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INFLUENCE OF SEASONALITY, WASTEWATER TREATMENT PLANT PROCESS, GEOGRAPHICAL LOCATION AND ENVIRONMENTAL PARAMETERS ON BACTERIAL COMMUNITY COMPOSITION IN SELECTED SOUTH AFRICAN DOMESTIC ACTIVATED SLUDGE WASTEWATER TREATMENT PLANTS

by

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DECLARATION

I, Mfundisi Phillip Thobejane, declare that the contents of this dissertation/thesis represent my own unaided work, and that the dissertation/thesis has not previously been submitted for academic examination towards any qualification. Furthermore, it represents my own opinions and not necessarily those of the Cape Peninsula University of Technology.

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ABSTRACT

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. Little is known about the underlying operational factors and geographical factors (ecophysiology) influencing the complex dynamics of the microbial populations in South African (SA) AS bioreactors, and how those dynamic interactions affect the systems' functional stability. Prior to this study, there were no studies detailing the overall bacterial composition in SA wastewater treatment plants (WWTPs).

In this study, we compared the bacterial communities in domestic WWTPs with different configurations from two different geographical locations in SA by analysing the bacterial metagenomes and physicochemical data. Samples (AS sludge and influent) and were collected from 3 WWTPs located in the city of Cape Town (CoCT) and 6 (WWTPs) located in the city of Ekurhuleni (CoE). The metagenomic bacterial community structures in 141 samples were first assessed using terminal restriction fragment polymorphism (T-RFLP). Based on the T-RFLP results, next generation sequencing (NGS) was performed by sequencing amplicons of the V4 region of the 16S rRNA gene using the Illumina MiSeq platform on a rationalised set of 50 samples. Univariate and multivariate statistical tools were used to analyze the output data and assess the influence of seasonality, WWTP process (configuration), geographical location (site) and environmental variables on bacterial community selection. Physiochemical analyses were performed in the bioreactors *in-situ* (temperature, pH, dissolved oxygen (DO)), and on AS and/or influent samples (dissolved sludge volume index (DSVI), volatile suspended solids (VSS), 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_4^+-N), nitrates and nitrites as nitrogen (NO_3^-/NO_2-N), total alkalinity (T.alk.) and volatile fatty acids (VFA) concentrations).

Good separation of the data points representing each geographical location (factor 'site') was noted on non-metric multidimensional scaling (nMDS plots) obtained using both the T-RFLP and

NGS data. Highly significant differences (P=0.001) were found between the 2 sites using 3-way fully nested analysis of similarity (ANOSIM), validating the nMDS results. As the geographical location played such a dominant role on the bacterial community selection, the factors 'season' and 'configuration' were analyzed separately for each geographical location. In the CoE WWTPs, no significant differences (P>0.05) were found for either of these factors when analysing the T-RFLP and NGS data using 2-way nested ANOSIM. In contrast, using the same methodology for the CoCT WWTPs, significant differences (0.05>p<0.01) were noted for the factor 'configuration'.

The highly significant differences in the overall bacterial structures from the 2 geographical locations could not be linked to climatic or WWTP operational differences. However, the there were highly significant differences (students t-test p<0.005) in some of the physicochemical parameters. With the influent from the CoE being generally more dilute in nature. It was hypothesized that this was the primary reason for the differences in the AS bacterial communities from each site. This was validated using BEST, 3-way ANOSIM and PCA. The BEST analysis indicated that the AS TP concentration was the primary driver for differences in the bacterial community structures from the 2 geographical locations. Concentrations of AS TP in the CoCT WWTPs and CoE WWTPs were 4.0 ± 6.4 and 9.5 ± 7.2, respectively.

High throughput sequencing generated a total of 2 645 unique zero radius operational taxonomic units (zOTUs) with a frequency of 530 594 across the 50 samples with median of 10 101 per sample (range 7592 to 14 890). In total, 27 identified phyla were observed. *Proteobacteria* was the most abundant phylum in all AS samples, accounting for 34.75 - 86.42% relative abundance (RA) at the CoCT and 37.133 – 77.89% RA at the CoE. The other two most dominant phyla were *Bacteroidetes* (5.08 – 32.72% RA at CoCT and 10.50 – 45.88% RA at CoE), and *Actinobacteria* (2.82 – 21.35% RA at CoCT and 1.43 – 14.16% RA at CoE). At the family level, a total of 277 families were identified, of which 256 were common to both CoCT and CoE. At this level, the taxonomic profile differed between the two geographic locations. *Pseudomonadaceae* was the most dominant family at the CoE, accounting for 0.53 – 51.21% RA while *Moraxellaceae* dominated at the CoCT accounting for 0.25 – 39.83% RA. A total of 832 genera were identified, of which 749 were common to both the CoCT and CoE WWTPs. *Pseudomonas* was the most dominant genus, with a mean estimated absolute abundance (EAA) of 23 358 ± 19 720 cells/ngDNA.gsludge^{-1} in the CoE and 20 582 ± 39 028 cells/ngDNA.gsludge^{-1} in the CoCT.

Overall, this study showed that geographically related physicochemical differences were more significant than season or WWTP configuration on bacterial community selection in the WWTPs in the CoE and CoCT. It was also shown that community fingerprinting using T-RFLP and high throughput sequencing yielded similar results. It was concluded that although T-RFLP is an older and is a less sophisticated fingerprinting technique than NGS and does not provide direct information on the taxa that are present, it can still be a valuable and reliable screening tool in cases where it important to include large datasets and funding is limited. It is recommended that future work is directed towards qualitative and quantitative analyses of the functional microbial species, particularly the nitrifying bacterial populations in South African AS WWTPs, and comparison of the results with global studies.

Keywords: Activated sludge, Wastewater treatment, Overall bacterial community structure, terminal restriction fragment length polymorphism, Illumina amplicon sequencing

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CLARIFICATION OF TERMS AND CONCEPTS

- **Activated sludge process:** A biological wastewater treatment process in which a mixture of wastewater and activated sludge is agitated and aerated.
- **Bio-flocculation:** is a complex phenomenon whereby the extracellular polymeric substances produced by bacteria induces aggregation of bacteria, organics and inorganics.
- **Biological oxygen demand:** The quantity of oxygen used in the biochemical oxidation of organic matter in a specified time, at a specified temperature, and under specified conditions.
- **Biomass**: Aggregation of biological material. In this thesis, biomass refers specifically to microorganisms.
- **Denitrification:** is a process by which certain species of bacteria under anoxic conditions reduce nitrate and nitrite nitrogen to nitrogen gas.
- **Food to Microorganism ratio:** is a measurement of the food (measured as biological or chemical oxygen demand) entering the activated sludge process and the microorganisms (bacteria) in the aeration tanks (measured as volatile solids).
- **Hydraulic Retention Time:** is the amount of time for a liquid is retained in a reactor. In this thesis, the term applies to domestic wastewater in different bioreactors.
- **Nitrification:** is the process by which ammonia is first converted to nitrite and, finally, to nitrate by nitrifying bacteria.
- **Sludge age:** is the time on average that biomass is retained in bioreactors and is dependent on the hydraulic retention time and the sludge recycle ratio.
- **Sludge bulking:** Sludge that settles and compacts slowly in the clarifier due to a number of factors, including over-proliferation of filamentous bacteria and inter-floc bridging.
- **Sludge Volume Index:** The measurement of settleability of the sludge after allowing the mixed liquor to stand for thirty minutes in a 1 L measuring cylinder.

LIST OF ABBREVIATIONS

- AB Absolute Abundance
- ALR Ammonia load Rate
- Ag_2SO_4 Silver sulfate
- ANOSIM Analysis of similarity
- AOB Ammonium oxidizing bacteria
- AS Activated sludge
- ASP Activated sludge process
- ATP Adenosine tri-phosphate
- A/O Anoxic/aerobic
- A/A/O Anaerobic/anoxic/aerobic
- BNR Biological nutrient removal
- BOD Biological oxygen demand
- C Carbon
- CAS Conventional Activated sludge
- cBOD Carbonaceous biological oxygen demand
- CO_2 Carbon dioxide
- COD Chemical oxygen demand
- CoE City of Ekurhuleni
- CoCT City of Cape Town
- CPUT Cape Peninsula University of Technology
- DNA Deoxyribonucleic acid
- DGGE Denaturing gradient gel electrophoresis
- DO Dissolved oxygen
- DSVI Diluted sludge volume index
- EAA Estimated absolute abundance
- EBPR Enhance biological phosphate removal
- EPS: Extracellular polymeric substances
- ESS Effluent suspended solids
- ERWAT: Ekurhuleni Water Care Company

- FAM Fluorescein amidite
- $Fe_2H_8N_2O_8S_2$ Ferrous ammonium sulfate
- FISH Fluorescence in situ hybridization
- F/M Food to mass ratio
- GAOs: Glycogen accumulating organisms
- GSB Green sulfur bacteria
- H_2S Hydrogen sulfide
- H_2SO_4 Sulfuric acid
- $HgSO_4$ Mercury sulfate
- HTS High throughput sequence
- HRT Hydraulic retention time
- MCRT Mean cell retention time
- ML Mix liquor
- MLE Modified Ludzack-Ettinger
- MLSS Mix liquor suspended solids
- MLVSS Mix liquor volatile suspend solids
- MWTPs Municipal waste treatment process
- N Nitrogen
- N_2 nitrogen molecule
- NBDSCOD Non-biodegradable soluble chemical oxygen demand
- NBDPCOD Non-biodegradable particular chemical oxygen demand
- nMDS non-metric multi-dimensional scaling
- NH_4^+ Ammonium
- NH_3 Ammonia
- NO_2^- Nitrite ions
- NO_3^- Nitrate ion
- NOB Nitrite oxidizing bacteria
- NGS Next generation sequencing
- O_2 Oxygen molecule
- OBCS Overall Bacterial Community Structure
- OLR Organic loading rate

- P Phosphorus
- PAOs Polyphosphate accumulating organisms
- PCR Polymerase chain reaction
- PHA Poly-β-hydroxyalkanoates
- PO_4 Phosphate
- PSB Purple sulfur bacteria
- qPCR Qualitative polymerase chain reaction
- RA Relative Abundance
- RAS: Return activated sludge
- RNA: Ribonucleic acid
- rRNA Ribosomal ribonucleic acid
- RBCOD Readily biodegradable chemical oxygen demand
- S Sulfur
- S^{2-} Sulfide
- $S_2O_3^{2-}$ Thiosulfate
- SA South Africa
- SAWS South African Weather Service
- SCOD Soluble chemical oxygen demand
- SBCOD Slowly biodegradable chemical oxygen demand
- SIMPER Similarity percentage
- SOB Sulfur oxidizing bacteria
- SRB Sulfur reducing bacteria
- SRT Sludge retention time
- SS Suspended solids
- SSCP Single stranded confirmation polymorphism
- SO_3^{2-} Sulfite
- SO_4^{2-} Sulfate
- SVI: Sludge volume index
- T-RFLP: Terminal restriction fragment length polymorphism
- VFA Volatile fatty acid
- WAS Waste activated sludge

- WRC Water Research Commission
- WWTPs: Wastewater treatment plants
- σ-P Orthophosphate
- 3SB 3 stage Bardenpho

1. CHAPTER ONE: INTRODUCTION

This chapter provides a short background on water resources in South Africa (SA) and its domestic and industrial applications (Section 1.1). It also briefly explains what wastewater is and how it is treated (Section 1.2). It gives a brief explanation of biological nutrient removal through the activated sludge (AS) process (Section 1.3). More importantly, this chapter lays a foundation in understanding the motives for this research (Section 1.4). It further highlights the research questions and the hypothesis (Section 1.5). Lastly, this chapter explains the aim and the objectives of the study (Section 1.6 and Section 1.7 respectively).

1.1. SOUTH AFRICAN WATER

Fresh water is an absolute need, not only for human activities, but also for all forms of living organisms and for the conservation of the ecosystem (Colvin et al., 2013; Hesham, 2012; Kılıç, 2020). Its necessity can influence the growth and sustainability of the economy. SA is a water scarce country. Potable water in SA (also in other countries) undergoes a treatment process before being used (Colvin et al., 2013; Pakharuddin at el., 2021). According to stats SA (www.statssa.gov.za, accessed 01/07/2017), the population of SA increased from 40.5 million in 1996 to 55.6 million in 2016. The increase in population has resulted in the generation of large quantities of domestic and industrial effluent. Water has a number of particular characteristics which call for specific usage and treatment in a resource account. Water not fit for human consumption may be satisfactory for other uses. The usage of water depends crucially on its quality e.g. water used for hydro-electric power generation, industrial purposes and transportation does not require high standards of purity, whereas other uses, such as drinking, recreation, and habitats for aquatic organisms, depend on higher levels of purity (stats SA, 2005; Shaltami and Bustany, 2021).

1.2. WASTEWATER AND WASTEWATER TREATMENT

Wastewater mainly consists of domestic waste, industrial waste and, in some municipal areas, storm sewage. The composition of the mixed liquor entering wastewater treatment plants is mainly water, comprising around 95%, with the remaining 5% being the solids fraction (Holder-Snyman, 2005; Crini and Lichtfouse, 2018). Wastewater generated from industrial activities, agricultural activities and domestic activities often contain high levels of organic

matter. Treatment of domestic and industrial wastewater to remove organic matter and toxic substances is important for the protection of the natural ecosystem and human health (Shchegolkova et al., 2016). Extensive treatment is required to convert waste materials into stable end products that can be safely disposed into inland waters (Merrangane, 2007; Crini and Lichtfouse, 2018). Depending on the effort given to the task, the treated water may still contain organics, toxins and some microorganisms which will be released to the rivers and streams (Willey et al., 2008; Crini and Lichtfouse, 2019). The treatment of domestic waste can be generalized, whereas industrial waste cannot, since the character of effluent from each industry is unique. Domestic wastewater typically consists of naturally occurring organic material. In contrast, industrial wastewater consists of chemicals which microorganisms may find difficult to metabolize, may not contain sufficient macronutrients for bioremediation, and may contain toxins (Coelho et al., 2015).

Environmental microbial communities are an important resource for biological processes such as wastewater treatment and soil remediation. Application of these microbial resources to treat wastewater requires an understanding of not just which organisms are present, but also their metabolic function and ecology (Saunders et al., 2016). The neutralisation of chemical pollutants (e.g. toxicants and xenobiotics) can be achieved by biological treatment at wastewater treatment plants (WWTPs) (Shchegolkova et al., 2016).

1.3. THE CONVENTIONAL ACTIVATED SLUDGE PROCESS

Biological nutrient removal (BNR) AS systems are composed of aerobic and anaerobic microorganisms such as bacteria, archaea, fungi and protists and the microbial diversity depends on the influent wastewater, environmental conditions such as pH and temperature, and prevalent operational conditions (Shchegolkova et al., 2016; Marrengane, 2007). Bacteria are the dominant population in the AS, and it is hypothesized that the dominant microorganisms play the most important roles in each stage of the system (Yang et al., 2014). The role of bacteria in the activated sludge process (ASP) is to remove the carbon (C) and other nutrients from wastewater and thus represent the most important component of every biological WWTP (Wagner and Loy, 2002; Peces at el., 2022).

1.4. MOTIVATION FOR THE STUDY

Bacteria are the dominant microorganisms in the ASP, and they play an important functional role in each stage of the system, contributing to the quality of the final effluent (Yang et al., 2014). Influent characteristics and operational conditions such temperature, pH and dissolved oxygen (DO) can affect the growth and prevalence of microorganisms in AS (Morgan-Sagastume and Allen, 2003). Establishing or monitoring operational parameters such as the sludge volume index (SVI), mixed liquor suspended solids (MLSS), chemical oxygen demand (COD), suspended solids (SS), and concentrations of ammonium (NH4), nitrates (NO3) and nitrites (NO2) aids in establishing the performance of the system.

Classical culture-dependent microbiological methodologies have been used to detect and quantify bacteria in wastewater. However, depending on the target species, the results may be unrepresentative and biased. Culture-independent molecular methods, including the polymerase chain reaction (PCR), quantitative polymerase chain reaction (qPCR), and fluorescent in situ hybridization (FISH), have also been used to identify microorganisms from ecological samples, including AS. However, these techniques depend on primers or probes that are typically designed to identify one genus or species. Microbial fingerprinting using terminal restriction fragment length polymorphism (T-RFLP) or denaturing gradient gel electrophoresis (DGGE) is useful for monitoring temporal and/or spatial community differences and changes. High-throughput sequencing (HTS) technologies, notably amplicon sequencing of metagenomic deoxyribonucleic acid (DNA) is currently the method of choice for assessing the relative abundance and diversity of microorganisms in environmental samples, as it is the only method capable of providing comprehensive insight into almost all of the species present (Huang et al., 2018). Little is known about the underlying operational factors and geographical factors (ecophysiology) influencing the complex dynamics of the microbial populations in SA's AS bioreactors, and how those dynamic interactions affect the system's functional stability. To date, there are no studies detailing the overall bacterial composition in SA WWTPs.

1.5. HYPOTHESIS AND RESEARCH QUESTIONS

It was hypothesised that different microbial communities are present in AS WWTPs with different configurations, from different geographical locations, and in different seasons.

To validate the hypothesis, the following questions needed to be answered:

- What are the core dominant bacterial species in selected AS WWTPs in the CoCT and the CoE?
- Are there statistically significant differences in the OBCS in AS WWTPs from different geographical locations, seasons and configurations?
- Which physico-chemical parameters are the primary drivers of the OBCS in the AS WWTPs included in the study?

1.6. AIM OF THE STUDY

To profile the microbial community structure in 3 domestic WWTPs in the CoCT and 6 domestic WWTPs in the CoE using bacterial community fingerprinting (T-RFLP) and 3 domestic WWTPs in the CoCT and 3 domestic WWTPs in the CoE amplicon sequencing.

1.7. OBJECTIVES

- To extract metagenomic DNA from activated sludge samples
- To amplify, digest and perform T-RFLP on 16S rRNA gene amplicons, and statistically analyse the results using appropriate software
- To sequence the 16S rRNA amplicons using amplicon sequencing (Illumina MiSeq) and analyse the results using appropriate software.
- To analyse the physico-chemical data
- To correlate the abundance of functional microbial taxa with selected physico-chemical parameters.

2. CHAPTER TWO – LITERATURE REVIEW

This chapter provides information about wastewater treatment processes to provide some background context to the research. It starts by defining wastewater treatment (Section 2.1) and then introduces microbial metabolism (Section 2.2) and the roles of microbial communities in biological nutrient removal (Section 2.3). Thereafter, AS components are described (Section 2.4), followed by an explanation of the AS process (Section 2.5), factors affecting/influencing the performance of AS (Section 2.6) and challenges or problems associated with the AS process (Section 2.7). The chapter ends with a description of the analytical methods used to identify the bacterial communities (Section 2.8).

2.1. WASTEWATER TREATMENT PROCESS

Water treatment is the process of removing all the matter (i.e. the biological, chemical and physical) that cause harm to the ecosystem (Pakharuddin at el., 2021). With the rapid population growth, urbanization and industrialization, the demand of water and the generation of wastewater has also increased significantly. Due to the water scarcity and shortage and most importantly, the protections of the ecosystem and biodiversity, satisfactory recycle of wastewater becomes imperative. Wastewater differ in composition (e.g. domestic and industrial wastewater) and depending on which type of composition, the treatment process will also differ (Fahad at el., 2019).

Wastewater treatment plants are infrastructures in which wastewater is treated to meet discharge standards. Activated sludge (AS) is a conventional treatment process that is used treat various types of wastewater. It is a unique artificial ecosystem with a high microbial diversity and a high concentration of biomass. This ecosystem mainly consists of eukaryotes, bacteria, archaea, and viruses, with bacteria dominating the system (Fahad at el., 2019; Xie at el., 2021). Highly diverse bacterial communities have superior treatment performance (Xie at el., 2021).

2.2. MICROBIAL METABOLISM

All microbial species need sources of C, energy, and electrons for growth and metabolism. Table 1 provides a summary of different sources of C, energy and electrons. Carbon serves as the backbone for all the organic molecules, energy is needed for the work to be performed, and the electrons are needed for electron transport chains and oxidation-reduction reactions (Willey et al., 2008; Bruslind, 2023).

Table 1: Sources of carbon, energy and electrons (adapted from Willey et al., 2008; Bruslind, 2023)

Nutritional type	Carbon Source	Energy Source	Electron Source
Organotroph Lithotroph			Organic molecule Inorganic molecule
Phototroph Chemotroph		Light Chemicals	
Heterotroph Autotroph	Organic molecule CO_2		

Five metabolic classes namely, photolithoautotrophy, photoorganoheterotrophy, chemolithoautotrophy, chemolithoheterotrophy and chemoorganoheterotrophy. Table 2 distinguishes all the metabolic classes of microorganisms (Willey et al., 2008; Chen et al., 2020). The main metabolic bacterial pathways involved in secondary wastewater treatment can be classified as heterotrophic or autotrophic. These are conducted by heterotrophs (or chemoorganotrophs) and autotrophs, respectively (Willey et al., 2008)

Table 2: Five metabolic classes of microorganism (adapted from Willey et al., 2008; Chen et al., 2020)				
Photolithoautotrophy	CO_2	Light	Inorganic e^- donor	Purple and green sulfur bacteria
Photoorganoheterotrophy	Organic carbon, but CO_2 may also be used	Light	Organic e^- donor	Purple non-sulfur bacteria
Chemolithoautotrophy	CO_2	Inorganic molecules	Inorganic e^- donor	Nitryifing bacteria – e.g. *Nitrosomonas, Nitrobacter*
Chemolithoheterotrophy	Organic carbon but CO_2 may also be used	Inorganic molecules	Inorganic e^- donor	
Chemoorganoheterotrophy	Organic carbon	Organic molecules	Organic e^- donor	Denitrifying bacteria – e.g. *Pseudomonas, Acidovorax, Comamonas, Acinetobacter*

2.2.1. HETEROTROPHIC METABOLISM

Microorganisms with chemoorganoheterotophic metabolism are commonly referred to as 'heterotrophs'. They use reduced organic molecules as a C source and energy for the synthesis of new cells. Heterotrophic metabolism involves the conversion of organic molecules to end products via a pathway that releases enough energy for it to be coupled with to the formation of adenosine tri-phosphate (ATP). Heterotrophs can generate ATP in two basic ways: respiration and fermentation (Table 3) and can be subdivided into three classes according to their dependence on free DO, namely aerobic (Equation 1), facultative (e.g. Equation 2) and anaerobic (Equation 3 and Equation 4). Strict aerobes require oxygen (O_2) as the terminal electron acceptor for the electron transport chain, which is also the preferred electron acceptor for facultative microbes. However, the latter are capable of switching from aerobic metabolism (Equation 1) in the absence of O_2, utilising other molecules such as NO_3^{2-} or NO_2^- (Equation 2) as terminal electron acceptors. Heterotrophs play an important role in the ASP, where aerobic heterotrophic metabolism dominates (Holder-Snyman et al., 2005; Chen et al., 2020; Winkel, M., 2013). Denitrifying bacteria (Section 2.3.3) are facultative heterotrophs that assist with removal of nitrogen (N) (Equation 2) in BNR WWTPs (Marrengane, 2007; Chen et al., 2020). Aerobic processes are typically biochemically more efficient, whereas anaerobic

processes are biochemically less inefficient and produce more complex chemical by-products (Gray, 2004).

Aerobes require free DO in order to utilise organic matter:

$$\text{Organics} + O_2 \xrightarrow[\text{Aerobic bacteria}]{} CO_2 + H_2O + \text{Energy} + \text{new cells} \qquad \text{(Equation 1)}$$

Facultative microbes can also use O_2 bound molecules to decompose organic matter (Gray, 2005):

$$\text{Organics} + NO_2 \xrightarrow[\text{Facultative bacteria}]{} CO_2 + N_2 + \text{Energy} + \text{new cells} \qquad \text{(Equation 2)}$$

(Gray, 2005).

Anaerobes do not require external electron acceptors in order to utilise organic matter

$$\text{Organics} \longrightarrow 2C_3H_6O_3 + \text{energy} \qquad \text{(Equation 3)}$$

$$\text{Organics} \longrightarrow 2C_2H_5OH + CO_2 + \text{energy} \qquad \text{(Equation 4)}$$

Table 3: Type of heterotrophic metabolism used to generate ATP (adapted from Chen et al., 2020)	
Respiration	Uses complete oxidation of organic compounds, requiring an external electron acceptor to balance the oxidation -reduction reactions used to generate ATP: much of the ATP is formed as a result of chemiosmosis based on establishment of a proton gradient across a membrane.
Aerobic respiration	Uses oxygen as the terminal electron acceptor in the membrane-bound pathway that establishes the proton gradient for chemiosmotic ATP generation.
Anaerobic respiration	Uses compounds other than oxygen e.g. nitrate as a terminal electron acceptor in the membrane-bound pathway that establishes the proton gradient for chemiosmotic ATP generation.
Fermentation	Does not require an external electron acceptor, achieving a balance of oxidation-reduction reactions using metabolic intermediates of organics substrate molecules. Various fermentation pathways produce different end products.

2.2.2. AUTOTROPHIC METABOLISM

Microorganisms with autotrophic metabolism use oxidised inorganic molecules for energy and carbon dioxide (CO_2) as their source of carbon (Willey et al., 2013; Gray, 2005). Autotrophs can also obtain the required energy for cellular function by photosynthesis i.e. the conversion of light energy to chemical energy (Table 4). In nature, autotrophs, including plants, play an important role as they synthesise organic molecules that are used by heterotrophs as their source of energy (Holder-Snyman et al., 2005; Chen et al., 2020). In AS systems, the most important autotrophs are the nitrifiers, which are chemolithotrophs. These organisms carry out the process of nitrification whereby ammonia (NH_3) is oxidized to NO_2^- and NO_3^- (Section 2.3.2) (Gerardi, 2006).

Table 4: Type of autotrophic microbial metabolism used to generate ATP (adapted from Chen et al., 2020.)

Oxygenic photosynthesis	Uses two connected photosystems and results in evolution of oxygen, as well as of generation of ATP; carried out by algae and cyanobacteria.
Anoxygenic photosynthesis	Uses one photosystem and does not result in the evolution of oxygen; carried out by anaerobic photosynthetic bacteria e.g. green and purple sulfur bacteria, and under some conditions, by cyanobacteria.
Chemolithoautotrophy	Uses oxidation of inorganic compounds such as sulfur, nitrite, nitrate and hydrogen to establish an electrochemical gradient across a membrane that results in generation of ATP by chemiosmosis.

2.3. MICROBIAL COMMUNITY STRUCTURE AND FUNCTION IN WASTEWATER

The microbial community structure in BNR systems is strongly influenced by the wastewater composition (Ogunlaja, 2015; Begmatov, 2022). Microorganisms can be classified as eukaryotes or prokaryotes based on their cellular structure, with prokaryotes consisting of the domains Bacteria and Archaea (Gerardi, 2006; Willey et al., 2008; Callieri et al., 2018). Bacteria are the most important of the microorganisms used in wastewater treatment and make up about 95% of the microbial community in AS (Gerardi, 2006; EPA, 1997; Wagnor and Loy, 2002; Holder-Snyman et al., 2005). Bioreactors contain a wide diversity of microbial species, which mainly grow within flocs (Section 2.4) (Seviour and Nielsen, 2010, Ismail, 2008; Begmatov, 2022). Microorganisms that fulfil a specific role in the AS are grouped together and

function in different zones of bioreactors (Table 5). Grouping allows the mass behaviour of a functional group to be modelled (Ismail, 2008; Ogunlaja, 2015).

Table 5: Principal organism groups included in models for activated sludge systems (modified from Ismail, 2008; Ogunlaja, 2015)

Organism	Biological Processes	Zone
Heterotrophs (all)	COD removal (organic degradation; DO uptake) Ammonification (organic N→$NH4^+$)	Aerobic
	Denitrification (organic degradation; NO_3^- →NO_2 →N_2)	Anoxic
	Fermentation (FRBCOD→ SCFA)	Anaerobic
Phosphate accumulating organisms (heterotrophic sub-group)	P release (SCFA uptake; PHA storage)	Anaerobic
	P release (SCFA uptake; PHA storage) P uptake (PHA degradation; denitrification)	Anoxic
	P uptake; P removal (PHA degradation; DO uptake)	Aerobic
Autotrophs-AO (nitrifiers)	Nitrification (NH_4^+ → NO_2 → NO_3^-; DO uptake)	Aerobic

COD - chemical oxygen demand, FRBCOD- fermentable readily biodegradable chemical oxygen demand, DO – dissolved oxygen, SCFA – small chain fatty acids, PHA - polyhydroxyalkanoates

2.3.1. THE REMOVAL OF ORGANIC MATTER

The organic material in effluent is present in soluble, colloidal or particulate forms (Drewnowski and Makinia, 2014). Some of the organic components are readily biodegradable, some slowly biodegradable, and some are non-biodegradable (recalcitrant). The biodegradability is generally expressed using chemical oxygen demand (COD) measurements: readily biodegradable COD (RBCOD), slowly biodegradable COD (SBCOD), non-biodegradable COD (NBCOD). During the AS process, when the influent enters the bioreactor, the activated sludge comes in contact with the pre-settled wastewater in an aerated tank and active microorganisms present in the sludge will oxidize the organics into CO_2 and water. Some of the influent organic materials are transformed into new cell material, leading to an increase in biomass concentration (Modin et al.,2016). Accepting the general formulation for protoplasm as $C_5H_7O_2N$ with glucose as an example substrate, the synthesis reaction can be summarized as Equation 5 (Ismail, 2008; Welz, 2008; Small and Abrahamsen, 2021).

$$5C_6H_{12}O_6 + 6NH_3 = 6C_5H_7O_2N + 18H_2O \quad \text{(Equation 5)}$$

The energy required for the synthesis/growth process is generated from the hydrolysis of organic molecules. This is achieved by the heterotrophs to give hydrogen ions, electrons and CO_2. For example, consider the organic molecule glucose, Equation 6 (Ismail, 2008; Mita, Srivastava and Sharma, 2018).

$$C_6H_{12}O_6 + 6H_2O \rightarrow 6CO_2 + 24H^+ + 24e^- \quad \text{(Equation 6)}$$

Therefore, in secondary wastewater treatment, the organic fraction is removed via two mechanisms. Firstly, conversion of organic material to biomass allows it to be removed from the final effluent by settling in a clarifier. Secondly, mineralisation takes place (Modin et al.,2016; Ismail, 2008; Shome et al.,2022).

Chemical oxygen demand and biological oxygen demand (BOD) are traditionally used to evaluate the organic pollutant removal efficiency of the WWTPs (Hana et al., 2018; Gray, 2004). The COD measures both the biodegradable and non-biodegradable organic compounds, and the BOD test measures the biodegradable compounds, so the COD concentration should always be higher that the BOD concentration. The COD test involves boiling potassium dichromate ($K_2Cr_2O_7$) with concentrated sulfuric acid (H_2SO_4), silver sulfate (Ag_2SO_4) and mercury sulfate ($HgSO_4$). In the reaction, the organics in the sample react with $K_2Cr_2O_7$ solution of known concentration so that the amount of oxidizable organic matter in the sample is proportional to the $K_2Cr_2O_7$ used up during the oxidation reaction. The excess dichromate is titrated with ferrous ammonium sulfide ($FeH_8N_2O_8S_2$) to calculate the amount of dichromate used (Gray, 2004, Nabbou et al., 2020).

The BOD test measures only the readily available organics. The BOD test measures the amount of oxygen consumed through biological oxidation or respiration over a period of 5 days at 20^oC. During the biological oxidation, organics are broken down to CO_2. However, not all the organic matter will be converted to CO_2, some will be converted to new cells (Alewi et al. -2021).

2.3.2. NITRIFICATION

The presence of nitrogenous waste in the final effluent can be detrimental to receiving waters. Nitrogenous waste includes ammonium ions (NH_4^+), nitrate ions (NO_3^-) and nitrite ions (NO_2^-) (Figure 2). If certain parameters are adhered to, sufficient nitrification can take place during the AS process to satisfy NH_3 discharge requirements. In circumstances where total N discharge limits are imposed, denitrification is also needed and more complex WWTP (BNR) configurations are required (Gerardi, 2002; Junaidi, Sumiyati and Sitinjak, 2020). Under oxic conditions, ammonium oxidising bacteria (AOB) and nitrite oxidising bacteria (NOB) will carry out nitrification in a sequential oxidation of NH_4^+ to NO_2^- (Equation 7), and NO_2^- to NO_3^- (Equation 8) respectively (Gerardi, 2006; Ogunlaja, 2015; Zhu et al., 2022). Nitrifying bacteria, notably *Nitrosomonas* (AOB) and *Nitrobacter* (NOB) are autrotrophic bacteria that obtain their energy from inorganic molecules and also obtain their C source from oxidized CO_2 (Manirakiza and Sirotkin, 2021). From the oxidation of NH_4^+ and NO_2, nitrifying bacteria obtain very little energy, which results in slow bacterial growth and low nitrifying biomass concentrations relative to heterotrophic biomass (Mehrani et al., 2022). To encourage growth of nitrifiers, which are sensitive to environmental factors, specific reactor conditions are required because of their slow growth rate, a relatively high mean cell residence times (MCRT) is required, sufficient bicarbonate alkalinity (HCO_3^-) is required, and plant operational parameters such as pH, temperature, DO level, organic loading rate (OLR), ammonia loading rate (ALR), C:N ratio, and hydraulic retention time (HRT) need to be kept within particular ranges (Awolusi et al., 2016; Gerardi, 2006; Sepehri and Sarrafzadeh, 2019).

$$2NH_4^+ + 3O_2 \xrightarrow[\textit{Nitrosomonas}]{} 2NO_2^- + 2H_2O + 4H^+ + \text{Energy} \qquad \text{(Equation 7)}$$

$$2NO_2^- + O_2 \xrightarrow[\textit{Nitrobacter}]{} 2NO_3^- + \text{Energy} \qquad \text{(Equation 8)}$$

(Willey et al., 2008)

2.3.3. DENITRIFICATION

Under anoxic conditions, denitrifying bacteria use NO_3 instead of O_2 as electron acceptors during their respiratory process (Rossi et al., 2014; Ogunlaja, 2015; Albina *et al.*, 2019). Denitrifying bacteria are heterotrophic facultative bacteria that are widely distributed in soil, freshwater, sea and in wastewater. In BNR systems, they promote N-removal by reducing NO_3 to N_2 (Maintinguer, 2013). Under anoxic conditions, the organic C sources act as electron donors and reduce NO_3 and NO_2 (terminal electron acceptors) to N_2 (Equation 9). The organics also provide energy to this functional group of bacteria. Ethanol, glucose and acetate are some of the external organic electron donors successfully used for denitrification. Three genera that contain the most species of denitrifying bacteria are *Alcaligenes, Bacillus*, and *Pseudomonas* (Maintinguer et al., 2013; Yang et al., 2020).

$$C_2H_5OH + NO_3^- + H^+ - C_5H_7NO_2 + N_2 + CO_2 + H_2O \qquad \text{(Equation 9)}$$

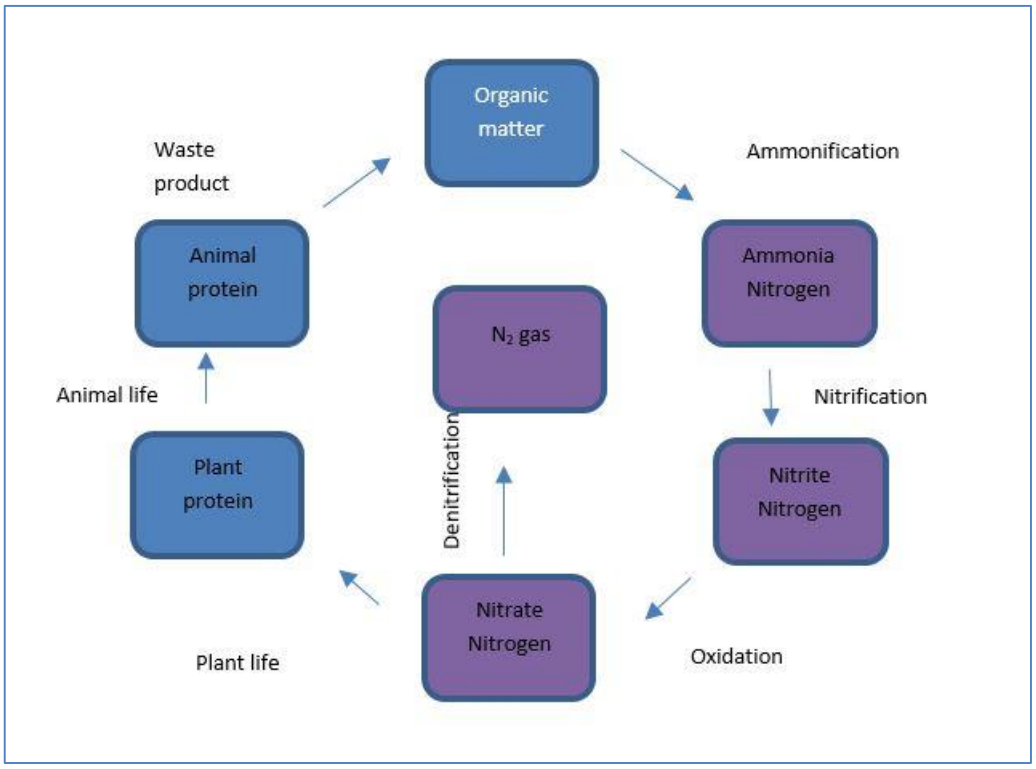

Figure 1: Schematic diagram of the nitrogen cycle. Purple boxes denote the processes taking place during biological nitrogen removal in wastewater (adapted from Curtin et al., 2011)

2.3.4. PHOSPHATE REMOVAL

Phosphates (PO_4^{2-}) are important macronutrients for living cells. They are essential components in ATP, DNA and ribonucleic acid (RNA), phospholipids, teichoic acids and teichuronic acids (Azizi *et al.*, 2021). The introduction of phosphorus (P) can have undesired effects on the quality of the receiving water. Being the main limiting nutrient responsible for eutrophication, it should therefore be removed/reduced if wastewater is discharged into sensitive aquatic systems (Gerardi, 2006, Zhang, Guisasola and Baeza, 2022). Phosphate removal can be achieved by chemical removal and also by enhanced biological phosphate removal (EBPR). The former is expensive and has led to intense focus on biological removal (Streichan et. al, 1990, Zhang, Guisasola and Baeza, 2022). Phosphate occurs in organic and inorganic forms (orthophosphate and polyphosphate). Orthophosphates (σ-P) are readily available nutrients for microbial growth and metabolism, and include phosphite (PO_3^{3-}), hydrogen phosphate (HPO_4^{2-}), dihydrogen phosphate ($H_2PO_4^-$) and phosphoric acid (H_3PO_4), with HPO_4^{2-}, $H_2PO_4^-$ being most commonly found in WWTPs (Tamás, 2020).

Phosphate is removed via EBPR from treatment systems by wasting cell biomass containing intracellular P in the form of polyphosphate (poly-p) (Figure 2). Under anaerobic conditions, heterotrophic bacteria known as phosphate accumulating organisms (PAO – *Acinetobacter*, *Aeromonas, Beggiato*, *Pseudomonas, Enterobacter* and *Moraxella*) store RBCOD such as volatile fatty acids (VFA) present in the influent as poly- β-hydroxyalkanoates (PHA). The energy in the form of ATP needed to transport VFA through the membrane and store them as PHA is derived from the hydrolysis of stored poly-P. The PAO use glycogen as the reducing agent to convert VFA to PHA. The hydrolysis of poly-P results in the release of σ-P into the bulk liquid in the anaerobic zone. Under aerobic or anoxic conditions, PAO can use the stored PHA as a source of C and energy. This gives them a competitive metabolic advantage over other heterotrophs when they first move from anaerobic into anoxic or aerobic zones. They readily absorb the σ-P in the bulk water for the glycogen synthesis, biomass growth and maintenance, leading to a reduction/removal of σ-P from the effluent. The biomass, which includes the PAO, is settled out in the clarifiers, and PO_4 removal is achieved through wasting the settled sludge (Shi, 2011; Van Loosrecht et al., 2016; Sidat et al., 1999; Ogunlaja, 2015, Campo *et al.*, 2020).

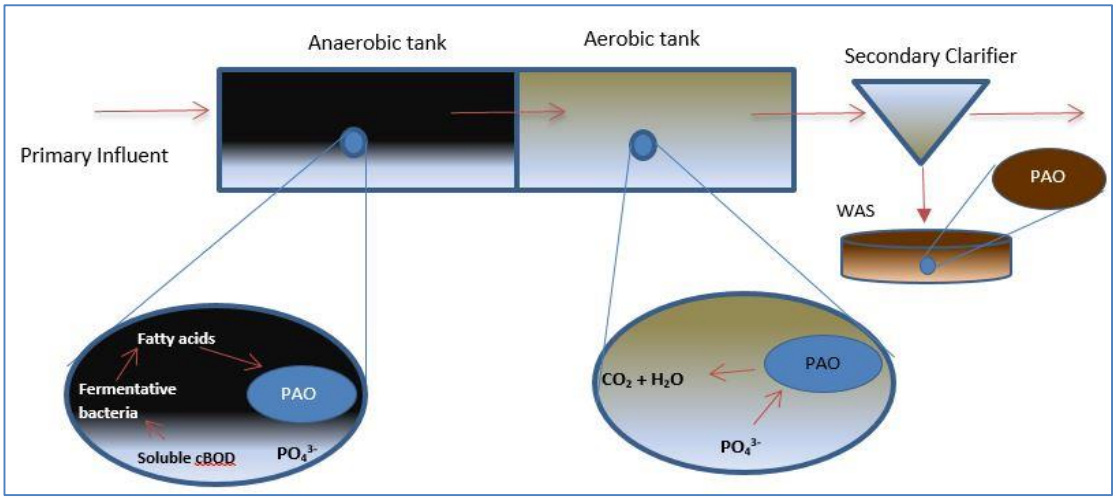

Figure 2: Schematic diagram of bacterial metabolic processes in the anaerobic and aerobic tanks in enhanced biological phosphate removal systems (modified from Shi, 2011)

2.3.5. SULFUR METABOLISM

Sulfur (S) is an essential element for all organisms, accounting for <1% of the dry weight of most. It is found in most proteins and is needed for the production of enzymes and enzyme cofactors such as thiamin and biotin . The S cycle (Figure 3) is complex, because S has a broad range of oxidation states, from –2 (completely reduced) to +6 (completely oxidized) (Muyzer and Stams, 2008). Sulfates (SO_4^{2-}) in wastewater are normally generated from paper and pulp industries, molasses-based fermentation industries and edible oil refineries. Sulfates can be removed from the wastewater via chemical precipitation and also biologically. The former is costly, thus the biological processes are given much attention (Gangagni Rao at el., 2003). The microbe-mediated biological S conversion applies three reaction processes: 1) assimilation of S, 2) dissimilation of organic S, and 3) oxidation and reduction of S compounds during which organic S compounds are decomposed into simple inorganic molecules such as SO_4^{2-}, sulfide $(S^2)^-$ and thiosulfate ($S_2O_3^{2-}$). A diverse range of microorganisms will use the inorganic S to synthesise important S-containing molecules such as amino acids following the reaction expressed by Equation 10 (Hao et al., 2014).

$SO_4^{2-} + 2e^- \rightarrow SO_3^{2-} + 6\ e^- \rightarrow H_2S \rightarrow$ Sulfur amino acids (Equation 10)

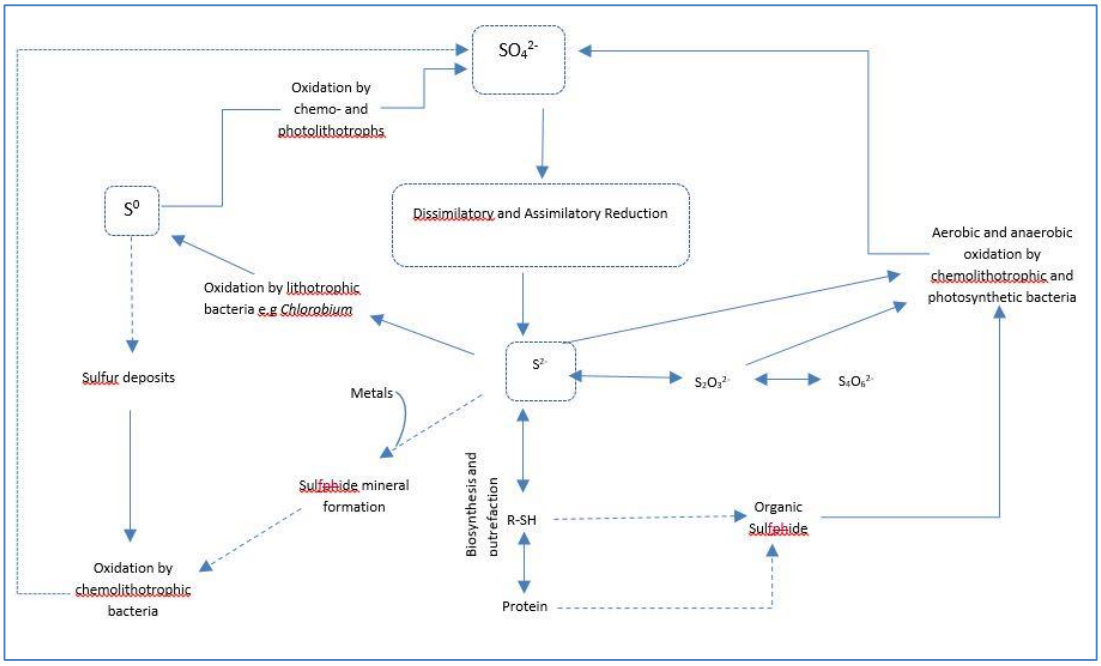

Figure 3: Schematic diagram of the sulfur cycle (adapted from Elliott and Whelan, 1980)

The two main groups of microorganisms involved in the biological S cycle are sulfur reducing bacteria (SRB) and sulfur-oxidizing bacteria (SOB) (Lin et al, 2018). The SRB use oxidized S under anaerobic conditions as electron acceptors and produce hydrogen sulfide (H_2S) while oxidizing organics for energy generation and cell growth. The S^{2-} that is generated, can be oxidized under oxic conditions by chemolithotrophic S bacteria or under anoxic conditions by phototrophic S bacteria. SRB are the main players in the anaerobic carbon cycle and are divided into two main groups: 1) those that incompletely degrade the organics to acetate and 2) those that degrade organics completely to CO_2 (Muyzer and Stams, 2008). The SOB use reduced forms of S or H_2S as electron donors to produce sulfuric acid (H_2SO_4) and are categorized into two groups based on their energy source and growth conditions: 1) phototrophic sulfur bacteria; and 2) chemotrophic colorless sulfur bacteria (Gerardi, 2006; Lin et al, 2018). The biological S conversion-based treatment processes were developed mainly to control the S^{2-} formation, volatilization of H_2S, chemical and biological oxidation of S^{2-} and precipitation of metal sulfides. Most of these processes combine one single biological step i.e. sulfate reduction. Based on the environmental conditions, the biological oxidation of S^{2-} to S and SO_4^{2-} can be performed by both the phototrophic sulfur bacteria and chemotrophic sulfur bacteria (Garcia et al, 2017). Phototrophic sulfur bacteria perform anoxygenic photosynthesis and are grouped into the green sulfur bacteria (GSB) which includes the genera *Chloribium* and purple sulfur bacteria (PSB) which includes the genera *Thiospirillum.* The biological

oxidation of S^{2-} to S and SO_4^{2-} given sufficient energy can be illustrated by Equation 11 and Equation 12 (Garcia et al, 2017; Lin et al, 2018). Most PSB contain S granules inside the cells and perform a complete oxidation to SO_4^2, whilst the GSB perform an incomplete oxidation of sulfide. The sulfur granules for GSB are deposited outside the cell (Garcia et al, 2017). Under anaerobic conditons, GSB and PSB require reduced form of sulfur and light energy to donate electrons (Lin et al, 2018).

Under limited light:

$$2H_2S + CO_2 – 2S^0 + CH_2O + H_2O \quad \text{(Equation 11)}$$

Under sufficient light:

$$H_2S + CO_2 + 2H_2O – SO_4^{2-} + 2CH_2O + H_2 \quad \text{(Equation 12)}$$

Chemotrophic SOB are commonly referred to as colorless sulfur bacteria. Without needing light, they oxidize almost all reduced S compounds, such as S^{2-}, polysulfide, elemental S, polythionate, $S_2O_3^{2-}$, sulfite (SO_3^{2-}), and in some cases organic S compounds such as methanethiol, dimethyl sulfide, and dimethyl disulfide using various electron acceptors (Lin et al, 2018).

2.4. ACTIVATED SLUDGE FLOCS

Activated sludge flocs are made up of living and dead bacterial cells (including filamentous bacteria), precipitation salts, trapped inorganic particles and organic particles. All these components are held together by chemical bonds and extracellular polymeric substances (EPS) which form a slime matrix (Eikelboom, 2000; Singh at el., 2022; Ferreira, 2022). There are three types of bacteria that constitute the floc structure. These bacteria include the floc formers, non-floc-forming bacteria and filamentous bacteria. The transformation of the biomass in the bulk solution occurs as the floc develops in the AS system. Typical AS flocs are composed of good balanced growth of filamentous bacteria and floc formers. Filamentous bacteria are bacteria that do not detach from one another during cell division. They are normally present in the AS whereby they form the macrostructure that will assist floc forming bacteria to adhere to, resulting to strong flocs that settle well (Marrengane, 2007; Ferreira, 2022; Deepnarain et al., 2019).

2.4.1. FLOC FORMING BACTERIA

Floc formation is a complicated phenomenon influenced by several factors. Floc forming bacteria initiate the formation of the floc in the AS process by EPS production. When the food to microorganism ratio (F/M ratio) in the bioreactor is high, the growth of floc formers is favoured. Conversely, when the F/M ratio is low, the growth of filamentous bacteria is favoured (Eikelboom, 2000; Marrengane, 2007; Gerardi, 2006; Ferreira, 2022). The transformation of the morphology of the floc occurs as the sludge ages. Table 6 provides the characteristics of young and old sludge flocs. The sludge age and the amount of biomass is a function of the concentration of the mixed liquor volatile suspended solids (MLVSS) in the system, which is controlled by the amount of sludge that is returned to the clarifier or wasted from the system i.e. decreasing the waste activated sludge (WAS) rate increases the sludge age, and vice versa (Gerardi, 2002; Ferreira, 2022).

Table 6: Characteristics of flocs particles at young and old sludge ages (adapted from Gerardi, 2002)

Characteristic of flocs particles	Young sludge age	Old Sludge Age
Fibril production	Few	Many
Fibril charge	Low	High
Polysaccharide production	High	Low
Polysaccharide strength	Weak	Strong
PHB deposition in floc particles	Core mostly	Core and perimeter
Floc shape	Spherical	Irregular
Filamentous organisms	Absent or few	Many
Floc strength	Weak	Usually strong
Floc size	Small	Medium to large
Floc settleability	Usually not desirable	Usually desirable
Ciliated protozoa	Few	Many
Dispersed growth	Significant	Insignificant
Particulate materials	Significant	Insignificant
Colloids	Significant	Insignificant
Diversity of bacteria	Small	Large
Diversity of enzymes	Small	Large

The bacterial growth curve has four phases (Figure 4) - lag, log, decline and endogenous phases which can all be related to the development of the floc. Table 7 characterizes each phase of the growth curve. Bacteria that enter the AS process through the inflow, which include soil and water bacteria, undergo a lag phase. During the lag phase, bacteria are metabolically active but are not yet reproducing. If a large fraction of bacteria are in the lag phase, insufficient EPS, polyhydroxybutyrate (PHB) granules and fibrils are available for floc formation and filamentous bacteria have not aged sufficiently to grow from single cells into chains. During the log phase, the bacteria have produced sufficient enzymes to synthesise cellular materials to enable them to reproduce. They absorb the biodegradable soluble organics, and the bacterial population (measured as MLVSS), increases (Gerardi, 2002; Muloiwa, Nyende-Byakika and Dinka, 2020).

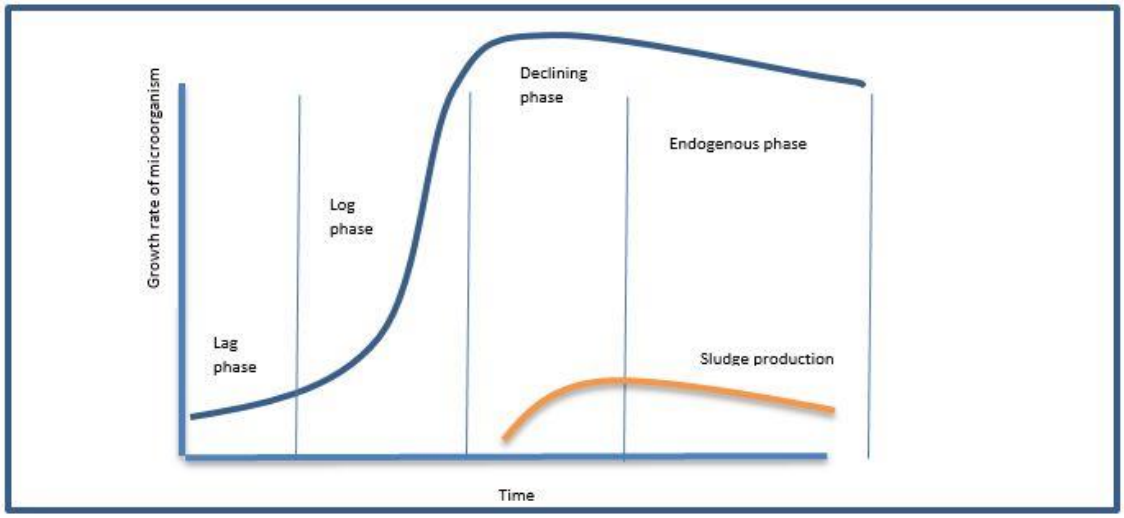

Figure 4: Graph showing the bacterial growth curve and the production of sludge (Gerardi, 2002)

Table 7: Microbial condition of each phase of the bacterial growth curve (adapted from Gerardi, 2002)				
Microbial condition	Lag phase	Log phase	Decline phase	Endogenous phase
Floc particle	Absent	Absent	Present	Present
Floc Shape	-	-	Spherical	Irregular
BOD	High	High	Moderate	Low
DO	Low	Low	Moderate	High
Number of bacteria	Low	Moderate	High	High
Location of bacteria	Dispersed	Dispersed	Flocculated	Flocculated
Dominant protozoan group	Amoeba and flagellates	Free swimming ciliates	Crawling ciliates	Crawling ciliates and stalked ciliates
Fine solids	Significant	Significant	Insignificant	Insignificant

BOD – biological oxygen demand; DO – dissolved oxygen

If the AS reaches a point where the nutrient supply is no longer sufficient to support the growth of all the bacteria in the system (low F/M ratio), the rate of bacterial growth begins to decline. This phase is called the decline phase. It is at this stage that the flocs begin to form. Floc-forming bacteria produce cellular fibrils, EPS and starch granules. The endogenous phase occurs at high sludge ages. At this stage, stored nutrients are depleted, and the energy obtained from the degradation of organics is used for cellular maintenance, with only limited cells undergoing reproduction. Dead bacterial cells are degraded and the contents also form part of the nutrient pool. The cellular reproduction and death rates are balanced and the bacterial population remains fairly constant (Holder-Snyman, 2005; Muloiwa, Nyende-Byakika and Dinka, 2020).

The growth curve is a good representation of a closed system. However, most biological treatment processes such as AS WWTPs are continuous systems where bacterial growth needs to be maintained in a particular growth phase (Holder-Snyman, 2005). The actual operation of AS systems is regulated by three factors: (1) the concentration of DO in the system, (2) the rate of recirculation of the AS and (3) the amount of AS wasted from the system. Sludge recirculating and wasting are important since they allow the bacterial population to be maintained in the required growth phase (Spellman, 2008; Marrengane, 2007).

2.4.2. FILAMENTOUS BACTERIA

Some microorganisms in AS grow in long, filamentous forms. Although some of these are algae and fungi, most are bacteria. Classification of filamentous bacteria in AS systems has been a major limitation with regards to accurate taxonomic groupings of the organisms. Most filamentous bacteria are still referred to as morphological types based exclusively on their microscopic features persisting from the study of Eikelboom (e.g. Type 021N) (Beer et al., 2002). Many filamentous bacteria are recalcitrant to cultivation, and hence were originally described on the basis of observable microscopic characteristics (Wagnor and Loy, 2002; Abusam, Al-Salmain and Mydlarczyk, 2020). The first identification keys were developed by Eikelboom whereby he characterized twenty-nine types of filamentous bacteria into several groups (Table 8). Morphotypes are typically identified by dichotomous keys, which have limitations. Some filamentous bacteria have similar morphologies but differ considerably in their physiologies and taxonomies (Marrengane, 2007; Ferreira, 2022). The presence of certain filamentous bacteria is important for proper floc formation and degradation of both complex and soluble carbonaceous BOD (cBOD). However, overgrowth of certain filamentous bacteria can be detrimental as it can cause problems such as filamentous bulking in the clarifier and foaming (Wagnor and Loy, 2002; Gerardi, 2006; Abusam, Al-Salmain and Mydlarczyk, 2020). Filamentous overgrowth can be associated with specific operational parameters (Table 9) (Gerardi, 2006; Abusam, Al-Salmain and Mydlarczyk, 2020).

Table 8: Eikelboom filamentous morphological types (adapted from Marrengane, 2007)

Morphological Description	Filamentous organisms
Sheath- forming, Gram negative filaments	*Sphaerotilus natans*, Type 1701, 1702, 0321.
Sheath- forming, Gram positive filaments	Type 0041, 0675, 1851.
Sheathless, curled, multicelled filaments	*Thiothrix* sp., *Nostocoida limicola*, Cyanobacteria, Type 021 N.
Slender coiled filaments	*M. parvicella*, Types 0581, 0912
Straight, short, multicelled filaments	Types 0803, 1091, 0092, 0961.
Gliding, motile filaments	*Beggiatoa* sp., Types 0941, 1111, 1501
Others	GALO*, Types 1863, 0411

* *Also previously referred to as Actinomyces, Nocardioforms, Nocardia amorae–like organisms (NALO) and more recently classified as GALO (Gordonia amarae-like organisms)*

Table 9: Operational conditions associated with undesired growth of filamentous bacteria (adapted from Gerardi, 2006)	
Operational Condition	Filamentous Bacteria
High MCRT (> 10 days)	Type 0041, 0092, 0581,0675,0803, 0961, 1851 and *M. parvicella*
Fats, oil and grease	Type 0092, *M. parvicella* and GALO
High F/M or slug discharge of soluble cBOD	Type 1863
High pH (>8,0)	*M. parvicella*
Low DO and high MCRT	*M. parvicella*
Low DO and low to moderate MCRT	*Haliscomenobacter hydrossis, S. natans* and Type 1701
Low F/M	*M. parvicella, Haliscomenobacter hydrossis,* GALO, Type 0041, 0092, 0581, 0675, 0803, 0961 and 021N
Low N or P	*H. hydrossis,* GALO, *S. natans*, *Thiothrix*, Type 0041, 0092, 0675, 1701 and 021N
Low pH (<6.5)	GALO
Organic acids	*Beggiatoa, Thiothrix* and Type 021N
Readily degradable substrates e.g. alcohols	*H. hydrossis , N. limicola, S. natans*, *Thiothrix,* Type 1851 and 021N
Septicity	*Beggiatoa, N. limicola, Thiothrix,* Type 0041 and 021N
Warm wastewater temperature	*S. natans*
Winter proliferation	*M. parvicella*

MCRT – mean cell residence time; F/M Food to microorganism; N – nitrogen; P - phosphorus

Domestic waste is normally rich in N and P whiles industrial waste can be N and P limited. These nutrients are important components of biomass growth. In AS systems with low F/M ratios, filaments including *H. hydrossis,* GALO, *Thiothrix* spp., and Eikelboom Types 0041, 0675, 0803, 0914 and 1851 tend to dominate over the floc formers (Marrengane, 2007).

The most common filamentous bacteria include *M. parvicella*, Type 0092, Type 0041, GALO, *S. natans* (Gerardi, 2006). The EPS produced by *Zoogloea ramigera* and other bacteria play an important role in the formation of flocs. Flocculation generally results into sedimentation which takes place during secondary settling, or clarification (Bitton, 2011). Most of the AS solids separation problems can be related to the nature of the AS flocs. Types of AS problems are listed in Table 10. Characterization of the AS flocs (shape, structure, strength and size) can assist with diagnosing settling problems. A wide range of particle sizes are found in AS - from single bacteria with dimensions of approximately 0.5 – 5 µm to large aggregates of > 1000 µm (Jenkins et al., 2003; Eikelboom, 2000; Ferreira, 2022). The shape of the flocs varies from irregular to round. The settling of the sludge is reduced when flocs are irregularly shaped and/or open. Open flocs are those in which water can flow throw whilst compact flocs are

those in which the bacteria are tightly adjoined. Flocs that are compact have the ability to settle quickly (Eikelboom, 2000).

Table 10: Activated sludge separation problems (adapted from Jenkins et al. 2003; Marrengane, 2007)

Name of the Problem	Description of the problem
Dispersed growth	Microorganisms do not form flocs as they are dispersed in the bulk solution resulting in formation of small flocs and/or single cells. This results in poor separation in the secondary clarifier subsequently leading to a turbid effluent.
Poor floc structure microstructure	The core of the floc that is normally created by the bacterial biomass is absent and results in the formation of loose, open flocs.
Unsettleable microflocs	These flocs develop from the disintegration of firm and sound flocs due to either low production of glycocalyx or consumption of the glycocalyx by bacteria or disintegration due to mechanical shearing. The disintegration of flocs leads to a turbid effluent.
Viscous bulking	Exopolysaccarides are essential components of the flocs produced by some bacteria (e.g *Zoogloeae*). Under stressful conditions, EPS is sometimes overly produced resulting in the AS becoming highly water retentive due to the hydrophilic character of the EPS. This results in poor sludge compaction, poor settling and foaming.
Foaming	Foaming can be visualized as a thick surface scum that causes solid separations problems. In can be caused by non-degradable surfactants and/or by filamentous bacteria. Non-filamentous biological foaming is caused by the production of lipids, lipopeptides, proteins and/or carbohydrates which act as biosurfactants.
Filamentous bulking	Overgrowth by filamentous bacteria occurs in BNR AS plants when conditions favour growth of filamentous over floc-forming bacteria. Incomplete denitrification favours filamentous bacteria. The proliferation of filamentous bacteria results in poor sludge settling. The measure of the sludge quality is determined by the sludge settleability which is expressed by SVI.

2.5. THE CONVENTIONAL ACTIVATED SLUDGE PROCESS

In 1914, Ardern and Lockett developed an activated sludge process (ASP) which later become an important system in the treatment of municipal and industrial wastewater (Rathore, Parde and Killedar, 2022). Activated sludge systems are suspended growth systems composed of microorganisms which are constantly supplied with nutrients (organic matter) and O_2 via mechanical aeration (Marrengane, 2007). Later, anoxic and/or anaerobic zones were incorporated in some systems for biological removal of N and/or P. Biological treatment methods, which usually involve the ASP, are the most widely applied technologies in WWTPs worldwide (Xia et al., 2018).

The overall process of wastewater treatment involves a number of stages that serve different roles. These stages include the primary, secondary and tertiary stages. The primary stage physically removes insoluble particles by gravity settling, screening, and/or addition of alum and other coagulating agents. About 20 to 30% of the BOD is removed (Willey et al., 2008; Tamás; 2020). The secondary stage promotes the biological transformations of dissolved organic matter to microbial biomass. About 90 to 95% of the BOD and many pathogens are removed. In addition to the ASP, examples include trickling filters, lagoons and anaerobic digesters (Willey et al., 2008; Naido, 2005). The tertiary stage provides a final treatment of the wastewater by removing inorganics, viruses and trace chemicals by using coagulation sedimentation and/or disinfection (Willey et al., 2008).

The conventional ASP (Figure 5) consists of four key phases – (i) aeration (the biomass is kept in suspension by continuous agitation in an aeration tank in order to remove soluble and particulates organic matter), (ii) clarification (the biomass is allowed to quiescently settle out in a clarifier in order to recover the biomass), (iii) collection of the return activated sludge (RAS) to supply the aeration tank with functional biomass, and (iv) the removal of excess sludge (waste) or waste activated sludge (WAS) in order to retain the correct amount of solids for a desired amount of time (Linden et al., 2001; Naido, 2005).

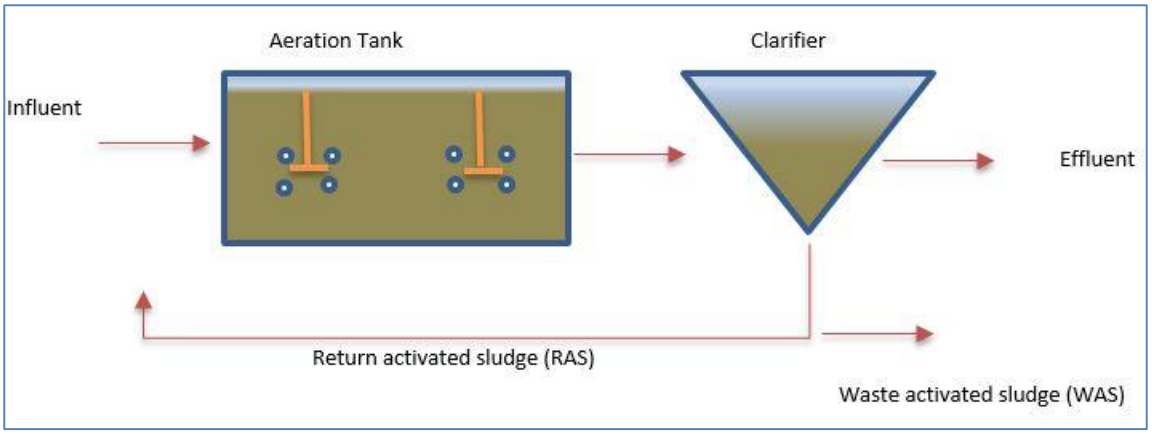

Figure 5: Schematic diagram of the configuration of a typical conventional activated sludge process (adapted from Grady et al., 1999)

The principle of the ASP is based on the ability of microorganisms to use organic waste as their source of carbon (C) for energy thereby playing an important role in the biodegradation of organic matter, transforming toxicants into harmless products and removing NO_3^-, SO_4^{2-} and PO_4^{3-} (Meerbergen et al., 2016). The ASP flocs are physically separated from the treated wastewater in a secondary clarifier (Naido, 2005; Sanin et al., 2006). The physical characteristics (size and morphology) of the floc particles influence their settleability in the secondary clarifier as described in Sections 2.3.1 and 2.3.2. Desirable floc characteristics are therefore crucial for the successful operation of the ASP (Burger et al., 2017; Jenkins and Wanner 2014).

2.5.1. WASTEWATER TREATMENT PLANT CONFIGURATIONS

The development of distinct reactor zones are important for effectively removing nutrients (Linden et al., 2001; Lilley et al, 1997; Ogunlaja, 2015). Wastewater treatment plants can incorporate aerobic zones (DO present), and/or anaerobic zones (absence of DO but not of NO_3 or SO_4^{2-}) to provide alternative biochemical environments for different microbial metabolic functions. The terminal electron acceptor utilized distinguishes between the zones i.e. O_2 is the electron acceptor in aerobic zones, NO_3^- or NO_2^- are usually the acceptors in anoxic zones, and neither O_2, NO_3^- or NO_2^- are present in anaerobic zones. Table 11 is a summary of the biochemical transformations occurring in various zones. Wastewater technologies incorporating different zones in different orders (configurations) have evolved over the years. Wastewater technologies such as the Wuhrmann process and the Modified Ludzack-Ettinger (MLE) process were established for N removal, while the Phoredox process, 3-stage Phoredox process, University of Cape Town (UCT) process and modified UCT process were established

for P and N removal, and configurations such as the 5-stage Bardenpho process were established for enhanced N and P removal (Ismail, 2008; Ogunlaja, 2015). Table 12 summarizes the primary benefits and drawbacks of several BNR systems.

Table 11: Summary of biological nutrient removal zones (adapted from Linden et al., 2001)

Zone	Biochemical transformation	Functions
Anaerobic	Utilization of stored polyphosphates by PAO Fermentation of organic matter	- Selection of PAOs - COD removal
Anoxic	Dentrification – conversion of NO_2^- and NO_3^- to N_2 Oxidation of organic matter Alkalinity generation	- Selection of denitrifying bacteria and N removal - COD removal
Aerobic	Nitrification- conversion of NH_4^+ to NO_2^- and NO_3^- P uptake and formation of polyphosphates by PAO Oxidation of organic matter Alkalinity consumption	- NH_4^+ removal - P removal - COD removal

PAO – phosphate accumulating organisms

Table 12: Benefits and drawbacks of some wastewater treatment plant process configurations (adapted from Grady et al., 1999; Ogunlaja, 2015)

Plant Configuration	Benefits	Drawbacks
Wuhrmann	- Moderate N removal - Moderate reactor volume - Low operational cost	- Limitation of C source at the anoxic zone - N_2 gas affects cause sludge to float in the clarifier - Poor alkalinity recovery
MLE	- Good N removal - Moderate reactor volume - Alkalinity recovery - Good solids settleability - Low operational cost	- Enhanced N removal not possible - Overall NO_3^- removal is limited by the recycle rate
4 Stage Bardenpho	- Excellent N removal - Alkalinity recovery - Good solids settleability - Reduced aeration requirement	- Large reactor volume
Modified 5 stage Bardenpho process	- Excellent N removal - Alkalinity recovery - Good solids settleability - Reduced aeration requirement	- Large reactor volume - Moderate to poor P removal
3 Stage Phoredox	- Good N removal - Moderate reactor volume - Alkalinity recovery - Good solids settleability - Reduced aeration requirement	- Moderate to poor P removal
University of Cape Town	- Good N & P removal - Moderate reactor volume - Alkalinity recovery - Good solids settleability - Reduced aeration requirements	- Enhanced N removal not possible - Reactor instability due to influent flowing directly into anaerobic zone

2.5.1.1. WUHRMANN CONFIGURATION

The Wuhrmann process configuration (Figure 6) is comprised of two zones - aerobic and anoxic. The influent is discharged directly to the aerobic zone where COD removal and nitrification takes place. The flow then moves to the anoxic zone where denitrification takes place. In the clarifier, the sludge settles and the RAS is returned to the aerobic zone, while excess sludge is wasted (WAS). Denitrification is limited by the amount of organic C (which is depleted in the aerobic zone). It also requires high alkalinity to maintain a steady pH in the aeration tank. Rising N_2 bubbles in the clarifier can negatively affect sludge settling (Ismail, 2008; Curtin, 2011).

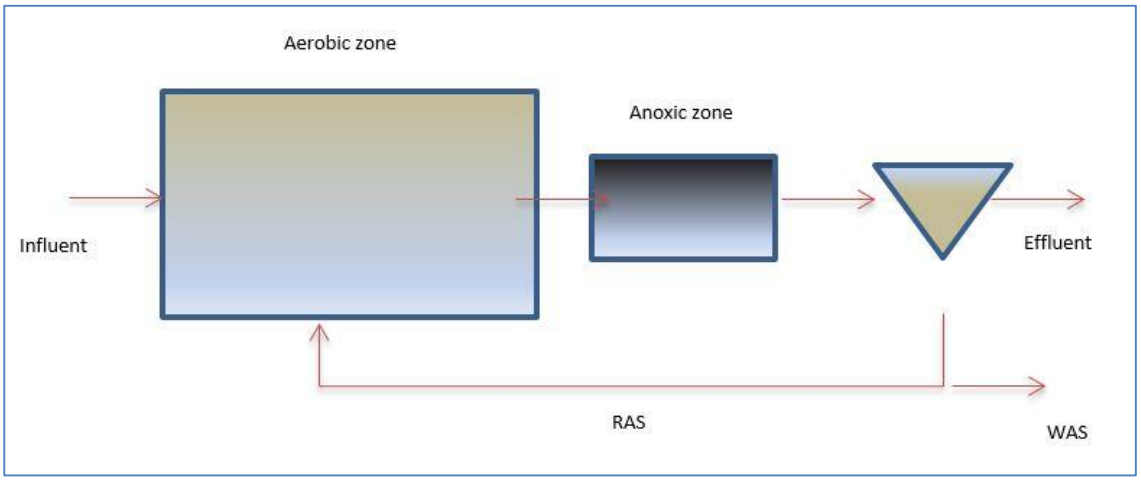

Figure 6: Schematic diagram of the Wuhrmann process configuration (modified from Linden et al., 2001)

2.5.1.2. MODIFIED LUDZACK-ETTINGER (MLE) CONFIGURATION

The Modified Ludzack-Ettinger (MLE) process configuration (Figure 7) is also comprised of two zones, but in contrast to the Wuhrmann process, the influent is directly discharged into the anoxic zone then flows to the aerobic zone. The RAS provides NO_3 formed by nitrification in the aerobic zone to the anoxic zone, where denitrification to takes place, releasing the N_2 to the atmosphere. In addition to nitrification, biodegradable organics are utilized and removed in the aerobic zone. The system is limited by the amount of NO_3^- returned through RAS. Complete denitrification is not possible and NO_3^- is discharged with the effluent (Shah, 2018; Lilley et al., 1997; Kumalo, 2022).

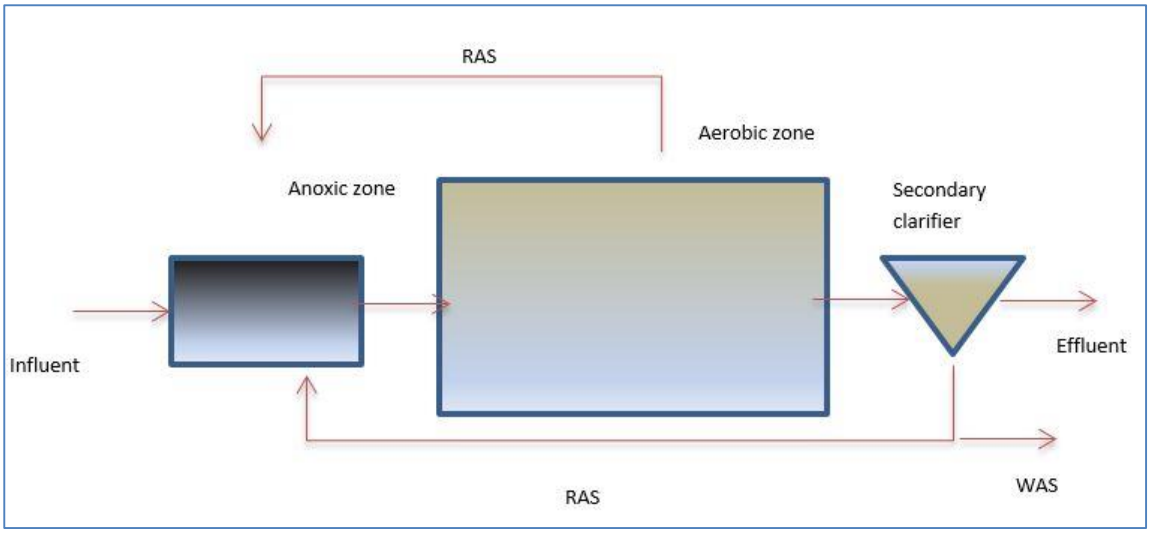

Figure 7: Schematic diagram of Modified Ludzack-Ettinger (MLE) process configuration (adapted from Linden et al., 2001)

2.5.1.3. FOUR STAGE BARDENPHO PROCESS CONFIGURATION

The Four Stage Bardenpho (4SB) process configuration (Figure 8) is comprised of four bioreactors aligned in series. The first and third reactors are anoxic zones, and the second and fourth are aerobic zones. The second anoxic zone is used to completely remove the NO_3^- remaining from the effluent of the first aerobic zone. The smaller second aerobic zone is used to allow the N_2 to volatilize and to oxygenate the mixed liquor before entering the clarifier. The RAS from the clarifier and the recycle from the second aerobic zone are all introduced at the first anoxic zone (Linden et al., 2001).

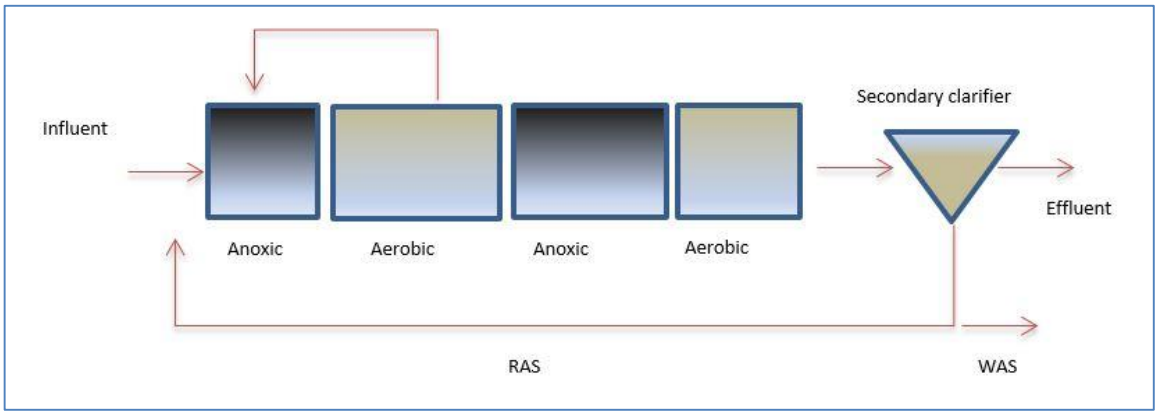

Figure 8: Schematic diagram of the 4 Stage Bardenpho process configuration (adapted from Linden et al., 2001)

2.5.1.4. MODIFIED BARDENPHO CONFIGURATION

The modified Bardenpho process configuration (Figure 9) provides conditions for enhanced N and P removal. The system consists of five reactors - anaerobic, anoxic, aerobic, anoxic and aerobic. Functionally, the reactors promote the growth of microorganisms that are responsible for nitrification and denitrification under aerobic and anoxic conditions, respectively. During the anaerobic stage, PAOs release σ-P is into the bulk solution and accumulate them during the anoxic/aerobic stages (Xue at el., 2019). Operational factors such as NO_3^- and O_2 present in the anaerobic stage and also a variation in wastewater composition can have detrimental effects on the process (Tykesson at el., 2004; Kumalo, 2022).

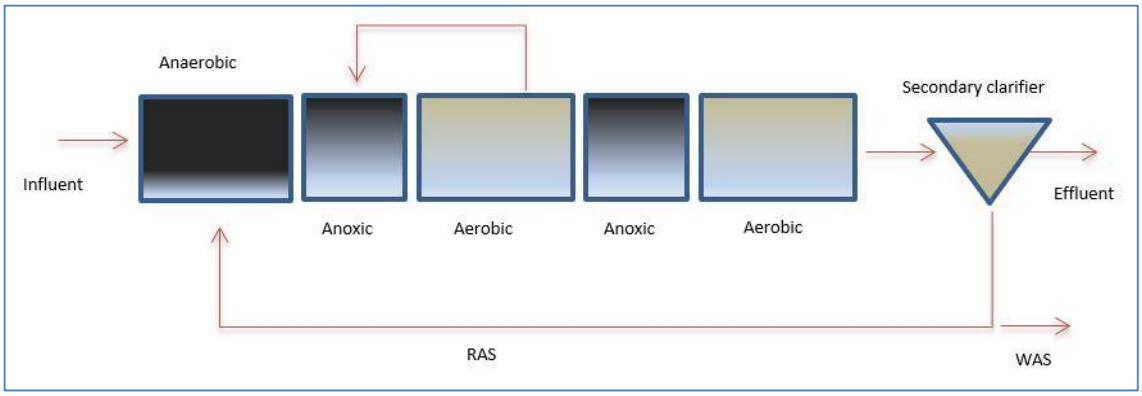

Figure 9: Schematic diagram of the 5 Stage Modified Bardenpho process configuration (adapted from Linden et al., 2001)

2.5.1.5. THREE STAGE BARDENPHO PROCESS CONFIGURATION

The three stage Bardenpho (3SB) process configuration (Figure 10) is a combination of MLE for N removal and anaerobic/aerobic (oxic) (A/O) treatment for P removal. The sludge retention time (SRT) in the anaerobic zone is similar to that in the A/O and the SRT in the anoxic zone is similar to that in MLE, the HRTs are also correspondingly similar, since the MLSS are a similar range. The recycle of mixed liquor from the aerobic zone to the anoxic zone enables substantial removal of N and the N removal rates are similar to those found in the MLE WWTPs. However, the P removal is lower than in the A/O process because NO_3^-/NO_2^- is returned to the anaerobic zone from RAS. In the up-front anaerobic zone, denitrifying organisms can therefore compete with the PAO for VFAs, retarding the proliferation of PAO (Grady et al., 1999; Linden et al., 2001).

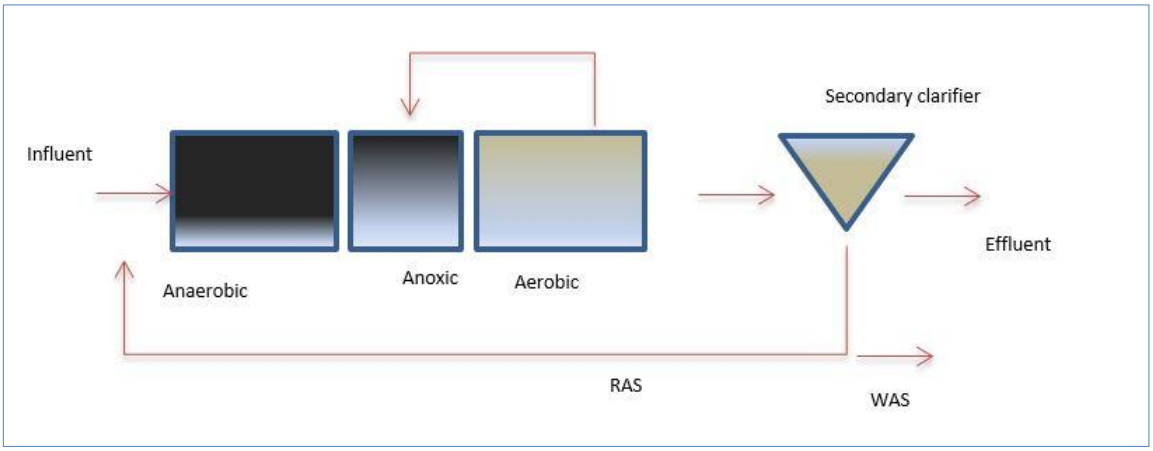

Figure 10: Schematic diagram of the 3 stage Bardenpho process configuration (adapted from Grady et al., 1999; Linden et al., 2001)

2.5.1.6. UNIVERSITY OF CAPE TOWN PROCESS CONFIGURATION

The University of Cape Town (UCT) process configuration (Figure 11) is a modification of the 3SB process that eliminates the return of RAS to the anaerobic zone. The process consists of anaerobic, anoxic and aerobic zones with the RAS recycled back to the anoxic zone. The nitrified mixed liquor from the aerobic zone is recycled back to the anoxic zone to be denitrified. As much of the COD is depleted in the anaerobic and aerobic zones, the electrons for denitrification are provided by endogenous decay (decaying microorganisms) from the aerobic zone and the RAS. The denitrified mixed liquor from the anoxic zone is in turn recycled back to the anaerobic zone (Linden et al., 2001; Kumalo, 2022). However, this still contains NO_3^-/NO_2^- and therefore has a similar detrimental effect on P removal as with 3SB WWTPs (Grady et al., 1999 and Linden et al., 2001). The modified UCT process configuration was designed to increase P removal. It is similar to the UCT process configuration, however, the anoxic zone is divided into two zones. The first zone receives the RAS and supplies the anaerobic zone, and the second zones receives the recycled mix liquor from the aerobic zone (Grady et al., 1999)

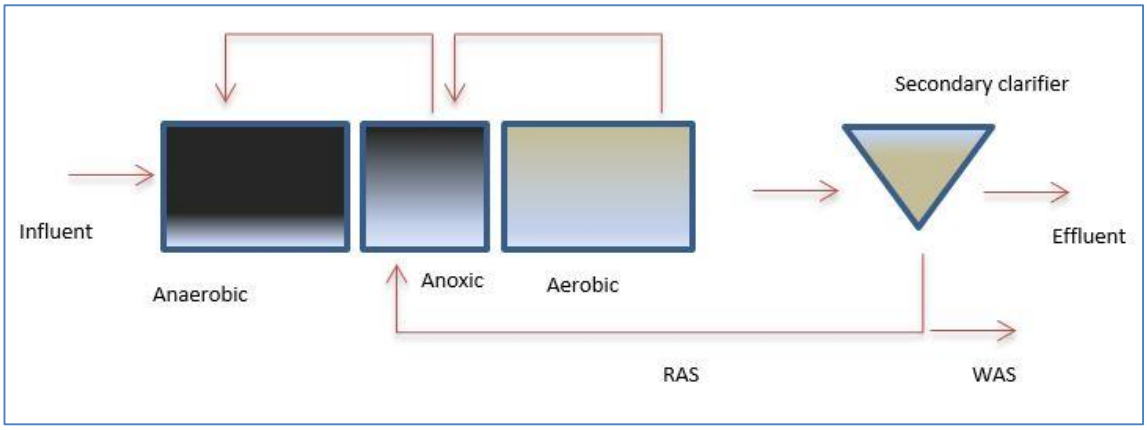

Figure 11: Schematic diagram of the University of Cape Town process configuration (adapted from Grady et al., 1999)

2.6. FACTORS AFFECTING THE PERFORMANCE OF THE ACTIVATED SLUDGE PROCESS

Non-steady state conditions in biological wastewater treatment are common, and are influenced by changes in the environmental conditions to which the biomass is exposed e.g. physicochemical factors (influent concentrations of substrates and nutrients, DO, temperature and pH) and biotic factors (predator–prey and symbiotic relationships) (Gerardi, 2006). The plant configuration and operational parameters such as HRT, sludge recycle and wasting rates, type of aeration, and OLR also play important roles in the prevalence of specific microbial populations (Van Loosrecht et al., 2016). Environmental changes are associated with system instability. The instability due to transients is becoming of greater concern as treatment systems are pushed to work at their treatment limits to meet more stringent regulations (Morgan-Sagastume and Allen, 2003).

2.6.1. PHYSICOCHEMICAL PARAMETERS

2.6.1.1. CONCENTRATION OF SUBSTRATE

Some of the bacteria proliferate under low nutrient condition in AS systems. Filamentous bacteria such as Type 1701 and Type 021N can grow in such conditions. The filamentous structure provides competitive advantages to obtain nutrients under limited nutrient supply. This is because filamentous organisms have more surface area exposed to the bulk solution than floc forming bacteria (Gerardi, 2006). High organic loading can cause bacterial growth to be dispersed which may subsequently lead to poor separation in the secondary settling tank (Merrangane, 2007).

2.6.1.2. pH

Bacteria have an optimum pH at which they grow best. For most bacteria the optimum pH usually is near neutral (pH 7), and most bacteria do not grow at values ±1 unit of their optimum pH and cannot tolerate pH values below 4 or above 9.5 (Gerardi, 2006). At non-optimal pH, the growth rate of biological systems is reduced because the catalytic activities and the function of membrane transport proteins are inhibited (Willey et al., 2008; Ghanizadeh and Sarrafpour, 2001). Nitrification is also affected by pH. The optimal pH for nitrification ranges from 7.5 to 8.5 and pH levels lower than 6.45 or higher than 8.95 may completely inhibit nitrification (Kumalo, 2022). An increase in pH of AS from 5.7 to 9 improves COD and suspended solids (SS) removal, and decreases the SVI (Ghanizadeh and Sarrafpour, 2001). Operation problems that may occur in AS systems when pH is below 6.8 or above 7.2 include decreased enzymatic activity, inhibition of nitrification, and interruption of floc formation (Gerardi, 2006).

2.6.1.3. TEMPERATURE

Temperature effects on the growth and functional role of microorganisms can be related to the temperature sensitivity of the enzymes. Enzymes have temperatures at which they function optimally and at some temperature below or above the optimum, they cease to be catalytic (Willey et al., 2008). Most microorganisms exist over a broad temperature range, however, there is usually an optimum temperature at which each species grows best (Manassra, 2006). Temperature transients in biological wastewater treatment can result from seasonal variations of which winter presents the most challenges due to reduced microbial activity. Temperature shifts have been related to decreased treatment performance and system instability e.g. lower activity, poor settling, high effluent suspended solids (ESS) (Morgan-Sagastume and Allen, 2003). Morgan-Sagastume and Allen (2003) increased temperatures in AS from 15°C to the upper mesophilic range of 35°C, which resulted in a reduction in soluble COD and ESS removal and caused deterioration of the sludge settling characteristics. Temperature transient also affects the structure of polymers and bacterial cell walls. This in turn causes a variation in the EPS and surface charge of bacteria, and at high temperature, the viscosity of EPS is decreased which results in reduction of biofloculation and settling (Ghanizadeh and Sarrafpour, 2001). Temperature also affects the growth rate of nitrifying bacteria. At low temperatures, the growth rate of nitrifying bacteria is slow which then causes the process to take longer to nitrify incoming NH_3/NH_4^+ (Wennerholm, 2014;

Kumalo, 2022). The reduction in performance of EBPR during summer periods or high temperature have been reported. The proliferation of glycogen accumulating organisms (GAO) at the expense of PAO is thought to be the underlying cause of reduced EBPR because at 20˚C PAO dominate, while at 30˚C GAO dominate (Shi, 2011).

2.6.1.4. DISSOLVED OXYGEN

Bacteria can grow in the presence or in the absence of O_2 and are placed in three groups based on O_2 requirements (Section 2.2.1) (Willey et al., 2008; Gerardi, 2006). The DO can be supplied by either mechanical means where large stirrers are used, or through bubble aeration. This ensures that the required concentration of DO is present (Holder-Snyman, 2005).

The DO concentration plays an important role in the performance of AS systems and its limitation frequently results in poor sludge performance in terms of settleability and NH_4^+ removal (Kumalo, 2022). The DO concentration within in the AS flocs is lower than that in the bulk solution around the flocs due to O_2 usage as it percolates through the flocs. However, some of the bacteria such as *S. natans* and *H. hydrossis* proliferate under low DO concentrations because they can out-compete most floc-forming bacteria for the limited quantity of O_2, which may result in filamentous bulking (Gerardi, 2006). Concentrations ≤0.2 mg/L are not sufficient for the growth of nitrifiers. For rapid and complete nitrification, the DO levels must be above 2 mg/L (Qasim and Zhu, 2017).

2.6.1.5. INHIBITORS OF GROWTH

Heavy metals have been found to be toxic to aerobic bacteria. Metals such as copper (Cu) and mercury (Hg) are considered to be the most toxic as they form complexes with the bacterial enzymes and other growth agents, resulting in microbial inactivity. High concentrations of salts and N compounds can also severely inhibit bacterial growth. Metal salt concentrations around 3000 mg/L or more results in increased osmotic pressure within the cell which may eventually cause the cell to burst (Holder-Snyman, 2005).

2.6.2. OPERATIONAL PARAMETERS

2.6.2.1. HYDRAULIC RETENTION TIME (HRT) AND SLUDGE RETENTION TIME (SRT)

The wasting and recirculation of sludge from the treatment process makes it possible to discriminate between hydraulic and solids retention times (HRT and SRT). Long SRT has been considered beneficial as it allows retention of slow-growing microorganisms such as nitrifies and results in lower sludge production because of endogenous decay. Long SRT can also improve the removal of emerging contaminants such as pharmaceuticals and endocrine disruptors, increase the O_2 transfer efficiency, and reduce the number of particles in the effluent. During operation at long SRT, most of the organic compounds entering the process with the wastewater will be oxidized to CO_2 and H_2O (Modin et. al.,2016; Kumalo, 2022).

The HRT of an aeration tank is the amount of time in hours it takes for wastewater to pass through (Gerardi, 2002). The HRT is an important control parameter in many WWTPs as it influences the hydraulic conditions and the contact time of different reactants within the reactor (Pan et al, 2004). Increasing HRT is correlated with a reduction in organics (BOD/COD), N and P content due to longer exposure of functional microbial communities to these nutrients (El Naker et al., 2018; Gerardi, 2002). Decreasing HRT adversely affects nitrification and the solubilization of particulate and colloidal organics, which results in the discharge of these in the final effluent (Gerardi, 2002).

2.6.2.2. F:M RATIO

The combination of the functional microbial communities and the wastewater is known as the mixed liquor (ML). The nutrient concentrations (food) in the ML decreases on a continuum from the influent to the effluent. As the nutrient level decreases, microbial growth becomes limited and floc formation is promoted (Section 2.4.1) (Ahansazan et al., 2014). In the secondary clarifier, the sludge settles at the bottom while the clarified effluent is discharged to the environment before or after tertiary treatment. Some of the settled sludge is returned to the system (RAS), while some is wasted (WAS) (Willey et al., 2008).

For the system to function efficiently, the biomass needs a steady balance of nutrients (food). If the biomass (microorganism) concentration is too high, the nutrient supply will be limited. Conversely, if the system has inadequate biomass, some of the nutrients will not be utilised,

and will be present in the final effluent. The balance between the nutrients and biomass is explained as the food-to-microorganism (F:M) ratio. (Ahansazan et al., 2014) (Equation 7). Since the operator usually has no control over the influent BOD concentration, the F:M is adjusted by manipulating the RAS and WAS rates to adjust the MLVSS. Equation 13 expresses the calculation of the F/M ratio (Welz et al., 2014).

FM = Q x [COD]/V x [MLVSS] (Equation 13)

Where:

Q = Flow rate into the reactor

V= bioreactor volume

Systems with high F:M ratios typically have low sludge ages (Section 2.4.1) and vice versa (Ahansazan et al., 2014). For nitrification to occur, the sludge must be old enough to establish sufficient growth of nitrifying bacteria (Section 2.3.2).

2.6.3. MICROBIAL INTERACTIONS IN ACTIVATED SLUDGE

2.6.3.1. COMPETITION AND PREDATOR AND PREY RELATIONSHIPS

There is always strong completion for nutrients between various microorganisms in WWTPs (Eikelboom, 2000). Competition in AS is described as competitive exclusion whereby bacteria competing for a common resource will lead to those with a growth advantage to dominate or co-exist. For example, some filaments can out-compete other bacteria when the concentration of N and P is low and *M. parvicella* is selected when wastewater has a high fatty acid concentration (Saikaly and Oerther, 2004; Merrangane, 2007). Predator and prey relationship exist in AS. The bacterial consortia are generally prey to some of the protozoa and metazoa species. This subsequently leads to the removal of free bacterial cells that cannot then be separated from the treated wastewater through settling (Burian et al., 2022). Protozoa and metazoa can also be used as indicator organisms. The presence of certain protozoa in the wastewater system can suggest the presence of specific condition (e.g. flagellates and amoeba indicate low DO conditions) (Eikelboom, 2000).

2.7. ACTIVATED SLUDGE SETTLING PROBLEMS: CAUSES AND MEASUREMENT

Mass occurrence of certain bacterial species can be detrimental for wastewater treatment by negatively influencing the settling properties of AS in the secondary clarifiers (bulking), by contributing to the formation of foam, or by outcompeting microorganisms required for nutrient removal (Wagner and Loy, 2002; Deepnarain et al., 2019). The sub-optimal performance of the ASP has been associated with the competition between beneficial and detrimental bacteria, such as PAOs and GAO, as well as floc-forming bacteria versus filamentous bacteria in WWTPs (Guo et al., 2017). Exocellular polymeric substances are produced by most floc forming bacteria and zoogloea bacteria produce large amounts that are important for the formation of firm flocs. Overproduction of EPS can cause the AS to be highly water retentive. Such glutinous sludge causes low settling and poor compaction velocity and mechanical problems as it foams when aerated (Marrengane, 2007). Filamentous bacteria play an important role in wastewater treatment as they directly affect the sludge settling. They provide a rigid support upon which floc forming bacteria can adhere and grow into activated sludge flocs. However, when filamentous bacteria are in excess quantity, they may be considered detrimental as they can cause sludge bulking. Sludge bulking is the sludge that settles and compacts slowly due to proliferation of filamentous bacteria and inter-floc bridging (Ramothokang et al., 2004; Deepnarain et al., 2019). Filamentous bacteria also affect the SVI. Among other parameters, the SVI is influenced by the floc structure and filament abundance in AS systems, and SVI > 150 mg/L may point to a bulking problem (Amaral et al., 2013).

The sludge volume index (SVI) is used to characterize the sludge settleability. The SVI is calculated using Equation 14. The volume occupied by the settled sludge component in a 1 L cylinder is measured after 30 min (Forster, 2003). After a set time the sludge volume is read off and the initial sludge concentration is established from which the volume of the settled sludge per gram of solids is calculated. The SVI is widely used to evaluate sludge settling. However, the calculation a major shortcoming in that the value is dependent on the initial sludge concentration and a true parameter for sludge settleability should be independent of the sludge concentration. To avoid this, the diluted SVI (DSVI) test was established. This test is based on the observation that when the sludge volume is less than 25% percent after settling, the calculated SVI is constant and does not depend on the initial sludge concentration. Testing for DSVI requires diluting sludge until the volume of the diluted

suspension is ≤ 200 ml/L initial volume after settling (Forster, 2003; Van Haandel and Van der Lubbe, 2007). Diluted sludge volume index is expressed by Equation 15.

$$\text{SVI}\left(\frac{\text{mL}}{\text{gram}}\right) = \text{Settled sludge}\frac{\text{volume(mL)}}{\text{MLSS(g)}} \quad \text{(Equation 14)}$$

$$\text{DSVI}\left(\frac{\text{mL}}{\text{g}}\right) = \text{diluted settled}\frac{\text{volume(ml)}}{\text{MLSS(g)}} * \text{Final}\frac{\text{Volume(mL)}}{\text{initial}}\text{Volume(mL)} \quad \text{(Equation 15)}$$

2.8. METHODS FOR CHARACTERISATION OF BACTERIAL COMMUNITIES IN WASTEWATER TREATMENT SYSTEMS

Traditionally, bacterial communities in WWTPs were analysed by light microscopy and cultivation-dependent techniques. However, these methods are inadequate for the description of the composition and dynamics of the bacterial communities actually present because many of the bacteria are difficult or impossible to culture or discriminate from one another using microscopy (Cydzik-Kwiatkowska and Zielin´ska, 2016; Wagner and Loy, 2002; Kumalo, 2022). In recent years, a variety of molecular approaches were developed and used to study bacterial diversity in WWTPs in a cultivation-independent way (Wagner and Loy, 2002; Ferreira, 2022). These molecular techniques are not limited by the problems inherent in the cultivation-dependent techniques (Marrengane, 2007). Molecular biological techniques commonly used in wastewater treatment include FISH and PCR based methods: PCR in conjunction with cloning of PCR amplicons, denaturing gel electrophoresis (DGGE), single strand conformation polymorphism (SSCP) and T-RFLP, and quantitative PCR (Lens et al., 2004; Huang et al., 2018).

2.8.1. POLYMERASE CHAIN REACTION

The PCR reaction is often the starting point for a longer series of experiments in which the amplification product is studied in various ways in order to gain information about the DNA molecule that acted as the original template (Brown, 2010). The PCR reaction allows the specific and exponential synthesis of a DNA region with the use of two primers which forms the two termini of the nucleic acid molecule to be amplified (Van Pelt-Verkuli et al., 2008). To carry out a PCR reaction, the target DNA is mixed with *Taq* polymerase, the two oligonucleotide primers, and a supply of nucleotides. The reaction starts by heating the mixture to 92°C - 94°C so that the hydrogen bonds between the nucleotides break (denature). The temperature is reduced to 50°C - 60°C to allow primers to anneal with the single strand DNA. The temperature is then raised to 72°C - 74°C to allow *Tag* polymerase to mediate the synthesis of new DNA from the template. The cycle of denaturing, annealing and synthesis is repeated (Lodge et al., 2007; Brown, 2010). Specific PCR primers have been employed to confirm the presence or absence of targeted microorganisms and have proved useful in detecting slow growing bacteria such *Mycobaterium tuberculosis* and *Helicobacter pylori*. The shortcoming of employing specific primers is that they cannot predict the presence or absence of non-targeted bacterial species in the tested sample. Detection and identification of non-

targeted bacteria will require the application of universal primers (Barghouthi, 2011). The 16S rRNA genes are highly conserved regions in the bacterial genome and can be selectively amplified and used to identify bacteria (Marrengane, 2007).

The composite characteristics of the bacteria are unlikely to have changed and thus they are good indicators of relationships. However, relationships are now well established by the sequencing of proteins and nucleic acids because it has been found that genotypic information provides more readily, reliably and precisely interpreted data and it is more informative of evolutionary relationships (Holder-Snayman, 2005). The ribosomal RNA (rRNA) gene, particularly the 16S rRNA gene of Bacteria and Archaea, has been widely utilized in the field of environmental microbiology because of its presence in all organisms and low mutation rates throughout Bacteria and Archaea evolution (Zhen et al., 2018). The prevalence of the rRNA enables oligonucleotide (probes) to be designed in order to identify virtually any organisms or group of related organisms (Holder-Snayman, 2005). The average bacterial 16S rRNA gene is a 1500 base pair coding for a region of RNA that is part of the 30S ribosomal subunit and has desirable properties that allowed it to become the most commonly used marker. It is present in all prokaryotic cells and has conserved and variable sequence regions, critical for both universal amplification and measurement of close as well as distant phylogenetic relationships (Srinivasan et al., 2015). The 16S rRNA sequences are similar among all known organisms, low stringency hybridization permits the identification of rRNA genes from unknown organisms (Holder-Snyman, 2005). Phylogenetic identification is achieved by the use of oligonucleotides that are complementary to phylogenetic group-specific 16S rRNA gene sequences.

2.8.2. TERMINAL RESTRICTION FRAGMENT LENGTH POLYMORPHISM

Terminal restriction fragment length polymorphism is a rapid, high throughput and cost-effective DNA fingerprinting method that is typically based on the 16S rRNA gene (Varma and Oelmüller, 2007; Hester and Harrison, 2008). The amplicons from the PCR amplification using fluorescent end-labelled primers are digested using restriction endonucleases with high specificity. Fragments are separated by electrophoresis with visualization of only terminal fragments as they contain the fluorescent label. The amplified fragments of the DNA originate from different organisms and consequently have sequence variations, ensuring that terminal restriction sites for different species in a community are unique (Hester and Harrison, 2008). One of the advantages with this technology is that it gives the relative amounts of bacteria of

a sample with good sensitivity by using fluorochome (Urrea-Valencia et al., 2021). The limitation of T-RFLP originates from the nucleic acid extraction and PCR amplification steps. Inhibitory compounds co-extracted with the DNA from compost samples have shown to decrease the microbial diversity seen in T-RFLP profiles. The main advantages of the automated method are the low cost in comparison to NGS, and the high speed (Lens et al., 2004). Apart DNA extraction and PCR biases inherent in many fingerprinting techniques, disadvantages include the lack of specificity and the fact that different results can be obtained with different restriction enzymes (Chauhan et al., 2011; Urrea-Valencia et al., 2021). The technology has been used for decades to analyze single organisms or whole environmental communities culture-free, however, the technique is limited with respect to resolution i.e. the information on the whole microbial consortia and their individual members is limited. Amplicon sequencing (NGS) provides more information, but is considerably more costly (Hassa et al., 2018).

2.8.3. NEXT GENERATION SEQUENCING

Next generation sequencing (NGS) is a high throughput technology that uses regular unmodified nucleotides so that the newly synthesised DNA strand is not permanently terminated but monitored as each base is incorporated. This technology makes it possible to conduct sequencing of millions of DNA fragments simultaneously allowing sequencing of the entire metagenome of interest at once giving a cross-section of the entire microbiota, including microorganisms minimally represented in the sample (Wang, 2016). The number of NGS methods is constantly increasing and the most common methods include genomics (whole-Genome sequencing, exome sequencing, *de novo* sequencing and target sequencing), transcriptomics (total RNA and mRNA sequencing, targeted RNA sequencing and small RNA and noncoding RNA sequencing) and epigenomics (methylation sequencing, ChIP sequencing and ribosome profiling) (Illumina, 2015). The commercially available NGS platforms NGS that are commonly used in the laboratories include 454 Genome Sequencer (GS) FLX, Illumina Genome sequencer and the applied Biosystems SOLiD™ System, Ion Torrent, PacBio and Oxford Nanopore (Low and Tammi, 2017; www.bio.libretext.org, 2022). NGS approaches have been used extensively for the detailed investigation of the microbial consortia of the human microbiome, marine ecosystems, or environmental microbiomes in general (Vierheilig et al., 2015). Studies using NGS technologies are providing new insights into the ecology of microbial mediated processes that influence freshwater quality such as algal blooms, contaminant

biodegradation, and pathogen dissemination (Tan et al., 2015). In an ever-developing field, no technique remains the same for long. Initially, the cost of the sequencing (Sanger sequencing) was prohibitively expensive, and the technique lacked the ability to multiplex. Less costly high-throughput, high-sensitivity NGS techniques were then developed that were able to multiplex and perform rapid sequencing while also addressing the need for shorter fragments (Black et al., 2015). Both whole genome sequencing and exome sequencing produce massive amounts of data that may be of little value in certain settings where information from a particular gene of interest is sufficient. The massive amounts of data (gigabytes) that are generated require high computational power and storage (Black et al., 2015). Illumina MiSeq has a lower cost per sequence than other platforms, enabling high-throughput that is comparatively cost-effective (Zhang et al., 2020). However, capital and operational costs of NGS technology is still more expensive than older techniques such as T-RFLP.

3. CHAPTER THREE - RESEARCH DESIGN AND METHODOLOGY

3.1. PLACE OF STUDY

The investigation/research was advanced in 3 domestic WWTPs located in City of Cape Town (Western Cape) and another 3 domestic WWTPs in City of Ekurhuleni (Gauteng) (Fig 12). For the purposes of this study, WWTPs from the CoE were Labelled E1, E2 and E3 while those from the CoCT C1, C2 and C3. Laboratory analyses were facilitated at Ekurhuleni Water Care Company (ERWAT) located in the City of Ekurhuleni and at the City of Cape Town Scientific Services department at the Athlone Wastewater Treatment facility located in the City of Cape Town. Table 13 provides information about the WWTPs used in this study and Figure 13 provides a schematic summary of the experimental plan as outlined in Sections 8.1.1 to 8.1.8.

Table 13: Wastewater treatment plants information

	City of Ekurhuleni						**City of Cape Town**		
Plant	**E1**	**E2**	**E3**	**E4**	**E5**	**E6**	**C1**	**C2**	**C3**
Hydraulic capacity	19 Ml/day	1 Ml/day	4.7 Ml/day	15 Ml/d	20 Ml/d	10 Ml/d	17.5 Ml/day	12.0 Ml/day	14.0 Ml/day
PST	No	No	No	Yes	Yes	No	No	No	No
Configuration	3SB	MLE	3SB	3SB	3SB	5SB	UCT	MLE	3SB
Industrial (%)	0	0	0	0	0	0	5	0	5

PST –primary settling tank Ml/day – mega litre per day MLE - Modified Ludzack-Ettinger

UCT – University of Cape Town 3SB – 3-stage Bardenpho 5SB – 3-stage Bardenpho

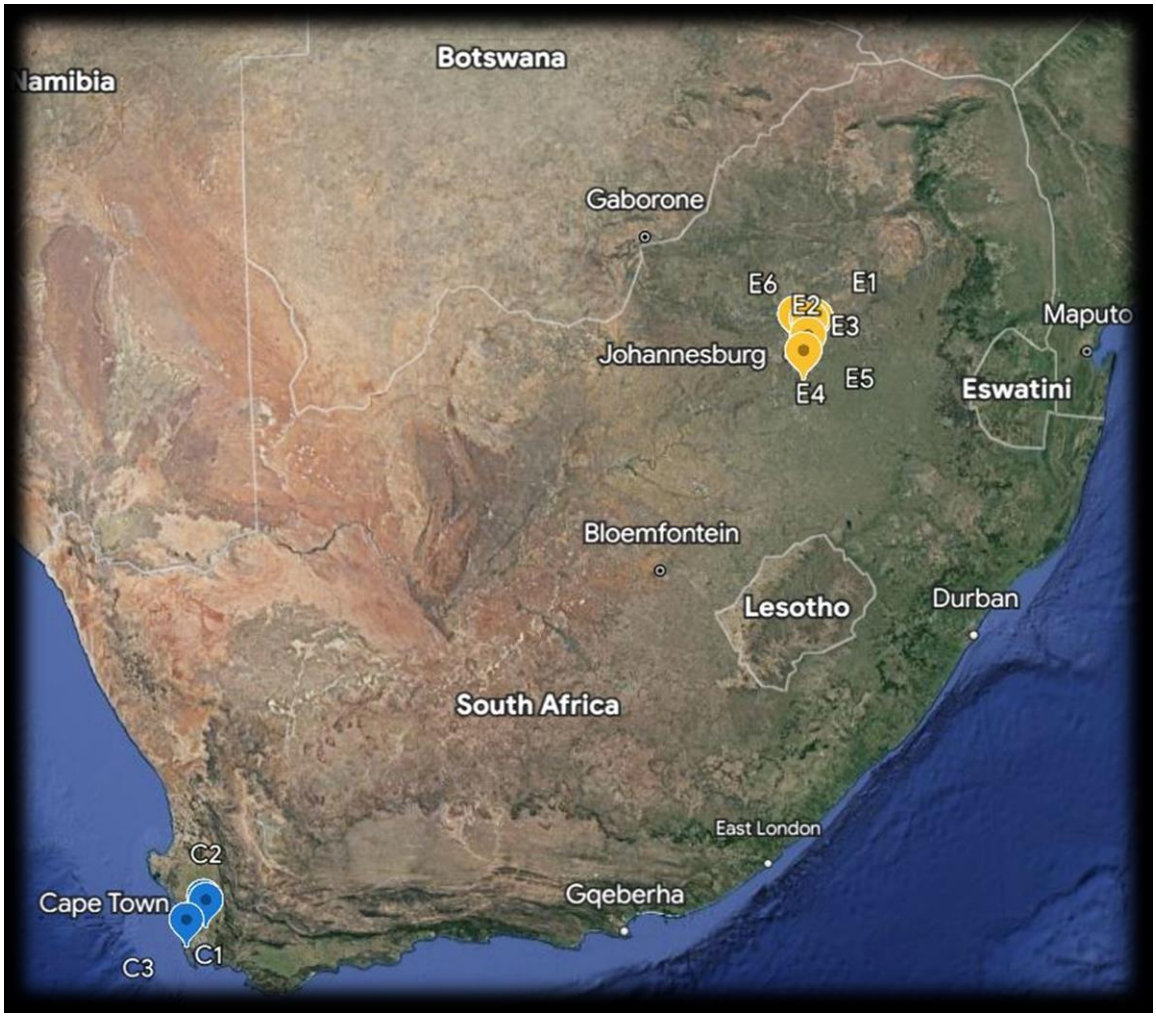

Figure 12: Location of wastewater treatment plants in the context of South Africa. C1, C2 and C3 wastewater treatment plants from the City of Cape Town and E1, E2, E3, E4, E5 and E6 from the City of Ekurhuleni. (Google Earth)

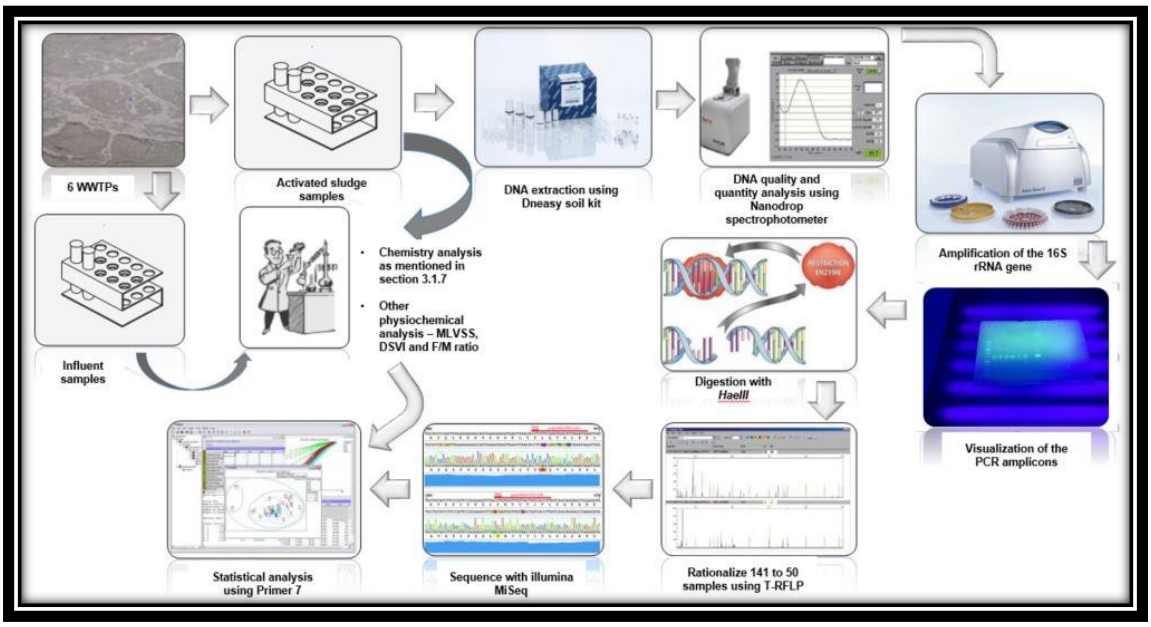

Figure 13: Flow diagram outlining the methodologies used to generate the study outputs

3.1.1. SAMPLING

Monthly sampling (from October 2015 until March 2017) was conducted during the course of Water Research Commission (WRC) Project K5/2471/3 entitled "National survey of filamentous bacterial populations in activated sludge". A total of 310 AS samples and 224 influent samples were taken. Of these, 141 AS samples and 110 AS were used in this study for microbial and physicochemical analyses, respectively (Table 14). Microbial screening was performed on all the AS samples that were taken, and the results used to rationalise the number of samples for NGS (Table 14). As far as possible, samples were taken during the same week of the month from each site. All samples were collected using the grab technique method. Since WWTPs did not have PSTs, 24 hr composite influent samples were taken. The AS (grab samples) were taken from the exits from the final aerobic zones of the reactors. Samples were transported to their respective laboratories (Ekurhuleni Water Care Company for the CoE samples, and City of Cape Town Scientific Services for the CoCT samples), stored in cooler boxes with ice packs.

Table 14: Activated sludge samples from the City of Cape Town and City of Ekurhuleni

	CoCT			CoE							
WWTP	C2	C1	C3		E3	E4	E2		E1	E5	E6
August 2015				19 August 2015				20 August 2015			
September 2015				14 September 2015				September 2015			
15 October 2015				21 October 2015				October 2015			
17 November 2015				November 2015				November 2015			
08 December 2015				December 2015				December 2015			
19 January 2016				20 January 2016				21 January 2016			
16 February 2016				15 February 2016				16 February 2016			
15 March 2016				18 March 2016				14 March 2016			
12 April 2016				14 April 2016				13 April 2016			
10 May 2016				16 May 2016				17 May 2016			
08 June 2016				10 June 2016				09 June 2016			
12 July 2016				13 July 2016				11 July 2016			
16 August 2016				16 August 2016				18 August 2016			
13 September 2016				16 September 2016				12 September 2016			
18 October 2016				14 October 2017				12 October 2017			
16 November 2016				18 November 2016				25 November 2016			
06 December 2016				December 2016				December 2016			
24 January 2017				January 2017				27 January 2017			
21 February 2017				February 2017				24 February 2017			
28 March 2017				17 March 2017				10 March 2017			

No sample Activated sludge sample Samples rationalized for NGS

3.1.2. SAMPLE PREPARATION AND STORAGE

Collected activated sludge samples were washed in equal volumes of 1X phosphate buffered saline (PBS) and stored at -20°C until required. Physicochemical analyses were performed on the day of collection.

3.1.3. DNA EXTRACTION

Activated sludge samples were defrosted at ambient temperature and thoroughly mixed by vortexing. Metagenomic DNA extractions were performed on all the AS samples obtained from the two sites. The 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 because the protocol is set for soil samples that have higher density. The final elution step was conducted using 100 µl eluent (buffer). A reagent blank control (elute solution) 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.

3.1.4. 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; Reysenbach and Pace, 1995). The forward primer E9F was FAM (fluorescein amidite dye) labelled. The PCR method described by Marchesi et al., 1998 was optimized and the final 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 to 20 ng of metagenomic DNA. The amplification was carried out using the Qiagen Rotor Gene 6000 2-plex thermal cycler with HRM capability. The PCR programme: 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.

The PCR amplicons were visualised on a 1% (w/v) agarose gel containing 8 µl of 10 mg/ml ethidium bromide (visualisation at 254 nm). 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 hours at 37°C using restriction enzyme *Hae*III and then inactivated at 80°C for 10 minutes. The digestion products were purified using the NucleoSpin gel and PCR clean up kit. Restriction fragment lengths and quality was determined via capillary electrophoresis at the Central Analytical Facility (CAF), Stellenbosch University. The profiles were analysed using the freeware, PeakScanner™ (version 1.0) (Applied Biosystems, https://products.appliedbiosystems.com) and a data matrix was generated. The data table was exported in .csv format and converted to tab-delimited text format for the online software, T-REX (http://trex.biohpc.org/; Culman et al., 2009), and a label file with sample names was created. These two tab-delimited text files were used to create large data

matrices through the use of the T-REX (Ver.1, Rev. 14) software. Peak height was used to characterise each unique terminal-restriction fragment (T-RF). Only peaks at positions between 30 and 500 bp were considered to avoid T-RFs caused by primer-dimers and to obtain peaks within the internal standard's linear range (Singh et al., 2006). The relative abundance of a T-RF in a T-RFLP profile was calculated by dividing the sample's peak height (a T-RF) by the total peak heights of all the samples (all T-RFs in the profile). T-RFs with intensities lower than 0.5%, which may have originated from background interference, were excluded from the matrices, thereby minimising the effect of the variations in the T-RFLP profiles caused by the starting quantities of DNA (Singh et al., 2006; Ding et al., 2013). Data matrices obtained from T-REX were exported to Microsoft Excel and analysed through the software, Primer 7 (Primer-E Ltd, UK) (Section 3.1.8.1).

3.1.5. AMPLICON SEQUENCING: Illumina MiSeq

The T-RFLP results were used to rationalise the number of AS samples from 141 to 50 (25 samples from the CoE and 25 from the CoCT). In order to be able to assess the influence of geographical location, WWTP configuration and season on the bacterial community composition, equal numbers of samples from each of the study site were taken during the same months, but different years were included (highlighted in dark blue in Table 14).

The rationalised samples were used for amplicon sequencing (NGS) 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) using Illumina MiSeq at MR DNA (Shallowater, USA) as described by Horn et al. (2020). Bioinformatic analyses were also performed at MR DNA as previously described (Horn et al., 2020). 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.

3.1.6. 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 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 minutes followed by 95˚C for 10 minutes. The cycling stage consisted of 40 cycles of 95˚C for 15 seconds, followed by 60˚C for 1 minute.

Bacterial numbers of *Escherischia 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 (Section 3.1.3) were calculated using Equation 16.

$$EAA = ((RA \times no.bacteria) \div DNA\ conc.) \div AS \qquad \text{(Equation 16)}$$

Where:

RA = relative abundance of taxa determined from amplicon sequencing

No. bacteria = number of bacteria in 1 µl template DNA

DNA conc. = DNA concentration of template DNA

AS = 1.0 g AS used for each sample extraction

3.1.7. PHYSICOCHEMICAL ANALYSES

Physicochemical analyses were performed during the course of WRC Project K5/2471/3 on the AS samples as well as influent samples taken on the same day as the AS. Physiochemical analyses performed included pH, BOD, COD, TP, σ-P, SO_4^{2-}-S, NH_4^+-N, NO_3^-/NO_2^--N. Total alkalinity and VFA measurements were performed only on influent samples. The BOD and COD measurements were performed on whole and filtered samples, with whole samples representing total COD/BOD, and filtrates representing the soluble fractions. The σ-P, SO_4^{2-}-S, NH_4^+-N, NO_3^-/NO_2^--N concentrations in the AS samples were only determined in the filtrates (soluble fractions). Chemical analyses were performed according to the methods in Table 15. In addition, DO measurements were taken 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. This historical data was used to correlate the bacterial community compositions in the AS with the physicochemical data (Section 3.1.8.2).

Table 15: Methods used to determine physiochemical parameters

		City of Cape Town	City of Ekurhuleni
COD	**Method**	Colorimetric: Dichromate reflux and titration	Colorimetric: Dichromate reflux and spectrophotometry
	Equipment	Gerhardt (Königswinter, Germany) COD digester Metrohm Titrando auto titrator (Herisau, Switzerland)	Hach (Loveland, USA) 45600-00 COD digester Hach DR 5000 UV/VIS Spectrophotometer
	Reference	ISO 15705 & APHA 5220-B	APHA 5220-B
BOD_5	**Method**	Respirometric	Respirometric
	Equipment	Oxitop (WTW, Weilheim, Germany) benchtop measuring device	Loviond Oxidirect® BSB/BOD instrument (Amesbury, UK)
	Reference	According to manufacturers' instructions	According to manufacturers' instructions
T. alk	**Method**	Automated potentiometric titration	Potentiometric titration
	Equipment	Aquakem Discreet Analyser [Thermo Fisher (Waltham, USA)]	Digital burette
	Reference	ISO 9963 & APHA 2320	APHA 2320
TP	**Method**	Colorimetric: Persulfate digestion with ammonium molybdate/ascorbic acid	Colorimetric: Persulfate digestion with ammonium molybdate/ascorbic acid
	Equipment	Lachat (Milwaukee, USA) QuikChem 8000 series FIA	Lachat QuikChem 8000 series FIA
	Reference	According to manufacturers' instructions based on ISO 15681	According to manufacturers' instructions based on ISO 15681
σ-P	**Method**	Colorimetric: Ammonium molybdate/ascorbic acid	Colorimetric: Ammonium molybdate/ascorbic acid
	Equipment	Lachat QuikChem 8000 series FIA	Lachat QuikChem 8000 series FIA
	Reference	ISO 15681	ISO 15681
SO_4^{-2}	**Method**	Turbidimetric: Barium chloride/hydrochloric acid	Turbidimetric: Barium chloride/hydrochloric acid
	Equipment	Lachat QuikChem 8000 series FIA	Lachat QuikChem 8000 series FIA
	Reference	According to manufacturers' instructions based on ISO 15923-1 & APHA 4500 SO4-E	According to manufacturers' instructions based on ISO 15923-1 & APHA
NH_4^+	**Method**	Colorimetric: Bertholot method	Colorimetric: Bertholot method
	Equipment	Lachat QuikChem 8000 series FIA	Lachat QuikChem 8000 series FIA
	Reference	According to manufacturers' instructions based on ISO 11732 & APHA 4500 NH3-F	According to manufacturers' instructions based on ISO 11732 & APHA 4500 NH3-F
NO_3^- /NO_2^-	**Method**	Colorimetric: hydrazine/sulfanilimide	Colorimetric: hydrazine/sulfanilimide
	Equipment	Lachat QuikChem 8000 series FIA	Lachat QuikChem 8000 series FIA
	Reference	According to manufacturers' instructions based on ISO 13395 & SM 4500 NO3-H	According to manufacturers' instructions based on ISO 13395 & SM 4500 NO3-H

ISO = International Standards Organisation, FIA = Flow injection analyser, APHA = American Public Health Association, American Water Works Association and Water Environment Federation (2005). Standard Methods for the examination of water and wastewater, 21st edition. Washington DC.

3.1.8. STATISTICAL ANALYSIS

3.1.8.1. BIOTIC DATA

The operational taxonomic units (OTUs) from processing of the T-RFLP data (Section 3.1.4) were used to determine Bray-Curtis similarity coefficients (Bray and Curtis, 1957) which were used to create similarity matrices and non-metric multidimensional scaling (nMDS) plots to determine the degree of similarity among the different bacterial community profiles from different sites, seasons and/or WWTP configurations using Primer 7® software (Clarke and Warwick, 2001). Primer 7® software was also used to analyse the processed Illumina MiSeq data saved on Microsoft Excel files (Section 3.1.5). The data was transformed (square-root) and Bray-Curtis similarity matrices based on the RA of the zOTUs were used to create nMDS plots, perform cluster analyses (group average linkages), calculate similarity percentages (SIMPER analyses), and analysis of similarity (ANOSIM, one-way, unordered, Spearman rank correlation).

3.1.8.2. PHYSICOCHEMICAL DATA AND COMBINED BIOTIC ABIOTIC ANALYSES

A student Test (Microsoft Excel) was performed on the abiotic (physiochemical) data to determine whether there were significant differences between the results obtained from different sites, seasons and/or WWTP configurations. The data was then 4th root transformed and normalised and analysed by principal component analyses (PCA) using Primer 7® software. In addition, one-way unordered ANOSIM (Spearman rank correlation) was performed on the Euclidean distance similarity matrices constructed from 4th root transformed and normalised data of the measured parameters.

Primer 7® was also used to determine which measured parameters most significantly contributed to the selection of microbial communities, 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 normalised data. The 'best' correlated parameters determined from BEST analyses were used to construct LINKTREE plots to visualise and assess the ranges of parameters that explained clustering of the functional microbial communities. 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).

4. CHAPTER FOUR – RESULTS AND DISCUSSION

4.1. THE INFLUENCE OF SITE, CONFIGURATION AND SEASON ON THE OVERALL BACTERIAL COMMUNITY STRUCTURE

A total of 136 AS samples were screened using T-RFLP, from which 50 samples were selected for analysis by NGS to profile the overall bacterial community structure (OBCS) in the AS from the WWTPS at the City of Cape Town (CoCT) and the City of Ekurhuleni (CoE). The OBCS between different configurations and seasons were also profiled. The physiochemical parameters were then analysed in conjunction with the microbial results to establish which parameters played a major role in selection of the OBCS.

4.1.1. THE INFLUENCE OF SITE ON THE OVERALL BACTERIAL COMMUNITY STRUCTURE

Figure 14 shows the nMDS with data points (dp) denoting the OBCS from samples taken from the CoCT, based in the Western Cape province and the CoE, based in Gauteng province. The nMDS plots (Figure 14) were constructed using the Bray-Curtis similarity matrices derived from the square root transformed data of both the T-RFLP and NGS datasets.

The nMDS plot (Figure 14a) generated with the T-RFLP data set showed that the dp from the two sampling sites (CoCT and CoE) were notably grouped apart or spaced apart (with the exception of few dp from the CoCT overlapping to the side of the CoE). The 2D stress was 0.13 indicating that the results are good, but it may be advisable to cross check them with an alternative method (Clark et al., 2014). The dp from CoCT were closely clustered, whilst those from the CoE were more openly clustered. The SIMPER (Table 16) between the two sampling sites was notably lower (8.96%) than within the two sampling sites (24.84 to 32.88%). To test whether the separation of the dp from the two sampling sites was significant or not, a one-way nested ANOSIM was performed from the Bray-Curtis similarity matrices derived from the square root transformed data of the T-RFLP dataset. Table 17 shows that the R value from the ANOSIM test was 0.771. The R value ranges from 0 to 1. Values close to 1 indicate that datasets are completely dissimilar, and values close to 0 indicate that datasets are highly similar. In this case, the R value indicated that the OBCS between the two sampling sites were dissimilar and statistically significant ($p < 0.01$). This confirmed that the separation of the dp noted on the nMDS was valid.

The nMDS plot (Figure 14b) generated with the NGS dataset showed that the dp from the two sampling sites (CoCT and CoE) were also grouped apart. The 2D stress was 0.12, again indicating that it may be advisable to confirm the results with an alternative method (Clark et al., 2014). The SIMPER between the two sampling sites was 56.63% (Table 18). A one-way fully nested ANOSIM was performed from the Bray-Curtis similarity matrices derived from the square root transformed data of the NGS dataset (Table 19). The R value was 0.538 and was statistically significant ($p < 0.01$). This indicated that the OBCS between the two sampling sites were moderately dissimilar and that the reasonably good separation of the dp visualised on the nMDS plot was valid. Similar results were found by Zhang et al. (2012), who demonstrated through cluster analysis that the microbial communities in AS from different geographic locations were different, and those from the same geographic location were similar, possibly due to the unique sewage composition at the different locations.

The two methods i.e. the T- RFLP and NGS produced similar nMDS plots with similar 2D stress values. The ANOSIM test results from the two techniques also produced similar results. However, results of the SIMPER test varied between the two techniques. The percentage values for the T- RFLP were lower than those of the NGS. The findings were consistent with studies by De Vrieze et al. (2018), who noted that T-RFLP and NGS data show some degree of similarity. Overall, all the results indicated that the bacterial communities in the WWTPs from the two sites showed moderate to high inter-site differences.

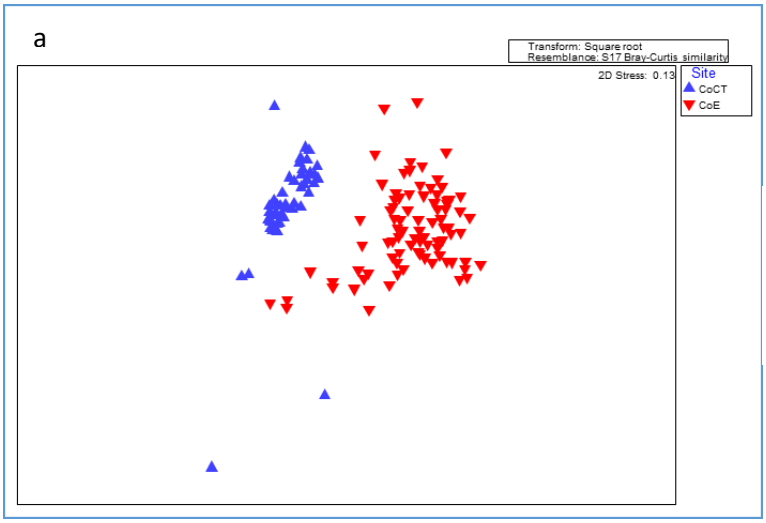

Table 16: Similarity percentage of the OBCS in the CoCT and CoE using T-RFLP data

	CoCT	CoE
CoCT	32.88	
CoE	8.96	24.84

Table 17: Statistical analysis of similarity for the CoCT and CoE sites using T-RFLP data

ANOSIM R value	0.771**

**significance (0.001 ≥ p < 0.01)

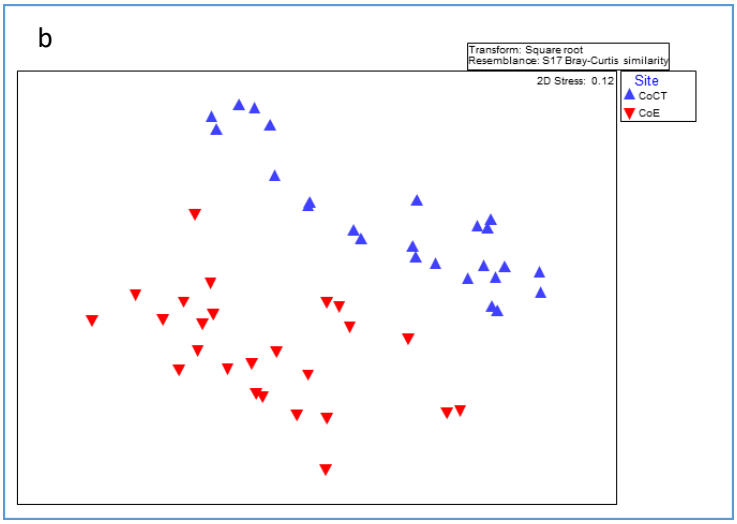

Table 18: Similarity percentage of the OBCS in the CoCT and CoE using NGS data

	CoCT	CoE
CoCT	64.43	
CoE	56.63	64.26

Table 19: Statistical analysis of similarity for the CoCT and CoE site using NGS data

ANSOIM R value	0.538**

**significance (0.001 ≥ p < 0.01)

Figure 14: Non-metric multi-dimensional scaling (nMDS) plot of the overall bacterial community structure (OBCS) data points (dp) at City of Cape Town and City of Ekurhuleni: dp denoting the OBCS from CoCT and CoE generated with T-RFLP data (a), dp denoting the OBCS from CoCT and CoE generated with NGS data (b)

4.1.2. THE INFLUENCE OF CONFIGURATION AND SEASON ON THE OVERALL BACTERIAL COMMUNITY STRUCTURE

A 3-way ANOSIM (Table 20) for the data from both the T-RFLP and NGS showed that overall, configuration and season did not have a significant influence on community structure. However, this did not mean that that configuration and/or season at each site would not necessarily be different as site was a confounding variable. Therefore, the influence of configuration and season were evaluated for each geographic location individually.

Table 20: The analysis of similarity (ANOSIM) for configuration and season the T-RFLP and NGS data

Factor	Technique	R value
Configuration	T - RFLP	-0.385
	NGS	-0.188
Season	T - RFLP	-0.069
	NGS	-0.103

*** Significance $p < 0.001$ **significance ($0.001 \geq p < 0.01$) *significance ($0.01 \geq p < 0.05$)

4.1.3. THE INFLUENCE OF CONFIGURATION ON THE OVERALL BACTERIAL COMMUNITY STRUCTURE AT THE CITY OF CAPE TOWN

The WWTPs from the CoCT had three different configurations i.e. the 3SB, MLE and the UCT. The nMDS plot (Figure 15a) generated with T-RFLP data set showed that the dp were closely clustered. However, the dp for each configuration were grouped together with few dp overlapping. The 2D stress was 0.12, an indication that the results are good, but it may be advisable to cross-check them with an alternative method (Clarke et al., 2014). SIMPER (Table 21) within and between the different configurations fell within a narrow range (30.57% to 36.85%). The ANOSIM R value was low ($R=0.08 – 0.176$) but significant ($p<0.01$) (Table 22). The results indicated that the OBCS from the three configurations were similar and also confirmed that the close clustering of the dp visualised on the nMDS plot was a valid visual representation.

The nMDS plot (Figure 15b) generated with the NGS dataset showed that the 3SB dp were clustered away from the other two configurations, whilst those representing the MLE and UCT configurations were closely clustered. The 2D stress was 0.06, indicating that the results are good, with no real need to cross check with an alternative method (Clark et al., 2014). The SIMPER test (Table 23) showed that the similarity between MLE and UCT was much higher (67.68%) confirming the close clustering visuals noted on the nMDS plot. The SIMPER between 3SB and MLE was 61.09% and between 3SB and UCT was 58.96%. The ANOSIM test (Table 24) showed moderate ($R=0.278$-0.473) but significant ($p<0.01$) dissimilarities between the bacterial communities in WWTPs with different configurations. Based on the results, it was deduced that the configuration played a small but significant role on microbial community selection.

The two techniques generated relatively similar nMDS patterns, i.e. the dp for each configuration were grouped together. The ANSOIM R values were higher for the NGS data.

This was expected, as the NGS is a much more sophisticated method. However, the results suggest that T-RFLP can still be a valuable cost-effective screening method.

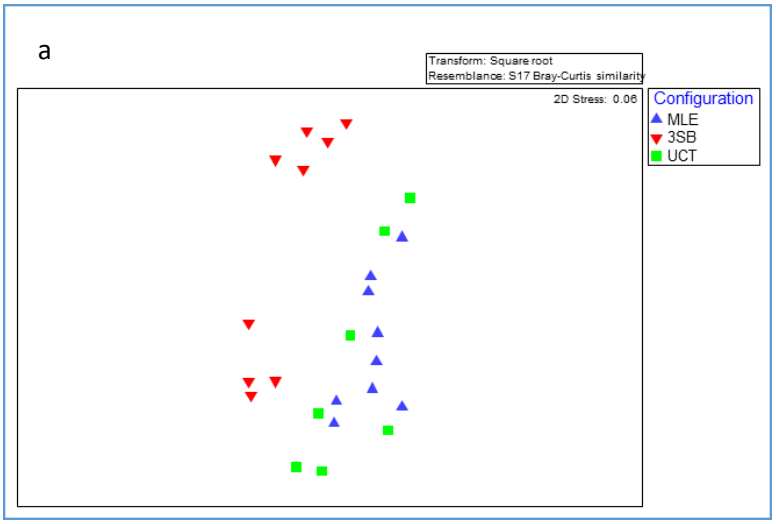

Table 21: Similarity percentage of the for 3SB, MLE and UCT configuration at the CoCT (T-RFLP data)

	3SB	MLE	UCT
3SB	36.85		
MLE	33.29	36.04	
UCT	30.57	31.8	32.66

Table 22: Statistical analysis of similarity for 3SB, MLE and UCT configurations at the CoCT (T-RFLP data)

ANOSIM R value: 0.127**	
Groups	ANOSIM R value
3SB, MLE	0.128*
3SB, UCT	0.176**
MLE, UCT	0.08*

**significance (0.001 ≥ p < 0.01) *significance (0.01≥ p < 0.05)

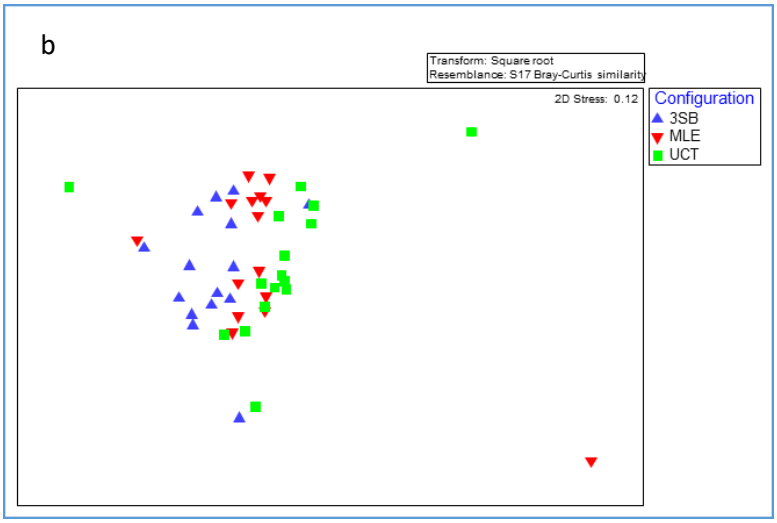

Table 23: Similarity percentage of the for 3SB, MLE and UCT configuration at the CoCT (NGS data)

	3SB	MLE	UCT
3SB	64.96		
MLE	61.09	73.84	
UCT	58.96	67.68	66.89

Table 24: Statistical analysis of similarity for 3SB, MLE and UCT configuration at the CoCT (NGS data)

ANOSIM R value: 0.367**	
Groups	ANOSIM R value
MLE, 3SB	0.473**
MLE, UCT	0.278*
3SB, UCT	0.325*

**significance (0.001 ≥ p < 0.01) *significance (0.01≥ p < 0.05)

Figure 15: Non-metric multi-dimensional scaling (nMDS) plot of the overall bacterial community structure (OBCS) data point (dp) for 3SB, MLE and UCT configuration: dp for 3SB, MLE and UCT configuration generated with T-RFLP data (a), dp for 3SB, MLE and UCT configuration generated with NGS data (b)

4.1.4. THE INFLUENCE OF CONFIGURATION ON THE OVERALL BACTERIAL COMMUNITY STRUCTURE AT CITY OF EKURHULENI

The WWTPs from the CoE had three different configurations i.e. 3SB, 5SB and MLE. The nMDS plot generated from T-RFLP data represented all three configurations and the nMDS plot generated from NGS data represented two configurations. The nMDS plot (Figure 16a) generated with the T-RFLP data showed that the dp were closely clustered and overlapping. The 2D stress was 0.18 indicating that the results are good, but it may be advisable to cross-check them with an alternative method (Clarke et al., 2014). The SIMPER test (Table 25) showed that the similarity between the three configurations were close (24.2% to 27.97%). The ANOSIM test (Table 26) showed low (R= -0.11 - 0.05) and insignificant dissimilarities between the bacterial communities in the WWTPS from the three different configurations.

The nMDS plot (Figure 16b) generated with the NGS data showed that the 3SB and MLE dp were clustered closer. The 2D stress was 0.14, indicating that the results are good, but it may be advisable to cross-check them with an alternative method (Clarke et al., 2014). The SIMPER Test (Table 27) showed the similarity between the 3SB and MLE configuration was 63.36% suggesting that the OBCS were fairly similar. The ANOSIM test (Table 28) showed a low (R=0.178) but significant dissimilarity between the bacterial communities in samples taken from the 3SB and MLE configurations.

The two techniques (T-RFLP and NGS) did not generate the same nMDS plots i.e. the dp for the different configurations in nMDS plot from the T-RFLP overlapped extensively, whilst those from the NGS were clustered together to some extent. The SIMPER values were once again notably higher for the NGS results than for the T-RFLP results. The ANOSIM results for both techniques showed that the OBCS were low in dissimilarity, significantly so for the NGS technique and insignificant for the T-RFLP. A larger number of WWTPs were included in the T-RFLP dataset which should theoretically provide more relevant statistical data.

Overall, for samples taken from the CoE WWTPs using NGS and T-RFLP technique generated somewhat inconclusive outcomes for the factor ‘configuration’, but generally suggested that configuration was not a major determinant for OBCS.

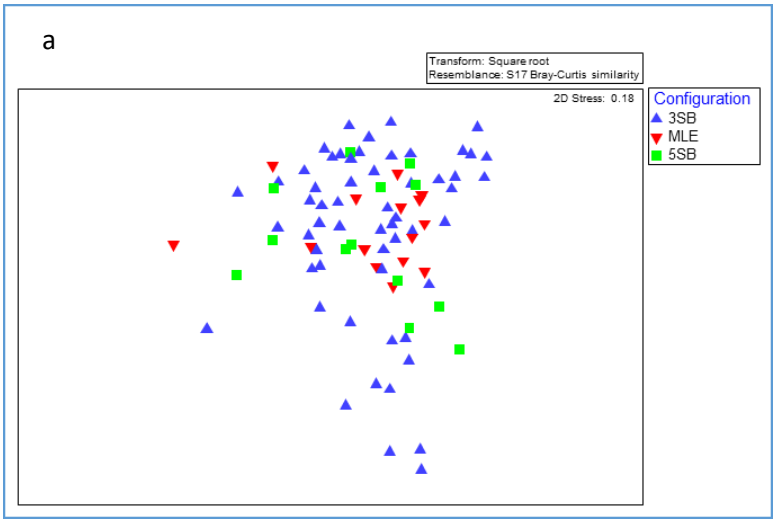

Table 25: Similarity percentage for 3SB, MLE and 5SB configuration at CoE (T-RFLP data)

	3SB	MLE	5SB
3SB	23.66		
MLE	26.11	29.84	
5SB	24.20	27.97	27.24

Table 26: Analysis of similarity for 3SB, MLE and UCT configuration at CoE (T-RFLP data)

ANOSIM R value: -0.069

Groups	ANOSIM R value
3SB, MLE	-0.11
3SB, 5SB	-0.02
MLE, 5SB	0.05

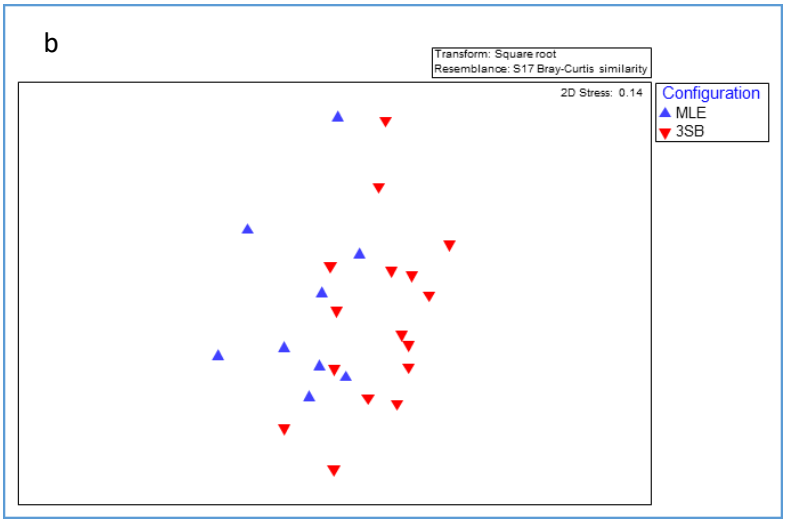

Table 27: Similarity percentage for 3SB and MLE configuration at CoE (NGS data)

	3SB	MLE
3SB	64.98	
MLE	63.36	65.47

Table 28: Analysis of similarity for MLE and 3SB configuration at CoE (NGS data)

ANOSIM R value	0.178*

**significance ($0.001 \geq p < 0.01$) *significance ($0.01 \geq p < 0.05$)

Figure 16: Non-metric multi-dimensional scaling (nMDS) plot of the overall bacterial community structure (OBCS) data point (dp) for 3SB, MLE and 5SB configurations: dp for 3SB, MLE and 5SB configuration generated with T-RFLP data (a), dp for 3SB and MLE configuration generated with NGS data (b)

4.1.5. THE INFLUENCE OF SEASON ON THE OVERALL BACTERIAL COMMUNITY STRUCTURE AT CITY OF CAPE TOWN

The nMDS plot (Figure 17a) generated with the T-RFLP results showed that dp for all four seasons were closely clustered and overlapping. The 2D stress was 0.12, indicating that the results are good, but it may be advisable to cross-check them with an alternative method (Clarke et al., 2014). The SIMPER test (Table 29) from the four seasons were very close (30.93% to 35.43%). The ANOSIM test (Table 30) showed low (R= -0.007 - 0.104) and insignificant dissimilarities between the bacterial communities during all four seasons with the exception of summer and winter which showed low (ANOSIM R=0.192) but significant ($p<0.05$) dissimilarity.

The nMDS plot (Figure17b) generated with NGS data also showed that dp for all four seasons were closely clustered and overlapping. The 2D stress was 0.06 indicating that the results are good, with no real need to cross-check with an alternative method (Clarke et al., 2014). The SIMPER test (Table 31) from the four seasons were very close (60.32 – 67.56%). The ANOSIM test (Table 32) showed insignificant dissimilarities between the bacterial communities in all four seasons with the exception of autumn and spring which also had a moderate dissimilarity (ANOSIM R=0.303, $p<0.05$).

The two techniques generated relatively similar nMDS patterns i.e. the dp from the nMDS were closely clustered and the ANOSIM results for both techniques showed that the OBCS were low in dissimilarity and mostly insignificant.

Overall, the results indicated only negligible seasonal effects between colder (autumn, winter) and warmer (spring, summer) seasons on the bacterial community composition in the CoCT WWTPs.

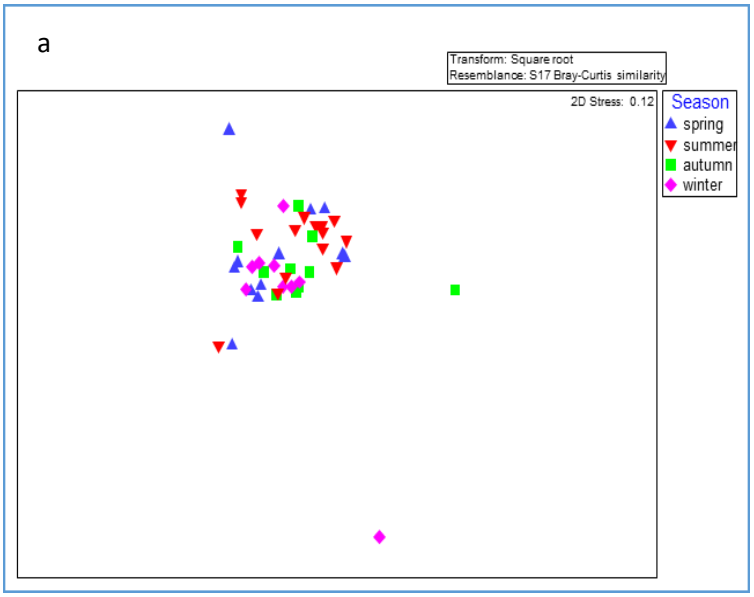

Table 29: Similarity percentage for the summer, autumn, winter and spring at CoCT (T-RFLP data)

	SPRING	SUMMER	AUTUMN	WINTER
SPRING	31.28			
SUMMER	30.93	35.04		
AUTUMN	31.78	33.53	36.43	
WINTER	33.02	31.16	35.43	35.49

Table 30: Analysis of similarity for the summer, autumn, winter and spring at CoCT (T-RFLP data)

Global ANOSIM R value 0.087*	
Groups	**ANOSIM R value**
spring, summer	0.104
spring, autumn	0.057
spring, winter	-0.007
summer, autumn	0.102
summer, winter	0.192*
autumn, winter	0.05

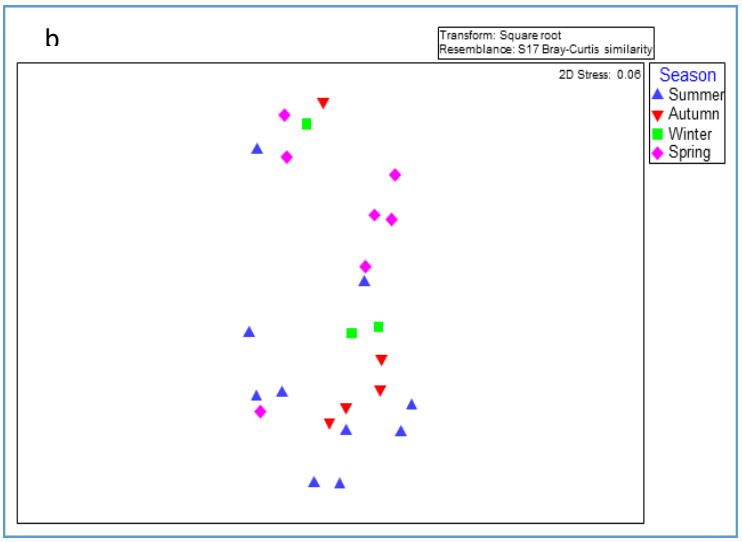

Table 31: Similarity percentage for the summer, autumn, winter and spring at CoCT (NGS data)

	SUMMER	AUTUMN	WINTER	SPRING
SUMMER	66.36			
AUTUMN	66.98	65.83		
WINTER	63.46	66.95	64.88	
SPRING	60.32	61.95	67.56	67.79

Table 32: Statistical analysis for summer, autumn, winter and spring at CoCT (NGS data)

Global ANOSIM R value 0.166*

Groups	**ANOSIM R value**
Summer, Autumn	-0.100
Summer, Winter	0.108
Summer, Spring	0.409
Autumn, Winter	0.015
Autumn, Spring	0.303*
Winter, Spring	0.004

**significance ($0.001 \geq p < 0.01$) *significance ($0.01 \geq p < 0.05$)

Figure 17: Non-metric multi-dimensional scaling (nMDS) plot of the overall bacterial community structure (OBCS) data point (dp) for summer, autumn, winter and spring at the City of Cape Town: dp for summer, autumn, winter and spring generated with T-RFLP data (a), dp for summer, autumn, winter and spring generated with NGS data (b)

4.1.6. THE INFLUENCE OF SEASON ON THE OVERALL BACTERIAL COMMUNITY STRUCTURE AT CITY OF EKURHULENI

The nMDS plot (Figure 18a) generated with T-RFLP data showed that the dp for all four seasons were closely clustered and overlapping. The 2D stress was 0.18 indicating that the results are good, but it may be advisable to cross-check them with an alternative method (Clarke et al., 2014). The SIMPER test (Table 33) showed that the similarity between the four seasons were very close (20.55 to 26.59%). The ANOSIM test (Table 34) showed low (R= 0.07 - 0.254) but significant dissimilarities between the bacterial communities during all four seasons with the exception of summer and winter which had an insignificant ($p>0.05$) dissimilarity.

The nMDS plot (Figure 18b) generated with NGS data showed that the dp were openly clustered and overlapping. The 2D stress was 0.14 indicating a reasonable representation of the dp requiring a second method for verification (Clarke et al., 2014). The SIMPER test (Table 35) showed that the similarities between the four seasons were very close (57.54% to 67.14%). The ANOSIM test (Table 36) showed both (i) moderate (R= 0.373 – 0.576) and significant dissimilarity between the bacterial communities in summer-winter, spring-winter and spring-summer and (ii) low (R= 0.044 – 0.151) and insignificant dissimilarities between the bacterial communities in spring-autumn, summer-autumn and autumn-winter.

The two techniques generated relatively similar nMDS patterns i.e. the dp from the nMDS were clustered and overlapping. The ANOSIM results for the T-RFLP indicated an overall low significant dissimilarity whiles the NGS indicated both a low insignificant and moderate significant dissimilarity.

The results indicated the negligible seasonal effects between summer-winter, spring-winter and spring-summer on the bacterial community composition in the CoCT WWTPs**.**

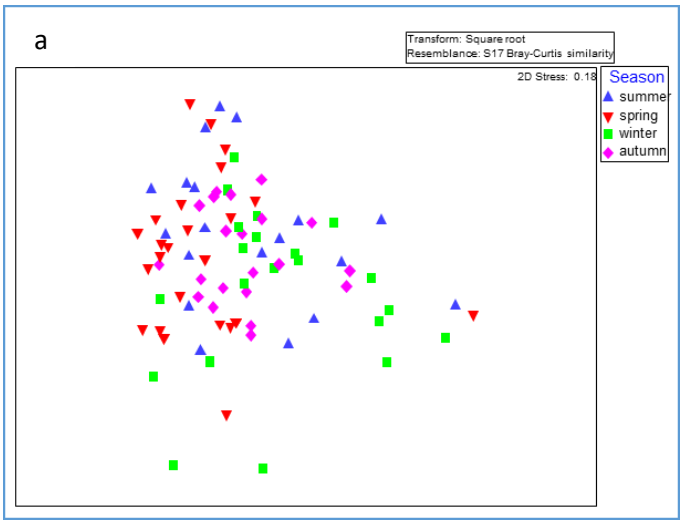

Table 33: Similarity percentage for summer, spring, autumn, winter at CoE (T-RFLP data)

	SUMMER	SPRING	WINTER	AUTUMN
SUMMER	24.27			
SPRING	23.59	27.79		
WINTER	23.15	20.55	24.08	
AUTUMN	26.59	25.53	26.14	30.78

Table 34: Statistical analysis of similarity for summer, autumn, winter and spring season at CoE (T-RFLP data)

Global ANOSIM R value 0.137*

Groups	ANOSIM R value
summer, spring	0.137**
summer, winter	0.046
summer, autumn	0.077*
spring, winter	0.254**
spring, autumn	0.205**
winter, autumn	0.070*

**significance (0.001 ≥ p < 0.01) *significance (0.01≥ p < 0.05)

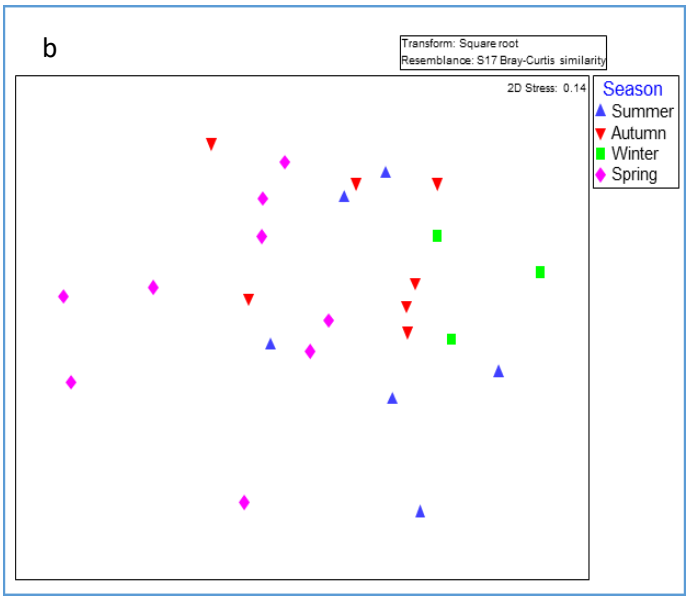

Table 35: Similarity percentage for summer autumn winter and spring season at CoE (NGS data)

	SPRING	SUMMER	AUTUMN	WINTER
SPRING	65.13			
SUMMER	61.38	67.18		
AUTUMN	63.85	66.64	68.00	
WINTER	57.54	65.49	67.14	72.94

Table 36: Statistical analysis of similarity for summer, autumn, winter and spring at CoE (NGS data) autumn, winter and spring at CoE (NGS data)

Global ANOSIM R value 0.235**

Groups	ANOSIM R value
Spring, Summer	0.373**
Spring, Autumn	0.151
Spring, Winter	0.576**
Summer, Autumn	0.094
Summer, Winter	0.444*
Autumn, Winter	0.044

**significance (0.001 ≥ p < 0.01) *significance (0.01≥ p < 0.05)

Figure 18: Non-metric multi-dimensional scaling (nMDS) plot of the overall bacterial community structure (OBCS) data point (dp) for summer, autumn, winter and spring at the City of Ekurhuleni. dp for summer, autumn, winter and spring generated with T-RFLP data (a). dp for summer, autumn, winter and spring generated with NGS data (b)

4.1.7. CRITICAL DISCUSSION OF FINDINGS

In this study, two high throughput DNA techniques (T-RFLP and Illumina MiSeq) were used to analyse the bacterial communities in AS samples from the CoCT and CoE. Both techniques demonstrated highly significant differences in the OBCS in AS samples collected from the two sites. In some instances, small, but significant differences in the OBCS were found between configurations and seasons at one or both of the sites independently.

The highly significant differences in the OBCS in the AS from the two cities were assumed to have been influenced by either the environmental conditions or the composition of the wastewater. Factors that contribute to microbial population selection in AS include the geographic location and the concentration and type of the pollutant which may be influenced by the diets and living habits of people in different areas (Zhao at el., 2014). Zhang et al., (2012) studied WWTPs from Asia (mainland China, Hong Kong and Singapore) and North America (the Unites states and Canada) to assess the similarities and differences in the bacterial community compositions using 454 pyrosequencing technology. The three WWTPs from North America use the conventional AS (CAS) processes and those from Asia use A/O (anoxic/aerobic) or A/A/O (anaerobic/anoxic/aerobic) process. Zhang et al. (2012) discovered that the samples from Asia had the richest diversity and those from North America had the lowest richness suggesting that the OBCS from the two regions (Asia and North America) were different. Another study was conducted by Zhao at el. (2014), who used 454 pyrosequencing technology to investigate ten WWTPs located in different geographic locations in China. The study found that the bacterial communities were strongly related to the geographic sampling location and the ANOSIM test showed that the bacterial communities were significantly different ($p<0.01$). Both of these studies were consistent with the findings of this study.

Wastewater treatment processes are designed by engineers to achieve specific treatment goals. The designers are influenced by the wastewater type they need to treat (domestic and/or industrial, type of industrial), wastewater characteristics, regulatory requirements, and receiving water body characteristics. Different AS designs tend to impose different selective pressures that have been reported to alter microbial community composition and physiological function. However, the design impacts on bacterial community composition are still poorly understood (Kim et al., 2021). Hu et al. (2012) used 454-pyrosequencing to investigate the OBCS in 12 municipal WWTPs (7 A/A/O membrane bioreactors, 3 A/A/O configured reactors, 2 A/O configured reactors and 4 oxidation ditches). They found no

clustering of dp on PCA plots and low Sorenson similarity indices between the OBCS in the WWTPs and concluded that configuration does not play a role in bacterial community selection. However, only one sample was tested from each WWTP and half of the WWTPs treated mixed domestic/industrial effluent with between 20 to 80% industrial composition. In contrast, Kim et al. (2021) found temporal variations, but also significant ($p<0.001$) differences in the OBCS in 7 A/O when compared with those in 10 CAS configured WWTPs. In addition, all the WWTPs treated domestic effluent and monthly samples were taken for a year, providing a more statistically representative study.

This study indicated that the WWTP configuration (design) had a negligible but significant effect on the OBCS. This effect was, however, noted only for configuration (3SB, MLE and UCT design) at the CoCT. In contrast to the CoCT, the design at the CoE (3SB, 5SB and MLE design) did not show any significant effect on the OBCS. When analysing the results in conjunction with literature findings, it appears that configuration designs can influence the OBCS, but may be overridden by other variables in some WWTPs.

This study also tested the hypothesis that different seasons profoundly affect the bacterial community compositions, especially knowing that temperature is a major driver for bacterial growth. Cai et al. (2020) found some repetitive seasonal patterns in the bacterial community composition in the AS from one of two WWTPS examined in Shenzhen, China over 3 years. They hypothesised that the microbial shifts in an A/O-configured reactor were related to temperature changes, and that changing environmental factors (wastewater composition) played a larger role than temperate in community selection in the second (UCT configured) WWTP. A study conducted by He et al. (2021) in Beijing, China on AS from different WWTPs using high-throughput sequencing technology showed that the RA of dominant bacterial genera in AS varied significantly between the WWTPs and different bacteria dominated in summer (ambient temperature: 28±2°C, water temperature: 24.9±1.1°C) and winter (ambient temperature: 0±3°C, water temperature: 16.8±1.3°C). Although samples were only taken at two instances in each season, there was a significant correlation between temperature and OBCS. The results were substantiated by those of a third Chinese study by Sun et al. (2021) who found that dp representing the OBCS determined using Illumina MiSeq in summer and autumn clustered together and those from winter and spring clustered together in nMDS plots. This SA study showed marginal seasonal effects that may have been related to other variables and was not entirely consistent with the Chinese studies. The anomaly could be related to the different climates, as the differentiation between summer and winter

temperatures are greater in the Chinese study sites than in the SA study sites (refer to Section 4.2 for climatic data).

In terms of the different methodologies employed in this study, T-RFLP is a simple and robust culture-independent tool that has been used for decades to analyse microbial community structures. More recently, high-throughput 16S rRNA gene amplicon sequencing has largely replaced methods such as T-RFLP. Although less samples were included in the NGS analyses in this study, the fact that similar overall results were obtained by both methods suggests that both techniques can be reliable for monitoring the OBCS in AS. These findings are consistent with studies conducted by De Vrieze et al. (2018) whom also compared T-RFLP and NGS (pyrosequencing) and found a high degree of similarity in the microbial diversity profiles. This study therefore demonstrated that T-RFLP can still generate reliable results for monitoring bacterial communities, albeit with taxonomic limitations. It is therefore recommended that NGS is the method of choice, but in cases where large numbers of samples require processing, T-RFLP can still be a valuable and cost-effective screening tool.

4.2. COMPARISON OF ENVIRONMENTAL (PHYSICOCHEMICAL) VARIABLES MEASURED IN INFLUENT AND ACTIVATED SLUDGE SAMPLES FROM THE TWO STUDY SITES

Highly significant differences in the OBCS were noted when "site" was used as a factor. As this was not related to season or configuration, it was hypothesized that it was either related to the climate, influent and/or operational differences. The physicochemistry of the AS is influenced by the influent character and the operational parameters. Physicochemical parameters of influent and AS from the two sampling sites are presented in Table 37. The influent physicochemical parameters were higher in the CoCT with the exception of the pH and SO_4^{2-} which were higher in the CoE. In the AS, the BOD_{tot}, BOD/COD_{tot}, BOD/COD_{sol}, pH, DO and σP were higher at the CoE and BOD_{sol}, $COD_{tot\ and\ sol}$, NH_4^+, TP, and SO_4^{2-} were higher at the CoCT. The Solids concentrations, which included the MLVSS and DSVI, were higher at the CoCT while the F/M ratio for both the COD and BOD were higher at the CoE. These results showed that the CoCT WWTPs were receiving a higher concentration of nutrients/waste than the CoE WWTPs. This could be due to human water usage habits and/or ingress of stormwater and/or groundwater into the sewerage pipelines in the CoE, which are known to be in a compromised state.

A student TTEST (Table 37) was performed and 7 (BOD, COD, T.Alk, NH_4^+, σ-P, SO_4^{2-}, and VFA) of 11 measured parameters from the influent, 7 (COD_{tot}, COD_{sol}, BOD/COD_{tot} ratio, NH_4^-, pH, TP and σ-P) of 13 measured parameters from the AS and all (MLVSS, DSVI and FM) measured solids parameters were significantly different between the CoCT and CoE samples.

To analyze the pattern of relationships, a PCA was performed on the physicochemical parameters. There was a grouping and separation of the dp from the CoCT and CoE in the PCA plot (Figure 19). The PCA plot showed that 24.6% and 13.3% of the variation could be explained by PC1 and PC2 (cumulative variation of 37.9%), suggesting a moderately good separation between the CoCT and CoE dp.

Most of the vectors on the PCA plot (Figure 19) were directed towards the CoCT sampling site (the vector indicates the direction in which the variables are changing). This showed that the influent (NH_4^+, Inf. Alk, Inf.COD, Inf. BOD, Inf. VFA, Inf. σP), solids (DSVI, MLVSS) and AS (TP) were higher at CoCT. ANOSIM results (Table 38) confirmed that these parameters were significantly different at each site.

Table 37: Comparison of environmental (physicochemical) variables measured in influent and activated sludge samples from the two study sites

INFLUENT				
Parameter	CoCT (ave ± SD)	CoE (ave ± SD)	T	T_{crit}
BOD ($mg.L^{-1}$)	475 ± 183	190 ± 124	**7.86*** **	2.00
COD ($mg.L^{-1}$)	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 ($mg.L^{-1}$)	395 ± 63	273 ± 73	**10.0*** **	1.99
NH_4^+ ($mg.L^{-1}$)	70.0 ± 21.0	43.2 ± 19.0	**7.84*** **	1.99
NO_3^-/NO_2^- ($mg.L^{-1}$)	0.23 ± 0.56	0.16 ± 0.45	1.24	1.98
TP ($mg.L^{-1}$)	13.9 ± 8.6	12.9 ± 27.1	0.153	1.98
σP ($mg.L^{-1}$)	9.0 ± 3.7	5.6 ± 4.9	**4.73** **	1.99
$SO_4^{-2}{}_{in}$ ($mg.L^{-1}$)	65 ± 30	71 ± 26	**2.49***	2.00
VFA ($mg.L^{-1}$)	93 ± 61	49 ± 26	**4.24** **	2.02
ACTIVATED SLUDGE				
Parameter	CoCT (ave ± SD)	CoE (ave ± SD)	T	T_{crit}
DO ($mg.L^{-1}$)	1.04 ± 1.04	1.21 ± 1.08	1.11	2.01
BOD_{tot} ($mg.L^{-1}$)	815 ± 421	983 ± 582	1.47	2.00
BOD_{sol} ($mg.L^{-1}$)	43 ± 42	22 ± 14	1.79	2.14
COD_{tot} ($mg.L^{-1}$)	6229 ± 1419	3727 ± 1523	**7.27*** **	1.99
COD_{sol} ($mg.L^{-1}$)	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^+ ($mg.L^{-1}$)	16.6 ± 20.3	6.5 ± 4.2	**3.26** **	2.02
NO_3^-/NO_2^- ($mg.L^{-1}$)	1.5 ± 2.6	0.6 ± 1.2	1.91	2.01
TP ($mg.L^{-1}$)	127.8 ± 40.2	20.8 ± 17.0	**15.5*** **	2.01
σP ($mg.L^{-1}$)	4.0 ± 6.4	9.5 ± 7.2	**4.07*** **	1.98
$SO_4^{-2}{}_{in}$ ($mg.L^{-1}$)	62 ± 19	52 ± 15	1.22	2.06
SOLIDS				
Parameter	CoCT (ave ± SD)	CoE (ave ± SD)	T	T_{crit}
MLVSS ($mg.L^{-1}$)	4426 ± 1018	2611 ± 1557	**7.49*** **	1.98
DSVI ($ml.g^{-1}$)	188 ± 106	111 ± 69	**3.66*** **	2.00
F/M ($mgBOD.mg\ MLVSS.day^{-1}$)	0.07 ± 0.05	0.30 ± 0.53	**3.73*** **	2.00
F/M ($mgCOD.mg\ MLVSS.day^{-1}$)	0.15 ± 0.07	0.69 ± 1.12	**4.12*** **	2.00

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

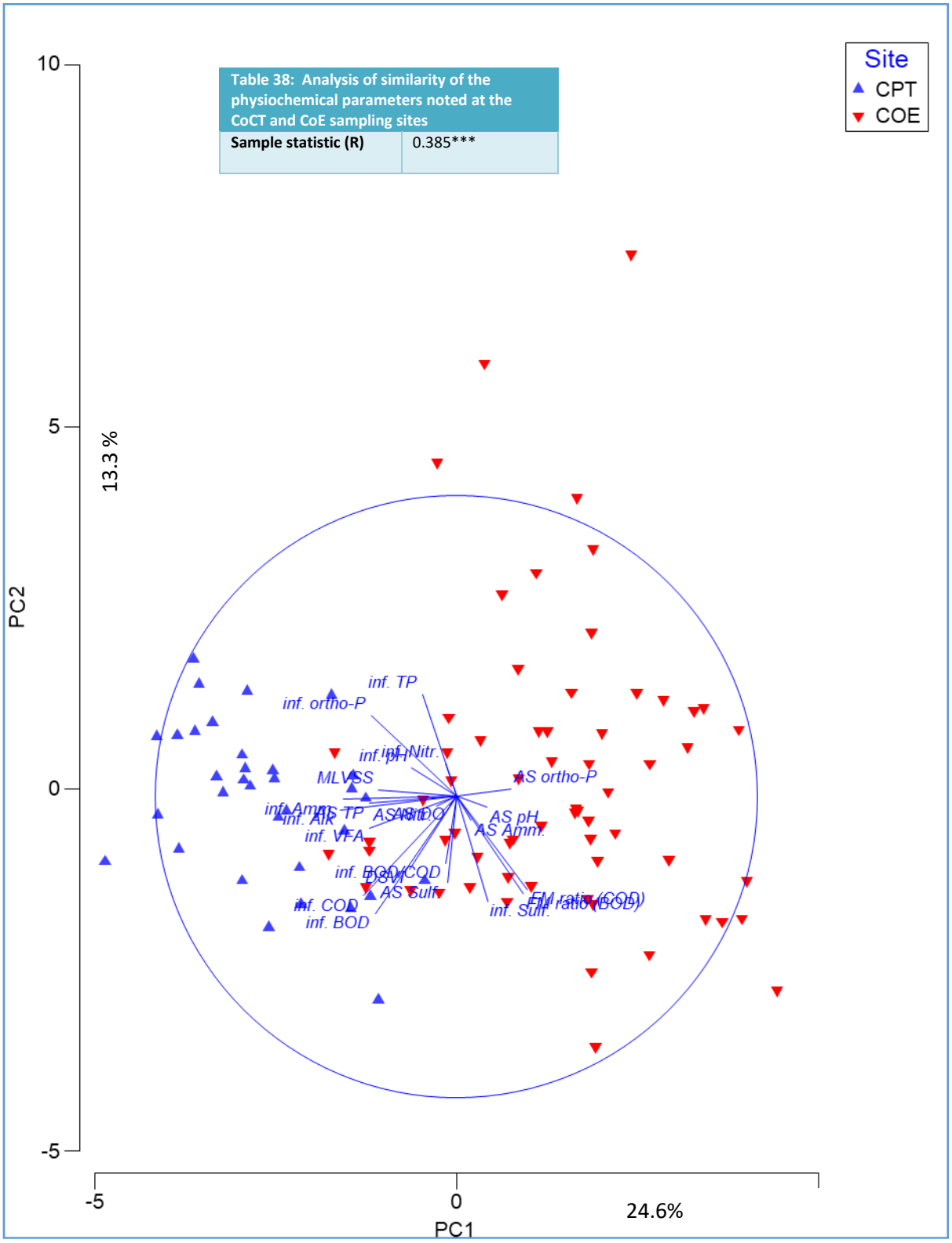

Table 38: Analysis of similarity of the physiochemical parameters noted at the CoCT and CoE sampling sites	
Sample statistic (R)	0.385***

Figure 19: Principal component analysis of the physiochemical parameters in samples from the City of Cape Town and City of Ekurhuleni wastewater treatment plants.

Climate is the average weather pattern of a region and includes temperature, rainfall, and wind (Abatenh et al., 2018). Data supplied by the South African Weather Service (SAWS) was extracted to assess and compare the historic climatic rainfall and temperature data from 2012 – 2021 between the two study sites (personal communication, September 2022). The hottest month was January with daily maximum (max) and minimum (min) temperatures of 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 CoCT and CoE, respectively (Figure 20A). July was the coldest month with recorded temperatures of 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. Overall, the average monthly temperatures were similar at both locations.

The annual rainfall is highly variable in SA. In the last decade, the CoE experienced higher rainfall (652±104 mm) than CPT (419±106 mm) (Figure 20B). The CoE is a summer rainfall area whiles the CoCT is a winter rainfall area.

These results showed that neither of the study sites experienced excessive cold winter or large seasonal temperature gradients, similar to studies conducted by Luo et al. (2020) and Maza-Márquez et al. (2022), where temperature effects on the microbial community compositions were also minimal. Rainfall was also discounted as a major driver of differences in the bacterial community compositions between the two sites because rainfall only has a diluting effect if the wastewater transport infrastructure (sewerage network) is compromised and/or the storm water drains into the sewerage network.

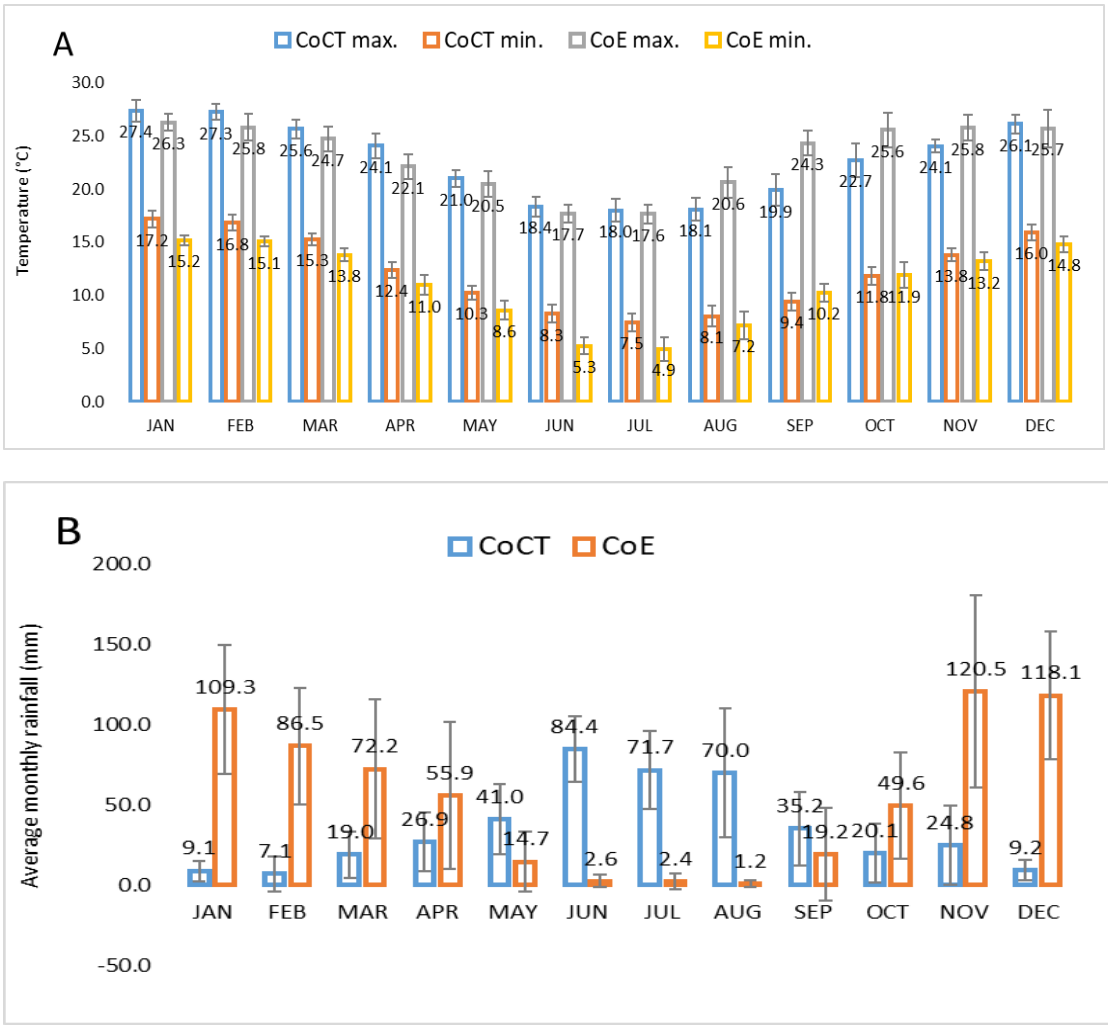

Figure 20: The average monthly temperatures taken from daily minimum (min.) and maximum (max.) recordings (A) and average monthly rainfall (B) in the City of Cape Town (CoCT) and the City of Ekurhuleni from 2012 to 2021 (South African Weather Service personal communication)

4.3. CORRELATION OF THE PHYSICOCHEMICAL PARAMETERS DATA AND THE OVERALL BACTERIAL COMMUNITY STRUCTURE

This study looked at factors that influenced the differences in OBCS between the two sampling sites (CoCT and CoE) and also within each sampling site. Both the influent and operational factors contributed significantly to driving bacterial community selection. Previous studies have also shown that microbes from the influent wastewater can proliferate in the AS, and that environmental and operational factors play an important role driving the structuring of the bacterial community (Kang et al., 2020).

A BEST analysis of Spearman rank correlation was performed on the Bray-Curtis similarity data of the NGS data and the Euclidian distance of the transformed and normalized data for the Influent, activated sludge and solids dataset of the measured parameters (Table 39). From the BEST analysis, the global Rho was 0.566 (a fairly good correlation) which was significant ($p<0.05$). AS TP on its own was the most important driver of the OBCS, and interactions of numbers of parameters did not increase the correlation.

A LINKTREE binary divisive cluster analysis (Figure 21a) was performed on the Bray-Curtis similarity data of the NGS data and the data for the Influent, AS and solids. The AS TP concentration was the primary parameter separating the bacterial communities in the AS from the CoCT WWTPs from those in the CoE WWTPs. The AS TP from the CoE was <47.4 mg/L while that from the CoCT was >60 mg/L. Figure 21b also shows that the AS TP concentrations in the CoCT samples were higher than those in the CoE samples.

Studies conducted by Zheng at al. (2019) demonstrated that the growth of the AS microbial populations through monitoring of the MLSS varied under different P sources. Phosphorus plays a significant role in the stability and function of the AS to an extent that when a suitable P source is absent in the influent, a decline in MLSS will result along with the poor removal of NH_4^+ and NO_2^- (Jobbágy et al.,2002). The AS contains a number of microbial groups involved in cycling of P, and it has been shown that changes in the concentration and source of P can lead to changes in the microbial diversity and functions (Zheng at al., 2019).

Table 39: BEST results of the microbial community structure and the Euclidian distance of the transformed and normalized data for the Influent and activated sludge (Region)		
Sample statistic (Rho): 0.566* Number of permutations: 99 (Random sample)		
No.Varables	Corr. Selections	Parameter(s)
1	0.566	AS TP
2	0.498	AS TP, AS Amm.
3	0.494	AS TP, AS Amm., inf. Alk
2	0.487	AS TP, inf. VFA.
2	0.483	AS TP, inf. Alk

*significance ($0.01 \geq p < 0.05$).

AS – Activated sludge, Inf – Influent, Amm. – Ammonium, AlK – Alkaline, VFA – Volatile fatty accids

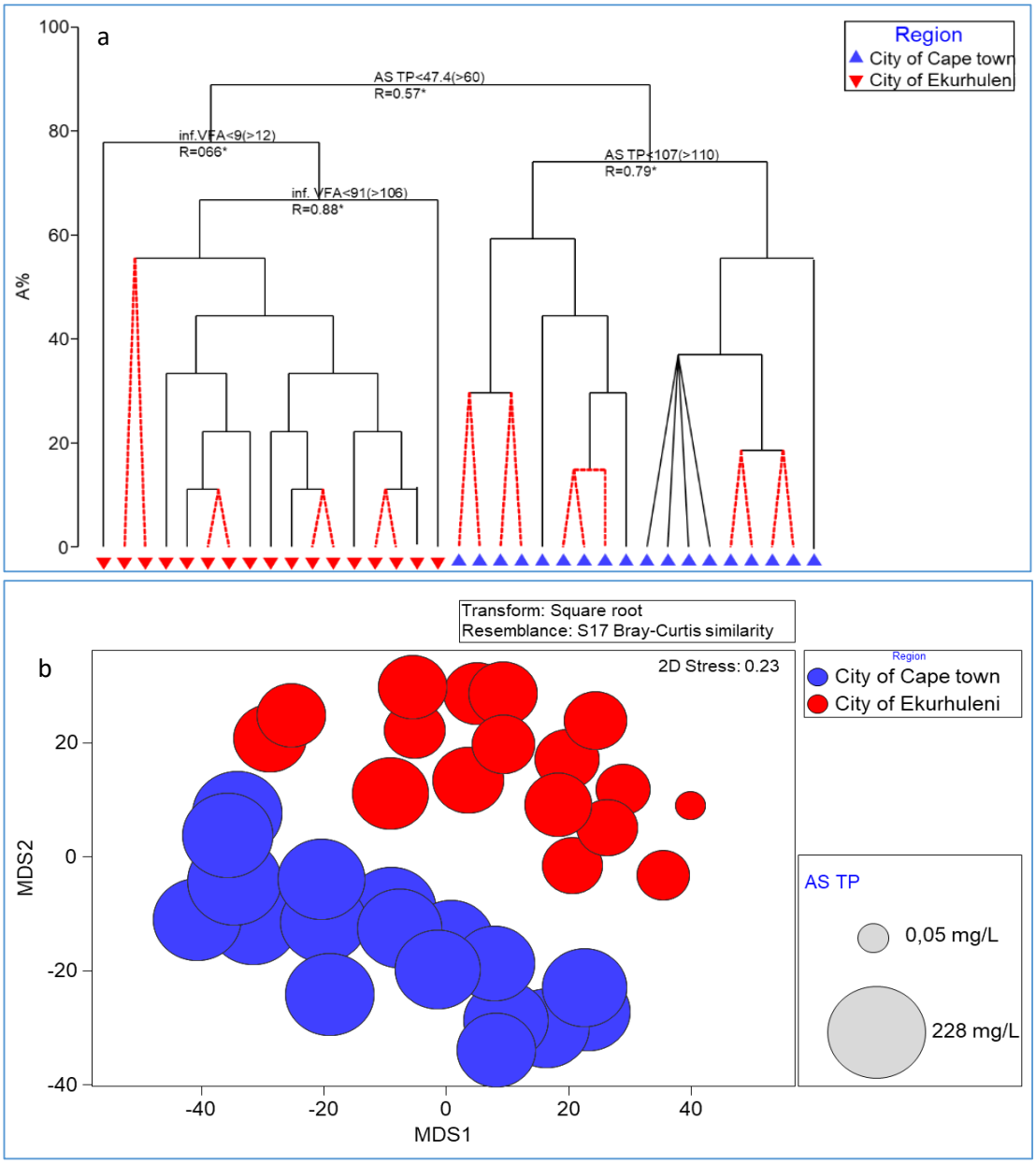

Figure 21: Plots for the physiochemical parameters for the City of Cape Town and City of Ekurhuleni: LINKTREE of abiotic and biotic data from the two sampling points – City of Cape Town and City of Ekurhuleni (a), bubble overlays of the most important physicochemical driver (activated sludge phosphorus concentration) of the microbial community structure determined using BEST (b)

BEST analysis performed on the Bray-Curtis similarity data of the NGS data and the Euclidian distance of the transformed and normalized data of the Influent, AS and solids from the CoCT's configuration (Table 40), showed that the global Rho was 0.643 (a good correlation) which was significant ($p<0.05$). An interaction of AS TP, AS DO, influent NH_4^+ and DSVI were the important influencers of the OBCS in the AS in the CoCT WWTPs.

A LINKTREE binary divisive cluster analysis (Figure 22a) was performed on the Bray-Curtis similarity data of the NGS data and the data for the Influent, AS and solids measured from the CoCT WWTPs using configuration as a factor. The results showed that inf. Amm, AS DO and DSVI (measure of bulking) were the important influencers of the OBCS between the MLE, 3SB and UCT configurations. The inf. Amm into the WWTP with 3SB configuration was <62.2 mg/L in comparison to >80.4 mg/L for MLE and UCT WWTPs (Figure 22b). The DO from the WWTP with 3SB configuration was <1.16 mg/L and that from the MLE and UCT was >1.22 mg/L (Figure 22c). The DSVI measured from the 3SB configuration was >216 and the one measured from the MLE and UCT configuration was <170 (Figure 22d).

Studies conducted by Wang et al. (2018) demonstrated that N in the form of NH_4^+ and NO_3^- reduced bacterial abundance and diversity. Another study by Yan at el. (2017) also demonstrated that N and seasonal changes significantly affect the diversity and abundance of the microbial community. DSVI is an expression of the overall quality of the activated sludge settleability. It has been determined that a DSVI >150 mgL^{-1} is indicative of bulking sludge. The DSVI from the 3SB configuration was higher (>216) than that from the UCT and MLE. This was because of the over-proliferation of *Thiothrix* spp. and Eikelboom Type 021N that was noted during the study period which resulted in severe filamentous bulking. Studies conducted by Lakay et al. (1999) demonstrated that the proliferation of low F/M filamentous bacteria increase the DSVI. The overgrowth of *Thiothrix* spp. and Eikelboom Type 021N was consistent with the fact that they have been associated with 'septic' sludge which can occur when there is insufficient aeration. The 3SB WWTP appeared to have suffered from insufficient aeration as evidenced by the low DO (<1 mgL^{-1}) value. Operators typically strive to maintain DO levels in the aerobic tank of AS WWTPs between 1-2 mgL^{-1} to ensure adequate removal of NH_4^+ (nitrification) and organics and to maintain good floc structure. The findings of this study were also consistent with studies conducted by Xu et al. (2019) who demonstrated that DO concentration affects the microbial community structure. At low DO, the volume of sludge

was reduced, and facultative bacteria, anaerobic bacteria, and filamentous bacteria were dominant.

Table 40: BEST results of the functional microbial community structure and the Euclidian distance of the transformed and normalized data for the Influent, activated sludge and solids (CoCT - Configuration)		
Sample statistic (Rho): 0.643* Number of permutations: 99 (Random sample)		
No.Varables	Corr. Selections	Parameter(s)
4	0.643	AS TP, AS DO, inf. Amm., DSVI
3	0.633	AS TP, inf. Amm., DSVI
5	0.626	AS TP, AS DO, inf. Amm., DSVI, inf. ortho-P
4	0.622	AS TP, AS DO, DSVI, inf. ortho-P
5	0.483	AS TP, AS DO, inf. Amm., DSVI, FM ratio (COD)

*significance ($0.01 \geq p < 0.05$).

AS – Activated sludge, Inf – Influent, Amm. – Ammonium, AlK – Alkalinity

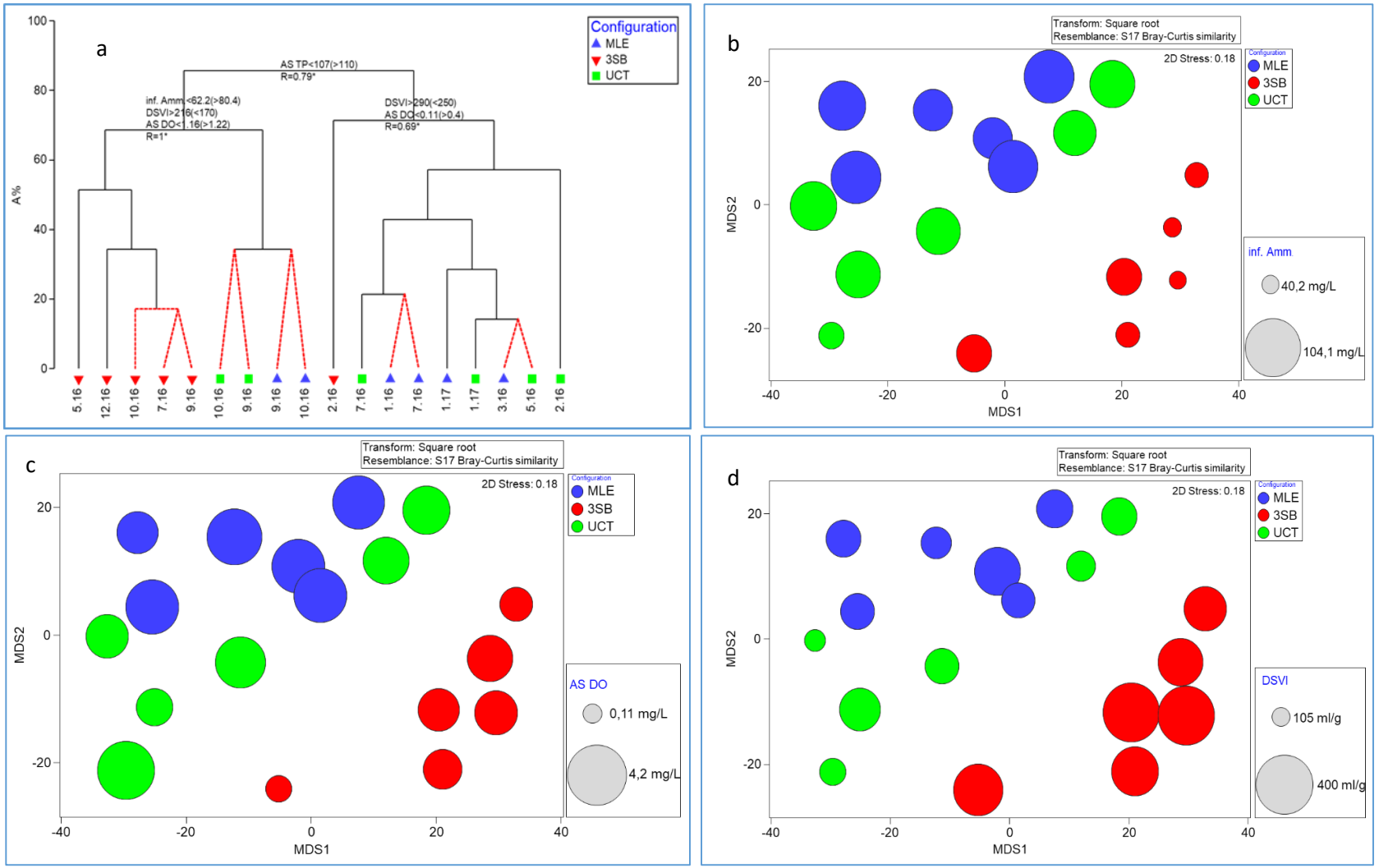

Figure 22: Plots for the physiochemical parameters from the configuration at City of Cape Town: LINKTREE of abiotic and biotic data from the three configurations at the City of Cape Town (a), bubble overlays of the most important physicochemical drivers (dissolved oxygen, ammonia and DSVI) of the microbial community structure determined using BEST (b, c and d)

BEST analysis performed on the Bray-Curtis similarity data of the NGS data and the Euclidian distance of the transformed and normalized data for the Influent, AS and solids measured from the CoE's configuration data (Table 41) showed that the global Rho was 0.456 (a fairly good correlation) but not significant ($p>0.05$). The LINKTREE (Figure 23a) showed no clustering related to the configuration. Although the dp denoting the OBCS in the MLE and 3SB configured WWTPs did cluster away from one another (Figure 23b), this did not appear to be driven by particular physicochemical parameters or combinations thereof (Figure 23c), the observation was not significant ($p>0.05$).

Table 41: BEST results of the functional microbial community structure and the Euclidian distance of the transformed and normalized data for the Influent, activated sludge (CoE - Configuration)

Sample statistic (Rho): 0.456
Number of permutations: 99 (Random sample)

No.Varables	Corr. Selections	Parameter(s)
5	0.456	AS pH, AS TP, AS Sulf. Inf. Alk, inf. VFA
4	0.452	AS pH, AS TP, AS Sulf., inf. VFA
5	0.447	AS pH, AS TP, AS Sulf. AS ortho - P, inf. VFA
5	0.440	AS pH, AS Sulf., AS ortho - P, inf. VFA, Inf. Alk
4	0.434	AS pH, AS Sulf., AS ortho - P, inf. VFA

AS – Activated sludge, Inf – Influent, Amm. – ammonium, AlK – Alkaline, VFA – Volatile fatty acids

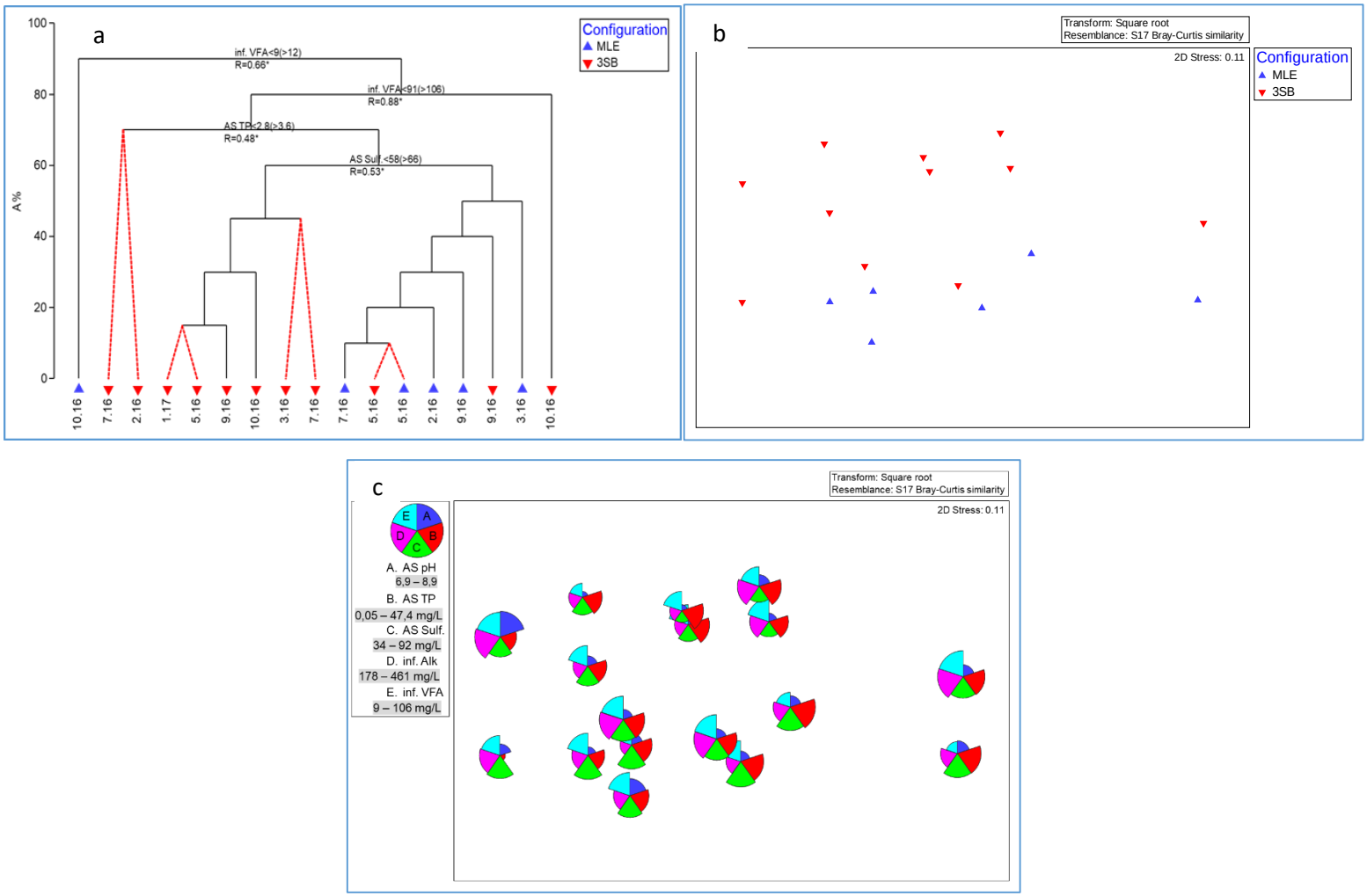

Figure 23: Plots results of the physiochemical parameters from the configuration at City of Ekurhuleni: LINKTREE of abiotic and biotic data from the two configurations at City of Ekurhuleni (a), the nMDS plot for the configuration at the City of Ekurhuleni (b), bubble overlays of the most important physicochemical drivers (pH, Tptal phosphate, sulfate, alkaline and volatile fatty acids) of the microbial community structure determined using BEST (c)

4.4. BACTERIAL COMMUNITY COMPOSITION IN WASTEWATER TREATMENT PLANTS AT THE CITY OF CAPE TOWN AND CITY OF EKURHULENI

A total of 2 645 unique zOTUs were generated with a frequency of 530 594 generated across the 50 samples with median of 10 101 per sample (range 7592 to 14 890). Horizontal asymptotes were generated for all samples in the rarefaction curves of sequencing depth plotted against richness (observed features/zOTUs) (Figure 24A) and the phylogenetic diversity measured using Faith's diversity index (Figure 24B). This indicated that the sequencing depth was sufficient to capture most of the bacterial diversity in each sample.

It is widely accepted that some biases will be introduced during one or more of the processes involved in obtaining the final amplicon sequencing output for final interpretation, starting right from sampling and DNA extraction (Keisam et al., 2016; Lee et al. 2012). Methods and analyses pipelines are constantly being changed and upgraded to minimize bias. Rarefaction is the practice of normalizing the sequencing output data after prior processing (including chimera removal and denoising) by randomly discarding reads so that all the samples contain the same number of reads as the sample with the lowest number of reads (Willis, 2019). Although data rarefaction is widely applied (e.g Travanty et al., 2019; Pramanick et al., 2022), in this study the data was not rarefied because it has been well argued that this practice discards vast amounts of potentially important information (McMurdie and Holmes, 2014) and introduces significant negative bias (Willis, 2019).

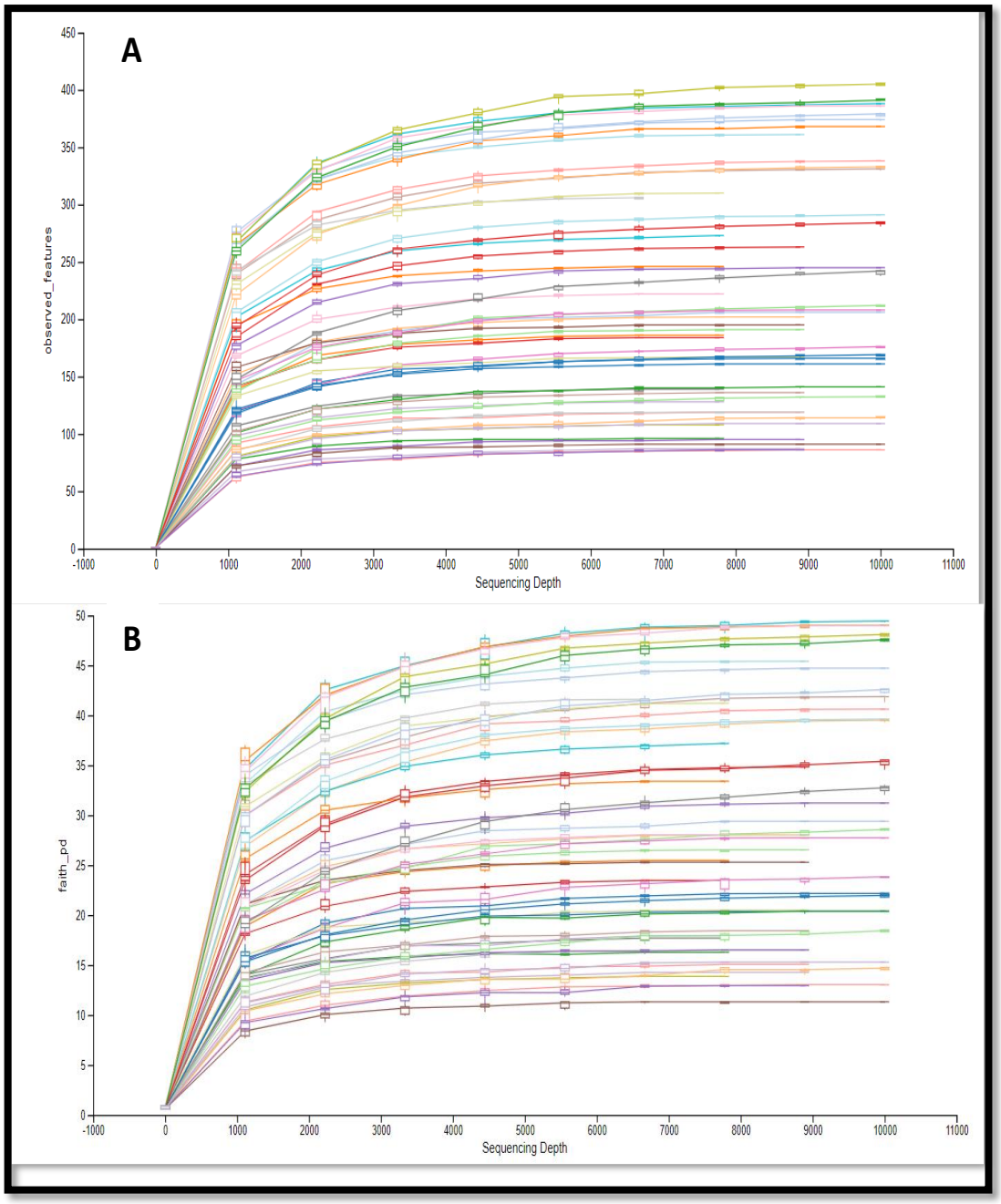

Figure 24: Rarefaction curves of the sequencing depths plotted against (A) richness and (B) phylogenetic diversity

A large number of samples (136) were screened for differences/similarities in the OBCS using T-RFLP. The T-RFLP results were used to rationalise the number of samples for amplicon sequencing (NGS) of the V4 region of the small subunit (SSU) of the 16S rRNA gene using the primer pairs 515F-Y (Parada et al., 2016) and revised 806-R (Apprill et al., 2015). These results were used to compile lists of taxa from the 50 selected samples. Distinct phyla, orders, classes, families, genera and species were reported as percentage or fractions of the total abundance,

which is equivalent to RA. A total of 27 phyla, 58 classes, 124 orders, 277 families and 832 genera were identified from all the WWTPs (CoE and CoCT). Some of the taxa were observed in high RA, some in low RA, and some were absent in some samples. Some taxa were prevalent in all the WWTPs, while others were not. This study focused mainly on analysing the families and genera because the primer set that was used does not discriminate between taxa well at species level (Apprill et al., 2015; Parada et al., 2016).

4.5. THE RELATIVE ABUNDANCE OF THE OF THE BACTERIAL PHYLA IN THE WASTEWATER TREATMENT PLANTS AT THE CITY OF CAPE TOWN AND CITY OF EKURHULENI

The RA of the dominant bacterial phyla at the two samplings sites (CoCT and CoE) are presented in Figure 25. The bacterial diversity was composed of 27 phyla. The most abundant bacterial phyla from the two sampling sites (CoCT and CoE) were *Proteobacteria* (Figure 25). *Proteobacteria* accounted for 34.75 - 86.42% RA at the CoCT and 37.133 – 77.89% RA at the CoE. The sub-dominating bacterial phyla were *Bacteroidetes* (5.08 – 32.72% at CoCT and 10.50 – 45.88% at CoE), *Actinobacteria* (2.82 – 21.35% at CoCT and 1.43 – 14.16% at CoE), *Firmicutes* (1.87 – 13.32% at CoCT and 1.30 – 5.90% at CoE), *Acidobacteria* (0.12 – 8.18% at CoCT and 0.10 – 3.93 at CoE), *Chloroflexi* (1.05 – 9.50 at CoCT and 0.30 – 9.92% at CoE) and *Planctomycetes* (0.13 – 5.22% at CoCT and 0.09 – 1.46% at CoE).

Proteobacteria was the most dominant bacterial phylum in both sampling sites (CoCT and CoE) and this is consistent with studies conducted by Wang et al. (2012), Zhang et al. (2012), Meerbergen et al. (2017) and Zhang et al. (2019), who also found that the most abundant bacterial phylum in municipal WWTPs was *Proteobacteria*. These studies also showed that *Bacteroidetes, Actinobacteria, Firmicutes, Acidobacteria, Chloroflexi* and *Planctomycetes* were present in high RA.

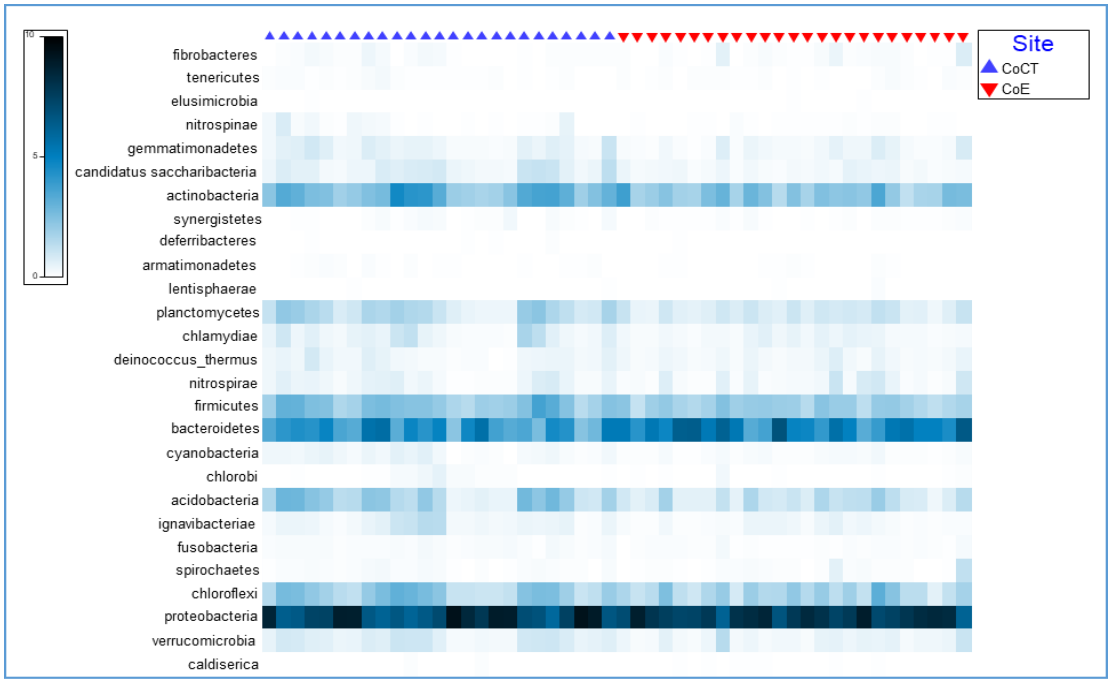

Figure 25: Shade plot (heat map) of the relative abundance of dominant bacterial phyla in the wastewater treatment plants at the City of Cape Town and City of Ekurhuleni.

4.6. THE RELATIVE ABUNDANCE OF THE DOMINANT FAMILIES IN WASTEWATER TREATMENT PLANTS AT THE CITY OF CAPE TOWN AND CITY OF EKURHULENI SAMPLING SITES

A total of 277 families were identified, of which 256 were common to both CoCT and CoE. A total of 269 and 264 families were identified from the CoE and CoCT, respectively. From the CoCT, a total of 165 and 109 families were common in all configurations during all four seasons, respectively. From the CoE, a total of 181 and 106 were common in all configurations and during all four seasons, respectively.

The taxonomic profiles between the CoE and CoCT differed (Figure 26) as expected from the nMDS and ANOSIM results (Section 4.1.1, Figure 14). *Pseudomonadaceae* dominated at the CoE, ranging from 0.53 – 51.21% and *Moraxellaceae* (0.25 – 39.83%) dominated at the CoCT. The sub-dominating families from the CoCT were *Pseudomonadaceae* (0.34 – 43.88%), *Saprospiraceae* (1.01 – 21.63%), *Chitinophagaceae* (1.01 – 7.93%) and *Acidobacteriaceae* (0.10 – 7.55%). The sub-dominating families from the CoE included *Flavobacteriaceae* (0.23 – 39.26%), *Moraxellaceae* (0.81 – 32.12%), *Xanthomonadaceae* (1.41 – 12.81%), *Saprospiraceae* (0.19 – 12.00%) and *Sphingobacteriaceae* (0.31 – 8.04%). The core bacterial family community structure (families >2%) varied notably between the two sampling sites. The variation in

taxonomic profiles supported the results shown in the nMDS plot (Figure 14b) and ANOSIM (Table 19), that the OBCS from the two sampling sites were distinctly different. These results are also supported by a study conducted by Zhang et al. (2012) from municipal WWTPs in China and North America. Their study showed through cluster analysis that the OBCS from WWTPs from different geographic locations group or cluster apart from one another. In their studies Zhang et al. (2012) found that 35 families accounted for 71-84% of the RA and that *Nocardioidaceae, Streptococcaceae, Cystobacteriaceae, Polyangiaceae, Rhodocyclaceae* and *Verrucomicrobiaceae* were found in all AS samples. In the WWTPs from North America, the RA of *Flavobacteriaceae, Comamonadaceae* and *Sphingomonadaceae* were significantly higher than in mainland China, where *Anaerolineaceae, Planctomycetaceae, Bradyrhizobiaceae, Desulfobacteraceae, Hyphomicrobiaceae* and *Rhodospirillaceae* were found in significantly higher RA (Zhang et al., 2012). In AS WWTP in Moscow, Russia, Shchegolkova et al. (2016) found *Planctomycetaceae, Opitutaceae, Verrucomicrobiaceae* and *Caldilineaceae* to be the most dominant families. In this (SA) study *Pseudomonadaceae* and *Moraxellaceae* dominated in the WWTPs in the CoE and CoCT respectively. These findings all support the hypothesis that WWTPs from different geographic region harbor different bacterial communities.

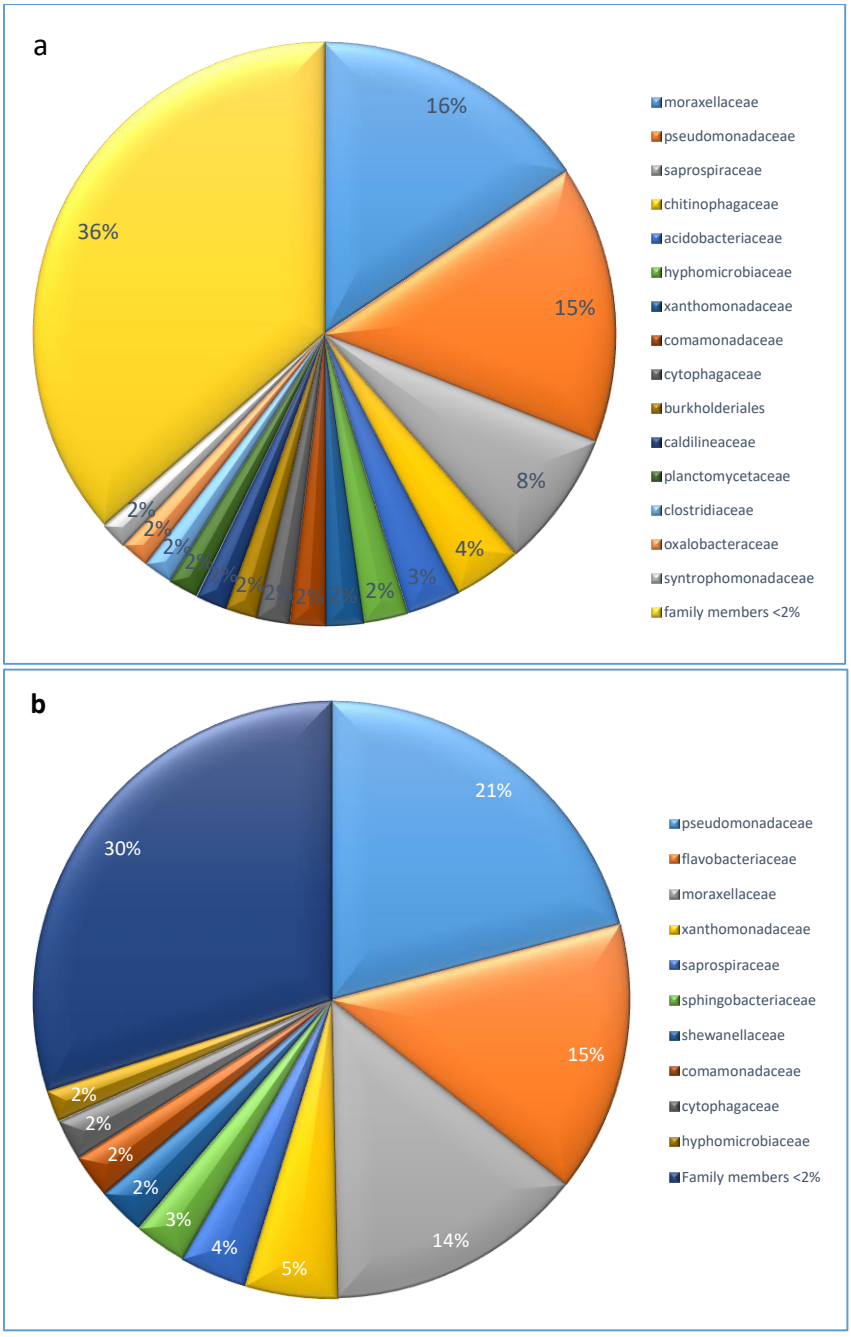

Figure 26: The relative abundance of the dominant bacterial families at the City of Cape Town (a) and the City of Ekurhuleni (b)

Quantitative evaluation of bacterial zOTU abundance was evaluated using diversity indices. Alpha diversity which is a diversity at the habitat level (intra-community diversity) is the most widely used and has two components i.e. species richness and evenness. Species richness measures the number of species per unit area or per sample. Species evenness gives the relative abundance of different species of a community (Thukral, 2017). Shannon-Weaver index (H') is one of the most widely used diversity indices (Konopiński, 2020). Univariate diversity measurements may also assist in establishing sampling coverage (Roswell at el., 2021). Univariate diversity indices (Table 42) for the bacterial families in the CoCT and CoE WWTPs showed that the CoCT WWTPs had a slightly higher diversity (Shannon) than the CoE WWTPs. It was postulated that this was due to the higher nutrient load in the influent, and the fact that the UCT and 3-SB configured WWTPs at the CoCT received on average 5% industrial effluent, while all of the WWTPs from the CoE received 100% domestic effluent. According to Ibarbalz et al. (2013), the characteristics of wastewater (domestic, industrial or a mixture of domestic and industrial) can have a decisive influence on the OBCS.

Table 42: Comparison of the univariate diversity indices of the bacterial families in the Cape Town and City of Ekurhuleni wastewater treatment plants

	Richness (*n*)	Evenness (J')	Shannon diversity (H)	Coverage
CoCT	264	0.87611	4.8851	0.99905
CoE	269	0.85623	4.7903	0.99733

The RA of bacterial families in the differently configured WWTPs from both sites is shown in Figure 26. In the CoCT WWTPs (Figure 27 a-c), the two most abundant families in all configurations were *Moraxellaceae* (0.39 – 39.83% at MLE, 0.46 – 39.06% at 3SB and 0.26 – 36.54% at UCT) and *Pseudomonadaceae* (0.50 - 30.89% at MLE, 0.41 – 43.87% at 3SB and 0.34 – 38.93% at UCT). The two sub-dominating families were *Saprospiraceae* (4.73 – 19.54% at MLE, 2.43 – 21.63% at 3SB and 1.01 – 6.39% at UCT) and *Acidobacteriaceae.* The taxonomic profile (families with average RA ≥ 3%) from the MLE configured WWTP included *Chitinophagaceae, Comamonadaceae*, *Xanthomonadaceae*, *Burkholderiales* and *Hyphomicrobiaceae*; from the 3SB configured WWTP they included *Chitinophagaceae,* and from the UCT configured WWTP they included *Chitinophagaceae, Cytophagaceae, Xanthomonadaceae*, *Hyphomicrobiaceae* and *Syntrophomonadaceae*. The univariate diversity indices (Table 43) showed that that the UCT configured WWTPs had a slightly higher bacterial family diversity (Shannon) followed closely by the MLE and then the 3SB configured WWTPs. The bacterial community structure in the MLE and UCT were slightly higher than the 3SB in

the evenness. It was postulated that the two drivers (i.e. AS DO and influent NH_4^+) noted on the LINKTREE (Figure 22a) might have influenced the bacterial community evenness.

Table 43: Comparison of the univariate diversity indices of bacterial families in the wastewater treatment plants with different configurations at the City of Cape Town

	Richness (*n*)	Evenness (J')	Shannon diversity (H)	Coverage
MLE	244	0.8810	4.843	0.9989
3SB	247	0.8616	4.747	0.9972
UCT	241	0.8878	4.869	0.9994

In the CoE WWTPs (Figure 27 d-f), the three most abundant families were *Pseudomonadaceae* (which ranged from 0.54 – 51.21% at MLE, 3.38 – 40.10% at 3SB_D and 0.53 – 49.56% at 3SB_R) followed by *Flavobacteriaceae (Weeksellaceace)* (0.23 – 32.26% at MLE, 2.53 – 39.26% at 3SB_D and 1.31 – 27.25% at 3SB_R) *and Moraxellaceae* (0.91 – 29.15% at MLE, 2.74 – 32.12% at 3SB_D and 0.81 – 30.73% at 3SB_R). The sub-dominating families (families RA ≥ 3%) from the MLE configured WWTPS were *Saprospiraceae, Xanthomonadaceae, Sphingobacteriaceae;* 3SB_E1 were *Xanthomonadaceae, Sphingobacteriaceae, Shewanellaceae* and *Comamonadaceae* and from the 3SB_E3 were *Xanthomonadaceae*, *Cytophagaceae* and *Shewanellaceae.* The univariate diversity indices for the different configurations are shown in Table 44. A similar diversities (Shannon) were seen in the bacterial families in both the 3SB configured WWTPs as well as the MLE configured reactors. The OBCS in all the configurations were evenly distributed however, the community in the 3SB_E3 was slightly more even than those in the other two configurations. This suggested that the environmental conditions that prevailed did not favour a specific bacterial family, which is supported by the BEST analysis (Table 41) and LINKTREE (Figure 23).

(**Note**: *The 5SB configuration samples were not represented as they were not part of the short-listed samples for the NGS analysis. The 3SB configuration accommodated two of the three WWTPs analysed for NGS. In this study, the 3SB WWTPS were analysed separately, designated as 3SB_E1 and 3SB_E3 to allow comparisons between 2 reactors with the same configuration and from the same site*.

Table 44: Comparison of the univariate diversity indices of bacterial families in the wastewater treatment plants with different configurations at the City of Ekurhuleni

	Richness (*n*)	Evenness (J')	Shannon diversity (H)	Coverage
MLE	249	0.8535	4.709	0.9967
3SB_E1	248	0.8594	4.738	0.9966
3SB_E3	246	0.8625	4.749	0.9971

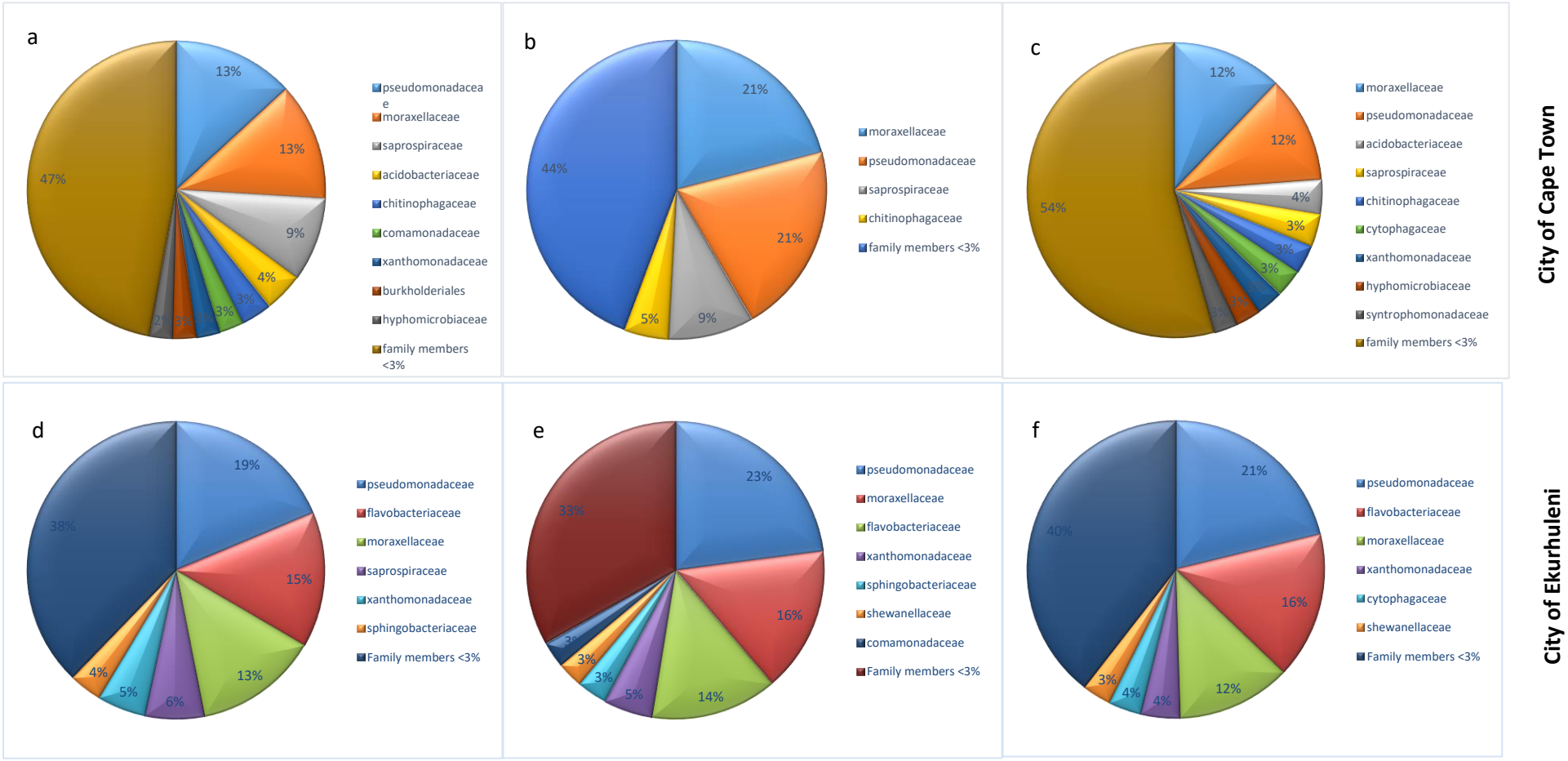

Figure 27: The relative abundances of the dominant bacterial families in the wastewater treatment plants configuration at the City of Cape Town and City of Ekurhuleni: Modified Ludzack Ettinger (a), 3-stage Bardenpho (b), University of Cape Town (c), Modified Ludzack Ettinger (d), 3-stage Bardenpho_E1 (e) and 3-stage Bardenpho_E3 (f).

The RA of bacterial families during the four seasons is shown in Figure 28. In the CoCT WWTPs (Figure 28 a-d), *Pseudomonadaceae* dominated in summer and autumn (0.39 – 41.28% in summer and 0.34 – 43.88% in autumn) whilst *Moraxellaceae* dominated winter and spring (14.87 – 39.06% in winter and 9.11 – 39.83% in spring). The two sub-dominating families during summer and autumn were *Moraxellaceae* (0.26 – 27.03% in summer and 0.42 – 37.52% in autumn) and *Saprospiraceae* (1.01 – 16.04% in summer and 2.94 – 19.54% in autumn). The two sub-dominating families during winter and spring were *Pseudomonadaceae* (11.36 – 24.31% in winter and 0.41 – 38.93% in spring) and *Saprospiraceae* (6.39 – 15.41% in winter and 2.61 – 21.63 in spring). Families that followed the top three (*Pseudomonadaceae, Moraxellaceae* and *Saprospiraceae)* varied with each season. Those with a RA ≥ 3% included *Chitinophagaceae, Hyphomicrobiaceae, Acidobacteriaceae* and *Xanthomonadaceae* in summer*; Acidobacteriaceae, Chitinophagaceae and Cytophagaceae* in autumn; *Chitinophagaceae* and *Acidobacteriaceae* in winter*;* and *Oxalobacteraceae* and *Chitinophagaceae* in winter. The univariate diversity indices for bacterial families during the different seasons are shown in Table 45**.** The highest diversity (Shannon) was found in summer, followed by autumn, winter and spring.

Table 45: Comparison of univariate diversity indices of bacterial families in the wastewater treatment plants during different seasons at the City of Cape Town

	Families (*n*)	Evenness (J')	Shannon diversity (H)
Summer	257	0.8882	4.929
Autumn	240	0.8886	4.870
Winter	217	0.8820	4.725
Spring	239	0.8566	4.691

In the CoE WWTPs (Figure 28 e-h), the most abundant families found during summer and spring were *Pseudomonadaceae* (14.63 – 51.21% in summer and 0.53 – 40.10% in spring), followed by *Flavobacteriaceae* (0.23 – 27.25% in summer and 1.31 – 20.06% in spring). In autumn and winter *Moraxellaceae* (2.74 – 30.73% in autumn and 25.81 – 26.48% in winter) was the most dominant family followed by *Flavobacteriaceae* (2.53 – 39.26% in autumn and 17.38 – 32.26% in winter). The remaining families with RA ≥ 3% were *Moraxellaceae, Xanthomonadaceae, Saprospiraceae* and *Sphingobacteriaceae* in summer*; Pseudomonadaceae, Sphingobacteriaceae, Saprospiraceae, Xanthomonadaceae, Shewanellaceae* and *Comamonadaceae* in autumn; *Pseudomonadaceae, Xanthomonadaceae, Comamonadaceae* and *Sphingobacteriaceae in winter; Moraxellaceae, Xanthomonadaceae, Cytophageceae, Shewanellaceae, Saprospiraceae, Hyphomicrobiaceae* and *Chitinophagaceae*

in spring. The univariate diversity indices during the four seasons are shown in Table 46**.** The highest diversity (Shannon) was found in spring, followed by autumn, summer and winter

Table 46: Richness and diversity indices of bacterial families in the wastewater treatment plants during different season at the City of Ekurhuleni

Sample	Families (*n*)	Evenness (J')	Shannon diversity (H)
Summer	240	0.84822	4.6488
Autumn	248	0.85078	4.6907
Winter	202	0.82032	4.3545
Spring	255	0.87448	4.8457

The species richness and diversity during the warmer and cool season varied. In the warmer season both species richness and diversity were high and in the cooler season were lower. Similar results were found by Fang et al. (2021), who showed that temperature significantly affected the alpha diversity of soil bacterial communities. The Shannon index values for bacterial community at 32^{o}C were significantly higher than those of other temperature gradients, and soil bacterial richness and diversity increased significantly ($p<0.05$). This study also showed that during winter, species richness decreased along with the Shannon index.

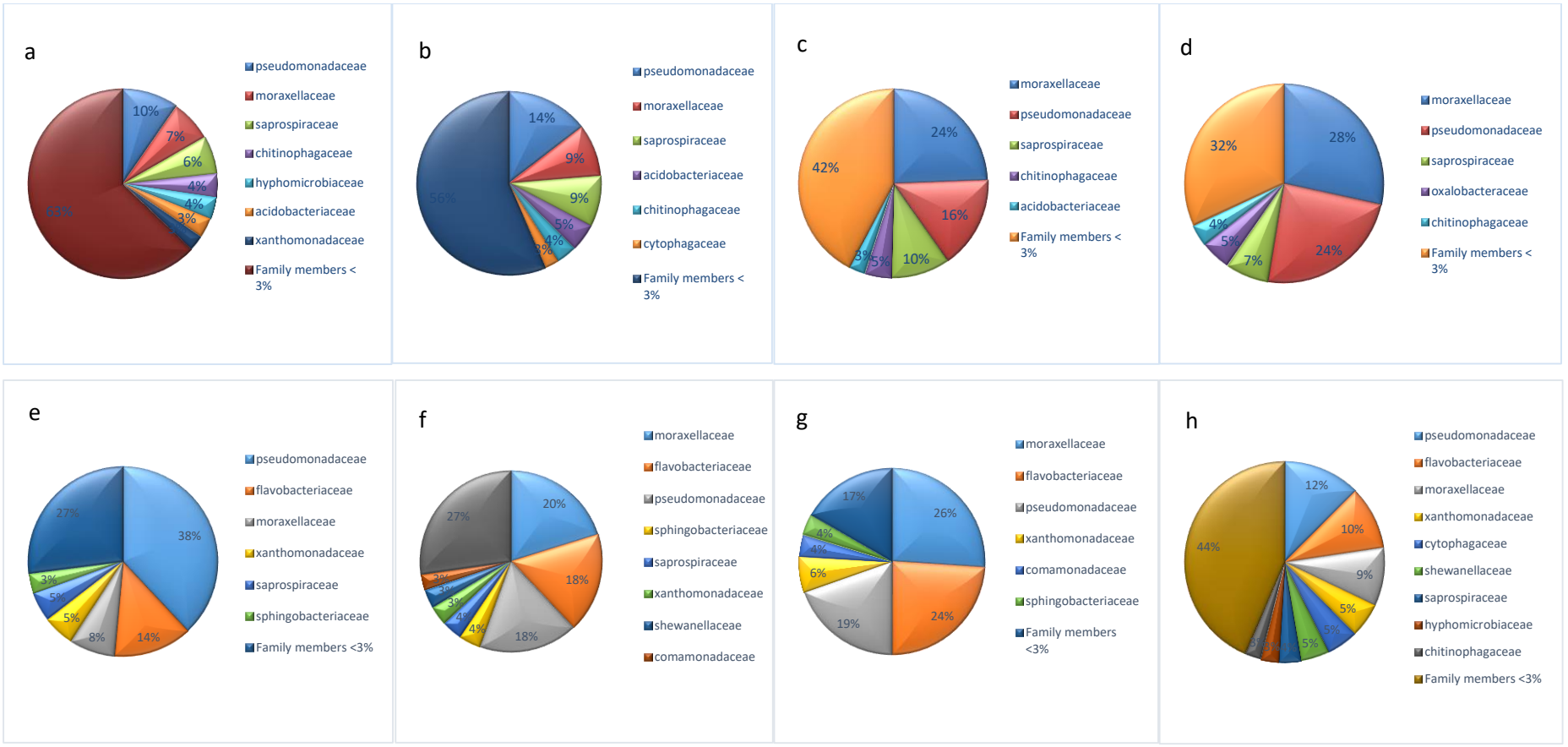

Figure 28: The relative abundances of the dominant bacterial families in all four seasons at the City of Cape Town and City of Ekurhuleni: summer, autumn, winter and spring at the City of Cape Town (a - d), and summer, autumn, winter and spring at the City of Ekurhuleni (e – h).

4.7. THE RELATIVE ABUNDANCE AND ESTIMATED ABSOLUTE ABUNDANCE OF THE DOMINANT GENERA IN WASTEWATER TREATMENT PLANTS IN THE CITY OF CAPE TOWN AND CITY OF EKURHULENI

A total of 832 genera were identified in all samples. Seventeen (17) genera accounted for 51.27 – 64.25% of the identified genera. Of the 832 genera, 749 were common to both the CoCT and CoE WWTPs. A total of 798 and 783 genera were identified from the CoE and CoCT, respectively. Of these, 404 and 157 genera were common in all configurations and all four seasons in the CoCT WWTPs, respectively. Similarly, 394 and 67 genera were commonly identified in all the configurations and in all four seasons at the CoE WWTPs, respectively.

The outputs of this study described in Sections 4.5 to 4.6 were provided in RA. Relative abundance is an expression of the relative proportion (%) of OTUs or taxa within each sample and is effective in establishing which microbial taxa dominate in different environments or change within environments on a temporal basis (Williamson et al., 2022; Tang, 2019). The actual abundance (AA) is the actual amounts of taxa in a unit volume of an ecosystem (Lin and Peddada, 2020). Although widely used, RA determinations are skewed because different bacterial taxa have different copy numbers of the 16S rRNA gene (Starke et al., 2021; Pereira-Flores et al., 2019). This has a knock-on effect if the AA is calculated from RA data and there is no reliable method to correct for these differences for either (Starke et al., 2021; Louca et al., 2018). Another inherent limitation of using RA to compare microbial data from different samples is that the RA of functional taxa may be similar in two samples, but the absolute abundance (AA) could be orders of magnitude lower in samples with less microbial biomass (Barlow et al., 2020; Finnegan and Droser, 2005). For example, Yao et al. (2022) assessed both the RA and AA of gut microbiota, and found that numbers of microbes were higher in the early fermentation stage than later in the fermentation, a pattern that was not reflected in the RA. This was expected as the substrate was readily degraded and used as an energy source for metabolism and growth during the early fermentation stage. Furthermore, if bacteria are not sourcing energy directly from substrate degradation, an increase in RA may be accompanied by a decrease in AA (Yao et al., 2022).

To overcome these limitations, AA needs to be calculated for accurate and comprehensive interpretation of the biological and ecological implications of bacterial community structure and function (Kong et al., 2021). The AA can be calculated using different methods and also

through the transformation of the RA, but methods have limitations (Barlow et al., 2020) or are too complex for routine use (Williamson et al., 2022).

In this study, a relatively simple method was used to estimate the AA (estimated absolute abundance (EAA) from the RA because in order to link structure with function in AS, the AA of functional species is important (Perreira-Flores et al., 2019). As exactly the same amount of sludge (1 g) was used for DNA extraction using the same method and reagent quantities for each extraction in this study, the AA was estimated using Equation 16. It is acknowledged that there are inherent drawbacks with the method employed. Notwithstanding the methodological limitations, some insight was provided into the actual amounts of bacterial taxa present in the different WWTPS in the CoE and the CoCT.

Table 47 provides the EAA of the 10 most dominant genera from each sampling site which were selected for inter-site and intra-site comparative purposes, giving a total of 17 genera. The EAA of top 10 genera displayed large variations between and within in each sampling site (Figure 29). The most dominant genus from the two sampling sites (CoE and CoCT) was *Pseudomonas* with a mean EAA of 23 358 ± 19 720 cells/ngDNA.gsludge^{-1} in the CoE WWTPs and a mean of 20 582 ± 39 028 cells/ngDNA.gsludge^{-1} in the CoCT WWTPs.

The EAA of the genera *Pseudomonas* and *Acinetobacter* was notably higher in the MLE and UCT WWTPs during spring in the CoCT WWTPs (Figure 29a). The EAA of *Pseudomonas* ranged from 60 953 – 79 624 cells/ngDNA.gsludge^{-1} in the MLE WWTP and 19 647 – 182 027 cells/ngDNA.gsludge^{-1} in the UCT WWTPs. *Acinetobacter* ranged from 46 929 – 162 440 cells/ngDNA.gsludge^{-1} in the MLE WWTP and from 24 845 – 125 200 cells/ngDNA.gsludge^{-1} in the UCT WWTP. In the CoE (Figure 29b), *Pseudomonas* was prevalent in all the WWTPs to varying degrees (1159 – 82 342 cells/ngDNA.gsludge^{-1}), with no distinct tends noted for either configuration or season, whilst *Acinetobacter* had a generally low EAA (82 – 27 729 cells/ngDNA.gsludge^{-1}).

Lewinella and *Acidobacterium* were generally more abundant (EAA range: 2 354 – 28 771 cells/ngDNA.gsludge^{-1} and from 1 498 – 31 791 cells/ngDNA.gsludge^{-1} respectively) in the MLE WWTP compared to the other configurations in the CoCT WWTPs. In the CoE, the EAA of *Lewinella* and *Acidobacterium* were relatively low. *Lewinella* ranged from 150 - 18 523 cells/ngDNA.gsludge^{-1} whilst *Acidobacterium* ranged from 86 – 15 256 cells/ngDNA.gsludge^{-1}.

The other two most prevalent genera at the CoE were *Pyschrobacter* and *Chryseobacterium*. The EAA of *Pyschrobacter* ranged from 64 – 73 466 cells/ngDNA.gsludge^{-1} whilst *Chryseobacterium* ranged from 71 - 76 832 cells/ngDNA.gsludge^{-1}. In contrast, the EAA of *Pyschrobacter* and *Chryseobacterium* was relatively low in the CoCT WWTPs. *Pyschrobacter* ranged from 34 – 21 228 cells/ngDNA.gsludge^{-1} whiles *Chryseobacterium* ranged from 30 - 880 cells/ngDNA.gsludge^{-1}.

Flexibacter and *Shewanella* displayed a fairly high EAA in spring in both the MLE and 3SB-E3 WWTPs in the CoE (Figure 29b). In the MLE WWTP, the EAA of *Flexibacter* was 142 163 cells/ngDNA.gsludge^{-1} whilst in the 3SB-E2 WWTP, the EAA was 35 984 cells/ngDNA.gsludge^{-1}. The EAA of *Shewanella* was 40 656 cells/ngDNA.gsludge^{-1} in the MLE WWTP and 64 388 cells/ngDNA.gsludge^{-1}) in the 3SB-E2 WWTP. The genus *Terrimonas* was found in fairly high EAA (28 337 cells/ngDNA.gsludge^{-1}) during spring in the 3SB-E2 WWTP. No year-on year seasonal or configuration trends were noted for any of the other most abundant genera either.

The bacteria in WWTPs play significant functional roles in biodegradation of organic matter and/or nutrient removal (Oluseyi Osunmakinde et al., 2019). Activated sludge is mostly dominated by heterotrophs that may remain stable under various environmental conditions (Kennes-Veiga *et al.*, 2021). Heterotrophs obtain their energy from the oxidation of organic matter. The release of energy through the aerobic reactions is more than from anaerobic reactions. The aerobic heterotrophs reproduce and stabilise the organic matter faster than the anaerobes. Because of the reproduction rate of the aerobic organisms being greater, sludge generation is also greater (Von Sperling, 2007).

These bacteria mineralise organic compounds using them as sources of energy as described in Section 2.2.1 (McIlroy et al., 2015; Galushko and Kuever, 2015). In this study, the heterotrophs *Pseudomonas, Chryseobacterium*, *Psychrobacter, Acinetobacter* and *Lewinella,* were the most abundant bacterial genera. The inter- and intra-site variability in the abundance of these genera in the AS samples suggest that they were in a constant state of flux with other genera that fulfil the same functional role in AS. Indeed, some of these genera exhibit similar metabolic functions with one another in addition to heterotrophy. For example, N-acyl homoserine lactone (AHL), a chemical that is associated with control of biofilm

formation via quorum sensing, is secreted by *Shewanella, Chryseobacterium, Acinetobacter* and *Pseudomonas* (Liébana et al., 2016).

Pseudomonas strains can be metabolically versatile (Michalska et al., 2020) and some species such as *P. aeruginosa, P. stutzeri* and *P. mendocina* may be involved in denitrification because they can use NO_3^- as an alternative electron acceptor to O2 (McIlroy et al., 2015; Galushko and Kuever, 2015). Some species are also denitrifying PAOs (Li et al., 2022). *Acinetobacter* are common in WWTPs, often due to high abundance in the incoming wastewater (McIlroyet al., 2015 and Shchegolkova et al., 2016). *Acinetobacter* are well known as PAOs in AS WWTPs (Ni et al., 2021; Li et al., 2022).

In studies conducted by Ye et al. (2017), *Psychrobacter*, a psychrophilic bacterium, had the highest potential for activity in the anaerobic tank of an AS WWTP in winter (13°C - 18°C). It was postulated that the cold conditions were selective for this genus. Duan et al. (2021) found that *Psychrobacter* had the ability to decompose grease, and that it proliferated selectively in particulate organid matter with high grease content. It has also been shown that *Psychrobacter aquimaris* assists with N removal and formation of sedimentary granular biofilms (Zhang et al 2021).

Lewinella is a filamentous bacterium and members of the genera have been isolated from the marine environment and require NaCl for growth (McIlroy et al., 2015; Khan et al., 2007). Members of the *Chryseobacterium* genera are inhabitants of soil and water and some are found in municipal water (Steinberg and Burd, 2015).

Terrimonas has the ability to decompose refractory organics and toxic substances and simultaneously reduce NO_3^{2-} nitrate in AS in WWTPs (Wang et al., 2022). The genera *Shewanella* is a promising catalytic agent in microbial fuel cells that convert chemical energy into electrical energy (Juliastuti et al., 2018).

Other studies that identified bacterial species in AS found the most dominant genera as *Caldilinea, Dechloromonas, Zoogloea, Prosthecobacter, Subdivision3_genera_incertae_sedis* and Gp4 amongst others (Shchegolkova et al., 2016; Kim et al., 2013; Wang et al., 2012; Zhang et al., 2012). They also showed that WWTPs from different geographic locations were dominated by different genera. In this study, neither *Caldilinea, Dechloromonas, Zoogloea, Prosthecobacter, Subdivision3_genera_incertae_sedis* or Gp4 dominated. Only *Caldilinea* was

identified amongst the dominant genera and ranged from 117 – 17 149 cells/ngDNA.gsludge^{-1} in the CoCT and 97 – 12 628 cells/ngDNA.gsludge^{-1} in the CoE WWTPs (Figure 29).

This study noted 15 genera that were only found in either the CoCT or CoE. Of the 15 genera, 6 were only observed in the CoCT WWTPs (*Anaerofilum, Laribacter, Oscillibacter, Shimazuella, Sporobacter* and *Thiocapsa*), while and 9 were only observed in the CoE WWTPs (*Epulopiscium, Magnetospirillum, Maribacter, Natronoanaerobium, Planococcus, Rehaibacterium, Sulfurovum, Taibaiella* and *Timonella*).

Table 47: Top 10 dominant genera in the AS WWTPS in the City of Cape Town and City of Ekurhuleni (SD= standard deviation from the mean)

	CoE	CoCT
	Average ± SD	Average ± SD
Acidobacterium	1588 ± 2980	4326 ± 6481
Acinetobacter	5141 ± 7074	20249 ± 39448
Caldilinea	1158 ± 2458	2451 ± 3545
Chryseobacterium	14359 ± 18532	214 ± 227
Clostridium	1668 ± 2275	2071 ± 2154
Flavobacterium	3312 ± 4865	290 ± 324
Flexibacter	7264 ± 29007	140 ± 224
Janthinobacterium	435 ± 1207	2163 ± 4795
Lewinella	4291 ± 5692	7253 ± 6849
Pseudomonas	23358 ± 19720	20582 ± 39028
Psychrobacter	15767 ± 19477	1742 ± 4489
Shewanella	5040 ± 14799	36 ± 47
Sphaerotilus	1354 ± 3071	2519 ± 3630
Sphingobacterium	2955 ± 4199	562 ± 1025
Stenotrophomonas	2631 ± 4328	165 ± 349
Syntrophomonas	746 ± 1329	2216 ± 3311
Terrimonas	1807 ± 5614	2350 ± 1922

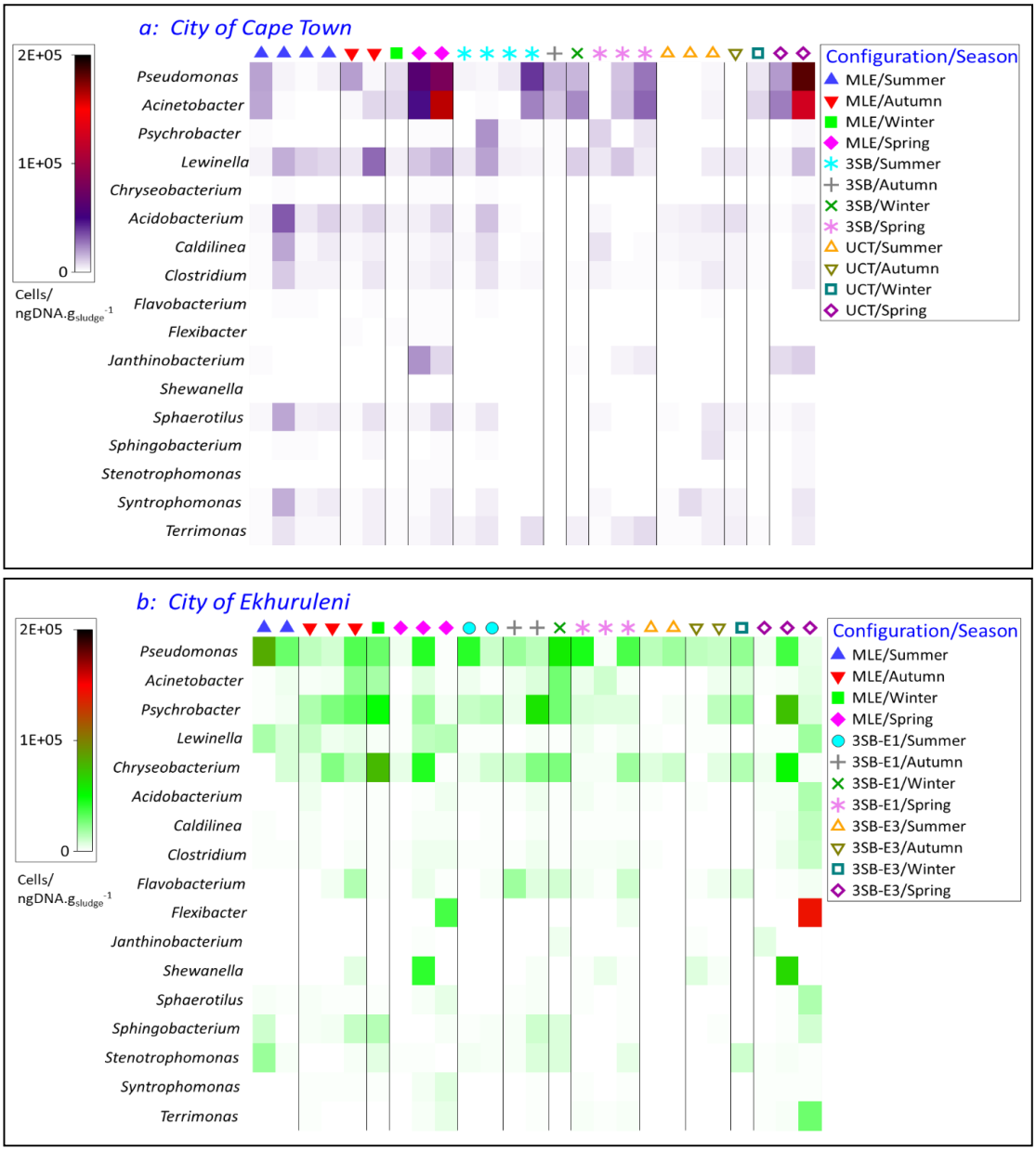

Figure 29: The estimated absolute abundance of the top 10 genera. The top genera observed at the City of Cape Town (a). The top genera observed at the City of Ekurhuleni (b)

5. CHAPTER FIVE - CONCLUSIONS

This study found that the T-RFLP and NGS both yielded similar trends when used as molecular fingerprinting tools to assess similarities and differences in the OBCS in AS samples. With the exception of 'configuration' for the CoE, the dp distribution on the nMDS plots generated using data from T-RFLP and NGS to assess the effects of site, configuration and season were highly similar. The processing cost per sample for T-RFLP is a fraction of the cost of amplicon sequencing (NGS). For the service providers used in this study, the cost of Illumina MiSeq is currently around R1598 per sample, while the local cost for T-RFLPs at Stellenbosch University is around R26 per sample. High throughput sequencing costs in SA are even higher, hence the need in this case to export samples for analysis to curtail costs and increase the number of analyses within allocated budgets. This study showed that, although T-RFLP is an older and is a less sophisticated fingerprinting technique than NGS and does not provide direct information on the taxa that are present, it can still be a valuable and reliable screening tool in cases where it important to include large datasets and funding is limited.

The study indicated that, in some instances, the factors 'configuration' (design) and 'season' had negligible but significant effects on the OBCS. However, a highly significant difference in the OBCS was noted for the factor 'site". It was shown that this was mainly related to differences in the physicochemical parameters in the influents and AS at each site (CoE and CoCT). Notably, the difference in the AS TP concentration was the primary driver for the differences in OBCS. The average AS TP concentration in samples from the CoCT and CoE were 127.8 ± 40.2 mg/L and 20.8 ± 17 mg/L, respectively. The LINKTTREE binary divisive cluster analysis indicated that the cut-off AS TP concentration for the CoE and CoCT clusters were < 47.4 mg/L and > 60 mg/L, respectively. Intra-site differences in the CoCT WWTPs that played the most significant roles in selection of the OBCS were AS DO (3SB was <1.16 mg/L and, MLE and UCT were >1.22 mg/L), influent NH_4^+ (3SB was <62.2 mg/L and, MLE and UCT were >80.4 mg/L) and DSVI (3SB was >216 mg/Land, MLE and UCT were <170 mg/L). It is postulated that that the highly significant difference for the factor 'configuration' in the CoCT WWTPs was largely as a result of the 3SB configured WWTP suffering low DO filamentous bulking during the study period, and not the design *per-se*. This was confirmed by the high DSVI readings (range: 145 - 663 mg/L) from observed overgrowth of filamentous bacteria. Overall, the findings were consistent with some studies, but not others conducted in other countries. Table 48 shows a summary of studies that found that geographical location and/or configuration and/or season played a role in microbial community selection in AS WWTPs.

From a community composition perspective, the bacterial profiles at phylum level were similar at both study sites. *Proteobacteria* was the most dominant bacterial phylum followed by *Bacteroidetes, Actinobacteria, Firmicutes, Acidobacteria, Chloroflexi* and *Planctomycetes.* At the lower family and genus taxonomic levels, the most abundant taxa differed between the 2 sites differed. The heterotrophs *Pseudomonas, Chryseobacterium*, *Psychrobacter, Acinetobacter* and *Lewinella* were the most abundant bacterial genera. These genera are all heterotrophic and exhibit similar metabolic functions.

It is recommended that future work is directed towards qualitative and quantitative analyses of the functional microbial species, particularly the nitrifying bacterial populations in SA AS WWTPs, and comparison of the results with global studies.

Table 48: Other studies that investigated the effects of season, location and process design on the bacterial community compositions in activated sludge from wastewater treatment plants treating primarily domestic wastewater based on 16S rRNA gene amplifications.

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., 2020
Cape Town, Ekhuruleni/South Africa	6/141	30 months	**No**	**Yes**	**No**	*This study*

*True seasonality over >1 year not established. NA = not applicable, ND = not determined

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