 1. nMDS plots of data (OTUs) obtained from TRFLP analyses in all study samples in terms of the geographical location (site) and process configuration (a), and season (b), and nMDS plots of data (zOTUs) obtained from HTS in terms of the geographical location (site) and process configuration (c), and season (d).

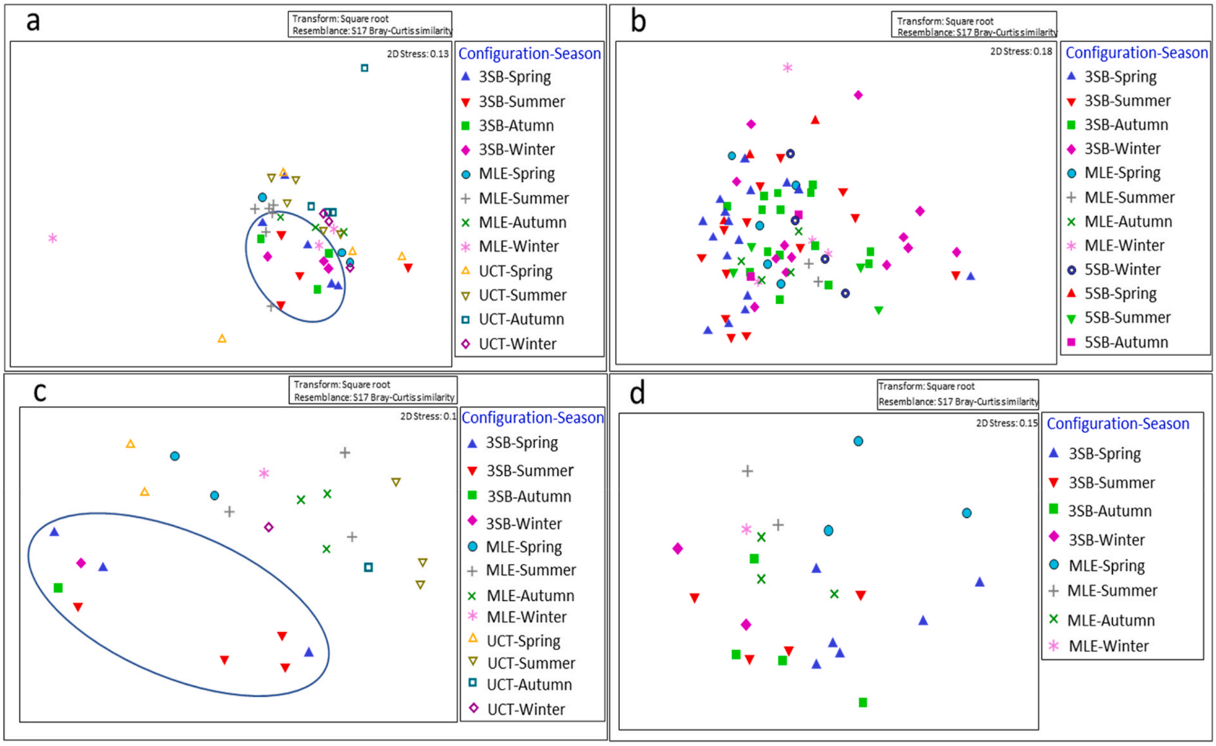

Fig. 2. nMDS plots of data obtained from TRFLP OTU data (a,b) and HTS zOTU data (c,d) of samples from WWTPs in the City of Cape Town (a,c) and the City of Ekurhuleni (b,d).

Table 2
R values and significance from one-way unordered ANOSIM.

SEASON	COE-TRFLP			COE-HTS		
	Spring	Summer	Autumn	Spring	Summer	Autumn
Summer	0.137**			0.331*		
Autumn	0.205**	0.077*		0.140	0.127	
Winter	0.254**	0.046	0.07	0.423*	0.235	0.040
SEASON	CPT-TRFLP			CPT-HTS		
	Spring	Summer	Autumn	Spring	Summer	Autumn
Summer	0.104*			0.347**		
Autumn	0.057	0.102		0.359*	0.130	
Winter	0.007	0.192*	0.05	0.020	0.008	0.067
CONFIGURATION	**COE-TRFLP**			**COE-HTS**		
	5SB	3SB	5SB	3SB		
3SB	0.020		ND			
MLE	0.050	0.110	ND	0.266		
CONFIGURATION	**CPT-TRFLP**			**CPT-HTS**		
	UCT	3SB	UCT	3SB		
3SB	0.176*		0.476**			
MLE	0.080*	0.128*	0.522**	0.617**		

Level of significance: *0.05 > p ≥ 0.01 **0.01 > p COE: City of Ekurhuleni; CPT: City of Cape Town; TRFLP: terminal restriction length polymorphism; HTS: high throughput sequencing.

3.3. Correlation of environmental parameters with overall bacterial community structures

As the environmental parameter profiles from each site were so notably different, it was hypothesized that this was the primary reason for the overall differences in the OTU/zOTUs profiles for the factor 'site'. This was statistically validated using the environmental and TRFLP data sets. The TRFLP was selected over HTS data because the dataset was larger, and the trends obtained from both methods for the factor 'site' were highly similar. BEST analysis was performed to ascertain which environmental variables were 'best' correlated with the OTU profiles (refer to Supplementary Table S3 for detailed results). The 3-way nested ANSOIM (site(configuration(season)) results validated the hypothesis, and indicated that the influent COD, AS TP and AS NH_4^+ were the most important drivers for differences in the bacterial community structures between the 2 sites (Table 4). This was confirmed by PCA analysis, with the PCA plot constructed from the environmental parameters (Fig. 3a) closely mirroring the nMDS plot constructed from the TRFLP data (Fig. 3d).

The Bray-Curtis similarity of the OTUs were linked to the environmental parameters selected by BEST using the binary divisive cluster analysis, LINKTREE (Fig. 4a). Apart from one outlier, all the COE sample data grouped together and apart from the CPT sample data. The primary driver separating the 2 'site' clusters was the AS TP concentration ('A' on the plot), which was notably higher in the CPT WWTPs (127.8 ± 40.2) than in the COE WWTPs (20.8 ± 17.0) (Table 3). According to

Table 3
Comparison of environmental (physicochemical) variables measured in influent and activated sludge samples from the two study sites (geographical locations).

INFLUENT				
Parameter	CPT (ave ± SD)	COE (ave ± SD)	T	T_{crit}
BOD (mg/L)	475 ± 183	190 ± 124	**7.86*****	2.00
COD (mg/L)	1051 ± 220	437 ± 258	**11.7*****	1.99
BOD/COD (ratio)	0.45 ± 0.14	0.44 ± 0.18	0.881	1.99
pH	7.6 ± 0.3	7.7 ± 0.6	0.602	1.98
T.Alk (mg/L as $CaCO_3$)	395 ± 63	273 ± 73	**10.0*****	1.99
NH_4^+ (mg/L as N)	70.0 ± 21.0	43.2 ± 19.0	**7.84*****	1.99
NO_3^-/NO_2^- (mg/L as N)	0.23 ± 0.56	0.16 ± 0.45	1.24	1.98
TP (mg/L as P)	13.9 ± 8.6	12.9 ± 27.1	0.153	1.98
σP (mg/L)	9.0 ± 3.7	5.6 ± 4.9	**4.73****	1.99
SO_4^{-2} (mg/as S)	65 ± 30	71 ± 26	**2.49***	2.00
VFA (mg/L)	93 ± 61	49 ± 26	**4.24****	2.02
ACTIVATED SLUDGE				
Parameter	CPT (ave ± SD)	COE (ave ± SD)	T	T_{crit}
DO (mg/L)	1.04 ± 1.04	1.21 ± 1.08	1.11	2.01
BOD_{tot} (mg/L)	815 ± 421	983 ± 582	1.47	2.00
BOD_{sol} (mg/L)	43 ± 42	22 ± 14	1.79	2.14
COD_{tot} (mg/L)	6229 ± 1419	3727 ± 1523	**7.27*****	1.99
COD_{sol} (mg/L)	65 ± 34	37 ± 26	**3.06****	2.06
BOD/COD_{tot} (ratio)	0.14 ± 0.07	0.29 ± 0.14	**5.32*****	2.00
BOD/COD_{sol} (ratio)	0.70 ± 0.75	0.81 ± 0.70	0.35	2.09
pH	6.8 ± 0.2	7.1 ± 0.4	**4.38*****	1.98
NH_4^+ (mg/L as N)	16.6 ± 20.3	6.5 ± 4.2	**3.26****	2.02
NO_3^-/NO_2^- (mg/L as N)	1.5 ± 2.6	0.6 ± 1.2	1.91	2.01
TP (mg/L)	127.8 ± 40.2	20.8 ± 17.0	**15.5*****	2.01
σP (mg/L)	4.0 ± 6.4	9.5 ± 7.2	**4.07*****	1.98
SO_4^{-2} (mg/L as S)	62 ± 19	52 ± 15	1.22	2.06
SOLIDS AND FM RATIO				
Parameter	CPT (ave ± SD)	COE (ave ± SD)	T	T_{crit}
MLVSS (mg/L)	4426 ± 1018	2611 ± 1557	**7.49*****	1.98
DSVI (mL/g)	188 ± 106	111 ± 69	**3.66*****	2.00
FM (mgBOD.mg MLVSS/day)	0.07 ± 0.05	0.30 ± 0.53	**3.73*****	2.00
FM (mgCOD.mg MLVSS/day	0.15 ± 0.07	0.69 ± 1.12	**4.12*****	2.00

P(T_{crit}<=T) two-tail *** significance $p < 0.001$ **significance ($0.001 = p < 0.01$) *significance ($0.01 \geq p < 0.05$).
BOD/COD_{tot} – total biological/chemical oxygen demand; BOD/COD_{sol} – soluble biological/chemical oxygen demand; NH_4^+ ammonium; N – nitrogen; NO_3^-/NO_2^- nitrates/nitrites; TP – total phosphorus; σP – ortho phosphate; SO_4^{-2} – sulfate; VFA – volatile fatty acids; MLVSS – mixed liquor volatile suspended solids; DSVI – diluted sludge volume index; FM – food to microorganism ratio.

Table 4
Global R values from analysis of similarity on environmental variables selected from BEST analyses.

Variable	Site	Configuration	Season
All	**0.644***	0.635	0.050
Inf. T.Alk	**0.153***	0.404	0.023
Inf. NH_4^+	0.107	0.288	0.107
Inf. COD	**0.428*****	0.481	0.001
Inf. VFA	**0.321****	0.308	0.091
AS NH_4^+	**0.862*****	0.173	0.149
AS pH	0.131	0.135	0.059
AS TP	**0.782*****	0.385	0.121
AS σP	0.288	0.192	**0.257***
MLVSS	0.053	0.327	**0.116***

3-way nested ANOSIM (site(configuration(season))) *** significance $p < 0.005$ **significance ($0.005 = p < 0.01$) *significance ($0.01 \geq p < 0.05$) Inf: influent; AS: activated sludge; T.Alk: total alkalinity; NH_4^+: ammonium; COD: chemical oxygen demand; VFA: volatile fatty acids; TP: total phosphorus; σP: ortho phosphate; MLVSS: mixed liquor volatile suspended solids.

LINKTREE, the influence of AS TP was highly significant ($R = 0.66$, $p = 0.001$) with cut-off values of <52 mg/L (COE cluster) and >59.8 mg/L (CPT cluster) ('A' on plot). For the COE, the next cluster division ('B' on plot, $p = 0.001$, $R = 0.71$) was driven by a combination of AS pH < 6.5 (>6.6), inf. T.Alk>474(<461) mg$CaCO_3$/L and inf. NH_4^+ >102(<94.9) mg/L, followed by AS NH_4^+ <21.3(>22.1) mg/L ('C' on plot, = 0.001, $R = 0.71$). The remaining descriptions of the parameters linked to each binary division in Fig. 4a are provided in the supplementary material (Table S4).

Significant differences were found for the factor 'configuration' for CPT (Section 3.1). In the LINKTREE plot, there was some clustering of data from samples taken from the 3SB-configured reactor from February to August (excluding June), but no other notable trends were apparent (Fig. 4b). The parameters responsible for the 3SB reactor sample clustering away from the others were AS σP (<13.8 mg/L, 'A' on plot), inf. VFA (<143 mg/L and >34 mg/L, 'B' and 'C' on plot), AS NH_4^+ (>66 mg/L, 'D' on plot), while the final division ('E' on plot) was related to the inf. NH_4^+ (<45.6 mg/L).

3.4. *Bacterial community composition*

The most abundant phyla in the AS samples were *Proteobacteria* (CPT: 57 ± 17%, COE: 59 ± 11%), *Bacteroidetes* (CPT: 17 ± 8%, COE: 26 ± 10%) and *Actinobacteria* (CPT: 8 ± 5%, COE: 5 ± 12%), with no notable trends being noted for 'season', 'site' or 'configuration' (Fig. S3). At genus level, apart from 2 samples from the COE (1 MLE in spring and 1 3SB in spring), the COE and CPT samples clustered away from one another (Fig. 5a). When analyzed separately, some 'season' groupings were noted in a cluster plot constructed from the COE sample database (Fig. 5b), and both 'season' and 'configuration' groupings were noted in the cluster plot constructed from the CPT sample database (Fig. 5c).

In contrast to the phlya, the RA order of genera in the COE and CPT AS differed from one another (Fig. 6). In order of RA, of the 833 genera that were identified in all the samples, *Pseudomonas, Psychrobacter, Acinetobacter* and *Lewinella* were found in the COE samples with RA>1%, and *Pseudomonas, Acinetobacter, Lewinella, Janthinobacterium* and *Psychrobacter* were found in the COE samples with RA>1%. Of the top 10 genera from each site, only five were shared between both sites (*Lewinella, Pseudomonas, Acinetobacter, Janthinobacterium, Psychrobacter*).

4. Discussion

Eighteen studies detailing bacterial community structures in municipal AS WWTPs were identified in a comprehensive SCOPUS literature search (Table 5). Terminal restriction length fragment polymorphism (TRFLP) was used as a molecular fingerprinting method in one study (Valentin-Vargas et al., 2012) and amplicon sequencing platforms were utilized to generate data for the other studies. The Illumina (San Diego, US) NovoSeq® HTS platform was used for one (Cai et al., 2020), the Roche (Basel, Switzerland) 454 pyrosequencing HTS platform for three (Zhao et al., 2014; Hu et al., 2012; Zhang et al., 2012), while the remainder employed the Illumina MiSeq® HTS platform. High throughput amplicon sequencing provides comprehensive fingerprinting and taxonomic data but is costly to perform. Cost constraints could be a reason why sample numbers are limited in many studies as >100 samples were only used in 6 of 18 reviewed studies (Table 5). Sampling numbers for the remaining 12 studies ranged from 7 to 72 (average 24 ± 19). It was therefore hypothesized that, although older, less sophisticated tools such as TRFLP and denaturing gradient gel electrophoresis (DGGE) are becoming obsolete, they may still be useful as cost-effective tools for screening larger datasets to rationalize numbers for HTS, while still providing some valuable information. Terminal restriction fragment length polymorphism provides robust information on similarities and differences in overall microbial community structures in ecological samples but can only provide limited

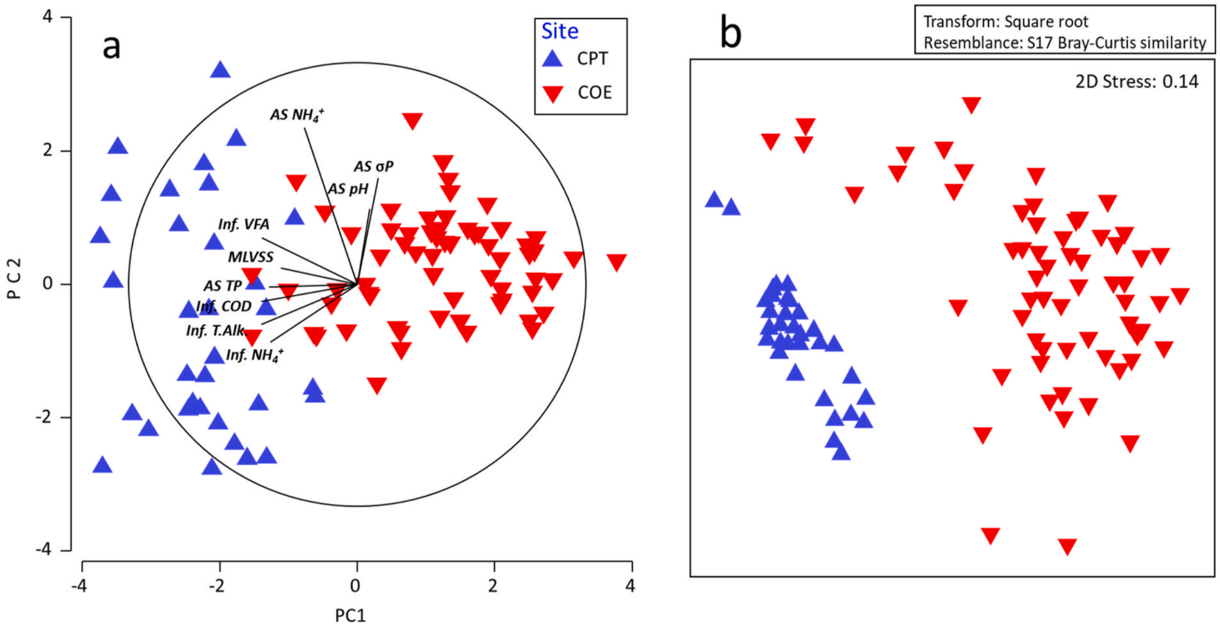

Fig. 3. PCA plot of (a) the Euclidian distance similarity matrix of selected environmental parameters aligned with (b) an nMDS plot of the Bray-Curtis similarity matrix of OTUs determined using TRFLP showing 'site' (geographical location) as a factor.

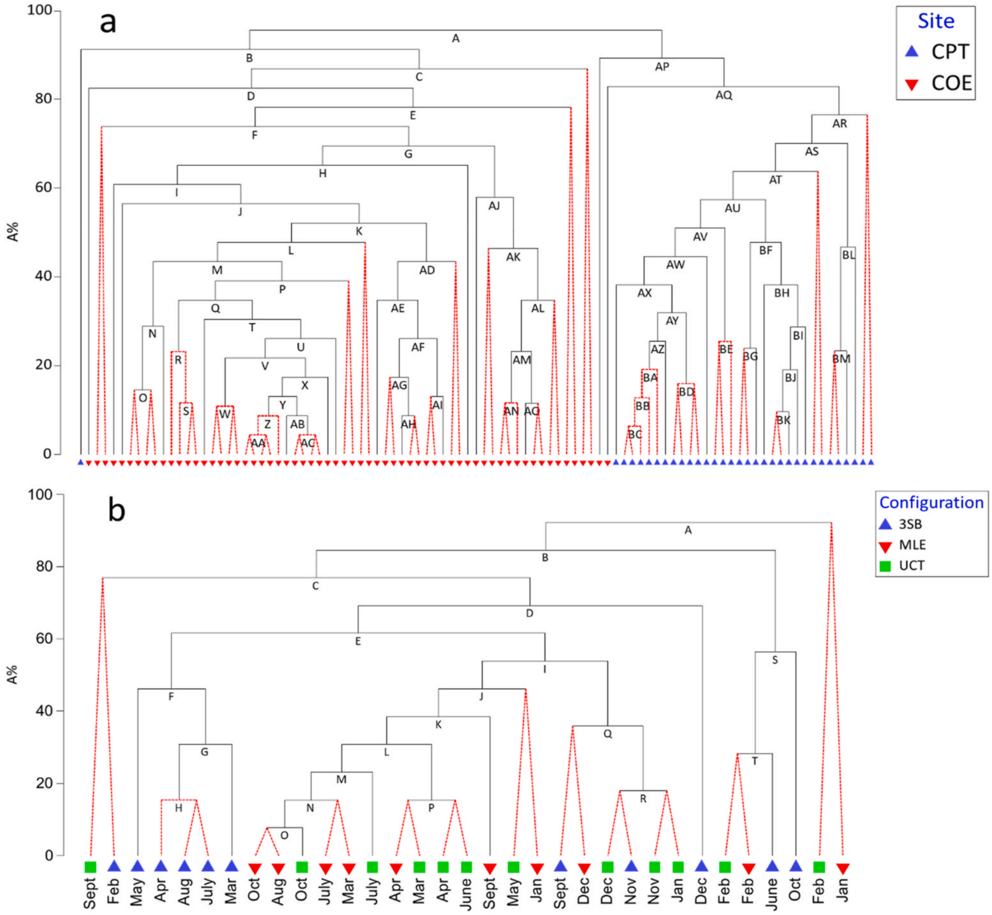

Fig. 4. LINKTREE plots of environmental parameters influencing the OTU profiles of the AS samples determined using (a) TRFLP from both study sites depicted with 'site' as the factor, and (b) from CPT depicting 'configuration' as the factor with labels denoting the month of sampling.

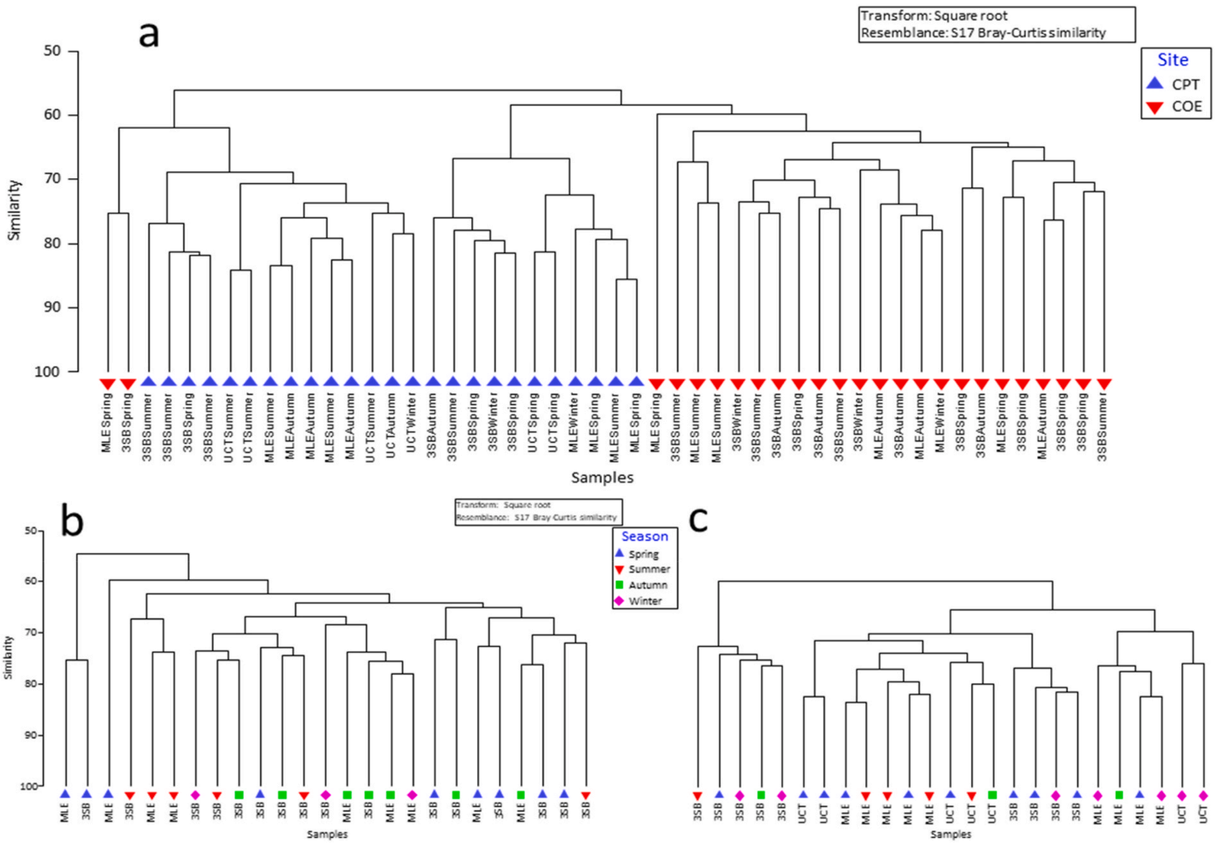

Fig. 5. Cluster plots (group average linkages) of the relative abundance of the genera in all samples (a), samples from the City of Ekurhuleni (b) and samples from the City of Cape Town (c). Note: x-axis similarity % and season key applicable for b and c.

taxonomic information (Ramond et al., 2013a, 2013b). In this study, TRFLP was performed on 141 AS samples and HTS was performed on 50 of these samples. The results in terms of the effects of geographical location (site), season, process configuration and environmental data on the bacterial community structures closely mirrored one another, validating the hypothesis that TRFLP is a useful screening tool for this type of study.

The geographical location (factor 'site') played a dominant role in bacterial community selection, masking any effects of process configuration and/or season when the data from both sites was analyzed simultaneously. Variations in AS microbial communities in WWTPS from different geographical locations may be associated with climatic and/or non-climatic factors (Dueholm et al., 2022). Similar to this study, other studies have also reported that the influent character can be the most significant driver of microbial community selection in AS (Begmatov et al., 2022; Wu et al., 2018). The taxonomic bacterial identity in AS develops over time and, although municipal WW (sewage) is rich in microbiota, there are only weak associations between the microbial community fingerprints in the influent and AS (Lee et al., 2015). Most studies (15 of 18 reviewed, Table 5) analyzed influent and effluent physicochemical parameters, usually including BOD and/or COD, total nitrogen (TN), ammonia nitrogen (NH_3–N) and total phosphate (TP). Some also correlated functional microbial abundance and composition with nutrient removal performance, while others also linked AS microbial community composition with process parameters such as solids and hydraulic retention times (S/HRT) (Jiang et al., 2018; Xu et al., 2018; Valentin-Vargas et al., 2012). To the best of our knowledge, no previous studies have investigated the link between a large range of AS physicochemical properties and bacterial community composition. The composition of AS reflects a combination of the microbial activity (performance), the influent composition, and the operational parameters, including the SRT and HRT simultaneously. It is therefore logical to use AS composition in studies focusing on bacterial community selection in AS. In this study, there was a highly significant link between the physicochemical (environmental) data and the bacterial community structures from each site. The three most significant parameters with the highest correlations with the microbial community structures for the factor 'site' were the influent COD and the AS TP and AS NH_4^+ concentrations (Table 4). Notably, there was no significant correlation of the influent TP and NH_4^+ concentrations and the bacterial community structures, so that TP and NH_4^+ would have been discounted as significant variables if the AS was not characterized. It is therefore strongly recommended that future studies should include AS environmental data if correlations are being sought between environmental variables and AS microbial community composition.

When the data was analyzed separately for each site, some moderate temporal 'season' effects on bacterial community selection were seen at both sites, while moderate effects of process configuration were only seen in the CPT WWTPs. Previous studies have shown that the WWTP process (configuration) can have a direct impact on the physicochemical make-up of the AS, and that the process configuration (Dueholm et al., 2022; Kim et al., 2021a) and WWTP capacity (Kim et al., 2019) can play significant roles in microbial community selection. For example, in studies conducted in Moscow (Begmatov et al., 2022), the US (Kim et al., 2021b), and Demark (Nierychlo et al., 2020), clear clustering of microbial communities and/or selection of certain taxa from differently configured WWTPs were demonstrated. However, as found in our study, it has been shown that the correlation between microbial community composition and WWTP process may be overshadowed by other factors, including the geographical location. The effect of geographical location has previously been ascribed to differences in environmental as well as regional, social and cultural differences, such as dietary habits, on the influent composition (Begmatov et al., 2022; Zhao et al., 2014). In a meta-study conducted for the MiDAS project, inter-continental

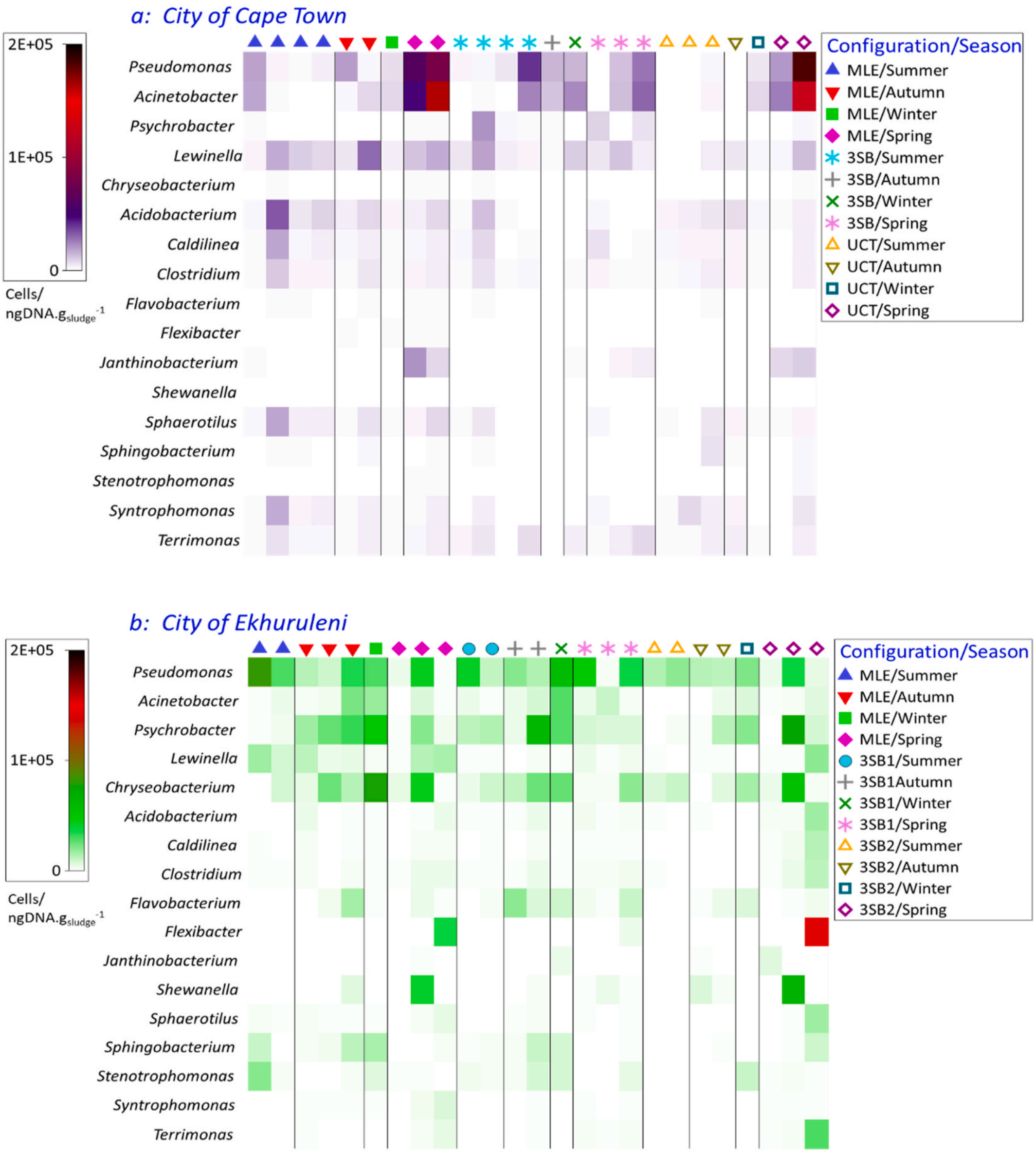

Fig. 6. Shadeplots of most abundant genera from samples from the City of Cape Town (a) and the City of Ekurhuleni (b) obtained from high throughput sequencing data.

geography and WWTP process were both shown to play significant roles in AS microbial community selection, with process exerting the greater influence (Dueholm et al., 2022). However, the authors pointed out that there were continental sampling biases which may have skewed these results as the study was Euro-centric (16 countries, 321 WWTPs) and Africa (1 country, 25 WWTPs), and South America (2 countries, 38 WWTPs) were particularly under-represented (Dueholm et al., 2022).

Temperature has been shown to be a key climatic driver for AS microbial community selection in some studies (Xie et al., 2021; Gao et al., 2016). The effect of temperature on microbial variability in AS WWTPs has been substantiated in a meta-analysis by Dueholm et al. (2022), who showed that high temperatures were more selective for certain taxa (*Competibactier, Thauera, Defluviicoccus, Azospira, Rhodoplanes, Ottowis, Phaeodactylibacter*) while low temperatures were more selective for others (*Flavobacterium, Tetraphpaera, Ferruginibacter, Trichococcus, Ca.* Epiflobacter, *Acinetobacter*). Logically, in areas experiencing cold winters and large seasonal temperature gradients, there should be more notable seasonal changes in the microbial community composition (Luo et al., 2020; Maza-Márquez et al., 2022) than, for example, in the tropics where temperature ranges between summer and winter are minimal (Gu et al., 2022). Studies have reported seasonal variations in microbial community compositions in AS WWTPs treating municipal WW (Kim et al., 2021b; Sun et al., 2021; Cai et al., 2020; Kang et al., 2020). However, unless a reasonable number of WWTPs are included in studies (Kim et al., 2021b) and/or long-term sampling is conducted (Cai et al., 2020; Kang et al., 2020), there is a possibility that temporal variations may not be truly seasonal (year-on-year) trends and could be ascribed to non-climatic factors. Some studies relying on once-off sampling instances have failed to establish links between temperature and microbial community composition (Jiang et al., 2018) or microbial diversity and abundance (Kang et al., 2020). Research that has established true seasonal trends in microbial community composition is rare (Cai et al., 2020; Kang et al., 2020), as most studies have been based on short-term sampling (Table 5). Even comprehensive meta-studies conducted as part of the MiDAS where 1186 AS samples (Wu et al., 2018) and 1480 samples (Dueholm et al., 2022) were tested, were unable to determine definitive seasonal effects on microbial community composition in individual WWTPs as the studies were based on once-off sampling

Table 5

Studies comparing bacterial community compositions in activated sludges from wastewater treatment plants treating primarily domestic wastewater based on 16S rRNA gene amplifications. Physicochemical (environmental) parameters excluded.

City/Country	WWTP/samples (*n*)	Duration	Significant influence established			References
			Season	Location	Process	
Moscow/Russia	9/21	Once-off	NA	NA	**Yes**	Begmatov et al., (2022)
Shenzen/China	2/72	38 months	**Yes**	NA	NA	Cai et al. (2020)
425 cities/31 countries	740/1480	Once-off	NA	**Yes**	**Yes**	Dueholm et al., (2022)
Shanghai/China	4/7	Once-off	NA	NA	NA	Gao et al. (2016)
Beijing/China	12/16	Once-off	NA	NA	**Yes**	Hu et al. (2012)
Hong Kong SAR	2/24	12 months	**No**	NA	**Yes**	Jiang et al. (2018)
Lanzhou & Xining/China	4/24	Bi-annual	**Yes**	NA	ND	Kang et al. (2020)
Seoul/North Korea; Ho Chi Min, Hanoi/Vietnam	8/48	5 months	NA	**Yes**	ND	Kim et al. (2019)
Minnesota USA (442 km radius)	20/120	12 months	**No**	**Yes**	ND	Kim et al., (2021a)
Midwest USA	24/480	12 months	**Yes**	No	**Yes**	Kim et al. (2021b)
Denmark	20/712	13 years	ND	ND	**Yes**	Nierychlo et al. (2020)
Beijing/China	2/103	12 months	**Yes**	NA	ND	Sun et al. (2021)
Global (23 countries)	269/1186	Once-off	NA	**Yes**	**Yes**	Wu et al. (2018)
Mayagüez & Adjuntas/Puerto Rico	2/24	12 months	ND	**Yes**	NA	Valentin-Vargas et al. (2012)
Shenzen/China	2/14	Once-off	NA	NA	**No**	Xie et al. (2021)
Xinjiang/China	4/8	Once-off	NA	ND	ND	Xu et al. (2018)
Global (China, Hong Kong, Singapore, Canada, USA)	14/14	Once-off	NA	**Yes**	ND	Zhang et al. (2012)
Wuxi, Lanzhou, Ma'anshan, Suzhou, Shijiazhuang, Urumchi, Hefei, Nanjin/China	10/10	Once-off	NA	**Yes**	ND	Zhao et al. (2014)
Cape Town, Ekurhuleni/South Africa	6/141	30 months	**Minor**	**Major**	**Minor**	*This study*

NA = not applicable, ND = not determined.

instances. In this study, in the coldest month (July), the recorded temperatures were: 18.0.4 ± 1.1 °C (max) and 7.5 ± 0.9 °C (min) and 17.6 ± 0.9 °C (max) and 4.9 ± 1.1 °C (min) in CPT and COE, respectively (Section 2.1, Fig. S1a), and AS temperature was not significantly correlated with bacterial community structure (Section 3.3). The results therefore suggest that the small to moderate correlations between 'season' and bacterial community composition were temporal changes unrelated to climatic factors.

In summary, highly significant differences were found in the bacterial community composition between two sites in SA, and these differences overshadowed any effects from temporal changes or differences in WWTP process configurations. It was found that the site differences were due to highly significant differences in environmental parameters, notably with the inclusion of comprehensive AS characterization for the first time. Furthermore, it was shown that TRFLP can be a useful and cost-effective tool to screen large datasets in order to rationalize the number of samples for HTS.

Credit author statement

PJW: Conceptualization, visualization, data curation, funding acquisition, formal analysis, investigation, methodology, project administration, supervision, validation, writing original draft. NvB: Conceptualization, methodology, supervision, validation, reviewing and editing original draft. MPT: Data curation, formal analysis, investigation, methodology, reviewing and editing original draft.

Funding

This work was supported by the Water Research Commission (WRC) of South Africa as part of Project K5/2471/3 (National survey of filamentous bacterial in activated sludge). Any opinion, findings and conclusion or recommendation expressed in this material is that of the authors and the WRC does not accept any liability in this regard.

Declaration of competing interest

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

Data availability

Raw sequence data obtained in this study has been deposited in the National Center for Biotechnology Information (NCBI) SRA database under BioProject PRJNA907162

Acknowledgements

The authors would like to thank the City of Cape Town Department of Water and Sanitation for access to wastewater treatment plants, as well as staff from Scientific Services at the City of Cape Town, particularly Ndumiso Sonjica, Ansie Smith, Mjikisile Vulindlu and Dr Swastika Surujlal-Naiker for advice and assistance with sample collection, sample preservation and physicochemical analyses as well as staff at the Ekurhuleni Water Care Company for advice and assistance with physicochemical analyses. The authors would also like to the South African Weather Service (SAWS, personal communication) for providing detailed climatic data from the City of Cape Town and the City of Ekurhuleni.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envres.2023.115394.

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