A combined metagenomics and metatranscriptomics approach to assess the occurrence and reduction of pathogenic bacteria in municipal wastewater treatment plants

Submitted in fulfillment for the Degree of Doctor of Philosophy in (Biotechnology) in the Department of Biotechnology and Food Technology, Durban University of Technology, Durban, South Africa

Thobela Conco

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Supervisor: Prof. Faizal Bux
Co-supervisor: Prof Sheena Kumari
Co-supervisor: Prof Thor Stenström
Co-supervisor: Dr Arshad Ismail

DECLARATION

I, Ms Thobela Precious Conco – 20823074, Prof. Faizal Bux (supervisor), Prof Sheena Kumari, Prof. Thor Stenström and Dr Arshad Ismail (co-supervisors) hereby declare that in respect of the following dissertation: Title: A combined metagenomics and metatranscriptomics approach to assess the occurrence and reduction of pathogenic bacteria in municipal wastewater treatment plants

1. As far as we ascertain no other similar dissertation exists.

2. All references as detailed in the dissertation are complete in terms of all published works consulted.

22/02/2023

Signature of student **Date**

22/02/2023

Signature of Supervisor **Date**

22/02/2023

Signature of Co-supervisor **Date**

22/02/2023

Signature of Co-supervisor **Date**

22/02/2023

Signature of Co-supervisor **Date**

APPROVAL

I hereby approve the final submission of the following dissertation.

Prof Faizal Bux
Supervisor
Doctoral Degree in Technology:
Biotechnology Durban Institute of Technology
(DIT)

Prof Sheena Kumari
Co-supervisor
PhD: Biosciences
Mangalore University, India

Prof Thor A. Stenström
Co-supervisor
PhD: Microbiology
Gothenburg University, Sweden

Dr Arshad Ismail
Co-supervisor
Doctoral Degree in Technology: Biotechnology
Durban Institute of Technology (DIT)

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DEDICATION

This thesis is dedicated to all who would dare to believe that “everything is possible to him who believes” (Mark 9v23). I especially dedicate it to my nieces Amahle Hadebe and Avela Conco, may my achievements be a launching pad for you do even greater and achieve more.

ABSTRACT

The emergence and spread of pathogens, antibiotic resistant bacteria (ARB) and antibiotic resistant genes (ARG) through insufficiently treated effluents from wastewater treatment plants (WWTP) pose a risk to human health and the environment. The present study focused on assessing the occurrence, prevalence and fate of dominant pathogenic bacteria, ARGs and mobile genetic element (MGE) in different WWTPs in Durban, Kwa-Zulu Natal, South Africa. The samples were taken from three wastewater treatment plants with different configurations, including trickling filter (TF), biological nutrient removal (BNR), and conventional activated sludge processes (CAS). Total genomic DNA and RNA were extracted from the samples for metagenomic and tracriptomic analysis.

A total of 23 pathogenic bacterial genera, including enteric and emerging opportunistic pathogens, were detected in the samples. *Acinetobacter* spp. and *Aeromonas* spp. were the predominant pathogens in influent metagenomes, while *Escherichia coli* and *Acinetobacter* spp. dominated influent transcripts. Based on Shannon-Wiener indices, the diversity of bacteria increased from influents to final effluents in two treatment plants. ARGs that confer resistance to aminoglycosides, beta-lactamases, tetracycline and sulfonamides were abundant in both influent and effluent samples. Results further exposed that MGE-ARG associations were the main drivers of ARG persistence to final effluents. This included 5 plasmids: R338-R151 (*sulI*), pRH-1238 (*strB*), pPM91 (*aadA*), pRH-1238 (*aadA4-5*), pRH-1238 (*sulII*); two class 1 integrons (*aadA* and *arr)* and 1 transposon Tn4351 (*tetX*). In transcripts, the MGE-ARG associations showed two plasmids: pRH-1238 (*aadA*) and pPM91 (*aadA*) and one hybrid plasmid R338-R151 (*sulI*).

The study investigated the potential impact of operational parameters (dissolved oxygen (DO), total suspended solids (TSS), pH and temperature) on selected bacterial pathogens (*Aeromonas* spp*, Acinetobacter* spp., *Pseudomonas aeruginosa* and *Klebsiella pneumoniae)* and their fates at different stages of the three WWTPs. Principal component analysis (PCA) showed that temperature, DO, and pH were the most relevant factors influencing pathogen abundance. Among the studied pathogens, *Acinetobacter* spp. was the most prevalent in the influent samples, followed by *Aeromonas* spp. As for the aeration samples, *Aeromonas* spp. was dominant in WWTP1 (CAS configuration) and WWTP2 (BNR configuration), while *Acinetobacter* spp dominated in WWTP3 (BNR configuration). *Acinetobacter* spp., *Aeromonas* spp., and *Pseudomonas aeruginosa* were the dominant ones in secondary effluents, with their dominance varying across the sampling period. In the final treated effluent, *Acinetobacter* spp., *Aeromonas* spp., and *P. aeruginosa* were dominant, with their dominance varying from sample to sample.

Additionally, free living amoebas (FLA) were also investigated for their contribution to the propagation and persistence of pathogens in secondary and final effluents. Using the conventional isolation technique, FLAs were isolated from different samples. The internalized bacteria and ARGs were further identified using metagenomic analysis. Metagenomic profiles identified nine species belonging to *Acanthamoeba* and two species belonging to *Entamoeba*. *A. castellini* was the most prevalent dominant species detected in effluent and final effluent samples of all three WWTPs. *P. aeruginosa*, *S. maltophilia*, *A. spanius*, *C. testosteroni*, and *E. cloacae* were the most dominant bacterial endosymbionts detected. Among these, *S. maltophilia* and *P. stutzeri* were detected in FLAs isolated from the final treated effluents indicating their prevalence in the chlorinated effluents. The presence of ARGs within FLAs were also ascertained. Genes conferring resistance to aminoglycosides (*aadA*); trimethoprim (*dfrA15* and *dfrA5*); sulfonamides (*Sul1* and

SulII), macrolides (*msrA, mphC*); rifamycin (*Arr*); quinolones (*qnrE1*) and tetracyclines (*TetA* and *TetG*). *SulI, dfrA5, AadA, dfrA15, SulII, TetA, TetG* and *qnrE1* were among the resistance genes that persisted into final effluents.

The results of this study have contributed significantly to our current understanding of pathogens, particularly the dominant pathogens and the role of FLAs in the dispersal of pathogens and ARGs into the environment via WWTPs. The study also indicates that the conventionally treated effluents may still contain human pathogens, ARGs, and MGEs, which may contribute to the propagation of emerging pathogens and antibiotic resistance in the receiving environment.

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ABBREVIATIONS

ARBs : Antibiotic resistant bacteria

ARGs : Antibiotic resistance gene

A2O : Anaerobic Anoxic Aeration

CAS : Conventional activated sewage sludge system

DAPI : 4′-6-diamino-2-phenylindole

DNA : Deoxyribonucleic acid

DO : Dissolved oxygen

FISH : Fluorescent *in-situ* hybridization

FLA : Free living amoeba

HRT : Hydraulic retention time

MLSS : Mixed liquor suspended solids

ng : Nanogram

NGS : Next generation sequencing

NNA : Non-Nutritive Agar

OUT : Operational taxonomic unit

pH : Potential of Hydrogen

PCR : Polymerase chain reaction

qPCR : Quantitative real-time

RNA : Ribonucleic acid

SBS : Sequencing by synthesis

SDS : Sodium dodecyl sulfate

SRT : Sludge retention time

TE : Tris-EDTA

Tris-HCl: Tris hydrochloride

TSS : Total suspended solids

WWTP: Wastewater treatment plant

OUTPUTS (PUBLICATIONS AND CONFERENCE PRESENTATIONS)

CONCO, T., KUMARI, S., AWOLUSI, O. O., ALLAM, M., ISMAIL, A., STENSTRÖM, T. A. & BUX, F. 2022. Profiling of emerging pathogens, antibiotic resistance genes and mobile genetic elements in different biological wastewater treatment plants. *Journal of Environmental Chemical Engineering*, 10, 107596.

• **Thobela Conco**, Sheena Kumari, Arshad Ismail, Thor Stenström, Faizal Bux (2019) Genomic and transcriptomic analysis of antibiotic resistance genes in biological wastewater treatment systems treating domestic and hospital effluents in Durban, South Africa (Small Water and Wastewater Systems 16th Specialised Conference, Murdoch University, Western Australia (Oral presentation)

• **Thobela Conco**, Sheena Kumari, Arshad Ismail, Thor Stenström, Faizal Bux (2020) Genomic and Transcriptomic profiling of antibiotic resistant pathogens in municipal wastewater treatment systems. Women in STEM Conference, University of Forte Hare, East London, South Africa from (Oral presentation)

• **Thobela Conco**, Sheena Kumari, Arshad Ismail, Thor Stenström, Faizal Bux (2020) Fate of antibiotic resistance genes in biological wastewater treatment systems treating domestic and hospital effluents. WISA Biennial Online Conference and Exhibition from 7-11 December 2020 (Oral)

CHAPTER ONE: INTRODUCTION

Wastewater treatment plants (WWTPs) serve as a hub for the growth of many fatal human pathogens and for developing multidrug resistant strains (Rizzo *et al*., 2013). In wastewater treatment facilities, wastewater from diverse sources such as hospitals, households and animal production farms, containing both pathogenic bacteria and traces of antibiotics enter and combine. Accordingly, the WWTPs provide ideal conditions for the multiplication of pathogens, development of antibiotics resistant bacteria (ARBs) and horizontal exchange of antibiotic resistant genes (ARGs) between a wide range of bacterial species, including human pathogens (Alexander *et al*., 2020). A poorly operated WWTP's therefore can potentially discharge high numbers of ARBs/ARGs and fatal pathogens into the environment.

Globally, studies have reported significantly high number of ARBs and ARGs in the final effluent (Sabri *et al.,* 2020, Amarasiri *et al*., 2020, Alexander *et al*., 2020). Similarly, in South Africa the problematic occurrence of pathogenic bacteria and resistance genes in treated final effluents discharged from WWTPs has been documented (Adefisoye *et al*., 2016, Osuolale and Okoh, 2017, Mbanga *et al*., 2020, Osunla *et al*., 2021, Mbanga *et al*., 2021). Moreover, the knowledge of pathogen occurrence and its distribution in different wastewater streams in South Africa is

however, limited mainly due to the limitations in the surveillance and screening techniques employed thus far. Using both culture and molecular methods, index pathogens (*Escherichia coli*) for monitoring water quality have been used to indicate the pathogen levels in treated wastewater (Motlagh and Yang, 2019). However, recent reports have shown severe challenges in using the above indicator organisms, especially their lack of correlation with other pathogens, including viruses and protozoa (Liang *et al*., 2015, Holcomb and Stewart, 2020). Indigenous protozoa namely, free living amoebas (FLA) are considered as reservoirs of pathogenic bacteria (so called amoeba resistant bacteria) such as methicillin-resistant *Staphylococcus aureus*, *Vibrio cholerae*, *Legionella* spp. and *Mycobacterium* spp. (Muchesa *et al*., 2017, Scheid, 2018, Samba-Louaka *et al*., 2018, Muchesa *et al*., 2018). The amoeba resistant bacteria engulfed during the treatment process are capable of multiplying within the protozoan cells. The incubation of these pathogens within protozoa, potentially affords protection from toxic conditions and further their ability to by-pass treatment steps of activated sludge treatment (Muchesa *et al*., 2018). Consequently, this has resulted in the increased discharge of fatal pathogens and pollutants to the receiving water bodies. Currently, South Africa is burdened with a three-layered threat of antibiotic resistance viz., drug resistant tuberculosis, human immunodeficiency virus (HIV) and ARBs (Department of Health 2015). There is a pressing need to identify current gaps that need to be bridged through research on development origin and dissemination of antimicrobial resistance. With the growing awareness of water reuse to augment freshwater supply, globally, research focus has shifted towards

monitoring and identifying emerging ARBs and mobile genetic elements (MGEs) from WWTPs and its receiving water bodies. However, traditional microbiological methods based on culture-dependent techniques for the identification of microbes in these environmental samples have become insufficient in identifying certain microbes. Moreover, the task of achieving suitable culture conditions for isolation and growth of each organism from the activated sludge is daunting. Molecular methods such as polymerase chain reaction (PCR) quantitative real time PCR (qPCR) and microarray have been widely used to detect pathogens and ARGs in sewage and natural waters (Liu *et al*., 2019). However, these technologies can only detect certain pathogens and cannot provide a comprehensive insight of potential pathogens in the environment. High throughput sequencing platforms such as Illumina have been used to overcome the aforementioned drawbacks of conventional culture techniques. Recent molecular techniques such as metagenomics a DNA based high throughput sequencing have been widely employed to profile microbial pathogens in wastewater samples (Li *et al*., 2019, Chen *et al*., 2022). This approach is however limited due to the basis of using genomic DNA which would fail in discriminating between viable and non-viable bacteria. While, the metatranscriptome approach based on analysis at RNA level could distinguish between viable and non-viable bacteria and may provide a better understanding of the fate of human pathogens during wastewater treatment (Ekwanzala *et al*., 2021). Therefore, a combined approach involving both metagenomics and metatranscriptomics would allow a comprehensive

understanding of the viability of these pathogens as well as their expressed genes in wastewater especially those that have not yet been investigated thus far.

1.2 RESEARCH AIM AND OBJECTIVES

The overall aim is to assess the occurrence, prevalence and fate of bacterial pathogens, ARGs and MGEs within the different wastewater treatment plants. In addition, further investigate the role of FLA-pathogen interactions aiding the survival and dissemination of selected pathogenic bacteria and resistant genes. To achieve the overall aim of this study, specific objectives were established and detailed asfollows:

I. Metagenomics and metatranscriptomics profiling of pathogenic bacteria in selected wastewater treatment plant treating municipal effluent in Kwa-Zulu Natal

II. Assessment of the impact of operational factors on the reduction of selected dominant pathogenic bacteria in wastewater treatment plants

III. *In vitro* evaluation of the role of protozoa as a potential host for the bacterial pathogens, resistant genes during secondary and tertiary treatment

1.2 OUTLINE OF THESIS

Chapter 1: Introduction, aim and objectives.

Chapter 2: Literature and background study of wastewater treatment process and wastewater microbiome (pathogenic bacteria, antibiotic bacteria, antibiotic resistance genes and mobile generic elements), detection and disinfection techniques.

Chapter 3: Presents a detailed metagenomics and metatranscriptomic profile of the fate of bacterial pathogens and antibiotic resistance genes in wastewater systems treating domestic and hospital effluents in and around Durban, South Africa.

Chapter 4: Focuses on monitoring and quantification of selected bacterial pathogens under specific process parameters in three wastewater configurations.

Chapter 5: Elucidates the role of free living amoeba in the harboring, propagation and dissemination of pathogenic bacteria and their antibiotic resistance genes via treated effluents.

Chapter 6: Highlights the general conclusions and significant findings of the study along with suggestions for future research.

CHAPTER TWO: LITERATURE REVIEW

2.1 Wastewater treatment

Since the beginning, wastewater treatment has been based primarily on the removal of soluble biodegradable compounds and pathogenic bacteria reduction by disinfection (Periasamy and Sundaram, 2013). The sequential process of urban wastewater treatment processes (e.g., mechanical/ physical, biological, chemical and physical–chemical) appropriates for the removal of organic compounds by means of biological activity and pathogen reduction by disinfection of final effluents (Rizzo *et al*., 2013). The primary treatment ensures the removal of particulate pollutants (debris, sand, oils, grease and particular wastes), which is achieved by grit screens and gravitational separation in large primary settling tanks. Subsequently, all dissolved wastes that pass through the primary treatment are therefore transferred into secondary treatment stage for biological elimination. Secondary treatment makes use of either the suspended growth or attached growth processes. The conventional sludge treatment process is the most widely used suspended growth treatment process. While attached biofilm on support processes (on fixed bed) are also the most classically used treatment processes. The removal efficiency in wastewater treatment is however, dependent on many factors such as process type, retention time, oxygen

concentration, pH, temperature and the process related biota present in activated sludge (Okoh *et al.,* 2007).

2.1.1 Conventional Activated systems

In South Africa the conventional activated system (CAS) is the most extensively used system for treatment of municipal waste from various sources which includes, households, hospitals and industry. CAS is based on the activated sludge process (ASP) which is a secondary treatment process that primarily focuses on the aerobic oxidation of organic pollutants, such as nitrogen (N) removal and chemical or biological phosphorus (P) of dissolved and suspended solids. Waste treatment via the activated sludge technology comprises of three major phases namely the biological treatment, clarification (solid-liquid separation) and disinfection (Fig 2.1). The biological treatment of waste is facilitated by a dynamic and phylogenetically diverse microbial community in the aeration basin (Parsley *et al.,* 2010). Subsequently biologically treated effluents are transferred to the secondary clarifier reactor for solid-liquid separation. Portions of the biomass is then recycled back to aeration reactor to maintain an active microbial community. The activated sludge has for the most part been able to efficiently remove suspended solids (SS) and biochemical oxygen demand (BOD), as well as nutrients (nitrogen and phosphorus) due to its design and process operation. This process nonetheless, still suffers many drawbacks, such as sludge bulking and foaming caused by the excessive growth of filamentous microorganism.

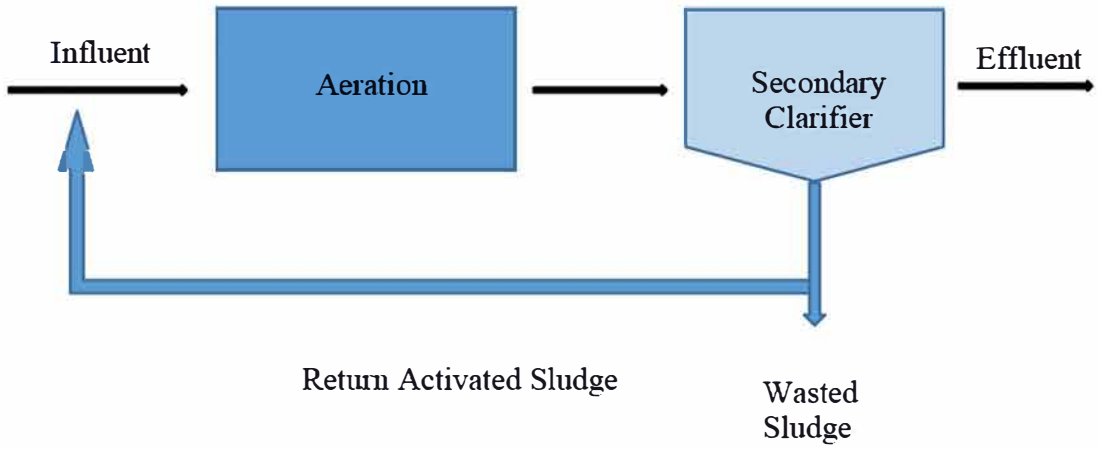


Figure 2.1 Diagram of activated sludge process with typical aeration and clarifier reactors.

2.1.2 Biological Nutrient Removal

Conventional activated sludge allowed an efficient removal of organics and contaminants from wastewater though its basic operation. However, due to the increase in population and industrialization arose concerns about eutrophication, stricter regulations forced its upgrading to improve nitrogen and phosphorus removal. The A2O process configuration which consists an anaerobic tanks followed by an anoxic and an aerobic reactor enabled the enhanced nitrogen and phosphorus removal (Fig 2.2). In this process enhanced nitrogen removal, is carried under a two-step nitrification-denitrification process. In the nitrification step reduced nitrogen compounds are converted to into oxidized forms nitrate/nitrite by nitrifying autotrophic bacteria under aerobic conditions. Subsequently mixed liquor suspended solids (MLSS) rich in nitrates is returned to the anoxic reactor at the end of aeration. Nitrites are then reduced into nitrogen gas by denitrification which is carried out by heterotrophic bacteria under anoxic condition.

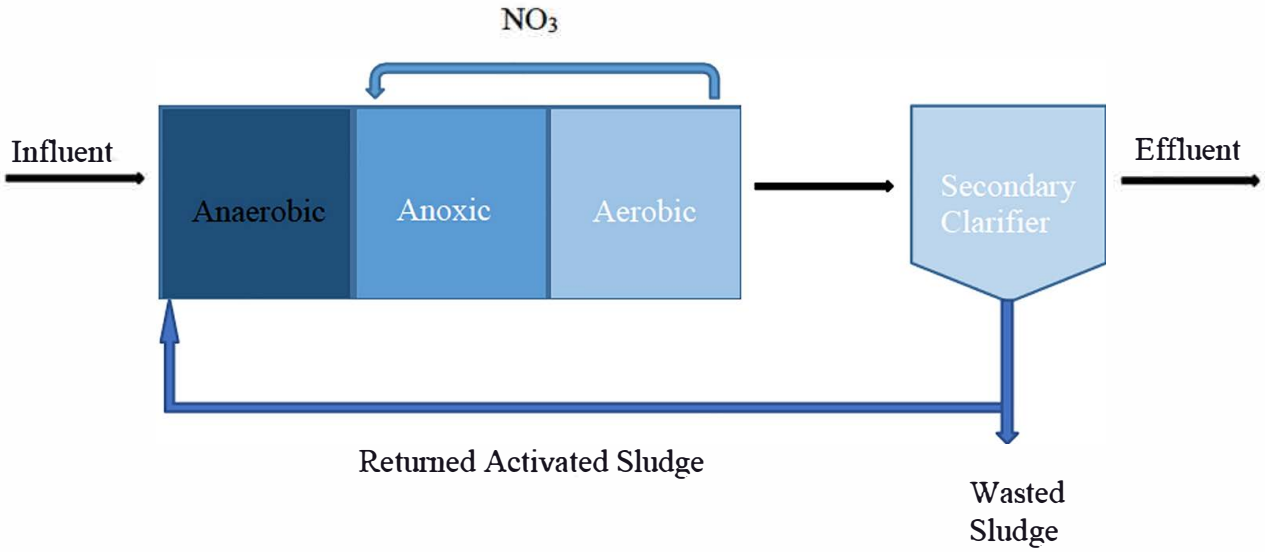


Figure 2.2 Diagram of an anaerobic-anoxic and aerobic reactor configuration.

2.1.3 Trickling filters

Conventional wastewater treatment is an energy intensive process, with the highest energy consumption occurring in aeration basins during biological wastewater treatment (Ali *et al*., 2017). In recent years the performance of the trickling filter design has become comparable to that of activated sludge plants (Guyer, 2014). Trickling filters (TFs) plants are non-submerged aerobic biofilm reactors that provide good performance with minimal maintenance and low energy requirements (Pearce, 2004, Vianna, 2012, Żyłka *et al.,* 2018, Bressani-Ribeiro *et al.,* 2018, Dąbrowski and Karolinczak, 2019). The main advantages of TFs include a high resistance to biomass wash-out, easy operation under variable wastewater flow-rates, effectiveness in treating high concentrations of organic material and ability to handle shock loads while producing less sludge (Dhokpande *et al*., 2014, Kopeć *et al*., 2018). A typical TF configuration consists of primary settling tanks, biological filtration beds, and clarifiers (Fig. 2.3). The operation of this system consists on passing pretreated wastewater through a distribution system on to a highly-permeable material covered with layers of biofilm. Formation of the biofilm is as a result of microorganisms aggregating, multiplying, and embedding themselves in a slimy matrix composed of the extracellular polymeric substances (EPSs) (Ali *et al*., 2017). The development and biological activity of the bacterial consortia within the biofilm layer is fundamental for the dispersion and complete degradation of organic pollutants in effluents as they filter through (Vianna, 2012, Ali *et al.*, 2017).

When a TF is combined with secondary sedimentation the expected reduction is reported as 1 -2 $\log_{10}$ for bacteria, 0-1 $\log_{10}$ for protozoa, 0 – 2 $\log_{10}$ for viruses, and 1-2 $\log_{10}$ for helminths (Vagadia,2018). Pathogens are removed by retention in the biofilm through adsorption, sedimentation of the slogged biofilm and by predation by other microorganisms in the biofilm.

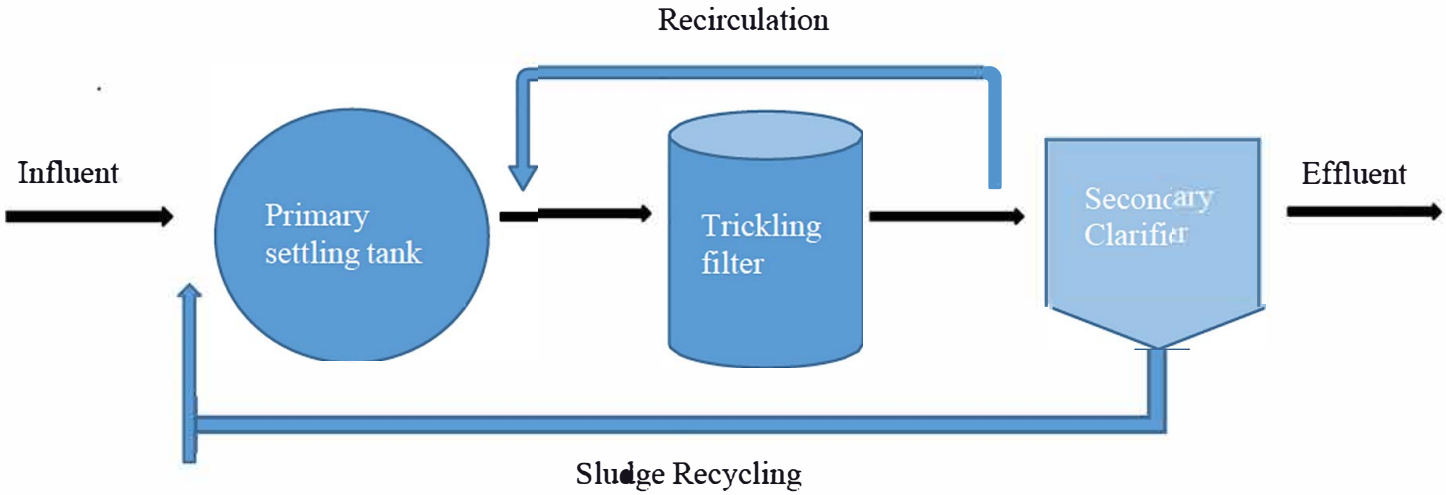


Figure 2.3 A trickling filter reactor configuration.

2. 2 Wastewater microbiome

2.2.1 Activated sludge flocs

Treatment of wastewater in the activated sludge process is facilitated by active biomass constituted by biological aggregates named flocs under aerobic conditions (Saunders *et al*., 2016, Tonanzi *et al*., 2021). Flocs as microbiological units play an essential role in activated sludge treatment process (Tu Vu, 2017). Flocs are composed of a dense microbial consortium in a matrix of extracellular polymeric substances. Floc-embedded microorganisms produce soluble microbial products and extracellular polymeric substances (EPS) due to biological or mechanical stress (Tonanzi *et al.,* 2021). EPS, a high molecular weight polymer is present on both outside microbial cells and within floc structures, are composed of proteins, polysaccharides, and humic substances, among other substances (Guo *et al.,* 2016). These biopolymers are resourceful, supporting several important cellular functions, which include binding of organic compounds, water retention, microbial adhesion to surfaces and protection against toxic substances (Sheng *et al*., 2010, Tonanzi *et al*., 2021). These flocs are constructed of both floc-forming bacteria which are both filamentous and non-filamentous organisms. Filamentous bacteria form the scaffold of which bacteria are embedded by a gel-like polymeric substance called extracellular polymeric substances (EPS).

2.2.2 Pathogenic bacteria

Wastewater treatment facilities contain many different types of pathogens that are harmful to the health of both human and animals. Enteric pathogens entering wastewater from human and animal are those mostly transmitted by the fecal or oral route, which includes bacteria, viruses and parasites (Chahal *et al*., 2016, Al-Gheethi *et al*., 2018, Wang *et al*., 2018). Bacterial pathogens in wastewater are of particular concern due to their ability to replicate independent of a host and thereby increase in the environment (Al-Gheethi *et al*., 2018, Yasir, 2021). Major bacterial pathogens and the diseases they are associated with are shown in Table 2.1. In the wastewater environment these pathogens and many others are provided ideal conditions for their proliferation and replication. Another important goal of wastewater treatment apart from biological nutrient removal is the reduction of pathogens during across the treatment train (Levantesi *et al*., 2010). Partial or lack of achievement of this goal is detrimental to the receiving environment. Moreover, where majority of pathogens exiting WWTPs with effluents have been associated with antibiotic resistance facilitated by genetic elements which increases the inevitability of gene transfer among bacterial communities.

Table 2.1 Major causative agents of diseases in municipal wastewater

Pathogen	Disease	Reference
Pseudomonas aeruginosa	Urinary tract infections, respiratory system	(Cai and Zhang, 2013a)
Escherichia coli	Gastroenteritis	(Osuolale and Okoh, 2017b)
Mycobacterium tuberculosis	Respiratory infections	(Cai and Zhang, 2013b)
Legionella pneumophila	Respiratory infections	(Cui and Liang, 2019)
Klebsiella pneumoniae	Respiratory infections	(King *et al*., 2020)
Vibrio cholerae	Cholera	(Varela and Manaia, 2013)
Aeromonas hydrophila	Meningitis	(Cui and Liang, 2019)
Acinetobacter baumanii	Urinary tract infections, respiratory system	(Zhang *et al*., 2009)
***Salmonella* spp.**	Salmonellosis, typhoid, paratyphoid	(Cai and Zhang, 2013a)
***Shigella* spp.**	Bacillary dysentery	(Chahal *et al*., 2016)
***Leptospira* spp.**	Leptospirosis	(Chahal *et al*., 2016)
Helicobacter pylori	Gastric ulcers	(Chahal *et al*., 2016)
Campylobacter jejuni	Gastroenteritis	(Strakova *et al*., 2021)
***Yersinia* spp.**	Gastroenteritis	(Chahal *et al*., 2016)
***Stenotrophomonas* spp.**	Meningitis	(Adegoke *et al*., 2017)

2.2.3 Occurrence of free living amoeba in wastewater treatment

The presence of protozoa, which includes amoebae is beneficial to the growth of bacteria because they excrete growth-stimulating compounds capable of enhancing bacterial activity (Motta *et al*., 2001, Madoni, 2011). Within the activated sludge community protozoa maintain a balance in bacterial population by preying on suspended microbes, which is crucial for final effluent quality. Since the inception of wastewater treatment the occurrence of free living amoeba (FLA) in the various processes has been observed Table 2.2. FLA are pleomorphic organisms with a diameter ranging from a few µm to 2 mm in diameter (Warren *et al*., 2016). A unique attribute of FLA is their biphasic lifestyle i.e. as a trophozoite (vegetative cell) or a cyst (Shawky *et al*., 2018). It is during their trophozoite stage, that FLA are metabolically active, feeding and replicating. However, under unfavorable environmental conditions such as nutrient depletion, fluctuating temperatures, changes in pH, osmotic stress, or the introduction of disinfectants, the amoeba enter a dormant phase of encystment (Tosetti *et al*., 2014). Amoeba cysts are hard structures composed of a multi layered thick protective wall made of lipids, glycoproteins, cellulose and chitin (Lambrecht *et al*., 2015). This protective wall enables cysts to survive desiccation and withstand hostile environmental conditions such as extreme temperatures, radiation (eg. gamma and UV), and biocide disinfection (Lambrecht *et al*., 2015). Upon return of favorable trophozoites emerge, using receptors target organisms are recognized and engulfed by means of pseudopodia (Delafont *et al*., 2016; Uribe-Querol and Rosales, 2017). Once phagocytosed organisms are enzymatically

digested within the phagolysosome (Uribe-Querol and Rosales, 2017). Certain bacterial species are however, capable of evading enzymatic digestion and thus us FLA as a ‘trojan horse’ for the colonization of new habitats (Balczun and Scheid, 2017).

Table 2.2 Amoeba species common to the wastewater environment

Amoeba species	**Environment**	**References**
***Acanthamoeba* spp.**	Wastewater	(Lu *et al*., 2015a, Belila *et al*., 2017, Cui *et al.,* 2017, Waso *et al*., 2017)
Naegleria fowleri	untreated and inadequately treated domestic water	(Lu *et al.,* 2015a Waso *et al.,* 2017,)
Hartmannella vermiformis	Wastewater	(Cui *et al*., 2017)
Balamuthia mandrillaris	Reclaimed domestic wastewater	(Cui *et al*., 2017)
Sappinia diploidea	Reclaimed domestic wastewater	(Cui *et al*., 2017)

2.2.4 FLA-pathogen interactions

Assimilation of bacterial pathogens by FLA in wastewater is achieved by means of phagocytosis (Uribe-Querol and Rosales, 2017) (Fig 2.4). Certain bacterial species, including *Legionella* spp. and *Acinetobacter* spp. are however, able to resist enzymatic digestion, oxygen radicles, and low pH of amoeba phagosomes (Tosetti *et al*., 2014, Strassman and Shu, 2017). Instead of being disintegrated by phagocytosis, these organisms modify amoeba vacuoles utilizing them as reservoirs in which they are able to proliferate (Fig. 2.4.b) (Van der Henst *et al*., 2016).

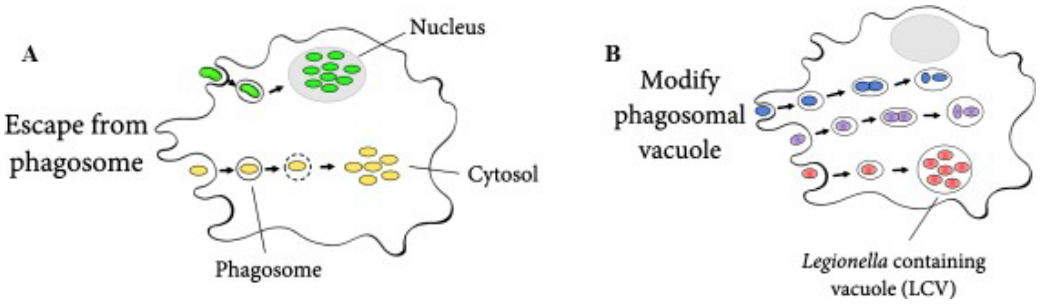


Fig 2.4 Survival mechanism employed by intracellular bacteria engulfed by FLAs. A) depicts intranuclear contained bacteria (green bacteria) and bacteria that have escaped the phagosome into in yellow. B) modification of vacuoles by pathogens (Strassmann and Shu, 2017).

This predation and survival of pathogen species has given rise to amoeba-bacteria interactions, where many FLA, including *Acanthamoeba castellanii*, *Dictyostelium discoideum*, *Hartmannella vermiformis*, and *Naegleria fowleri* have been found to harbor bacteria (Strassmann and Shu, 2017). Pathogens having associations with FLA in wastewater treated effluents and finaleffluents, Table 2.3. *Acanthamoeba* a common wastewater resident harbors bacteria as endosymbionts as well as act as a reservoir for facultative pathogens (Siddiqui and Khan 2012). Among pathogens colonizing FLA are antibiotic resistant human pathogens such as *Legionella* spp, *Mycobacterium* spp and Chlamydia-like bacteria (Scheid, 2016, Cui *et al*., 2017). The evasion of enzymatic digestion by human pathogens colonizing FLA has enabled them to use amoeba phagosomes as training ground for invasion of human macrophages (Balczum and Scheid, 2017).The ability of FLA to resist the harsh wastewater environment (Delafont *et al*., 2016), has furtherprovided internalized pathogens the convenience of replication and safe passage through the wastewater treatment process (Van der Henst *et al*., 2016; Balczum and Scheid, 2017). Under favorable conditions colonizing bacteria are capable of amoeba lysis (Van der Henst *et al*., 2016),thus making FLA vectors for colonization of new environments. The role of FLA in the dissemination of pathogens into the environment poses a severe threat to public health.

Table 2.3 FLA- pathogen associations detected in wastewater treatment

Pathogen/ARB	Disease	Source	Reference
***Legionella* spp.**	Legionnaires disease	Industrial water	(Loret and Greub 2010) (Ji *et al.* 2014) (Waso *et al.* 2017) (Lienard *et al.* 2017)
Legionella pneumophila	Pnuemonia	Wastewater	(Delafont *et al.* 2013) (Cui *et al.* 2017)
Mycobacterium avium	Mycobacterial infection	Wastewater	(Delafont *et al.* 2013)
Parachlamydia	Lower respiratory tract disease in a bovine		(Delafont *et al.* 2013)
Pseudomonas aeruginosa	Gastrointestinal infections Urinary tract infections Dermatitis	Wastewater	(Moritz, Flemming and Wingender 2010) (Li *et al.* 2016) (Cui *et al.* 2017; Waso *et al.* 2017)

Escherichia coli	Urinary tract infections	Wastewater	(Wingender and Flemming 2011) (Moustafa 2017) (Cui *et al.* 2017)
Stenotrophomonas maltophilia	Pulmonary infections	WWTP Chlorinated effluents	(Cateau *et al.* 2014)
***Salmonella* spp.**	Gastro illnesses	Wastewater	(Moustafa 2017) (Waso *et al.* 2017)
***Vibrio* spp.**	Diahorrea /gastro illnesses	Wastewater	(Moustafa 2017) (Waso *et al.* 2017) (Cui *et al.* 2017)
***Shigella* spp.**	Gastro illnesses	Wastewater	(Moustafa 2017) (Waso *et al.* 2017)
Chlamydiales	Chlamydia pneumonia	Wastewater	(Corsaro *et al.* 2010) (Lienard *et al.* 2017)
Klebsiella pneumoniae	Urinary tract infection	Surface water	(March *et al.* 2013)
***Aeromonas* spp.**	Gastroenteritis	Drinking water	(Wingender and Flemming 2011) (Waso *et al.* 2017)

2.3 Antibiotic resistance in wastewater treatment

Modern disease management strategies as well as the extensive use in veterinary medicine have resulted in increased pharmaceutical use, of antibiotics (Hendriks and Pool, 2012). WWTPs are connected to public and private hospitals, households, and other non-point sources pollution where antibiotics are used (Aali *et al*., 2014). A large part of the antibiotics consumed ends up in wastewater, and in the wastewater the antibiotics may exert selective pressure for or maintain resistance among microorganisms (Börjesson, 2009). Consequently, WWTPs receive wastewater laden with antibiotics, antibiotic resistant bacteria (ARBs), and the ARGs that they carry (Miller *et al*., 2016). The nutrient-rich, microbial dense nature of WWTP biomass, in concert with the presence of antibiotics has created probable hotspots for antibiotic resistance gene transfer because hot spots for exchange and sharing of genetic material (Shwartz *et al.,* 2003; Parsley *et al.,* 2010; Rizzo *et al.,* 2013; Miller *et al.,* 2016). Antibiotic resistance genes may be shared between pathogen and non-pathogenic microbes through horizontal gene transfer in high bacterial density and diversity found in in biofilms of wastewater systems (Shwartz *et al.,* 2003; Pruden *et al.,* 2006; Miller *et al.,* 2016). It is now understood that even non-pathogenic organisms, such as commensals, can be of concern and can serve as a source of ARGs to pathogenic bacteria of clinical significance (Miller *et al.,* 2016).

2.3.1 Mechanisms of resistance in bacteria

Antibiotic resistance can occur as a result of chromosomal mutations which is passed on through replication of the mutated organism or acquired through horizontal gene transfer of foreign genetic material. These antibiotic resistant bacteria have the potential to transfer the antibiotic genes that they now possess between other organisms which includes pathogenic, and non-pathogenic organisms of the same or different species (Zhang *et al*., 2016a). There are three mechanisms by which an organism can acquire foreign genetic material by horizontal gene transfer. These mechanisms are transformation, transduction, and conjugation. Transformation refers to the assimilation of unprotected naked DNA, transduction is phage mediated, and conjugation is the cell-to-cell transfer of genes by means of mobile genetic elements (Munita and Arias, 2016). Mobile genetic elements refer to DNA segments that promote intracellular and enable intercellular mobility of DNA. Transposons, insertion sequences, integrons, and plasmids are examples of these mobile genetic elements. The key mobile genetic elements involved in the dissemination of antibiotic resistance are plasmids and transposons (Munita ad Arias, 2016; Partridge *et al*., 2018). Plasmids which are self-replicating, extrachromosal DNA are the most common preferred MGEs within bacterial communities in wastewater treat environment (Munita ad Arias, 2016; Partridge *et al*., 2018).

2.3.2 Resistance within FLA

The assimilation of antibiotic resistant organisms by FLA encourages for the intimate interaction at cell-to-cell level where sharing of genes is inevitable (Wang and Wu, 2017; Denet *et al*., 2017; Muchesa *et al*., 2018;). This interaction between pathogenic and non-pathogenic organisms of the same or different species may facilitate the sharing of antibiotic resistance genes between bacteria in a niche environment provided by FLA colonization (Zhang *et al*., 2016). The presence of mobile genetic elements within pathogenic bacteria hosted by FLA can facilitate the uptake of resistance genes via horizontal gene transfer (HGT) (Xiong *et al*., 2015; Wang and Wu, 2018). Where the transfer of such genetic material among viable bacteria and possibly bacterial remains within amoeba hosts may be facilitated by mobile genetic elements (MGEs), such as integrons, transposons, and plasmids (Partridge *et al*., 2018). Amoeba lysis by endosymbionts may consequently, contribute to the dissemination of fatal pathogenic resistant bacteria or MGE elements encoding resistance (Van der Henst *et al*., 2016; Balczum and Scheid, 2017). The possible release of MGEs originating from dead bacteria from within amoeba cells, thus makes FLA Trojan horses for ARGs as well.

2.4 Pathogen detection methods

Traditional microbiological methods based on culture-dependent techniques for the identification of microbes in environmental samples have become insufficient in identifying certain microbes. Moreover, the task of achieving suitable culture conditions for isolation and growth of each organism from the activated sludge is daunting. The drawbacks suffered by culture dependent methods include the lack of differentiation between the target and other non-target endogenous microorganisms of the same samples, false negative/positive results, time consumption and tedious procedures, and the inability to detect viable but non-cultivable (VBNC) cells (Yergeau *et al*., 2016; Zhang *et al*., 2021). Circumventing these limitations by molecular techniques promises reliable rapid analysis with high accuracy and specificity. Molecular-based methods, i.e., polymerase chain reaction (PCR), and fluorescence visualization (Fluorescence in situ hybridization (FISH), have been identified to detect bacterial communities in the activated sludge community (Kuo *et al*., 2021). Further to that the application of quantitative polymerase chain reaction (qPCR), a powerful tool that made possible the successful quantification of identifiable microbial populations. Advances in molecular techniques in recent years has seen the emergence of a highly accurate detection technique such as next generational sequencing (NGS) which has made the detection of pathogenic bacteria in wastewater more rapid (Chahal *et al*., 2016).

2.4.1 Fluorescence microscopy

Biological specimens pose a unique challenge to imaging due to their low refractive indexes comparable to water, resulting in reduced contrast. Advancements in microscopy has led to the development of fluorescence microscopy to circumvent the challenges of traditional detection techniques. The introduction of fluorescence microscopy, using fluorescent indicators that can be tailored in terms of their specificity for targets such as proteins, lipids, or ions has enabled the identification of bacterial species in complex environments (Korzeniewska and Harnisz, 2012). Fluorescence microscopy has thus become a widely used tool in cell biology, dueto its ability which to illuminate whole specimen while exciting the fluorophore contained withina specimen resulting in simultaneously viewing and capturing an image with a camera (Korzeniewska and Harnisz, 2012). Imaging of organisms from the wastewater community is however limited by low refractive indexes of specimen in comparison to water, resulting in diminished contrast. Fluorescent stains such as 4′,6-diamidino-2-phenylindole (DAPI) which intercalates to double-stranded DNA increasing the fluorescence of DNA by approximately 20- fold thereby allowing the labelling of DNA and easy visualization of both morphology and cellularcontent (Chazotte, 2011). Advances in fluorescence microscopy has led to the simultaneous use of multiple fluorescence dye. Fluorescence *in situ* hybridization (FISH) with rRNA-targeted probes is a staining technique which uses multiple fluorescence dyes for the phylogenetic identification of bacteria in mixed community (Korzeniewska and Harnisz, 2012). Fluorescence *in situ* hybridization (FISH) has

been widely used in studying the community structure of activated sludge(Kim *et al.,* 2004; Khan and Faheem, 2013, Benakova and Wanner, 2013, Lukumbuzya *et al.,* 2019). Hybridization in FISH is based on the binding of rRNA-targeted oligonucleotide probes, which are covalently linked to fluorescent dyes with specific microbial populations for their identification and quantification in their natural habitat (Wolf, 2017; Lukumbuzya *et al*., 2019).

2.4.2 Fluorescent *in situ* hybridization (FISH) for the detection of pathogenic bacteria in wastewater

Human sewage is a great source of pathogenic bacteria discharged into WWTPs with fecal matter. Valid identification of many well-known and emerging pathogens, is often difficult due to challenges in achieving appropriate culture conditions for their enrichment (Girones *et al*., 2010). Furthermore, many human pathogens have been found to exist in a viable but non-culturable (VBNC) state under stressful conditions (Li *et al*., 2014). For these reasons, FISH has been used for therapid detection and identification of viable but non-cultivable emerging pathogens from water, sewage and sludge (Girones *et al*., 2010, Aistleitne *et al*., 2020, García-Hernández *et al*., 2021). Some of the available rRNA oligonucleotide probes used for the detection of emerging bacterial pathogen are outlined in Table 2.4. Initial drawbacks suffered by FISH application in environmental samples included the presence of low levels of targeted rRNA, low probe permeability of cells, and poor probe hybridization efficiency (Garcia-Armisen and Servais, 2004, Kubota, 2013). Over the years many advancements have been devised to circumvent the shortfalls in application to environmental samples viz. catalyzed reporter deposition (CARD)-FISH, direct viable count (DVC)-FISH, FISH- confocal whole slide imaging (Frankenstein *et al*., 2021, Garcia-Armisen and Servais, 2004, Kubota, 2013, Wagner and Haider, 2012).

Table 2.4. Applied FISH probes for the detection of pathogens.

Pathogen	Specificity	Reference
E. coli	5'GAGTAAAGTTAATACCTTTGCTC3'	(Korzeniewska and Harnisz, 2012a, Kuo *et al*., 2021)
K. pneumoniae	5'-CGGTGAGGTTAATAACCTCTCGA3-'	(Korzeniewska and Harnisz, 2012b, Kuo *et al*., 2021)
E. aerogenes	5'-GCGAGTAACGTCAATCGCCAAG-3'	(Kuo *et al*., 2021)
C. freundii	5'-AAGGCGTTGTGGTTAATAAC-3'	(Kuo *et al*., 2021)
M. tuberculosis	5'-AGAATGAGCCTGCGAGTCAG-3'	(Shah *et al.*, 2017)
V. parahaemolyticus	5'-TGCAATTCCGAGGTTGAGCCCCGG -3'	(García-Hernández *et al*., 2021)
Enterobacteriaceae	5'-CTC TTT GGT CTT GCG ACG-3'	(Korzeniewska and Harnisz, 2012a)
***Salmonella* spp.**	5′-CACTTCACCTACGTGTCA-3	(Santiago *et al*., 2018)
***Acinetobacter* spp.**	5'-TTA-GGC-CAG-ATG-GCT-GCC-3'	(Reitz *et al*., 2018)
S. maltophilia	5'-GTC-GTC-CAG-TAT-CCA-CTG-C-3	(Reitz *et al*., 2018)

2.4.2 Polymerase chain reaction (PCR)

PCR is the most widely used molecular-based technique for the detection of pathogens in the wastewater community. Complex microbial communities are studied using PCR on the basis of targeting the 16S rRNA gene. The 16S rRNA gene has several conserved regions which are common to a large number of bacterial species, and variable regions, which are shared by fewer species (Fredriksson *et al*., 2013). The reflection of most bacteria availed by the conserved regions has made the 16S sequence an ideal proxy to achieve a trustworthy level of taxonomic and genetic information (Järvinen *et al*., 2009, Rosselli *et al*., 2016). The principle of PCR is based on the use of DNA polymerase which is an *in vitro* replication of specific DNA from a DNA template (Atawodi *et al*., 2011, Zhang *et al.,* 2021). A typical amplification process includes thermal cycling, a process which includes the repeated heating and cooling of sample to melt the DNA, facilitate the anneal of primers to the complementary DNA target, and finally accomplish enzymatic replication of the primer-bound sequences using temperature-dependent DNA polymerases (Caetano-Anollés, 2013) (Fig 2.6). PCR as a qualitative detection method has shown many advantages, its drawback however lies with its inability to ascertain the exact concentrations of targeted genes present in a sample. The efficiency of the wastewater treatment process is hinged on the presence andconcentrations of resident microbes and thus the need for supplementing the PCR method is imperative.

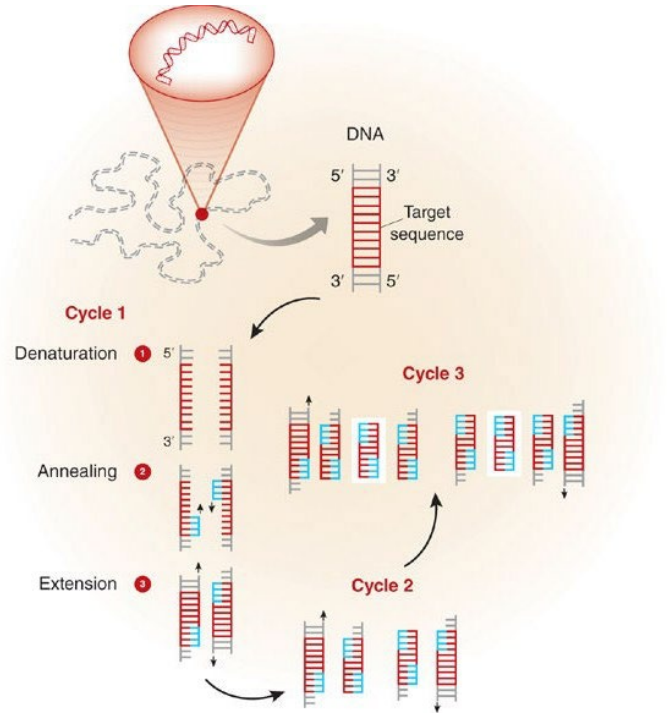


Figure 2.5 Diagrammatic representation of the polymerase chain reaction process (Garibyan and Avashia, 2013).

2.4.3 Quantitative real-time PCR (qPCR)

In recent years quantitative real-time PCR (qPCR) has become a widely used technique to quantify microbes in complex environment including that of wastewater. This method is based on the detection of a fluorescent signal that is produced in proportion to the amplification of the PCR product, cycle after cycle. Currently there are two commonly used methods for the detection and quantification of amplified products i.e the SYBR green assay that intercalates with double-stranded DNA and the TaqMan probe systems with sequence-specific DNA probes consisting of fluorescently labeled reports (Cao and Shockey, 2012, Garibyan and Avashia, 2013, Pereira-Gómez *et al*., 2021) (Fig 2.6). Presently SYBR-Green-based detection is the most widely used method and thus presents several advantages over the probe based method, as being cheaper and not requiring the synthesis of specific probes (Pereira-Gómez *et al.,* 2021).

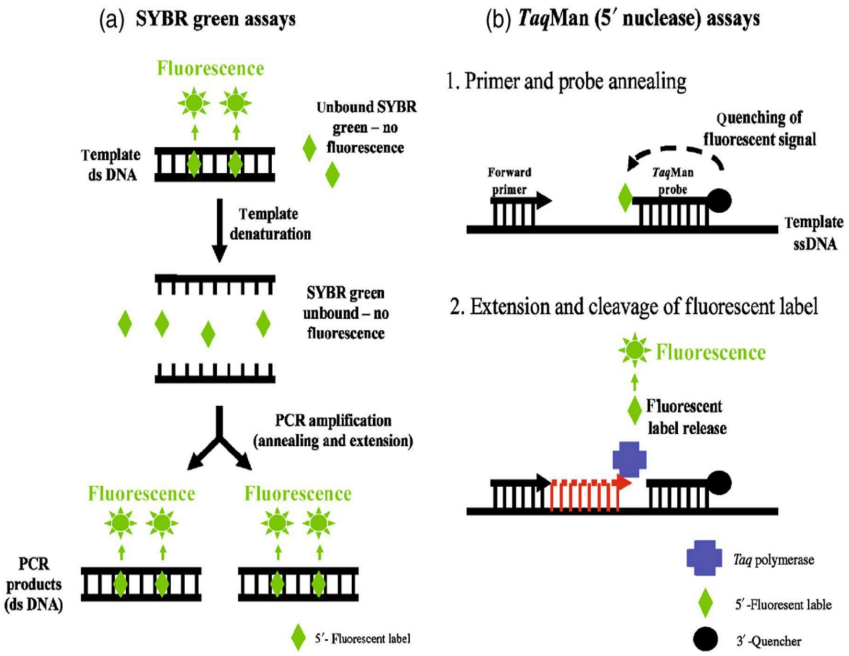


Figure 2.6 Illustrative representation of the quantitative polymerase chain reaction process. A) Diagrammatic stepwise process of the SYBR green method. B) Stepwise flow illustrating the probe based method (Smith and Osborn, 2009).

Real-time PCR has circumvented the drawbacks of PCR by providing real-time detection and quantitative analysis of target DNA sequences with higher specificity and sensitivity (Zhang *et al.,* 2021). The quantification of targeted genes during the exponential amplification, has proven to be beneficial and thus extensively applied in studies monitoring the fate of microbial species and genes across the wastewater treatment train (Garibyan and Avashia, 2013). Many pathogens that have been successfully quantified in wastewater are listed in Table 2.5. Real-time PCR is not without its own limitations. A major drawback of the q-PCR-based approaches is the requirement for prior sequence data of the specific target gene of interest, which has confined its specificity to only known genes (Smith and Osborn, 2009). Therefore, the use qPCR as an analysis tool for detecting environmental microorganisms is inevitably limited to the analysis of known and characterized.

Table 2.5 Pathogens quantified in wastewater from influents to final effluents

Name of pathogen	Sample type	Reference
E. coli	Influent, final effluent	(Cui and Liang, 2019, Mbanga *et al*., 2020)
E. faecalis	Influent, final effluent	(Mbanga *et al*., 2020)
***Salmonella* spp.**	Influent, secondary effluent Final effluent, Sludge	(Levantesi *et al*., 2010, Dungan *et al*., 2012) (Tsai *et al.,* 1998)
***Campylobacter* spp.**	Influent, secondary effluent Final effluent	(Levantesi *et al.,* 2010, Dungan *et al*., 2012, Vadde *et al.,* 2019)
P. aeruginosa	Sludge	(Tsai *et al.,* 1998)
L. monocytogenes	Influents	(Dungan *et al*., 2012)
A. hydrophila	Influents, final effluents	(Cui and Liang, 2019)
***Arcobacter* spp.**	Influents, final effluents	(Lu *et al.,* 2015b, Cui and Liang, 2019)
K. pneumoniae	Influents, final effluents	(Lu *et al*., 2015b)

2.4.4 Next generation sequencing

Traditional molecular testing methods greatly relied on use primary technology such as the Sanger sequencing technology which is based on chain-termination method (Liu *et al.,* 2012, Kanzi *et al*., 2020). Though Sanger sequencing is efficient for sequencing few short DNA fragments, it has a limited application when sequencing large sequences such as those of wastewater samples due to the presence of thousands of DNA templates. Over the years advances in genome sequencing have paved the way for the development of next generation sequencing (NGS) technologies. NGS, also known as massively parallel sequencing describes a DNA sequencing approach capable of producing millions of short read sequences in a much shorter time,at a much cheaper cost and with higher throughput compared to Sanger sequencing (Reis-Filho, 2009, Behjati and Tarpey, 2013, Kanzi *et al*., 2020). By use of different approaches, NGS sequencing methods have significantly overcome the limited scalability of traditional Sanger sequencing by attaching the DNA molecules to be sequenced to solid surfaces or beads, allowingfor millions of sequencing reactions to happen in parallel (Reis-Filho, 2009). Further NGS has provide the possibility of quantifying every generated short read of each DNA molecule, allowingthe identification of mutations in non-modal populations of cells and accuracy in copy number assessments of all genomic regions (Kanzi *et al*., 2020). In addition to the ability to sequence DNA,massively parallel sequencing can be applied to sequencing RNA, where the combination of both provide a wealth of information on the presence and expression of genes (Reis-Filho, 2009).

The ability to define pathogens based on their genomes revealing their identity, interaction within otherpathogens and possible gene they may harbor makes NGS a powerful tool with endless benefits inin molecular diagnostics of pathogens in the wastewater microbiology. Currently available NGS platforms apply different approaches to achieve high-throughput sequencing. The Illumina sequencing platforms have been most extensively used sequencing platforms in wastewatercommunity studies, due to lower per-base costs and error rates and greater data output (Takahashi*et al*., 2014, Chan *et al*., 2019, Zahedi *et al*., 2019, Kanzi *et al*., 2020 Yasir, 2021).

2.4.4.1 Illumina platform

The basis of Illumina chemistry is the reversible-termination sequencing by synthesis (SBS) with fluorescently labeled nucleotides on a flow cell (Escobar-Zepeda *et al.,* 2015, Liu *et al*., 2012, Shuikan *et al.,* 2019). The principle of Illumina sequencing is that the DNA fragments with barcoding primer (adaptor) are attached to the flow cell, followed by bridge amplification to form clusters which contains clonal DNA fragments (Fig. 2.7) (Morozova and Marra, 2008). The sequencing reaction is performed in the flow cell by adding labeled nucleotides. When the labeled nucleotide is incorporated, subsequent excitation of fluorescent molecules occurs and resultant signal generated is then recorded by optical sensors. After that, the fluorescent molecules are removed and the next labeled nucleotide incorporated. DNA fragments can be sequenced from one or both sides giving single end or pair-end sequencing, respectively, with a maximum read length of 300 base pairs per read (Escobar-Zepeda *et al*., 2015).

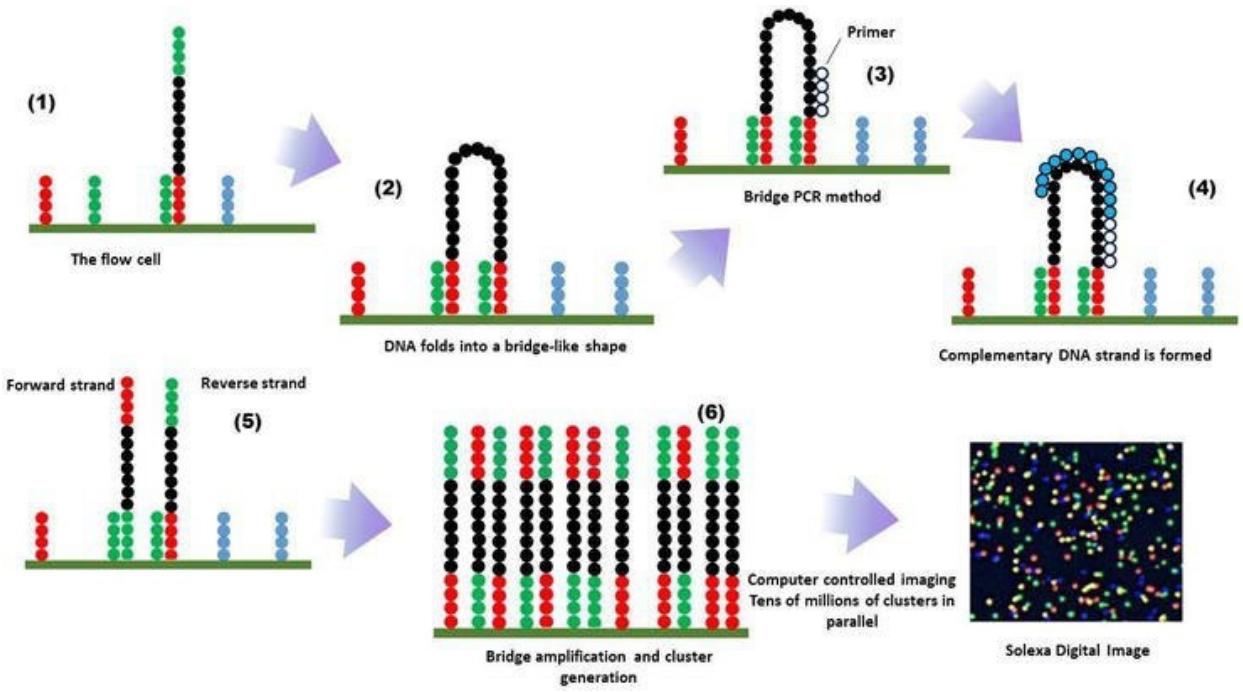


Figure 2.7. Schematic diagram of Illumina sequencing (Shuikan *et al*., 2019).

CHAPTER THREE: METAGENOMICS AND METATRANSCRIPTOMICS PROFILING OF PATHOGENIC BACTERIA IN SELECTED WASTEWATER TREATMENT PLANT TREATING MUNICIPAL EFFLUENT IN KWA-ZULU NATAL

3.1 Introduction

The primary objective of conventional wastewater treatment is the removal of organic matter and pathogens present in wastewater (Levantesi *et al*., 2010). Over the years, studies have made apparent the limitation of conventional treatment plants in the removal of pathogens and resistant determinants (Aali *et al*., 2014, Ben *et al*., 2017, Li *et al*., 2017). The activated sludge system serves as suitable hotspots for the increase, transfer and spread of ARGs via horizontal gene transfer and their subsequent release with effluents (Zhuang *et al*., 2015, Zhang *et al*., 2016, Zhang *et al*., 2016b, Alexander *et al*., 2020, Jäger *et al*., 2018, Sabri *et al*., 2020). Thus making wastewater treatment plants important anthropogenic sources for the significant increase, proliferation and release of ARB, ARGs and MGEs to the aquatic environment (Rafraf *et al*., 2016, Zheng *et al*., 2017, Nnadozie *et al.,* 2017, Chu *et al*., 2018, van den Honert *et al*., 2018, Sabri *et al*., 2020). In a recent study conducted by Yoo and Lee (2021) they have reported relatively high levels of ARGs and MGEs across the treatment trainof WWTPs. Thus, the sharing of antibiotic resistance genes between organisms by means of HGT in aquatic environments is inevitable (Shwartz *et al*., 2003; Parsley *et al*., 2010; Rizzo *et al*., 2013; Miller et al., 2016, Zhang *et al.,* 2016b). Consequently,

the development of resistance by different microbes in the aquatic environment poses a serious harmto human and environmental health (Zheng *et al.,* 2017).

Conventional culture-based techniques for tracking the presence of ARBs in final effluents are tedious and have been met with the challenge of non-cultivability of certain pathogenic bacteria (Neelakanta and Sultana, 2013; Costa´n-Longares *et al.,* 2008). This limitation posed by culture dependent methods has resulted in un-cultivable bacteria, ARGs and MGEs, being unaccounted during routine final effluent analysis. Advancement in molecular approaches, high throughput sequencing technique such as metagenomics are regarded as the most efficient method for species composition analysis of environmental samples (Cydzik-Kwiatkowska and Zielin´ska, 2016; Neelakanta and Sultana, 2016). Metagenomics, a DNA based high-throughput sequencing has proved reliable in microbial community analysis, however suffers the limitation of differentiating between viable and non-viable cells. The prospects made available by the metatranscriptomics approach have aided in overcoming the above challenge (Cydzik-Kwiatkowska and Zielin´ska, 2016). Metatranscriptomics using mRNAs has made possible the detection of functionally active microbial members that are present in biological reactors (Delforno *et al*., 2019). Therefore, application of both metagenomic and metatranscriptomic allows the assessmentof the diversity, functionality and fate of ARBs, ARGs and MGEs (Cydzik-Kwiatkowska and Zielin´ska, 2016).

Among various wastewater treatment processes, conventional activated sludge (CAS) anaerobic-anoxic-oxic (A2O) and trickling filter (TF) are the most popular processes adopted by municipal

WWTPs in South Africa. The fate of microbial pathogens, ARB and ARG across the treatmentprocesses have been rarely compared. Thus, this study aims to comprehensively determine the diversity, distribution and removal of pathogenic bacteria, ARG and MGE in three full-scale WWTPs, treating domestic and hospital wastewater using both metagenomic and transcriptomicsapproaches. Three WWTPs with different design configuration (CAS, A2O, TF) were selected forthe study to assess the impact of process configuration on their fate and removal.

3.2. Materials and Methods

3.2.1 Wastewater treatment plants description

Raw sewage, secondary effluents and post chlorinated wastewater samples were collected from three municipal wastewater treatment plants (WWTPs) treating domestic and hospital effluents in and around Durban, South Africa. The three treatment systems were selected mainly based on their source and design configuration. WWTP1 configured with (A2O) receives influent mainly from domestic sources from a population of approximately 26 000 and has an average in- and outflow of 4800 m^3/d. WWTP2 configured with a CAS system receives domestic and hospital effluents from a population of approximately 56 000, and has an average inflow/outflow of 2283 m^3/d. WWTP3 with a TF configuration receives influent from both domestic and hospital sources. It serves a population of about 74 000 and inflow/outflow of 12143 m^3/d. Treated effluents from these plants are discharged directly into the rivers located next to each WWTP.

3.2.2 Sampling procedure

Samples of the raw influent, secondary clarifier and post chlorinated chambers were collected from the three treatment plants. One liter of the influent sample was collected from each of the wastewater treatment plants due to their turbidity, and 5 L samples collected from clear secondary effluents and post-chlorinated effluents. The samples were directly transported to the laboratory at 4°C for further processing.

3.2.3. Sample Preparation and Extraction of Total Nucleic Acids

Precisely 0.25 gram of biomass was recovered from each sample using 0.45 µm pore size cellulose nitrate membrane filters (Sartorius Stedim Biotech, Goettingen Germany) with the aid of a vacuum filtration unit. The concentrated biomasses from influent, clarified and post chlorinated samples were used for nucleic acid extraction. Biomass for RNA isolation was immediately mixed with RNAlater (ThermoFisher Scientific, MA, USA) and kept at -80°C before RNA extraction. From each concentrated biomass, total DNA and RNA were extracted using DNeasy PowerSoil kit and RNeasy PowerMicrobiome kit (Qiagen, Germany) respectively, according to the manufacturer's instructions. The RNA samples were further treated with DNase 1 (Qiagen, Germany) to eliminate DNA contamination. Subsequently, the RNA was purified using MEGAclear transcription clean-up kit (ThermoFisher Scientific, MA, USA), followed by enriching bacterial mRNA using MICROBEnrich kit (ThermoFisher Scientific, MA, USA) (Płaczkiewicz *et al*., 2019). The

enriched mRNA samples were converted to cDNA by using Maxima H minus double-strand cDNA synthesis kit (ThermoFisher Scientific, MA, USA). The DNA, RNA, mRNA and cDNA samples were stored at -20 ºC for further analysis. The concentrations of the extracted DNA and RNA samples were determined using Implen N80 NanoPhotometer (Implen, Muenchen, Germany) and the quality was checked by running the samples on 0.8% and 1% agarose gel electrophoresis for DNA and RNA respectively.

3.2.4. Library construction, sequencing, and assembling of sequences

Total DNA and cDNA samples (10 µg each) were stored on ice and transported to the National Institute for Communicable Diseases (NICD) Sequencing Core Facility, Johannesburg, South Africa for high-throughput sequencing using the Illumina MiSeq. Multiplexed paired-end libraries (2×300 bp) were prepared using the Nextera XT DNA Library preparation kit (Illumina, San Diego, CA, USA) before DNA sequencing. For quality control, resulting paired-end reads were checked and trimmed using CLC Genomics Workbench version 11.0 (CLC Bio, Aarhus, Denmark) (Singh-Moodley *et al*., 2017). Taxonomic classification was performed using Kraken-2 (https://github.com/DerrickWood/kraken2) and visualized using Krona (Ondov *et al*., 2011). Genera classified reads were *de novo* assembled using the metagenomic *de novo* assembler Megahit (https://github,.com/voutcn/megahit) (Li *et al*., 2018) via Shovill (https://github.com/tseemann/shovill).

3.2.5 ARG Annotation

Following the assembly ARGs were annotated against Abricate (https://github.com/tseemann/abricate) which makes use of a combination of databases for the accurate screening of antimicrobial resistance genes which included Resfinder (Ng *et al*., 2019), Comprehensive Antibiotic Resistance Databases (CARD) (Jia *et al*., 2016), ARG-ANNOT (Antibiotic Resistance Gene-ANNOTation) (Gupta *et al*., 2013). PlasmidFinder database was used to detect plasmid associated ARGs (Carattoli *et al*., 2014). Identified resistome along the treatment stages of three WWTPs were further visualized using Circos (Krzywinski *et al*., 2009). Further, genes that conferred resistance to clinical antibiotics (e.g., beta-lactams, sulfonamides, aminoglycosides, tetracyclines, trimethroprim, and macrolides), were analyzed.

3.2.6 Operational taxonomic units clustering analysis

The OTU table in the BIOM format (Biological Observation Matrix 1.22.0) generated by the CLC Bio Genomics Workbench was imported into R using Biomformat (McMurdie and Paulson, 2021) for visualization and further analysis. The Alpha diversity indices (i.e., Chao1, Shannon, and Simpson) were calculated using R-package Vegan 2.5–7 from rarefied samples used for richness and diversity indices of the overall bacterial community. The principal component analysis (PCA) was employed to evaluate the community structure of the potential pathogens and ARGs across the treatment of the different configurations using XLSTAT version 2021.2. (Addinsoft, https://www.xlstat.com/). Furthermore, statistical analysis for comparison and correlation between samples; heatmaps of potential pathogens, ARGs and MGE profiles were done using the Origin Pro 2018 software (Origin Lab Corporation, Northampton, MA).

3.3 Results

3.3.1 Microbial profiling and community richness

After quality filtration and trimming of raw reads, resulting clean sequences were clustered into operational taxonomic units (OTUs) based on 97% similarity (Table 1 and Supplementary Table 1). The rarefaction curves obtained for the samples at 0.03 distance and the plateaus, suggested that the sequencing depths were sufficient to saturate the curve and therefore, the actual diversity was likely fully covered in each sample (Supplementary Fig. 2). The alpha diversity was estimated by the Shannon-Wiener Index (H'), Simpson's Index (λ), Chao1, and Pilou. Significant overall diversity and richness was apparent across metagenomes from influent to final effluent samples in all the three WWTPs.

Table 3.1. Diversity metrics of total bacteria community in samples collected from influents to final effluents of three WWTPs.

Sample	OTUs	Diversity		Pilou Evenness (J)	Richness
		Shannon-Wiener Index (H')	Simpson's Index (λ)		Chao1
WWTP 1 INF	515368	4.46	0.96	0.16	452
WWTP 1 S.EFF	136052	5.37	0.99	0.17	364
WWTP 1 F.EFF	220784	5.40	0.99	0.16	444
WWTP 2 INF	540578	4.67	0.97	0.15	527
WWTP 2 S.EFF	196782	5.47	0.99	0.16	467
WWTP 2 F.EFF	307882	4.81	0.97	0.16	440
WWTP 3 INF	529810	4.19	0.95	0.16	444
WWTP 3 S.EFF	219760	5.34	0.99	0.16	431
WWTP 3 F.EF	299688	5.38	0.99	0.16	469

The Shannon indices in final effluent metagenomes across all three WWTPs displayed a similar pattern of higher diversity compared to that of influents. WWTP1 and WWTP3 shared a similar pattern of increasing diversity from influents to final effluents, while inter-compartmental variations occurred across the treatment train of WWTP2. In WWTP2 species diversity increased from influents to secondary effluents, which was followed by decrease in final effluents.

Based on Chao1 (Table 3.1), influent metagenomes of both WWTP1 and WWTP2 showed higher bacterial richness compared to secondary and final effluents in both these plants. Interestingly

WWTP3 final effluent metagenomes, exhibited the highest species richness compared to influent metagenomes.

Analysis of the whole microbial community was conducted and Krona plots constructed to illustrate the distribution of genera across the treatment train of all three WWTPs (Supplementary Fig. 3.1). The top three phyla in all the samples across the three WWTPs (Supplementary Fig. 3.1) were Proteobacteria, Terrabacteria group and FCB group, with the highest abundance of Proteobacteria ranging from 91-94% in influents, 71-82% in secondary effluents and 81-87% in final effluent samples. In WWTP1 influents Proteobacteria constituted about 93% of all bacteria followed by the Terrabacteria and FCB groups that constituted 3% each of the total bacteria. After sedimentation Proteobacteria still dominated secondary effluents accounting for 71% of total bacteria, while an increase was apparent in both Terrabacteria and FCB group which account for 17% and 5% of all bacteria respectively. Final effluents 84% of all bacteria was constituted bythe Proteobacteria phyla, followed by Terrabacteria group at 9% of total bacteria and FCB group at 3% of all bacteria. In WWTP2 Proteobacteria the top most dominant phyla decreased from influents to secondary and finally increased in final effluents accounting for 91%, 77% and 87% of all bacteria respectively. Both the Terrabacteria and FCB groups displayed a similar pattern of increase from 3% and 4% in influents to 10% and 7% secondary effluents of total bacteria in respectively. Similarly, a shared pattern of decrease in both Terrabacteria and FCB groups to 6% and 4% respectively in final effluents was apparent. In WWTP3, Proteobacteria which made up

94% of total bacteria in influents, decreased to 82% in secondary effluents and finally 81% in final effluents. Both the Terrabacteria and FCB groups which accounted for 2% each of all bacteria influents, increased to 8% and 6% respectively of all bacteria in secondary effluents. While in final effluent metagenomes of WWTP3, Terrabacteria remained stable at 8% while the FCB group increased to 7% of all bacteria in final effluent samples.

The top most dominant families across the samples included *Moraxellaceae, Pseudomonadaceae*, *Aeromonadaceae*. In influents of WWTP1, WWTP2 and WWTP3 the genera *Aeromonas* was the most dominant accounting for 31%, 19% and 33% of all bacteria respectively. The second most dominant genera, *Pseudomonas* which make up 7%, 17% and 8% of all bacteria in WWTP1, WWTP2 and WWTP3 in influents metagenomes respectively. The distribution and percentages of the various genera however, varied across the treatment train of all three WWTPs. A similar pattern in the shift of dominance between genera occurred in secondary effluents of WWTP1 and WWTP2, compared to WWTP3. In both secondary effluents of WWTP1 and WWTP2 *Pseudomonas* displayed a dominance over the *Aeromonas* genera as compared to influents, while secondary effluents of WWTP3, *Acinetobacter* (7%) dominated over *Pseudomonas* (6%). In WWTP1 *Pseudomonas* and *Aeromonas* accounting for 3% and 2% respectively. While *Pseudomonas* (11%)and *Aeromonas* (3%) were present in secondary effluents of WWTP 2. Final effluents of WWTP 1 displayed a growth in the dominance of *Pseudomonas* assuming 15% and *Acinetobacter* making up 4% as the second most dominant genus. In final effluents of WWTP 2 a

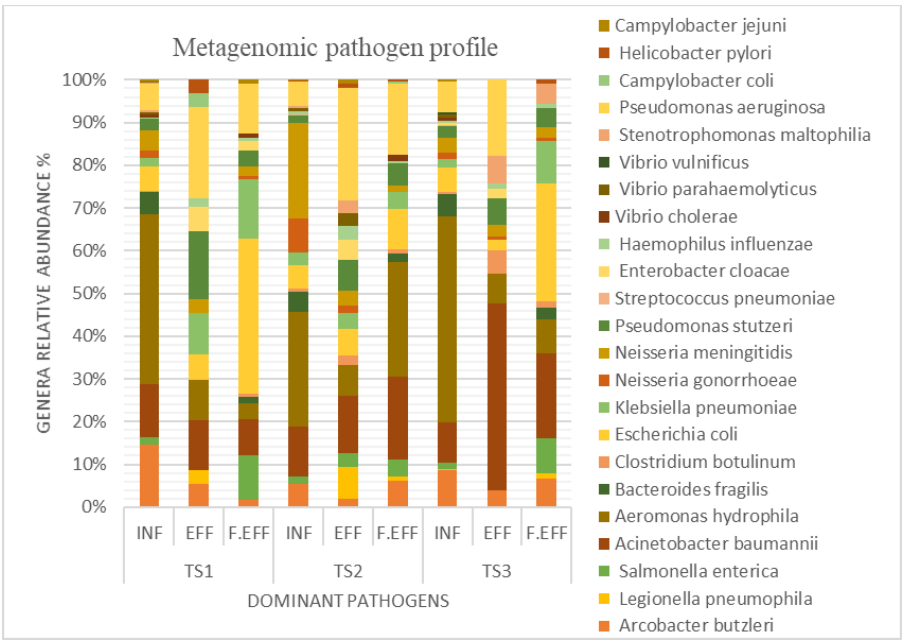
shared co-dominance between *Pseudomonas* and *Aeromonas* occurred with each genera constituting 11%. It was interesting to note another of dominance between *Pseudomonas* (10%) and *Acinetobacter* (3%) infinal effluents of WWTP3.

3.3.2 Fate of pathogenic microbiome from influents to final effluents

A comprehensive profile of the diversity of potential human pathogens was conducted from both metagenomics and transcripts across the treatment stages of the WWTPs investigated. The distribution of potential pathogenic bacteria is shown in Fig. 3.1. The most prevalent genera identified in the three treatment systems included common enteric pathogens such as species of *Arcobacter, Enterobacter*, *Escherichia, Campylobacter, Salmonella and Vibrio* as well as emerging opportunistic pathogenic bacterial species from various genera viz., *Acinetobacter, Aeromonas, Bacteroides, Legionella, Mycobacterium, Pseudomonas, Klebsiella, Stenotrophomonas and Neisseria*). *A. butzleri, L. pneumophila, S. enterica, A. baumanii and A. hydrophila* were the dominant bacterial pathogens in all samples in both the metagenomes and transcripts. However, the abundance of each pathogen from influent to final effluent samples was different. Influent metagenomes across the three WWTPs showed a similarity in the relative abundance of *A. hydrophila* which was higher followed by *A. baumanii* and *A. butzleri*. In contrast secondary effluents metagenomes showed a difference in relative abundances. In WWTP1 secondary effluents *P. aeruginosa* had a higher abundance followed by *P. stutzeri* then *A. baumanii*. WWTP2 *P. aeruginosa* was higher, followed by*, A. baumanii* then *L. pneumophila*. While, in WWTP3 secondary effluents *A. baumanii* had a higher abundance followed by *P. aeruginosa* and *S. maltophilia*.

In the transcripts the relative abundance of *E. coli* was higher followed by *A. hydrophila* in influents of WWTP2 and WWTP3 compared to WWTP1 which was dominated by *A. hydrophila* followed by *N. meningitides.* In secondary effluents the different samples pathogens displayed different dominance, for instance effluents of WWTP1 were dominated by *E. coli,* while WWTP2 was dominated *by M. leprae* and WWTP3 *A. baumanii*. Similarly, final effluent transcripts showed different dominance. *C. botulinum, E. coli,* and *A. baumanii* dominated final effluents of WWTP1, WWTP2 and WWTP3 respectively.

A



B

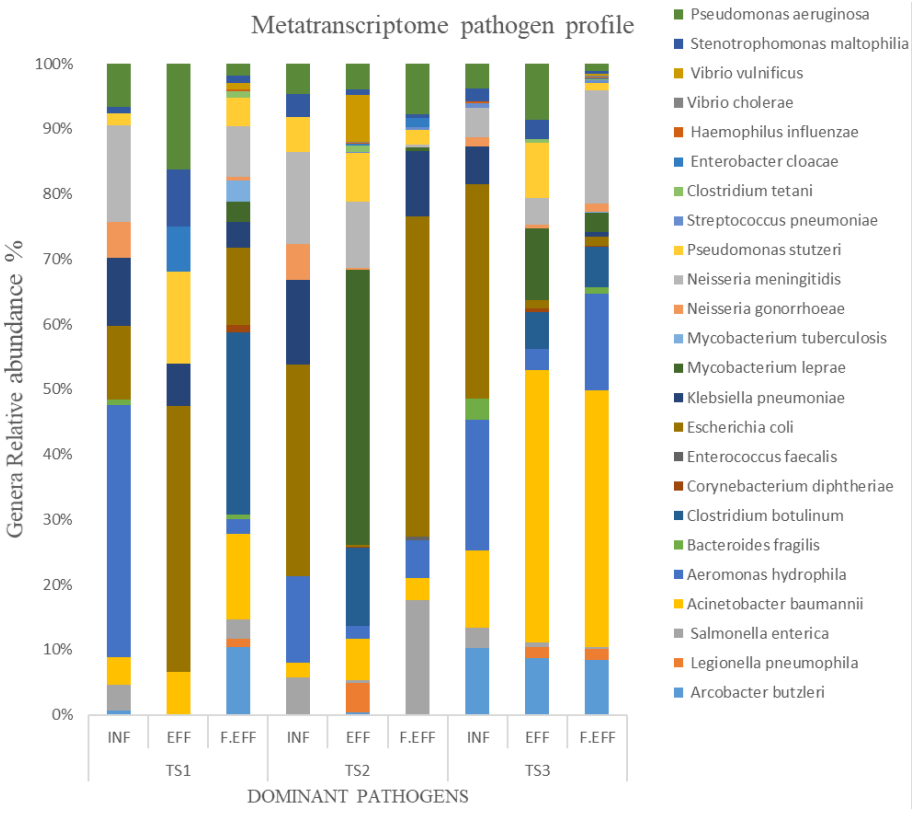


Figure 3.1. Stacked bar graphs showing (A) taxonomic distribution of dominant pathogenic species in metagenomes and (B) taxonomic distribution of dominant pathogenic species in transcripts.

A Principal Component Analysis (PCA) bi-plot was used to compare the pathogenic bacterial community distribution across all samples to demonstrate the similarities and differences among communities along the treatment stages of the three WWTPs (Fig. 3.2). The PCA plot showed similarities between the bacterial communities of WWTPs receiving their influents from similar sources. The result showed that influent metagenomes samples from WWTP2 and WWTP3 (receiving domestic and hospital wastewater) had similar pathogenic community profile compared to that of WWTP1 that received no hospital wastewater. Interestingly the final effluents of WWTP1 and WWTP2 had a similar pathogen profile even though their influent sources differed. Similarly, secondary effluents of WWTP1 and WWTP3 shared similarity in pathogen profile, though the influent types received by these two plants varied. Transcript influents of WWTP2 and WWTP 3 showed a similar profile compared to influent transcripts of WWTP1. While transcripts of secondary effluents of WWTP1 and WWTP3 shared similar pathogen profile compared to that of WWTP2. A variation was apparent between the final effluents of WWTP2 and WWTP3, though they shared a similar influent source as well as a similar pathogen profile in influent transcripts.

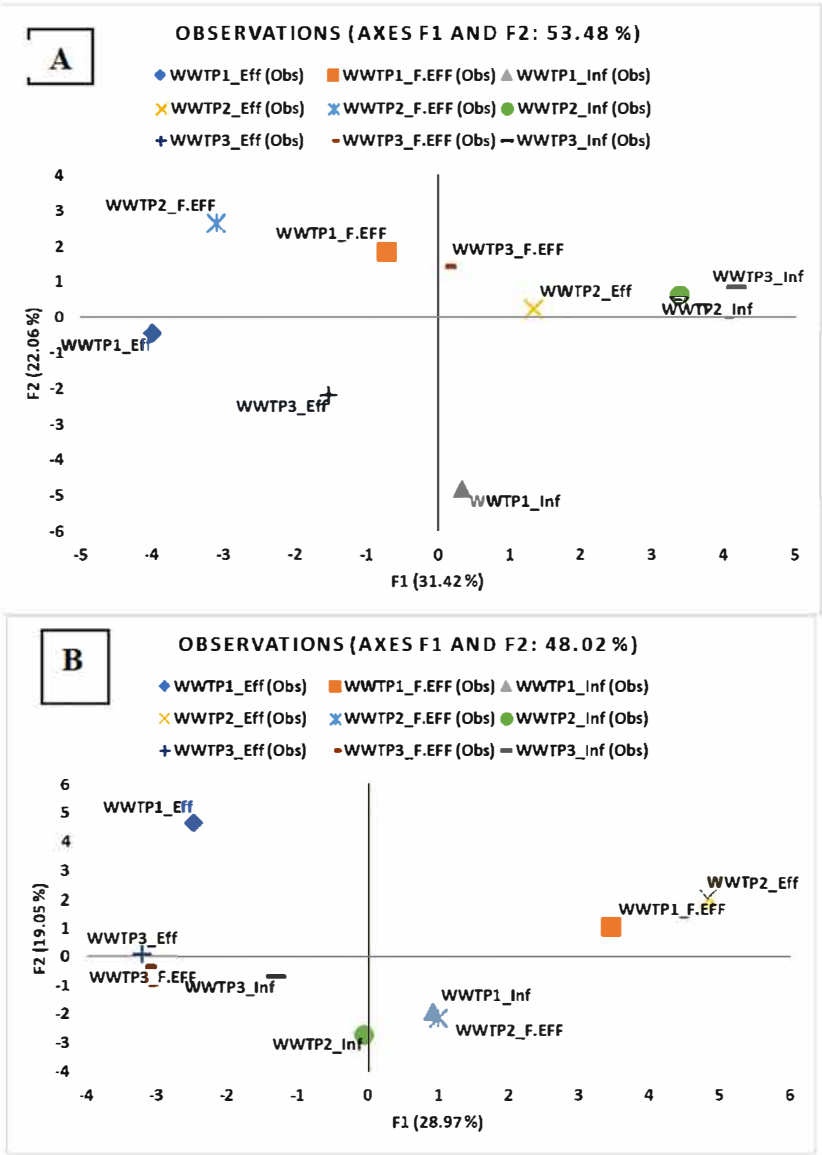


Figure 3.2. Principle component analysis (PCA) for the variances in the distribution of pathogenic bacterial communities from influents to final effluents of three WWTPs. (A) Pathogen distribution across treatment stages in metagenomes. (B) Pathogen distribution across treatment stages in transcripts.

Further analysis showed that a total of 31 species of potential pathogens were present samples. In WWTP1 metagenomes 12, 9 and 19 pathogens were present in influents, secondary and final effluents respectively. *A. butzleri, E. cloacae, S. maltophilia, P. aeruginosa, P. strutzerii,* and *A. hydrophila* were present in lower abundance in final effluents than both influents and secondary effluents (Fig. 3.3a). WWTP2 metagenomes showed 21,19 and 16 pathogens were detected in influents, secondary and final effluents respectively. *N. meningitides, N. gonorrhoeae*, *V parahaemolyticus*, *C. jejuni,* and *H. influenza* which had a higher abundance in secondary effluents were removed in final effluents (Fig. 3.3a). Metagenomes of WWTP3 displayed 22, 12, and 17 pathogens in influent, secondary and final effluents respectively. *C. testosterone* which was higher in abundance in secondary effluents compared to influents was removed completely in final effluents (Fig. 3.3a).

Transcripts of WWTP1 displayed 17, 9, 21 in influent, secondary and final effluent samples respectively (Fig. 3b). *A. hydrophila, E. coli, P stutzeri, S. pneumoniae, E. cloacae* and *L. pneumophilia* which were present in secondary effluents in high abundance persisted into final effluents. In WWTP2 transcripts 17, 22, 17 were present in influent, secondary and final effluent samples respectively. *P stutzeri, S. pneumoniae, E. cloacae, A. hydrophila, E. coli, A. baumanii, N. meningitides,* and *P. aeruginosa* into final effluents. WWTP3 transcripts displayed 12, 13 and 16 in influent, secondary and final effluent samples. Among the bacteria that persisted from

influents to final effluents were *N. gonorrhoeae*, *P stutzeri, S. pneumonia, A. hydrophila, E. coli, A. baumanii, N. meningitidis*.

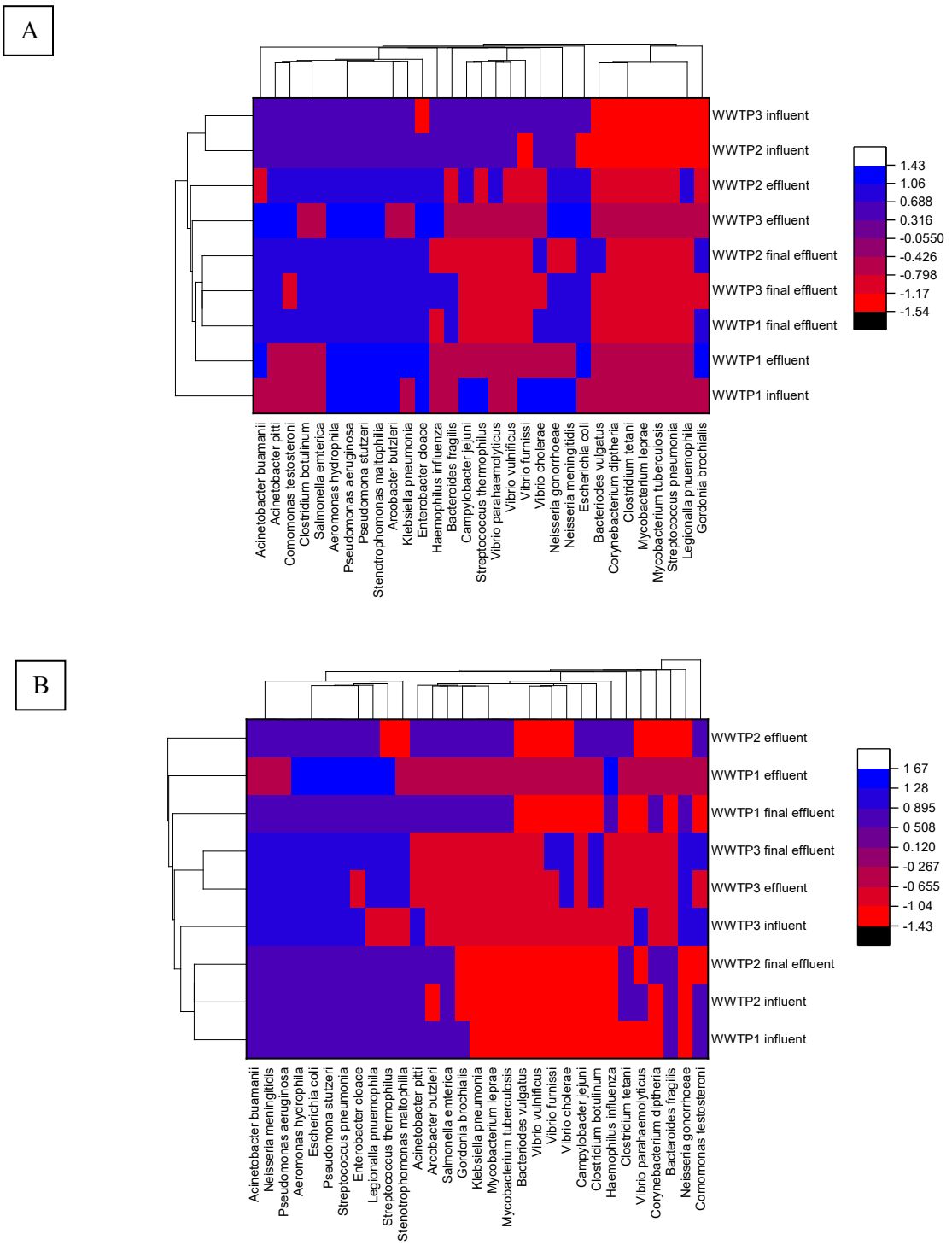
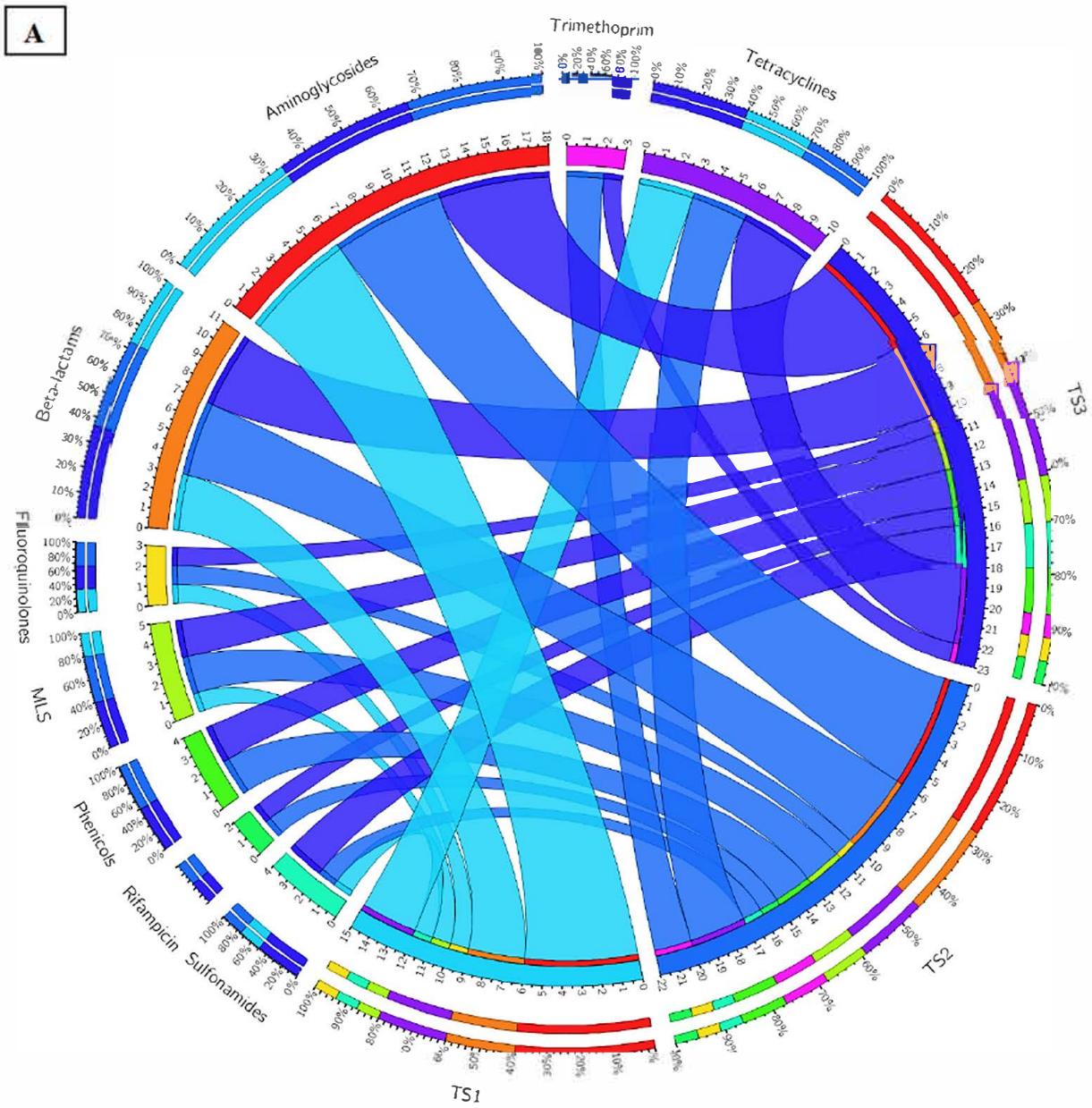


Figure 3.3 Heat map illustrating the relative abundance of potential pathogens in (A) metagenomes and (B) transcripts. The top dendogram clusters OTUs with the closest bacterial affiliation. The left dendogram clusters samples based on shared OTUs across the three WWTPs. Blue and red.

3.3.3 Abundance and diversity of ARG across the treatment systems

Metagenomic data sequences were aligned against a combination of databases for the overall screening of antibiotic classes and their resistance genes. This method detected the overall presence of ARGs belonging to nine antibiotic classes from influent to effluent across the 3 WWTP (Fig. 3.4a). The average diversity of ARG subtypes was higher in all influent samples. Genes conferring resistance to aminoglycoside, beta-lactams and tetracycline were more abundant and widespread than other detected ARGs across the three treatment systems. Observation of transcriptional activity in the transcripts showed that out of the nine identified classes of antibiotics, only seven classes were expressed from influent to secondary effluents across the threesystems (Fig. 3.4b). These included genes conferring resistance to aminoglycosides, trimethoprim, beta-lactams, tetracyclines, trimethoprim, macrolides and sulfonamides. Among the expressed genes, aminoglycoside was detected with the highest transcript abundance. Variation of ARG subtypes between metagenomes and transcripts was apparent, where selected subtypes that dominate metagenomes were not expressed and therefore not detected in transcripts. Furthermore, this phenomenon was observed across all the three WWTPs investigated. The metagenomes of WWTP2 and WWTP3 exhibited a higher ARG diversity compared to WWTP1, which treated domestic effluents only. However, in transcripts, the most expressed genes were displayed by WWTP1 and WWTP3.


A
Trimethoprim
Tetracyclines
TS3
TS2
TS1
Sulfonamides
Rifampicin
Phenicols
MLS
Fluoroquinolones
Beta-lactams
Aminoglycosides

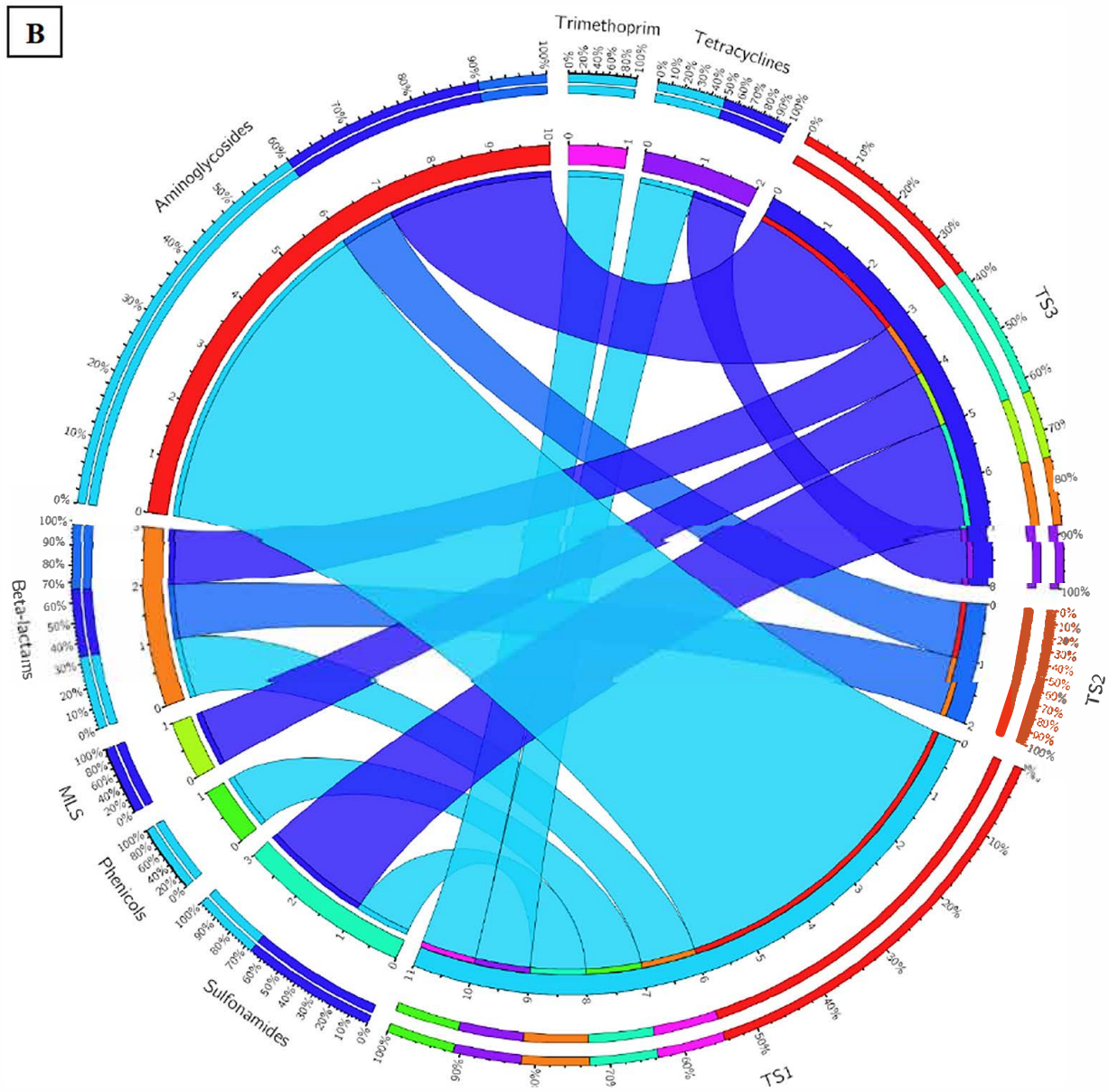


Figure 3.4. Distribution of antibiotic classes in the three WWTP's. A) In metagenomes and B) In transcripts. Length of the outer ring represents the abundance of the of antibiotic classes across the 3 treatment plants in metagenome and transcripts.

3.3.4 Occurrence and distribution of ARGs from influent to final effluents

Further ARGs screening at various sampling points across the treatment train of the three WWTPs was conducted. ARG distribution was determined using PCA bi-plot to compare similarities and variations along the treatment stages of the three WWTPs (Fig. 3.5). The PCA plot showed similarities in the ARG resistome of WWTPs receiving influents from similar sources. PCA showed that the core resistome in metagenomes was divided into two main clusters, one consisting of influents and another represented by secondary and final effluents samples. While the core resistome of transcripts had two main clusters, consisting of secondary and another represented by final effluents samples. Influent metagenomes of WWTP2 and WWTP3 showed a similar ARG profile contrasting that of WWTP1 (Fig. 5a), while a great dissimilarity was apparent in influent transcripts of all three plants (Fig. 5b). The clustering together of WWTP1 and WWTP2 secondary and final effluents showed a shared resistome in metagenomes. In transcripts a similar resistome in secondary effluents was shared between WWTP1 and WWTP3. Resistome profiles of final effluent samples across the three treatment plants clustered indicating a shared ARG profile.

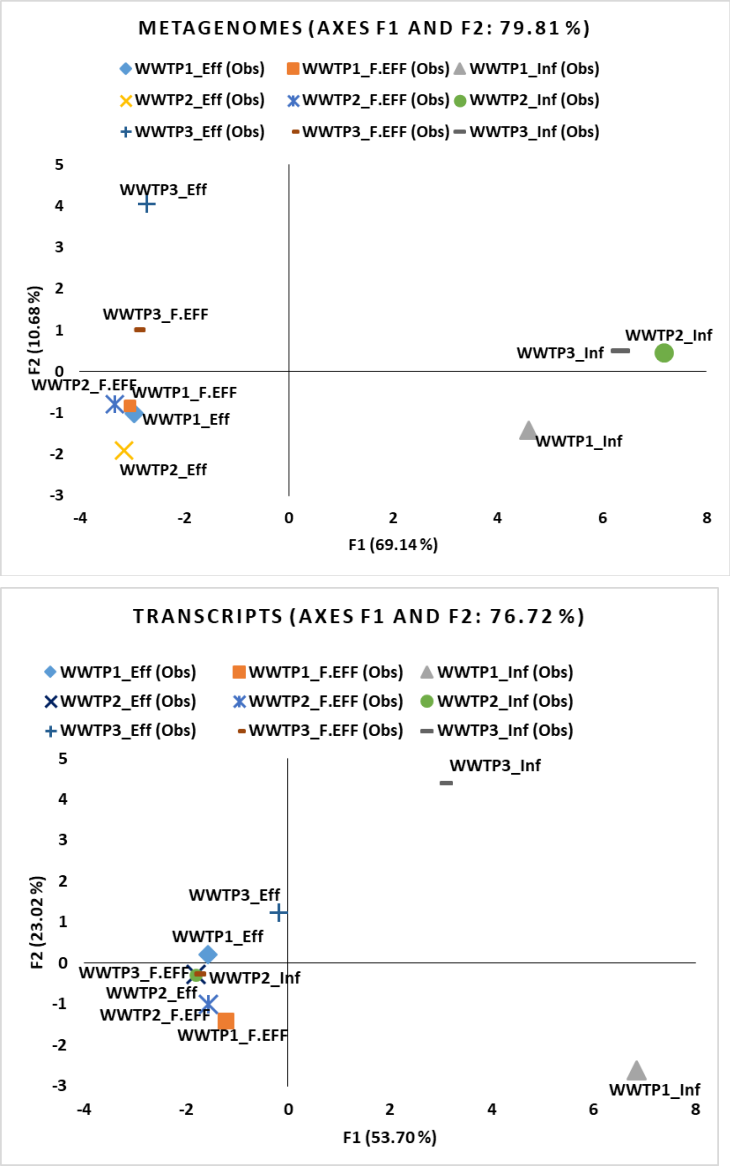


Figure 3.5. Principle component analysis (PCA) for ARG profile from influent to final effluents of three WWTPs. A) Shows the clustering of similar genes in metagenomes. B) shows the clustering of similar genes from transcripts. Axis values indicate the percentage of variance.

The relative abundance of the ARGs significantly decreased across the treatment of all three WWTPs in both metagenomes and transcripts. Metagenomes of WWTP1 displayed 19, 2 and 2 ARGs detected in influents, secondary and final effluent samples respectively. The *SulI* was present in high abundance in both secondary and final effluents (Fig. 3.6a). Among the genes detected in WWTP2 25, 1 and 1 were present in influent, secondary and final effluents respectively. In WWTP2 secondary effluents the *dfrB* gene was present in high abundance. While the *SulI* gene dominated final effluent samples. 23, 6 and 5 ARGs were detected in influent, secondary and final effluents of WWTP3 respectively. Both the *SulI* and *aadA4-5* gene were persistent from influents to final effluents metagenomes (Fig. 3.6a).

Similarly, in WWTP1 transcripts, 11, 3 and 1 ARG subtypes were detected in influents, secondary and final effluent samples respectively. Aminoglycoside resistance gene *aadA4-5* was the dominantly expressed gene in final effluent transcripts of WWTP1 (Fig. 3.6b). WWTP2, beta-lactam gene *TEM*-D which was absent in metagenomes, was the only gene dominantly expressed from influent to final effluents in transcripts (Fig. 3.6b). While in WWTP3 transcripts 7, 3 and 1 ARGs were detected in influents, secondary and final effluents respectively (Fig. 3.6b).

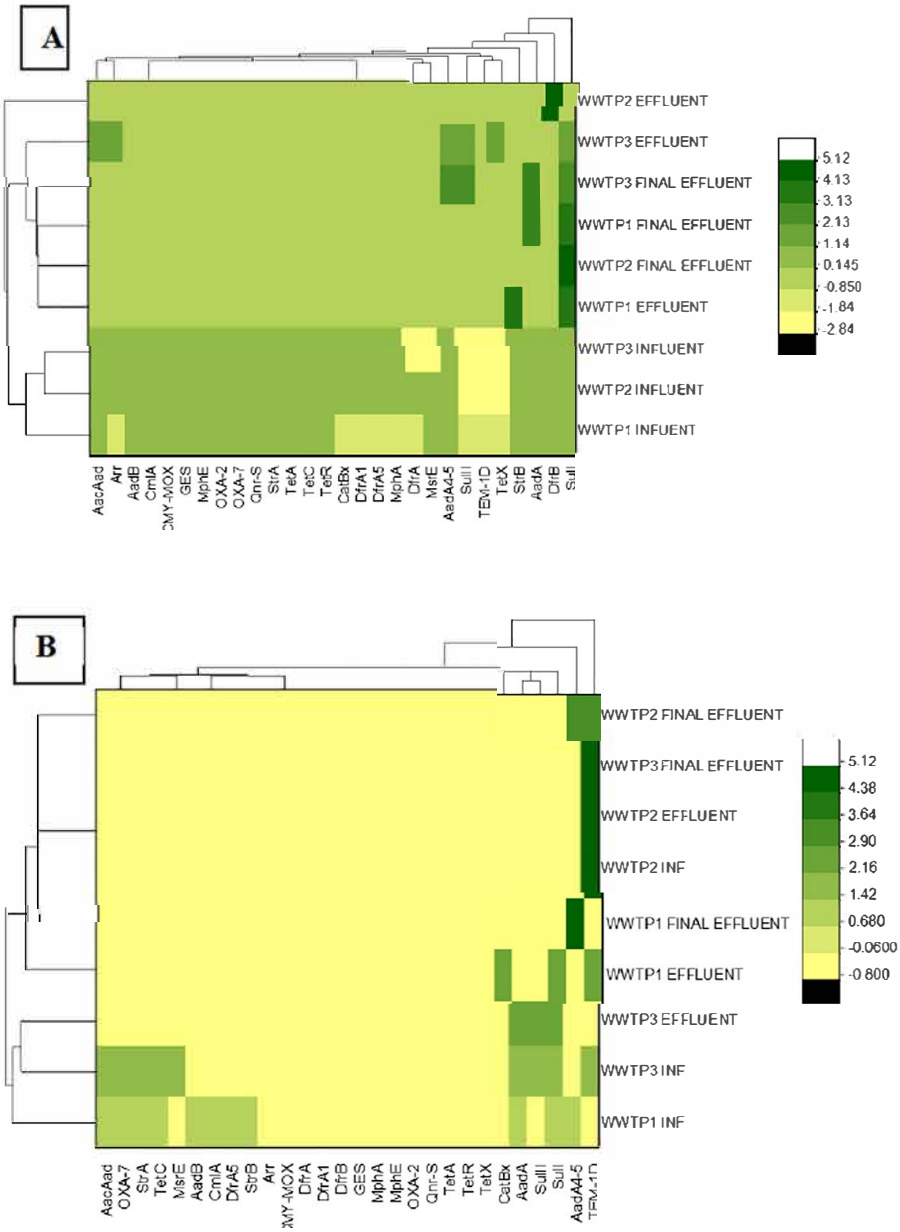


Figure 3.6. Heat maps showing the distribution of ARGs for each sample. A) Distribution of ARGs from influent to final effluent metagenomes and B) ARG distribution in transcripts from influent

3.3.5 ARG associations and co-occurrences in sewage

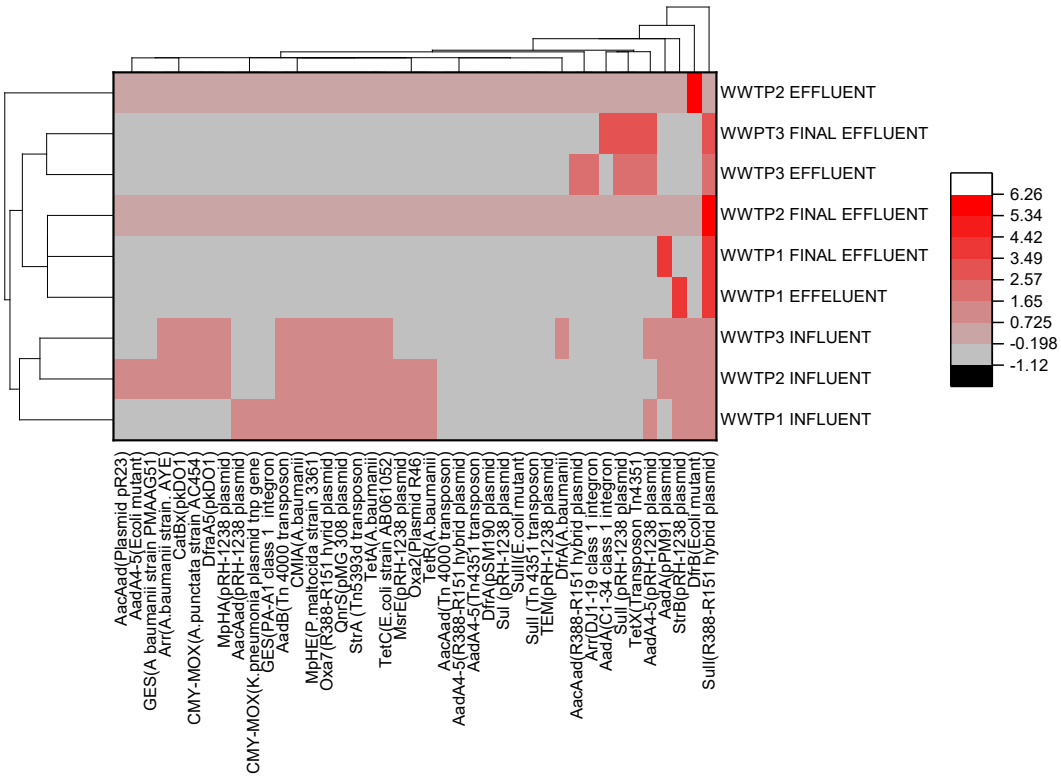
Following assembly of metagenomic and transcript data, a total of 42 ARGs belonging to 9 antibiotic resistance exhibited ARG-MGE co-occurrence from influents to final effluent samples across the three WWTP. Plasmid-ARG associations were the most predominant co-occurrences (Fig 3.7 and Supplementary Fig. 3). The most frequent co-occurrence, were between *SulI* and the R388-R151 hybrid plasmid all samples particularly influents across the three treatment plants. The persistence of the *SulI* gene into final effluents was predominantly linked this MGE. Aminoglycoside ARG subtypes also showed co-occurrence with various plasmids (Fig. 3.7). The reduction in MGEs from influent to final effluents significantly impacted the persistence of selected ARGs across the treatment stages (Fig. 3.7a) in metagenomes. The persistence of certain ARGs in into final effluents in both genomes and transcripts was predominantly facilitated by Plasmid-ARG associations compared to other mobile elements. The co-occurrence between *SulI* and R388-R151 hybrid plasmid borne on *P. aeruginosa* was observed to persist from influents to final effluent samples across all three WWTPs. Similarly, the co-occurrence between a sulfonamide subtype *SulII* and pRH-1238 plasmid carried by *S. enterica* was detected in secondary effluents and final effluents of WWTP3 (Fig.3.7a). The propagation of ARGs was not limited to ARG-MGE co-occurrences as certain ARGs were observed to have associations with chromosomal DNA. Influent metagenomes displayed a shared core resistome of chromosomal associated ARGs which included, *DfrB- E. coli* mutant, *TetC-E. coli*, *MphE- P. multocida*, *CmlA-*

A.baumannii, *TetA- A. baumannii*, *TetR- A. baumannii* across all three WWTPs. Another shared resistome with chromosomal associated ARGs was between WWTP 2 and WWTP3 this included associations between *CMY-MOX- A. punctate* and *Arr-A. baumannii* str. AYE in influent metagenomes. Certain chromosomal-ARG associations were only detected in specific WWTPs, such as associations between *GES –A. baumannii* strain PMAAG51, AadA4-5-*E. coli* which was only detected in WWTP2 influent metagenomes. Similarly, *AacAad- P. aeruginosa*, *Qnr-S- S. enterica*, *GES- P. aeruginosa,* and *A. baumannii* str. AYE with *DfrA* were only detected in influent metagenomes of WWTP3. The persistence of chromosomal associated ARGs into secondary and final effluents of both WWTP2 and WWTP3 was noteworthy. While no such persistence was observed in WWTP1. The association between *DfrB- E. coli* was also detected in WWTP2 secondary effluents, while *Arr-E. coli* and *AadA-P. aeruginosa* were detected in secondary and final effluents respectively.

ARG associations in transcripts showcased the presence of both MGE and chromosomal associations across the treatment train of the three WWTPs. MGE-ARG associations detected in influent transcripts included *strA*-transposon Tn5393d (*A. faecalis)*, *AadA*-plasmid pPM91 (*P. mirabilis*) which were common to WWTP1 and WWTP3 influent transcripts. Other MGE-ARG associations detectable in WWTP1 influent transcripts included *DfrA5*- plasmid pKDO1 (*K. pneumoniae*), *StrB*- plasmid pRH-1238 (*S. enterica*), *OXA-7*- R388-R151 hybrid plasmid (*P. aeruginosa), SulI*-R388-R151 hybrid plasmid (*P. aeruginosa*) and *AadB*- transposon TN4000 (*K.*

pneumoniae). In WWTP3 macrolide resistance gene *MsrE* was actively expressed on plasmid pRH-1238 borne on *S. enterica*. Chromosomal linked ARGs in influent transcripts included *TetC-E. coli* (detected in WWTP1 and WWTP3), *TEM-1D-E. coli* (all three WWTPs), *CmlA-A.baumannii* and *AacAad-K. pneumoniae* (WWTP1), *OXA-7-P. aeruginosa* (WWTP2). Selected ARGs of varied associations persisted into secondary and final effluents. ARG-MGE associated gene *SulI*-R388 borne on *P. aeruginosa* hybrid plasmid R151, previously detected in influents of WWTP1 and WWTP3 persisted into secondary effluents in both treatment plants. *AadA*- plasmidpPM91 (*P. mirabilis*), and *SulII* plasmid pRH-1238 (*S. enterica*) was detected in secondary effluents of WWTP3. *AadA4-5*-plasmid pRH-1238 (*S. enterica*) was persistent in final effluents of WWTP1 and WWTP2. *TEM-1D* borne on *E. coli* was the only chromosome associated ARG topersist to final effluents of WWTP2 and WWTP3.

A


WWTP2 EFFLUENT
WWPT3 FINAL EFFLUENT
WWTP3 EFFLUENT
WWTP2 FINAL EFFLUENT
WWTP1 FINAL EFFLUENT
WWTP1 EFFELUENT
WWTP3 INFLUENT
WWTP2 INFLUENT
WWTP1 INFLUENT
6.26
5.34
4.42
3.49
2.57
1.65
0.725
-0.198
-1.12
AacAad(Plasmid pR23)
AadA4-5(Ecoli mutant)
GES(A.baumanii strain PMAAG51)
Arr(A.baumanii strain_AYE)
CatBx(pkDO1)
CMY-MOX(A.punctata strain ACA54)
DfraA5(pkDO1)
MphA(pRH-1238 plasmid)
AacAad(pRH-1238 plasmid)
CMY-MOX(K.pneumonia plasmid tnp gene)
GES(PA-A1 class 1 integron)
AadB(Tn 4000 transposon)
CMIA(A.baumanii)
MphE(P.maltocida strain 3361)
Oxa7(R388-R151 hyrid plasmid)
QnrS(pMG 308 plasmid)
StrA (Tn5393d transposon)
TetA(A.baumanii)
TetC(E.coli strain AB061052)
MsrE(pRH-1238 plasmid)
Oxa2(Plasmid R46)
TetR(A.baumanii)
AacAad(Tn 4000 transposon)
AadA4-5(R388-R151 hybrid plasmid)
AadA4-5(Tn4351 transposon)
DfrA(pSM190 plasmid)
Sul (pRH-1238 plasmid)
SulII(E.coli mutant)
SulI (Tn 4351 transposon)
TEM(pRH-1238 plasmid)
DfrA(A.baumanii)
AacAad(R388-R151 hybrid plasmid)
Arr(DJ1-19 class 1 integron)
AadA(C1-34 class 1 integron)
Sul (pRH-1238 plasmid)
TetX(Transposon Tn4351)
AadA4-5(pRH-1238 plasmid)
AadA(pPM91 plasmid)
StrB(pRH-1238 plasmid)
DfrB(Ecoli mutant)
SulI(R388-R151 hybrid plasmid)

B

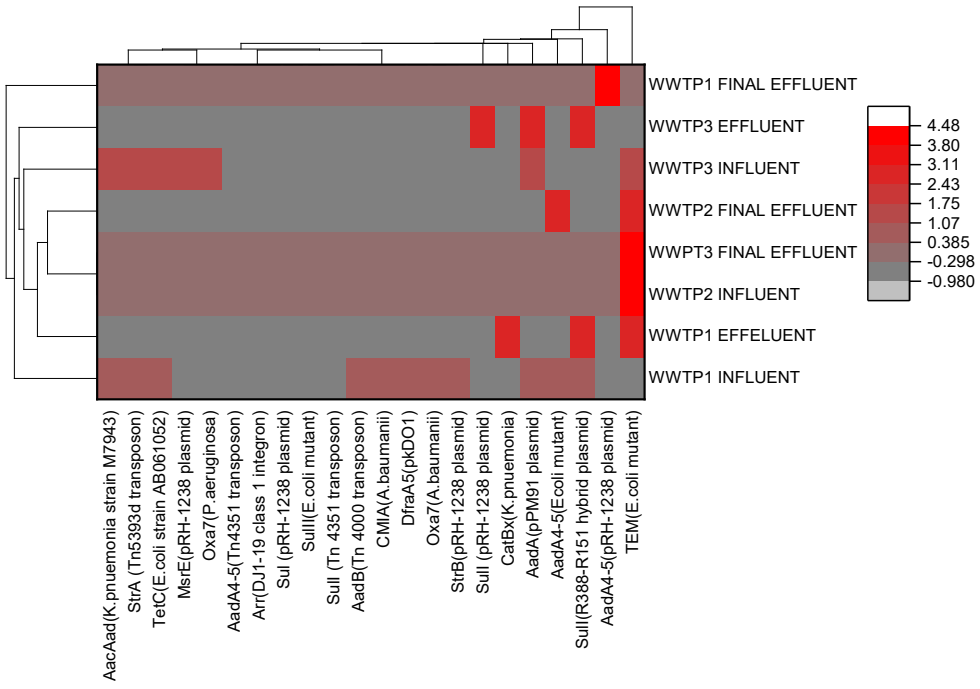

WWTP1 FINAL EFFLUENT
WWTP3 EFFLUENT
WWTP3 INFLUENT
WWTP2 FINAL EFFLUENT
WWPT3 FINAL EFFLUENT
WWTP2 INFLUENT
WWTP1 EFFELUENT
WWTP1 INFLUENT
4.48
3.80
3.11
2.43
1.75
1.07
0.385
-0.298
-0.980
AacAad(K.pnuemonia strain M7943)
StrA (Tn5393d transposon)
TetC(E.coli strain AB061052)
MsrE(pRH-1238 plasmid)
Oxa7(P.aeruginosa)
AadA4-5(Tn4351 transposon)
Arr(DJ1-19 class 1 integron)
Sul (pRH-1238 plasmid)
SulII(E.coli mutant)
SulI (Tn 4351 transposon)
AadB(Tn 4000 transposon)
CMIA(A.baumanii)
DfraA5(pkDO1)
Oxa7(A.baumanii)
StrB(pRH-1238 plasmid)
Sull (pRH-1238 plasmid)
CatBx(K.pnuemonia)
AadA(pPM91 plasmid)
AadA4-5(Ecoli mutant)
SulI(R388-R151 hybrid plasmid)
AadA4-5(pRH-1238 plasmid)
TEM(E.coli mutant)

Figure 3.7. Heat map illustrates the relative abundance of MGE's across the treatment train of the3 treatment plants. The color gradient from Red to Grey in (A) metagenomics and (B) transcripts represents lower to higher abundance of MGE's harboring ARG subtypes.

3.4 Discussion

3. 4.1 Diversity of pathogenic bacteria in the influent and effluent.

The alpha diversity of the overall bacterial communities was investigated in three WWTPs using Chao1, Shannon, Simpson indices and Pilou Evenness. Across the three WWTPs the species richness decreased from influents to secondary effluent samples. Nonetheless, increasing species richness from secondary effluents to final effluents occurred in WWTP1 and WWTP3 which is an indication of the impact of the biological treatment on microbial community enrichment. Poor sludge settle-ability resulting from filamentous bulking and foaming has been the greatest contributor of solid particle carryover in treated effluents in biological wastewater treatment systems (Nierychlo *et al*., 2020). The presence of solid particles in treated effluents significantly impacts microbial communities in effluents as well as the effluent quality.

The diversity results however, revealed slight differences in microbial communities within the individual plants. Based on Shannon and Simpson's indices, WWTP1 and WWTP3 had a lesser bacterial diversity in influents compared to WWTP2, however a higher diversity was exhibited by both plants in final effluents samples (Table 3.1). The increase in diversity within these two configurations may potentially suggest the occurrence of enrichment across the treatment stages which significantly influences a varied biodiversity. The increase of diversity observed in WWTP1, a BNR configured treatment plant in this study is in accordance with conclusions of (Freitas *et al*., 2009, Nnadozie *et al*., 2017) that conditions within the BNR configuration could

allow for higher enrichment of microbial communities. While the biofilm WWTP3 a bio-filter configured treatment plants, actively influences the growth of a more diversified bacterial community by providing bacteria with protection against unfavorable conditions, and nutrients for growth (Marques da Silva and Casetta, 2019). It was interesting to note bacterial diversity in chlorinated final effluents was higher compared to influents as displayed by both WWTP1 and WWTP2. This suggested that chlorine disinfection has very little effect, if any on microbial populations. According to Netshidaulu (2015) the reaction of chlorine with organic and inorganic compounds in water creates a high chlorine demand, leaving very little free chlorine for the adequate disinfection of wastewater.

The dominance of Proteobacteria phyla ranging from 91% - 94% in influents of WWTP1, WWTP2 and WWTP3 respectively was in agreement with previous studies documenting the dominance of Proteobacteria in wastewater (Osunmakinde *et al*., 2019, Monaco *et al.*,2020, Xie *et al*., 2021, Yasir, 2021). Further the persistence in dominance of the Proteobacteria phyla from influentsto final effluents across all three WWTP is consistent with observations from a previous study (Osunmakinde *et al.,* 2019). The top most dominant genera across the three treatment systems belonged to *Aeromonas*, *Pseudomonas* and *Acinetobacter*. *Aeromonas* spp.*,* which are considered as opportunistic human pathogens were detected in higher proportion in influents across all three WWTPs in this study. It was however, interesting to note that *Aeromonas* in WWTP2 which was significantly reduced after biological treatment later increased in final

effluent. The presence of low concentrations of residual chlorine in final effluents contributes to the insufficient inactivation of pathogenic bacteria (Martínez-Hernández *et al*., 2013), and further allows the proliferation bacteria in the absence of the inhibitory effect of biocides. Furthermore, *Aeromonas* spp. are capable of altering both the cell membrane and cell surfaces to make the cells less permeable to biocides (Nzimande, 2013). Therefore, the potential release of *Aeromonas* spp. with effluents is of great concern as this genus has been linked to high antimicrobial resistance patterns, increasing the public threat, especially in cases of immunocompromised individuals (Olaniran *et al*., 2015, Piotrowska *et al*., 2017). Other prevalent pathogens in the WWTPs investigated included *Pseudomonas* and *Acinetobacter.* Both these genera previously observed in wastewater influent and effluents have been identified as potential ARG vectors (Osunmakinde *et al*., 2019).

Dominance of *A. hydrophila* and *A. baumanii* (Fig 3.1a) in influents of all the three treatment plants was apparent, while in influent transcripts *E. coli* and *A. baumanii* were more dominant among the viable pathogens profiled in all three systems (Fig 3.1b). The fate of pathogens that initially dominated influent metagenomes and transcripts varied across the treatment stages, however their persistence to final effluents was apparent. This finding suggested that though the various WWTP could successfully reduce pathogens, their incomplete elimination is difficult as previously reported by (Lu *et al*., 2015b, Cui and Liang, 2019).

The presence of *A. hydrophilia* an emerging contaminant noted in treated effluents in both metagenomes and transcripts in all WWTPs surveyed in this study has been previously observed after conventional treatment and disinfection (Che *et al*., 2019, Cui and Liang (2019). According to (Al-Jassim *et al*., 2015) and (Hou *et al*., 2019) bacteria are capable of overcoming oxidative stress of chlorination, thereby maintaining acceptable intracellular residual chlorine concentration by regulation of efflux pumps. Pathogens such as *A. baumanii,* and *P. aeruginosa*, detected in all influent samples were not only persistent to final effluents but increased compared to the influents (Fig 3.1). These observations are in line with the previous study conducted by Cui and Liang (2019) where they indicated that pathogens in treated effluents were higher in concentration in the treatment system surveyed. The ability of *P. aeruginosa* to regrow in water after chlorine disinfectionhas been also reported earlier (Nguyen *et al*., 2017, Al-Gheethi *et al*., 2018, Isaac *et al*., 2020).

3.4.2 Distribution of ARGs

The profiles showed the presence of 20 ARG subtypes in these treatment systems which conferred resistance to aminoglycoside, beta-lactams (class A, class C, class D beta-lactamases) chloramphenicol, macrolides, quinolone, rifamycin (*arr*), sulfonamide, and tetracycline. The ARG profile was common across the treatment systems with the exception of genes which conferred resistance to rifamycin which were only detected in treatment systems that received hospital effluents. A significant reduction was observed in all the three plants investigated based on both metagenomics and transcript datasets. Based on the WWTP1 metagenomics, a reduction across the treatment train occurred from 19 ARGs detected in influents to only 2 ARGs persisting to final effluent (Fig. 3.6a), while the 11 ARGs detected in influents transcripts were reduced to 1 ARG in final effluents (Fig. 3.6b). According to earlier studies, the reduction of bacteria via sludge wastage and the impact of biological stress on bacterial populations significantly influences the reduction of ARG abundance (Wéry *et al*., 2008, Yang *et al*., 2014). The substantial reduction of ARGs displayed in this treatment plant can therefore be attributed to sludge adsorption and sludge wastage. Similarly, the same reduction pattern as that of WWTP1 was observed in the WWTP2 metagenome, where the ARGs were reduced from the initial 25 in the influents to 1 to final effluents (Fig 3.6a). The reduction in activated sludge systems may be due to adhesion of ARGs to sludge flocs, thereby facilitating their removal by adsorption (Zhang *et al.* 2016a; Lee *et al.* 2017). Interestingly, an increase of ARGs in final effluent was apparent in WWTP2 transcripts

were 2 ARGs were detected in the final effluents, yet only 1 of the genes was initially detected in influent samples (Fig 3.6b). The possible occurrence of HGT may be considered for the increase observed. Previousstudies have also reported that, the chlorine disinfection may select and increase the concentrationof ARGs in the final effluents (Shi *et al*., 2013; Zhang *et al*., 2019). In WWTP3 influent metagenomes, the 22 ARG subtypes detected were reduced to 5 subtypes which were detected insecondary effluents. WWTP3, based on the metagenome analysis, showed a lesser reduction compared to both WWTP1 and WWT2. According to previous studies conducted on ARG removalin trickling filters, it has been concluded that the ARG removal, which is usually biofilm adsorptiondependent, is significantly influenced by the biofilm quality (Zhang *et al.* 2016a; Lee *et al.* 2017).No significant reduction was observed after the disinfection stage in post chlorinated effluents (Fig3.6a). However, in the transcripts, ARGs that had been detected in the inflow were substantially reduced to 1 detectable in final post chlorinated effluents (Fig 3.6b).

A shared core resistome of 20 ARG subtypes belonging to 9 ARG types was observed across the treatment train of the three systems. The most predominantly persistent ARGs were those that encode for aminoglycosides and sulfonamides resistance. The *SulI* gene was the most detected in both genomic and transcriptomic datasets of all configurations investigated (Fig 3.6a and b). The persistence of *SulI* gene observed in final effluents in this study correlates with the previous observation by Goa *et al* (2012) where a high concentration of *SulI* was detected in final effluents after treatment. According to earlier findings, the removal efficiency of *SulI* is dependent on the

free chlorine present in the water. Liu and Hu (2020) observed that free chlorine below 20 mg/L had no significant reduction effect on the *SulI* genes, whilst (Zhang *et al*., 2015) and (Zhuang *et al*., 2015) observed that only free chlorine dose above 30 and 40 mg/L could result in significant reduction.

Aminoglycosides have been reported to be readily degraded in the environment, and genes encoding resistance to this antibiotic group rarely persist into final effluents (Börjesson 2009). However, the persistence of genes encoding resistance against aminoglycosides were found in final effluents in this study (Fig 3.6a and b). The findings on aminoglycosides persistence into thefinal were contrary to the common trait of ARGs associated with aminoglycosides.

3.4.3 Persistence/Removal of MGEs

Influent metagenomes samples were dominated by MGEs across the three WWTPs. The presence of a high abundance of MGEs play a crucial role in facilitating the exchange of resistance genes between organisms (Gupta *et al*., 2018). A highly efficient removal of MGE linked resistance determinants across the treatment train was apparent in both metagenomics and transcripts (Fig. 3.6). However, selected genes including those encoding resistance to sulfonamides (*SulI*), aminoglycoside (*AadA5*, *AadA*) and tetracyclines (*Tet X*) in metagenomes persisted (Fig 3.7). For the *SulI* gene this was associated with plasmids in this study and also documented in previous studies (Che *et al.,* 2019; Yoo and Lee, 2021). Across all three WWTPs, aminoglycoside (*AadA5*, *AadA*) ARGs associated with plasmids were persistent into final effluents. The presence of *AadA* detected in effluents in this present study is consistent with findings of previous work (Thornton *et al*., 2020). Tetracycline resistance gene *Tet X* previously observed by Liu *et al* (2012) was detected in final effluents of WWTP3 having associations with Tn4351 transposon. In transcripts resistance determinants associated with MGEs were also detected in final effluents. These included aminoglycoside (*AadA5)* and sulfonamide (*SulI* and *SulII*). Aminoglycoside resistant gene *AadA4-5* encoded resistance on plasmid pRH-1238 from *S. enterica* in WWTP1. In WWTP3 gene *AadA* encoded resistance on plasmid pPM9 from *P. mirabilis* strain EYG91. *SulII* encoded resistance on plasmid pRH-1238 from *S. enterica* and *SulI* encoded resistance on R388-R151 hybrid plasmid on *P. aeruginosa*.

Interestingly MGE mediated resistance remained persistent over post-chlorination treatment (Figure 3.7a and b). Previous studies assessing the effect of chlorine on ARGs have shown that chlorine has little or no effect on the elimination of ARGs carried on MGEs (Shi *et al*., 2013, Furukawa *et al*., 2017, Quach-Cu *et al*., 2018). This limitation of chlorination disinfection to reduce ARG carryover may contribute to the dissemination of ARGs, especially those on mobile elements, into the receiving water bodies (Rafraf *et al*., 2016, Chu *et al*., 2018, Karkman *et al*., 2018). Gene transmission between genera in the environment as a result of the release of insufficiently treated effluents is inevitable and will ultimately have human health risks (Razavi *et al*., 2017, Zheng *et al*., 2017, *Su et al.,* 2017).

3.5. Conclusions

ARGs and pathogenic bacteria which host resistance genes in most cases, were comprehensively profiled from both metagenomics and transcripts across different treatment stages of the WWTPs. The results clearly showed an overall reduction in the pathogenic bacteria and diversity of the ARGs across the treatment train of the three municipal treatment systems, which suggested that the various treatment processes had the ability to partially remove both pathogens and ARGs. However, complete removal of bacterial pathogens was not achieved across the treatment systems investigated. Additionally, the persistence of selected ARGs into final effluents of the 3 systems was also observed. The role of MGEs in ARG persistence across the treatment stages was also noted where their influence in HGT through the release of insufficiently treated final effluents was inevitable. Plasmid-ARG associations were observed to be the most common means of ARG persistence in final effluents, followed by transposons and integrons. These findings clearly indicated the persistence of viable pathogens as well as ARGs on extracellular DNA fragments is of great concern downstream where the possibility of gene transfer is inevitable. This, therefore, suggest the need for exploration of supplementary technologies such as cold plasma technology, ozone treatment that can enhance the degree of removal prior to the release of effluents downstream.

CHAPTER FOUR: THE FATE OF PATHOGENIC BACTERIA DURING WASTEWATER TREATMENT IN DIFFERENTLY CONFIGURED TREATMENT SYSTEMS

4. 1 Introduction

Wastewater treatment facilitates show a variable potential for the reduction of selected pathogens during the different municipal wastewater treatment processes and during the conditions when they are working well they have the ability of decreasing the rate of potential health risks (Olaolu *et al*., 2014). However, operational and physicochemical factors may affect their survival and removal during the treatment process. Factors such as temperature, total suspended solids (TSS), dissolved oxygen (DO) and pH are known to affect the survival of the pathogens in wastewater (Wang *et al*., 2021). Various pathogenic microorganisms have proven resistant to wastewater treatment processes, their survival capacities are reflected in their release with treated effluents. These include human pathogens and opportunistic pathogens such as *P*. *aeruginosa*, *Aeromonas* spp., *Acinetobacter* spp. and *K*. *pneumoniae* (Limayem *et al*., 2019, Skwor *et al*., 2020, Serwecińska *et al*., 2021, Teban-Man *et al*., 2021, Rocha *et al*., 2022). Moreover, pathogens released with inadequately treated effluents have been identified as sources of antibiotic resistant organisms and as potential vectors of ARGs disseminated into the environmental microbiome (Manaia *et al*., 2018). The continuous release of pathogens, ARBs, and ARGs in insufficiently treated effluents

has contributed greatly to outbreaks linked to emerging and reemerging pathogen diseases in both humans, animals and (aquatic) animal health (Wen *et al*., 2009; Aw and Rose 2012). This has therefore informed the need for effective monitoring of the microbiological quality of effluents before discharging into the receiving environments (Dungeni *et al.*, 2010, Becerra-Castro *et al*., 2015; Noel and Rajan, 2015; Shakir *et al*., 2017). Culture independent techniques such as real-time quantitative polymerase chain reaction (qPCR) has made possible for the detection and enumeration of an extended array of pathogens in treated effluents. This allows for new approaches for efficient effluent quality assessment and compliance with regulations (Al-Gheethi *et al*., 2018). In this chapter the aim was to evaluate and compare the performance in the removal of selected bacterial pathogens in three full scale WWTPs by employing real-time quantitative PCR enumerations. *P. aeruginosa*, *Aeromonas* spp., *Acinetobacter* spp. and *K. pneumoniae* previously detected in high abundance from influents to final effluent genomes in chapter 3 were selected and monitored across the treatment train of the three WWTPs.

4.2. Materials and Methods

4.2.1 Sampling and operational parameters

The process configurations and wastewater description characteristics for the plants under investigation as previously described in section 3.2.1. In addition to the detailed sampling procedure as presented in section 3.2.2, aeration samples of 500 mL volume and 50 g of stones were collected from the TF bed. Plant operational parameters is shown in (Supplementary Table 4).

4.2.2 Sample preparation and genomic DNA extraction

Genomic DNA was extracted from the collected samples as described in section 3.2.3. The concentrations and quality of the extracted DNA samples were determined using Implen N80 NanoPhotometer (Implen, Muenchen, Germany) and the quality was checked by running the samples on 0.8% agarose gel electrophoresis for DNA.

4.2.3 Polymerase chain reaction (PCR)

Polymerase-chain reaction (PCR) identification of the selected pathogens *Aeromonas* spp. *Acinetobacter* spp., *P. aeruginosa,* and *K. pneumoniae* were carried out using specific primers as listed in Table 4.1. PCR amplification was conducted in a T100 Thermal Cycler (Bio-Rad) using 25 µL PCR reactions containing 12,5 µL Taq 2X mastermix (New England Biolabs), 1 µL of each primer (0.5 M), 2 µL of DNA (10 ng/uL) and 8.5 µL sterile water. Optimized annealing temperatures for each primer are listed in Table 4.1. PCR products were visualized using agarose gel (1%) electrophoresis at 80 volts for 60 min and examined under UV illumination using the molecular Chemi Doc XRS+ (Biorad).

Table 4.1: Primers used for PCR and qPCR in this study

Primer	Sequence(5'-3')	Target organisms	Annealing Temp	Fragment size (bp)	References
gyraB-F	GAAGGCCAAGTCGGCCGCCAG	*Aeromonas* spp.	52℃	198	(Anbazhagan *et al*., 2011)
gyraB-R	ATCTTGGCATCGCCCGGGTTTTC				
efp-F	AGCCAGGCCTTAAGGTCATG	*Acinetobacter* spp.	52℃	422	(Anbazhagan *et al*., 2011)
efp-R	GCCAGAAGTATCACCACGTA				
PA-SS-F	GGGGGATCTTCGGACCTCA	P.*aeruginosa*	58℃	196	(Spilker *et al.,* 2004)
PA-SS-R	TCCTTAGAGTGCCCACCCG				
KP-27F3	GGATATCTGACCAGTCGG	K. *pneumoniae*	56℃	202	(Dong *et al*., 2015)
KP-27B3	GGGTTTTGCGTAATGATCTG				

4.2.4 Standard curve preparation

PCR amplicons were purified using GeneJET PCR Purification Kit (Thermo Scientific) following the manufacturers' instruction. The concentrations (ng/µL) of the purified DNA (purified 16S rRNA gene fragments) used as templates for the standard were determined using Implen N80 NanoPhotometer (Implen, Muenchen, Germany). Subsequently, the calculation of copy numbers was done, based on specific molecular weight and Avogadro's number were as follows:

$$\text{Number of copies} = \frac{(\text{Amount in ng} \times \text{Avogadro}'\text{s number})}{\text{Length in bp} \times 1 \times 10^9 \times 650}$$

4.2.5 Quantitative real-time PCR analysis

Quantification of specific gene copy numbers in total DNA was performed using real-time PCR (C-100 Touch, CFX 96, Bio-Rad Laboratories Pty Ltd, USA) with the primer sets targeting different pathogens (Table 4.1). The qPCR reaction mixture (10 µL) contained of 5 µL Luna® Universal qPCR Master Mix (New England Biolabs), 1 µL of each primer (0.5 µM), 2 µL of template DNA and 3 µL sterile distilled water. Appropriate negative controls (without template DNA) were amplified using respective primer conditions. Melting curve analysis was used to confirm specificity of the primer.

4.2.6 Statistical analysis

Data analysis and statistics were performed using XLSTAT software (Addinsoft). Evaluation of principal components was performed to display similarities between treatment plants and any correlation between selected pathogens and parameters. Correlations were calculated using the Pearson coefficient.

4.3 Results

4.3.1 Process performance and operational parameters

Water samples from all the treatment plants were collected from June to September 2021 (once a month over four months). The temperature recorded during the sampling period across ranged from 22 ℃ to 25 ℃in influents, 24 ℃ to 26℃ in aeration basins, 20 ℃ to 24 ℃ in secondary effluents and 17 ℃ to 24 ℃ in final effluents. Variations in the physico-chemical characteristics of the influents across all WWTPs investigated were apparent, and significantly influenced the final effluent water quality. The efficiency of TSS removal in all the plants investigated showed average removals ranging from 66.7% to 96.0 %. WWTP1 showed a more efficient overall reduction of TSS compared with WWTP2 and WWTP3, considering TSS concentrations recorded in 3 months of the study period viz. June, August and September were observed to be relatively higher than the original TSS (4500 mg/L) for this WWTP1 plant design.

It was however, interesting to note that higher than permitted TSS concentration discharge limit range of 30-40 mg/L were recorded in final effluents of WWTP1 in the months of July (48 mg/L) and September (76 mg/L). TSS concentrations in WWTP3 final effluents remained considerably higher than the permissible range (30-40 mg/L). TSS in final effluents of WWTP3 measured 49 mg/L, 63 mg/L and 43 mg/L in June, July and August respectively.

4.3.2 Quantification of specific bacterial pathogens in samples collected from different stages of the treatment train

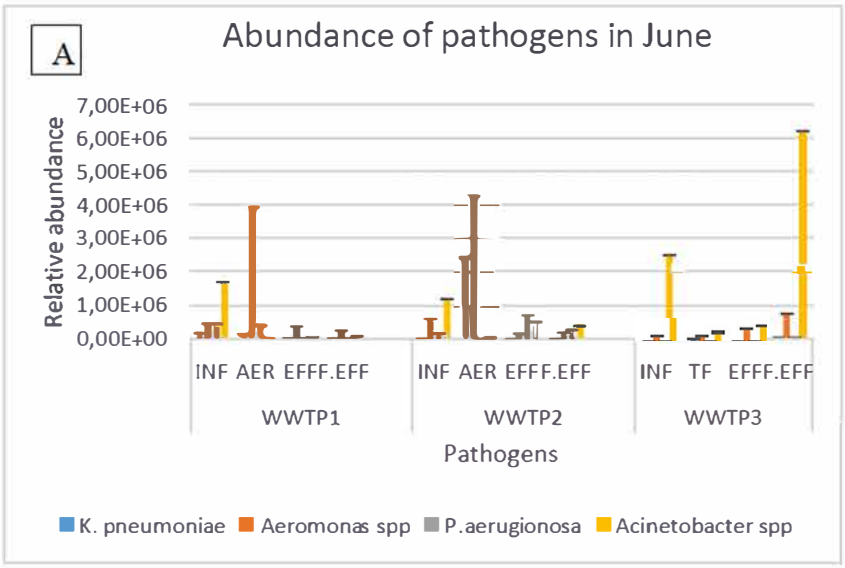
The four opportunistic bacterial pathogens studied (*Aeromonas* spp., *Acinetobacter* spp., *P. aeruginosa* and *K. pneumoniae*) were detected in all samples, including from the final treated effluent. In order to determine the reduction effects of different treatment stages the fate of these pathogens, was assessed by quantification. The qPCR results of the of target pathogens are presented in Fig. 4.1. The abundance of *Aeromonas* spp. and *Acinetobacter* spp. were detected within the range of $1.51 \times 10^5 - 2.17 \times 10^7$ copies/L and $7.44 \times 10^3 - 1.42 \times 10^7$ copies/L respectively. While *P. aeruginosa* and *K. pneumoniae* were in the range $6.78 \times 10^3 - 2.46 \times 10^6$ copies/L and $7.62 \times 10^3 - 2.58 \times 10^7$ copies/L respectively across the WWTPs.

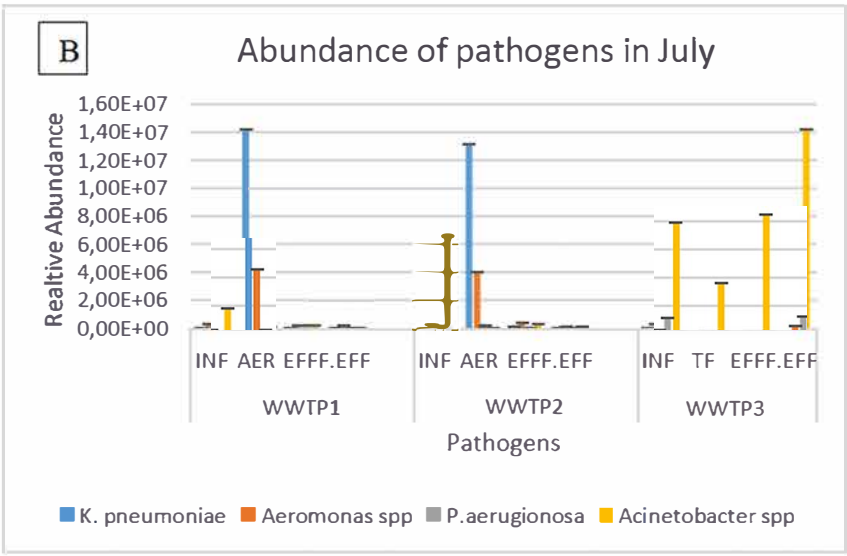
WWTP1 showed an overall reduction of selected pathogens from influent to final effluent compared to WWTP2 and WWTP3. Pathogen concentrations in the influent and final effluent water samples varied for each treatment plant, as seen in Fig.4.1. *Acinetobacter* spp. abundance was the highest in all influents. The second most abundant pathogens belonged to *Aeromonas* spp. which was observed in June, July and September, while *P. aeruginosa* assumed the second highest dominance in August. Variations in the abundance of pathogens were apparent in the aeration basins of the individual plants. In June, both WWTP1 and WWTP2 showed a similar pattern in the highest abundance of *Aeromonas* spp. which was followed by *P. aeruginosa* and *K. pneumoniae* respectively. While in trickling filter samples of WWTP3 *Acinetobacter* spp. exhibited a higher abundance by one order of magnitude over *Aeromonas* spp. A similar pattern of the abundance of

K. pneumoniae followed by *Aeromonas* spp. was apparent in the aeration samples of WWTP1 in July, August and September as well as WWTP2 in July. While the trickling filter basin of WWTP3 showed abundance of both *Acinetobacter* spp. and *Aeromonas* spp. which has decreased from influents. WWTP2 aerations basins showed a high abundance of *K. pneumoniae* followed by *Aeromonas* spp. in both August and September samples. Both these species had increased from influents to aeration samples in the respective months. However, in TF (WWTP3) samples *Acinetobacter* spp. followed by *P. aeruginosa* were abundant during the above months. Both species showed a decrease in abundance compared to influent samples, however were significantly abundant in trickling filter samples. Similarly, in September, *Acinetobacter* spp. was present with high abundance in WWTP3 trickling filter basin, followed by *Aeromonas* spp. which had decreased from influent samples.

In secondary effluents for the month of June *Aeromonas* spp., *P. aeruginosa* and *Acinetobacter* spp. show cased abundance in WWTP1, WWTP2 and WWTP3, respectively. While the second most abundant in both WWTP1 and WWTP2 was *Acinetobacter* spp. and *Aeromonas* spp. in secondary effluents of WWTP3. *Acinetobacter* spp. was highly abundant in final effluents of both WWTP2 and WWTP3. In WWTP1 *Aeromonas* spp. though decreased by one order of magnitude from secondary effluents was highly abundant in final effluents. In July WWTP1 showcased the abundance of *Aeromonas* spp. followed by *P. aeruginosa* in secondary effluents. Both pathogens had decreased by one order of magnitude from aerations samples. Secondary effluents of WWTP2

and WWTP3 showed shared co-dominance of *Acinetobacter* spp. and *Aeromonas* spp. in varying abundances. Both species had decreased from aeration to secondary effluents in WWTP2, while both species has increased from trickling filters samples to final effluents in WWTP3. A high abundance of *Aeromonas* spp. was common to both WWTP1 and WWTP2 final effluents, where the species was seen to decrease from secondary effluents. In WWTP3 *Acinetobacter* spp. which has increased by one log order of magnitude from secondary effluents was dominant. In the month of August *Acinetobacter* spp. abundance was high in all secondary effluents, followed by *P. aeruginosa* in all three WWTPs. In final effluents *Acinetobacter* spp. showed high abundance in both WWTP1 and WWTP2 but varied in copy numbers. In WWTP1 *Acinetobacter* spp. increased from secondary effluents, while the same species decreased in WWTP final effluents. *P. aeruginosa* which was decreased from secondary effluents was abundant in final effluents in WWTP3. In September high abundance of *Acinetobacter* spp. was common in all secondary effluents. This was followed by the abundance of *Aeromonas* spp. in WWTP1 and WWTP2. While *K. pneumoniae* which increased from trickling filter was the second most abundant in WWTP3. Both WWTP2 and WWTP3 shared a high abundance of *Acinetobacter* spp. which had decreased from secondary to final effluents in both plants. In WWTP1, *Aeromonas* spp. which had decreased from secondary to final effluents had a high abundance.


A
Abundance of pathogens in June
Relative abundance
7,00E+06
6,00E+06
5,00E+06
4,00E+06
3,00E+06
2,00E+06
1,00E+06
0,00E+00
INF AER EFFF.EFF
INF AER EFFF.EFF
INF TF EFFF.EFF
WWTP1
WWTP2
WWTP3
Pathogens
K. pneumoniae
Aeromonas spp
P.aerugionosa
Acinetobacter spp


B
Abundance of pathogens in July
Realtive Abundance
1,60E+07
1,40E+07
1,20E+07
1,00E+07
8,00E+06
6,00E+06
4,00E+06
2,00E+06
0,00E+00
INF AER EFFF.EFF
INF AER EFFF.EFF
INF TF EFFF.EFF
WWTP1
WWTP2
WWTP3
Pathogens
K. pneumoniae
Aeromonas spp
P.aerugionosa
Acinetobacter spp

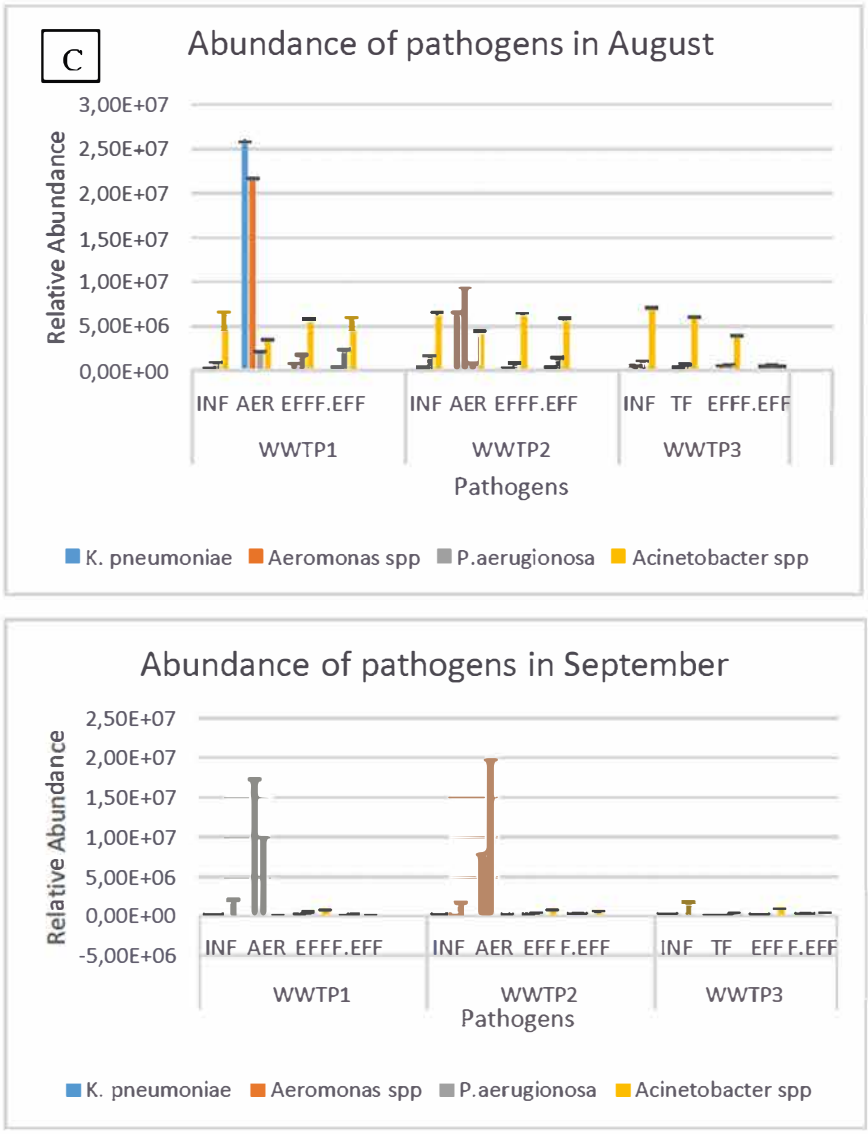
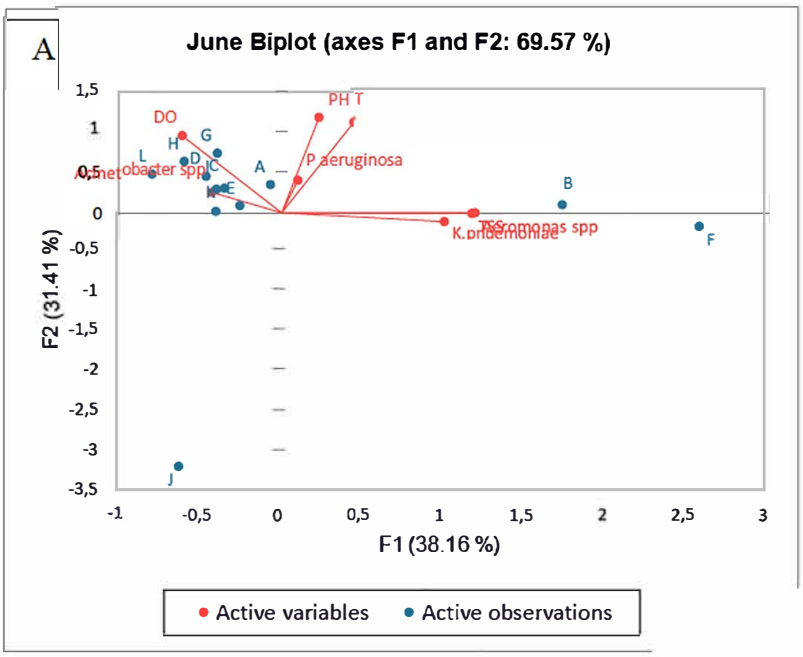


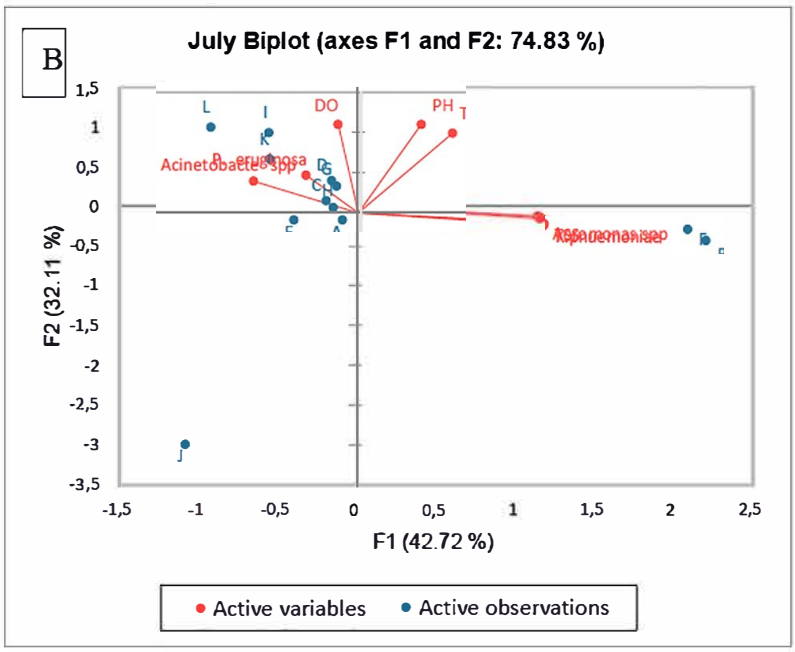
Figure 4.1 Quantification of pathogens from influent to final effluent of BNR, CAS and TF configured wastewater treatment plants. A, B.C and D depicts the fate of pathogens in June, July, August and September respectively.

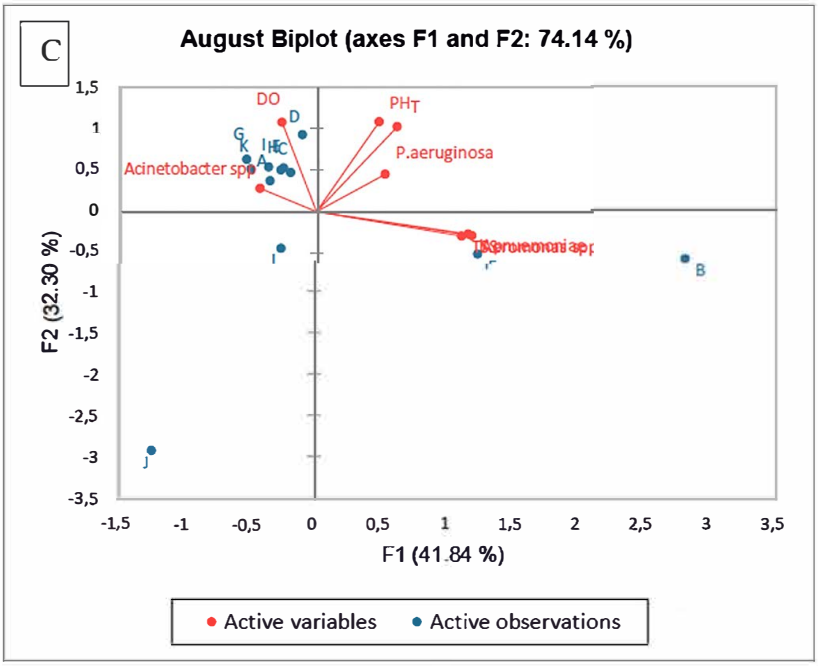
4.3.3 Pathogen composition vs process parameters

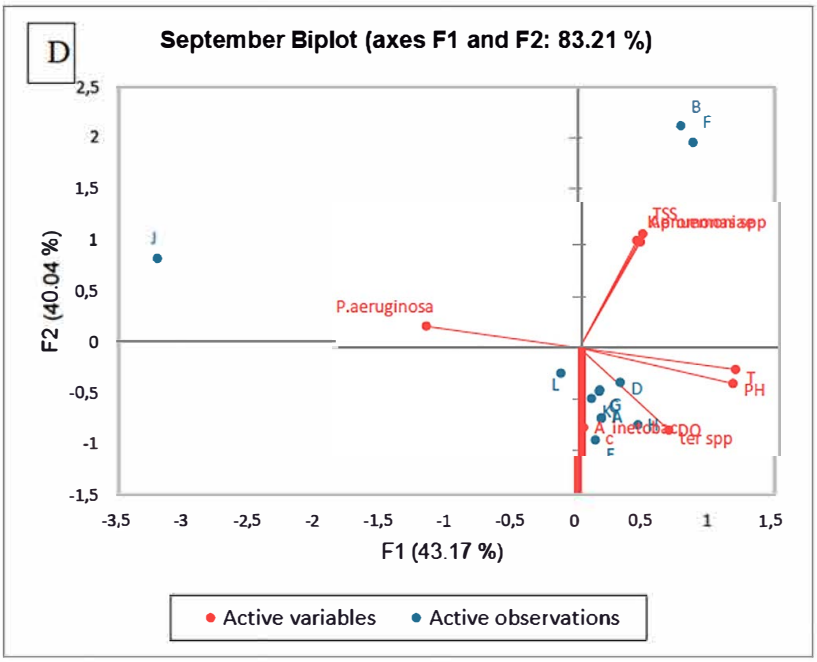
PCA bi-plots were employed for the demonstration of the similarities and correlations among bacterial pathogens and selected plant operational parameters (Fig. 4.2). PCA showed that the results were divided into two main clusters, one consisting of similarities between samples and another represented by affinity between pathogens and selected process parameters. Results of the June biplot (Fig. 4.2a) showed the abundance of both *Aeromonas* spp. and *K. pneumoniae* tend to be larger with higher TSS particularly in aeration basins. While the abundance of *P. aeruginosa* correlated with high temperature and pH. *Acinetobacter* spp. was present in samples that showed a high affinity to high DO concentration, thus correlating the abundance of this species with increase in DO concentration. In the month of July, the abundance of *Aeromonas* spp. and *K. pneumoniae* correlated with high TSS levels in aeration samples of both WWTP1 and WWTP2. Another positive correlation was observed between high DO concentrations with *Acinetobacter* spp. and *P. aeruginosa* in WWTP3 (influents, secondary and final effluents) and both WWTP1 and WWTP2 secondary and final effluents. Temperature and pH showed no significant effect on pathogens (Fig.4.2b). In August a similar trend of correlation between high TSS levels and both *Aeromonas* spp. and *K. pneumoniae* in aerations of WWTP1 and WWTP2 was apparent. The second ordination axis showed correlation between high temperature and pH. *Acinetobacter* spp. tends to be high in higher DO concentrations (Fig. 4.2c). Similarly, in September the abundance of *Acinetobacter* spp. correlated to the presence of high DO concentration as well as high temperature and pH in influent,

secondary effluents and final effluents of both WWTP1 and WWTP2, while in WWTP3 this correlation was observed in influent and secondary effluents. A consistent pattern of correlation between high TSS concentrations and both *Aeromonas* spp. and *K. pneumoniae* was still observed in aerations of WWTP1 and WWTP2 (Fig 4.2.d).









*A-1:INF, B-1: AER, C-1: SEC EFF, D-1:FIN EFF,
E-2: INF, F-2:AER, G-2:SEC EFF, H-2: FIN EFF,
I-3:INF, J-3:TF, K-3:SEC EFF, L-3:FIN EFF.

Figure 4.2. PCA Biplot diagram depicting the effect of process parameters on pathogens across the treatment train of three WWTPs (here denoted as 1, 2 and 3). Summarizes the effects of process parameters on pathogens in the month of June, July, August and September (A, B, C and D) respectively. Axis values indicate the percentage of variance. Abbreviations, INF- influent: AER-aeration, SEC EFF-secondary effluent, FIN EFF-final effluent.

4.4 Discussion

4.4.1. Fate of *Aeromonas* spp from influents to final effluents

Across the three WWTPs the behavior of this species varied from influents to final effluents. In both WWTP1 and WWTP2, *Aeromonas* spp. was observed to increase from influents to aeration basins followed by a decrease in secondary effluents. A variation was however, observed in this populations behavior in the final effluents of WWTP1 and WWTP2. In WWTP3, *Aeromonas* spp. showcased a completely different behavioral pattern with a decrease from influent to trickling filter tanks followed by an increase in both clarifies and final effluents. A significant relationship between the *Aeromonas* spp. and TSS concentrations (Fig. 4.2) was apparent across the sampling period. The increase in TSS concentrations from influents to aeration basins in both WWTP1 and WWTP2 had a significant influence on the *Aeromonas* spp. population (Fig. 4.2). The abundance and dominance of *Aeromonas* spp. in activated sludge communities have been documented in a previous study (Piotrowska *et al*., 2017). Further the conditions within the BNR configuration have been known encourage the enrichment of selected microbial communities (Freitas *et al*., 2009). A gradual decrease of *Aeromonas* spp. from effluents to final effluents of WWTP1 was apparent suggesting the BNR configured system was effective in the partial removal of this population. While in WWTP2 and WWTP3 the concentration of *Aeromonas* spp. increased into final effluents across the study period was noted. This observation was found to be in line with the findings of

Martone-Rocha *et al.,* (2010) who observed the decrease, fluctuation and incomplete removal of *Aeromonas* spp. across the wastewater treatment system (Martone-Rocha *et al*., 2010). The survival and proliferation of *Aeromonas* spp. in chlorinated effluents of all WWTPs investigated in this study may be owing this species moderate resilience to chlorine treatment (Skwor *et al*., 2020).

4.4.2. Fate of *Acinetobacter* spp. from influents to final effluents

Relative abundance of *Acinetobacter* spp. assessed across the treatment train of three WWTP. With the increase in TSS concentration and decrease of DO levels the concentration of *Acinetobacter* spp. were observed to decrease particularly in aeration basins of WWTP1 and WWTP2 and trickling filters of WWTP3. This decrease of the concentration of *Acinetobacter* spp. may be owing to this population being outcompeted by obligate aerobes in substrate utilization within the highly competitive aerobic environment of activated sludge (Wentzel *et al*., 1986). According to Lotter *et al*., (1986), under aerobic conditions *Acinetobacter* strains with an Entner-Doudoroff pathway are capable of utilizing glucose as a substrate thus grow aerobically in competition with other heterotrophs, while absence of pathway results in the usage of acetate as an alternate substrate and are thus at a severe disadvantage due to the shortage of this substrate in waste water (Lotter *et al*., 1986). Furthermore, the limited and or incomplete penetration of nutrients or electron acceptors into the biofilm resulting from poor diffusion has contributed to the slow and spatial heterogeneous growth of microorganisms (Stewart, 2003). A strong relationship was observed between the increasing DO levels and the increasing concentration of *Acinetobacter* spp. in clarified secondary effluents across the study period of all three WWTPs (Fig 4.2). This observation of this present study is in line with that of Chen *et al*., (2019), in a study the authors observed a correlation between the increase in DO levels and the growth of *Acinetobacter* spp. (Chen *et al*., 2019). The presence of *Acinetobacter* spp. varied in final effluents of the three WWTPs across the study,

nonetheless the incomplete removal of this population was apparent. According to Jumat *et al*., (2018) *Acinetobacter* spp. undergo a shift in metabolism in activated sludge, which influences their survival and persistence in wastewater. Further the presence and possible enrichment of *Acinetobacter* spp. chlorinated effluents in this study corroborates with the findings of a previous study by Jumat *et al*., (2018) where *Acinetobacter* spp. underwent an enrichment in final effluents (Jumat *et al*., 2018).

4.4.3. Fate of *Pseudomonas aeruginosa* from influents to final effluents

The relative abundance of *P. aeruginosa* varied from plant to plant across the study period. In the months of July, August and September a positive relationship was observed betweenincreasing TSS concentrations and increasing *P. aeruginosa* populations from influents to aeration basins in WWTP1. The enrichment of in *P. aeruginosa* may be owing to the its predominance denitrification in activated sludge during activated sludge treatment (Drysdale *et al*., 1999, Chen *et al.,* 2003). *P. aeruginosa* populations showed a strong relationship with DO levels in all three WWTPs across the study period (Fig. 4.2). Decrease in DO levels from influents to aeration basininfluenced an increase in the concentrations of *P. aeruginosa*, while the increase of DO levels influenced the decrease of *P. aeruginosa* concentrations in effluent and final effluent samples across the three WWTPs (Fig. 4.2). This finding was observed to be in line with that of previous studies where *Psuedomonas aeruginosa* achieved optimal growth under microaerobic growth (Sabra *et al*., 2002, Alvarez-Ortega and Harwood, 2007). According to Sabra *et al*., (2002), *P. aeruginosa* is capable of creating growth conditions by blockage of the transfer of oxygen which plays an important role in the defense of this pathogen against reactive oxygen intermediates (Sabra *et al*., 2002). The presence of *P. aeruginosa* in chlorinated final effluents varied across the treatment all three WWTPs during the study period. In both WWTP1 and WWTP2 the decrease of this populations concentration in selected months was observed, however complete elimination of the species was not achieved in both treatment systems. The incomplete removal of *P. aeruginosa* has been

documented in previous studies (Slekovec *et al*., 2012, Ng *et al*., 2019). An increase in the concentrations of *P. aeruginosa* in final effluents in WWTP 3 was apparent in the duration of the study period (Fig. 4.1). This enrichment may be due to *P. aeruginosa* having the ability to regrow in water after chlorine disinfection earlier (Nguyen *et al*., 2017, Al-Gheethi *et al*., 2018, Isaac *et al*., 2020). Furthermore, the capsular extracellular polymericsubstances (EPS) of *P. aeruginosa* have a high resistance to chlorine treatment (Sabra *et al*., 2002, Xue *et al*., 2013).

4.4.4. Fate of *Klebsiella pneumoniae* from influents to final effluents

Increase in TSS concentrations and decrease of DO levels from influents to aeration basins (WWTP1 and WWTP2) and trickling filter (WWTP3) significantly impacted *K. pneumoniae* by increasing concentrations. Temperature was another key parameter that showed a strong relationship with *K. pneumoniae* populations. Increase in temperature in aerations basins of both WWTP1 and WWTP2 showed a correlation with increase in the population concentration. In a study conducted by Konopacki *et al*., (2020) mathematically evaluating the growth dynamic of *K. pneumoniae* concluded the optimal growth was within the range of 25-33℃ (Konopacki *et al*., 2020). This observation correlates with the findings of this present study. Similarly, the decrease of temperature in effluent and final effluents of both WWTP1 and WWTP2 (Annex. Table 4.1) significantly affected the decrease of *K. pneumoniae* concentrations (Fig 4.1). Interestingly an increase in *K. pneumoniae* concentrations in final effluents of WWTP3 was observed. The abundance of *K. pnuemoniae* in final effluents has been linked with possible colonization of microplastics in wastewater (Kelly *et al*., 2021). *K. pneumoniae* have been documented for their high adhesive activity in producing of biofilms (Lenchenko *et al*., 2020), which may facilitate influence bacterial association with particles promoting growth status of the bacteria (Chahal *et al*., 2016). According to bacteria associated with particles are more resistant to chlorine compared with microorganisms free in suspension (Chahal *et al*., 2016). Even though the concentrations of *K. pneumoniae* in final effluents were reduced in both WWTP1 and WWTP2 while enriched in

WWTP3 the species remained incompletely elimination *K. pneumoniae* across the three WWTPs. The persistence of *K. pneumoniae* into final effluents as observed in this present is in line with previous studies.

4.5 Conclusion

The application of quantitative polymerase chain reaction for monitoring bacterial pathogen abundance under different plant operational parameters has proven to be beneficial in ascertaining the fate of the pathogens across the treatment train of various WWTPs. The exact mechanisms employed by bacteria in their survival, reduction and proliferation within the specific reactors is poorly understood, and thus an approach combining both the process conditions and concentrations of the individual selected pathogen could be used as an effective tool to improve wastewater treatment efficiency in removing pathogens. In this study, significant relationships between plant operational parameters such as DO, TSS, temperature and pH and selected bacterial pathogens were assessed from influents to final chlorinated effluents.

The results clearly showed an overall reduction of selected pathogenic bacteria from influents to final effluents and subsequent enrichment in selected final effluents. An increases of TSS concentrations and decrease in DO levels had a great influence on the *Aeromonas* spp. population, whereas the increase in TSS concentration (in aeration basin) and the increasing DO levels (effluent and final effluents) had a strong relationship with *Acinetobacter* spp. *P. aeruginosa* seemed to thrive under high TSS and low DO in aerations, while it was negatively impacted by increasing DO in effluents and final effluents. A strong relationship between increasing temperature and increase in *K. pneumoniae* populations was apparent. Additionally, the persistence of the selected pathogens in varying concentrations in the final effluents was also

observed. These findings clearly indicated that reduction of selected pathogens is achieved, however, complete elimination was not successful. Further the disinfection method used in these plants were insufficient in the elimination of pathogens, while it has influenced the enrichment of certain species. This, therefore, suggest the need for further research particularly longitudinal studies to assess the impact of tertiary treatment methods on pathogen removal.

CHAPTER FIVE: EVALUATION OF THE INTERACTION BETWEEN SELECTED BACTERIAL PATHOGENS AND FREE LIVING AMOEBA IN WASTEWATER TREATMENT

5.1 Introduction

Free-living amoeba (FLA) are widely distributed in wastewater systems due to the presence of high concentrations of organic matter and microorganisms (Rubeniņa *et al*., 2017, Denet *et al*., 2017). FLAs are natural predators that engulf other microorganisms, such as bacteria, algae, viruses, and fungi. In wastewater systems FLA predominantly graze on sludge flocs and planktonic bacteria which are taken up through phagocytosis (Strassmann and Shu, 2017). Ingestedorganisms are digested within the phagolysosomes of the amoeba (Tosetti *et al*., 2014). However,certain bacteria including pathogens, have developed mechanisms to elude the phagocytic pathway(Paquet and Charette, 2016). These amoeba-resistant bacteria (ARB), can survive, replicate and finally exiting the amoeba host through lysis (Van der Henst *et al*., 2016). In wastewater, *Acanthamoeba* species, *Naegleria fowleri,* and *Entamoeba histolytica* have been documented to be reservoirs for a variety of amoeba-resistant bacteria which include pathogens viz. *L. pnuemoniae, K. pneumoniae, P. aeruginosa, Mycobacterium* spp, and *A. baumanii*. Within the amoeba's they will exhibit a much higher degree of antibacterial resistance (Tosetti *et al.,* 2014). In addition, antibiotic resistant genes expressed by these ARB's have been associated with mobile genetic elements (MGE's) such as plasmids, integrons, and transposons (Poirel *et al*., 2017). The

co-existence of bacterial genera and their intimate cell-to-cell interactions within the amoeba cells may encourage the potential sharing of genetic material through horizontal gene transfer (HGT). The role of MGE's such as bacterial plasmids in facilitating the transfer of various genes including antibiotic resistance among bacteria have been widely documented (Partridge *et al.,* 2018).

This, therefore, suggests that cell-to-cell interaction between pathogenic and non-pathogenic bacteria with MGEs whilst in amoeba hosts may encourage the uptake of genes and further promote the emergence of multi-drug resistant bacteria capable of passing disinfection of wastewater treatment. The release of antibiotic resistant bacteria from amoeba hosts may contribute to the dissemination and propagation of resistance genes in the receiving environment (von Wintersdorff *et al*., 2016, Muchesa *et al*., 2018).

Traditional conventional culture dependent techniques capable of detecting the presence ofselected pathogenic bacteria in treated final effluents are however limited in accounting for pathogens engulfed by free living amoeba. Furthermore, these conventional techniques fail to detect the presence of genes encoding antibiotic resistance released from lysed amoeba cells and disseminated through treated effluents. The need for culture independent techniques for rapid detection and quantification of endosymbionts and ARGs in FLA is crucial. The aim of this study was, to isolate and enrich FLA from different wastewater samples through conventional methods and to profile the bacterial endosymbionts and ARGs mediating antibiotic resistance within FLA via metagenomics.

5.2. Materials and Method

5.2.1 Sampling and FLA enrichment

The description of wastewater treatment plants under investigation is shown in section 3.2. The detailed sampling procedure and preparation for this study is also shown in section 4.2.1 and 4.2.2. Secondary and final effluents of 5L volume were vacuum filtered using 0.45 µm pore size cellulose nitrate membrane filters (Sartorius Biotech, Goettingen Germany). Membrane filters containing concentrated biomass of secondary and final treated effluents from respective treatment processes, were subsequently inverted onto non-nutrient agar as outlined by Muchesa *et al*., (2017). Modification to the protocol by Muchesa *et al*., (2017) was the placement of heat-killed *E. coli* seed culture as a well opposite to the inverted membranes. The media was supplemented with 10 μg of ampicillin to inhibit the growth of bacteria, and 10 μg of cycloheximide to avoid the growth of fungi. Inoculated plates were incubated for 3 days at 37ºC to allow the migration of free-living amoeba trophozoites towards *E. coli* seed culture. Thereafterthe incubation plates were examined using a cell imager (BioRad) to assess amoeba cellsmigration. Upon detection of trophozoites, excisions were made on agar to capture migrating cellsbefore they reached *E. coli* seed culture. Agar pieces containing amoeba cysts and trophozoites were sub-cultured onto non-nutrient agar plates containing the same components as the initial media. Sub culturing was carried out three times for axenization of amoeba trophozoites. To confirm purity of axenized amoeba cultures, cells were harvested, stained with safranin and

examined at 1000x magnification under a light microscope to confirm the absence of bacteria. Concentrated axenized amoeba trophozoites from each sample investigated were harvested, divided into three batches and stored at -80 ℃ for further analysis.

5.2.2 Preliminary detection of intracellular bacteria using fluorescence microscopy DAPI (4′,6-diamidino-2-phenylindole)

The first batch of axenic samples was fixed in 4% Paraformaldehyde (PFA) and stored at -20ºC for further analysis as per the protocol by Amann (1995a). Fixed samples of 10 µL volume were spotted on teflon-coated slides. Slides were air dried and dehydrated in ethanol series (50%, 80% and 100%) for 3 min to remove of excess water. Samples were then stained with 3µL of DAPI 32 (4’,6-diamidino-2-phenylindole) and allowed to stand at room temperature in the dark for 10 min and thereafter rinsed with warm milliQ water. DryVector shield mounting agent drops were added to wells and coverslips were placed on. The slides were viewed using an Epifluorescence microscope (Olympus BX51).

5.2.3 Genomic sub-culturing and DNA isolation

Concentrated axenized amoeba trophozoites were harvested and co-cultured with heat-killed *E. coli* in 10 mL TSY broth containing 10 μg ampicillin, following incubation at 37ºC for 24 h to induce amoeba lysis. Trophozoite lysis was monitored by staining and observation at 1000x magnification under light microscopy (Zeiss Primo Star, Germany). Lysate volume of 2 mL was pelleted by centrifugation at 7500 rpm for 5 min. Subsequently total genomic DNA was extracted from lysates using the DNeasy PowerSoil kit (Qiagen, Germany), as described in section 3.2.3.

5.2.4 Library construction and ARG annotation

Construction of multiplexed paired-end libraries, followed by sequencing and assembling was carried out as detailed in section 3.2.4. Additional amoeba genomes classification was conducted using the K2_eupathdb48_20201113 database (Aurrecoechea *et al*., 2017). Following the assembly ARGs were annotated as described in section 3.2.5.

5.3 Results

5.3.1. Isolation of free-living amoeba

The initial growth and presence of FLA in all the samples were the emergence of amoeba cysts which migrated away from the inverted membrane towards the heat-killed *E. coli* (Fig. 5.1a). Amoeba cysts were harvested by excising pieces of media containing cyst cells and subsequently subcultured which gave rise to the gradual emergence of trophozoites cells (Fig. 5.1b). Further harvesting and sub-culturing of vegetative trophoziotes resulted in the complete emergence and concentration of vegetative cells (Fig. 5.1c). Non-nutritive media was further excised and sub-culturing of concentrated trophozoites was conducted for axenization of trophozoites and elimination of extracellular bacteria. The microscopic images (Fig. 5.1d)) confirm the axenicity of the isolated FLA from secondary and final treated effluents.

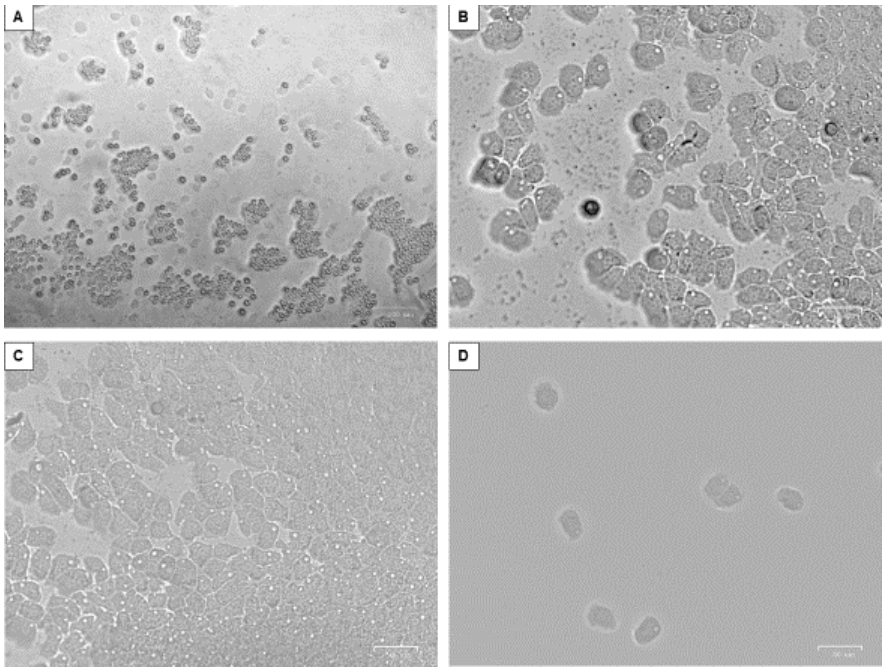


Figure 5.1. Depicts the presence and growth of FLA in pre-chlorinated secondary and final chlorinated effluent samples. a) Migration of amoeba cysts. b). Shows the presence of both vegetative trophozoite and few cysts c). Displays the presence of concentrated amoeba trophozoites. d) Displays the axenization amoeba trophozoite.

5.3.2 Detection of intracellular bacteria using fluorescence microscopy

Axenized amoeba trophozoites cells were harvested and stained with DAPI (4′,6-diamidino-2-phenylindole) for the detection of intracellular bacteria as shown (Fig. 5.2 a and b). The FLA examined all contained bacterial cells. However, the distribution pattern of endosymbionts varied among the FLA hosts, where certain endosymbionts were localized within the cytoplasm (Fig. 5.2a) while some were observed on the periphery of trophozoite cells (Fig. 5.2b).

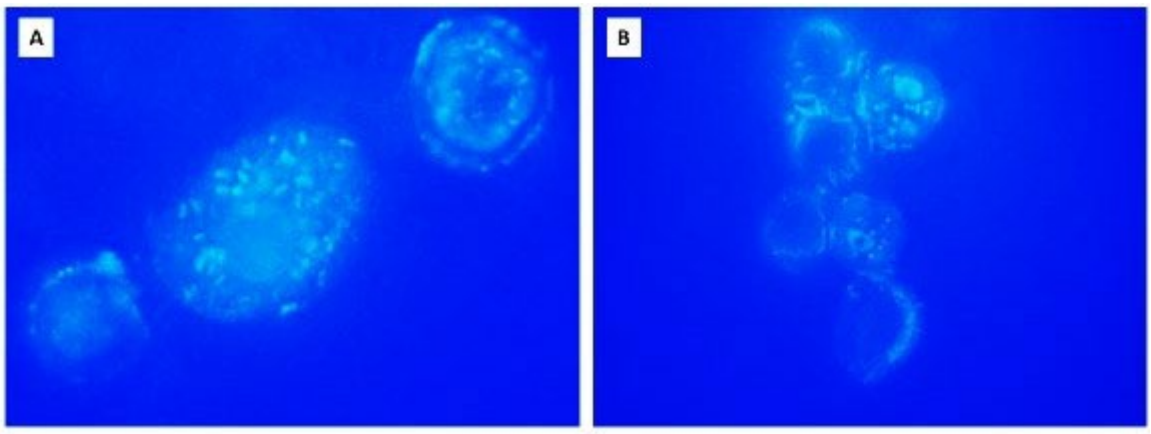


Figure 5.2. DAPI (4′,6-diamidino-2-phenylindole) stained intracellular bacteria distributed within FLA hosts. a). Shows the presence of intracellular bacteria centrally localized within the cytoplasm of FLA host cells. b). Displays the presence of endosymbionts mostly localized on the periphery of FLA host cells.

5.3.3 Isolation of intracellular bacteria via amoeba lysis

Following the positive detection of intracellular bacteria from axenized amoeba cultures in section 5.2., the amoeba trophozoites were further stained with safranin and observed at 1000x magnification to assess amoeba cell intergrity. The cell membrane of the axenized trophozoites were observed to be intact and uncompromised (Fig. 5.3 a and b). Subsequently axenized uncompromised throphozoite cells were co-cultured with heat-killed *E.coli* in TSY broth and incubated at 37 ℃ for 3-5 days to induce lysis. Monitoring of trophozoites was conducted periodically to ascertain amoeba cell lysis and potential release of endosymbionts. After the 5 day incubation period, cell membrane lysis was observed in isolates from both effluents and final effluents samples (Fig. 5.3 c and d). Further the release of endosymbionts was apparent from lyzed cells.

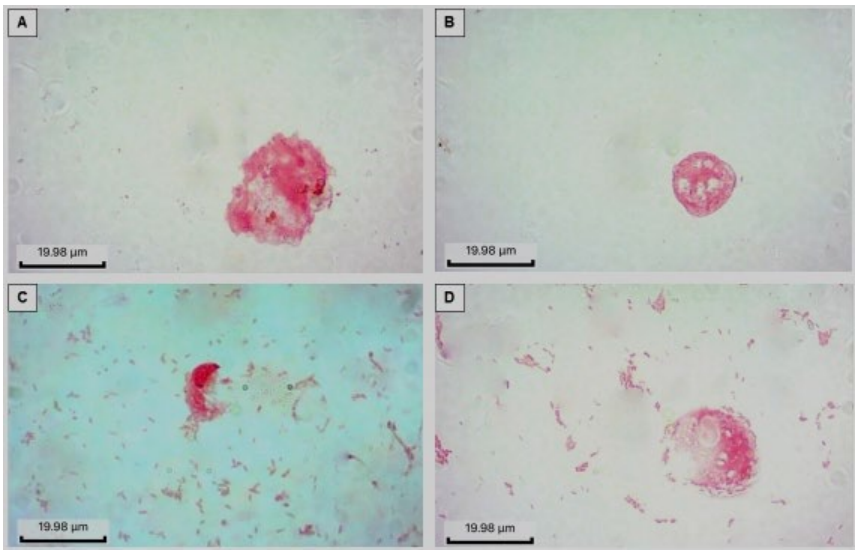


Figure 5.3. Conventional staining of amoeba trophozoites cells from TSY broth. (a) and (b) Depicts light micrographs of intact amoeba trophozoites at 1000x magnification. While (c) and (d) micrographs shows distinct cell membrane lysis and release of intracellular bacteria at 1000x magnification.

5.3.4 Identification of amoeba spp.

Genomic DNA extracted from axenized cultures was used to produce a comprehensive metagenomic profile of the diversity of FLA species isolated from different WWTPs. The distribution of the amoeba species in different samples are shown in Table. 5.1. A total of 9 genera belonging to *Acanthamoeba* and 2 *Entamoeba* spp. was detected. *Acanthamoeba castellini* was the most prevalent dominant species detected in effluent and final effluent samples of all three WWTPs. This was followed by *A. healyi* and *A. lenticulata* which had a similar prevalence across the samples. A similar FLA profile was apparent between WWTP2 and WWTP3, even though the presence of FLA varied from effluents to final effluents in each plant. WWTP3 was the only plant with the most persistence of FLA from effluents to final effluents in all treatment plants investigated.

Table 5.1 FLA detected in secondary and final effluent metagenomes

FLA	WWTP1	WWTP2		WWTP3	
	S.EFF	S.EFF	F.EFF	S.EFF	F.EFF
A. castellini	+	+	+	+	+
A. healyi	+	-	+	+	+
A. lenticulata	+	-	+	+	+
A. qui	+	-	-	-	-
A. culbertsoni	-	+	-	+	+
A. astronyxis	-	+	-	-	-
E. histolytica	-	+	-	+	+
A. palestinensis	-	-	+	+	-
A. royreba	-	-	+	+	-
A. lugdunensis	-	-	-	+	-
E. moshkovskii	-	-	-	+	-

5.3.5 Identification of endosymbiont bacteria

A comprehensive metagenomic profile of the diversity of potential pathogen endosymbionts was conducted from both secondary and final effluent samples across the three WWTPs investigated. The distribution of potential pathogenic endosymbiont bacteria is shown in Fig. 5.4. The most dominant bacterial endosymbionts in both secondary and final effluent metagenomes were *P. aeruginosa*, *S. maltophilia, A. spanius, C. testosteroni,* and *E. cloacae*. Variations in the abundance of each endosymbiont pathogen from effluent to final effluent samples were apparent. Effluent metagenomes of WWTP2 and WWTP3 showed a similarity in the relative abundance of *P. stutzeri* which was higher followed by *P. putida* and *P. aeruginosa* respectively. While in effluent metagenomes of WWTP1 *C. freundii* displayed relative abundance followed by *E. cloacae*. Difference in relative abundances of bacterial endosymbiont occurred in final effluents. The relative abundance of *P. stutzeri* was higher followed by *S. maltophilia* in the final effluents of WWTP2. In contrast, *S. maltophilia* relative abundance was higher followed by *P. stutzeri* in the final effluents of WWTP3.

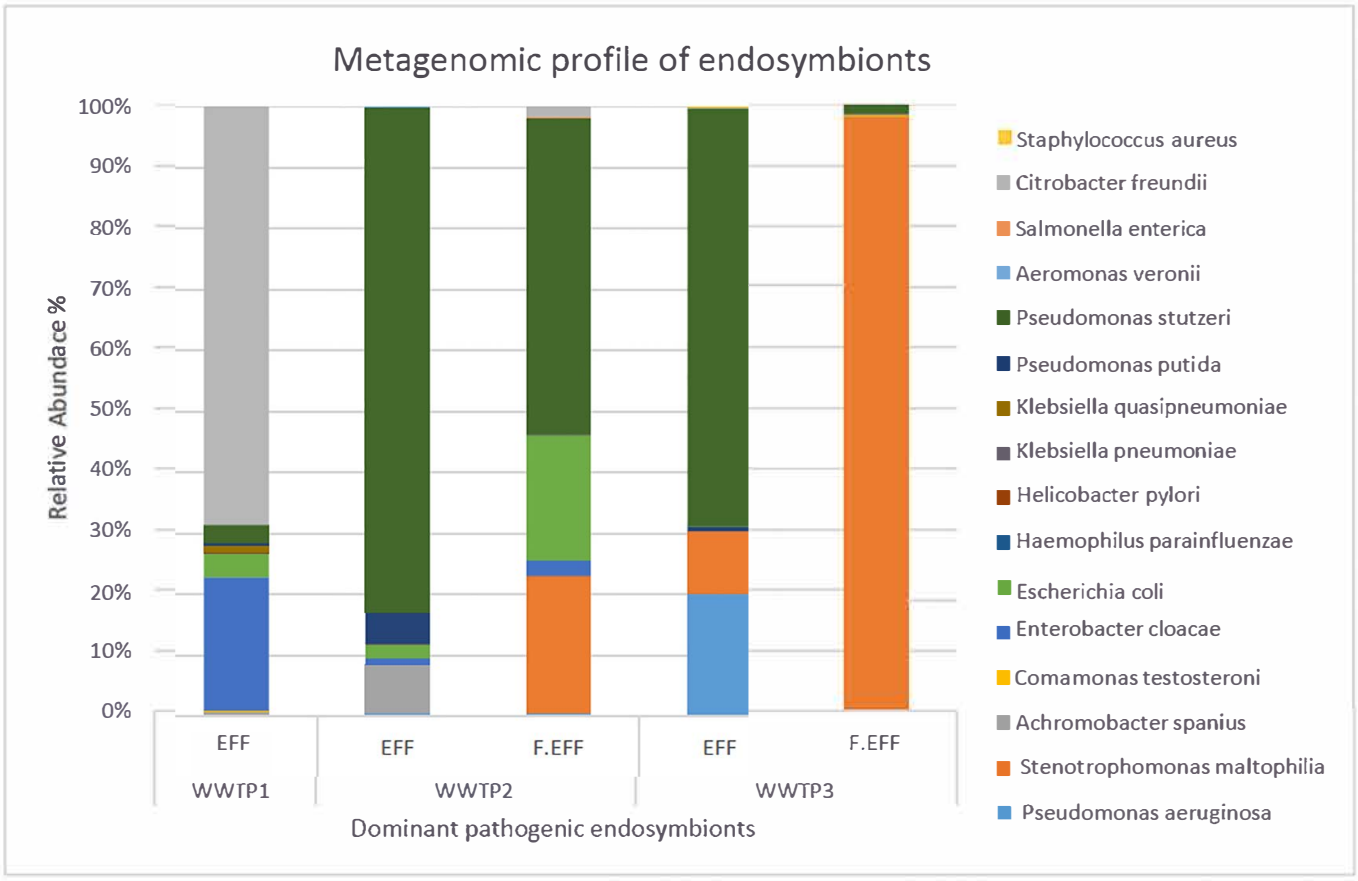


Figure 5.4. Display of the taxonomic distribution of dominant pathogenicendosymbiont species in metagenomes.

5.3.6 ARG association within FLA

Metagenomic data sequences from lysed axenized FLA were aligned against a combination of databases for the screening of antibiotic resistance genes. The presence of ARGs belonging to aminoglycosides, sulfonamides, tetracyclines, trimethoprim, macrolides and rifamycin antibiotic classes were detected from FLA isolated from secondary effluent to final effluent across the 3 WWTP (Table 5.2). Among the detectable genes were genes conferring resistance to aminoglycosides (*aadA*); trimethoprim (*dfrA15* and *dfrA5*); sulphonamides (*Sul1* and *SulII*), macrolide (*msr A, mphC*); rifamycin (*Arr*); quinolone (*qnrE1*) and tetracyclines (*TetA and TetG*). Interestingly the *Sul1* gene detected in secondary effluents of WWTP1 as observed in section 3.3.4 was also detected in FLA from secondary effluent of the same WWTP1. Aminoglycosides gene *aadA*, detected from FLA isolated from WWTP1 has been previously detected in transcripts of the same plant. *Arr* resistance gene detected in FLA from final effluents of WWTP3 has been previously noted in section 3.3.4 as having ARG-MGE association in *E. coli* detected in final effluent metagenomes.

Table 5.2 Diversity of ARGs detected in FLA from secondary to final effluents

ARG	WWTP1	WWTP2		WWTP3	
	S.EFF	S.EFF	F.EFF	S.EFF	F.EFF
SulI	+	-	-	-	-
dfrA5	+	-	-	-	-
AadA	+	-	+	-	-
dfrA15	+	-	+	-	-
SulII	+	-	+	-	-
TetA	+	-	-	-	-
TetG	+	-	-	-	-
msr	-	-	-	+	-
mph	-	-	-	+	-
arr	-	-	-	+	-
qnrE1	-	-	+	-	-

*SEC EFF- secondary effluents, * FIN EFF- final effluents

5.4 Discussion

The purpose of this study was to investigate the presence of endosymbionts and mobile genetic elements, particularly ARG associations within FLA within WWTPs.

Concentrated secondary and chlorinated effluent samples from WWTPs investigated were cultured by inversion using FLA isolation media (Muchesa *et al*., 2017), which allowed for the initial FLA growth and migration in the form of cysts across all samples investigated (Fig 5.1a). Interestingly FLA were abundantly present in both pre and post chlorinated samples as cysts compared to vegetative cells (Fig 5.1a). It has been reported that unfavorable conditions, such as nutrient starvation and the presence of toxic substances significantly influence the differentiation of vegetative cells into dormant cysts (Muchesa *et al.,* 2014, Lambrecht *et al*., 2017, Samba-Louaka *et al*., 2019). In the cystic phase, FLA can bypass the rigor of the wastewater treatment regiment, while also providing shelter for internalized bacteria (Lambrecht *et al*., 2015). Amoeba cysts are capable of monitoring changes in their environment by means of minute pores known as "ostioles", which has a double function, also as exit points for trophozoites during excystation when favorable growth conditions return (Chávez-Munguía *et al*., 2005, Lakhundi et al., 2014, Siddiqui *et al*., 2019). In this study, a series of sub-culturing yielded the emergence of trophozoites active in all samples investigated (Fig 5.1b, c and d).

Acanthamoeba spp. been reported to be the most frequently isolated amoeba from treated effluents (García *et al*., 2011, Salahuldeen *et al*., 2021). Metagenome data sets made apparent the presence of *Acanthamoeba* spp. was the dominant FLA isolated from all samples including final chlorinated samples (Table 5.1). *Acantamoeba* spp. has been previously identified as host to various microorganisms in wastewater including *Pseudomonas, Vibrio, Klebsiella, Salmonella,* and *E. coli* (Riquelme *et al*., 2016; Van der Henst *et al.,* 2016; Cui *et al*., 2018;). *Acanthamoeba* spp which included *A. castellanii*, *A. culbertsoni*, *A. astronyxis*, *A. healyi*, and *A. lenticulata* that are associated with central nervous system diseases (Xuan *et al*., 2017), were detected in effluents and final effluents of WWTP2 and WWTP3 which shared hospital influent source. The persistence of selected FLA, particularly those identified as causative agents from effluents to final effluents was commonly observed in WWTP3 which operated on a biofilm configuration. According to Del Olmo *et al*., (2021) the presence of biofilms, not only provide amoebae with food but also the protection for their persistence. *Entamoeba histolytica* is an anaerobic parasitic protozoan and is well known as a human pathogen was detected in both WWTP2 and WWTP3 (Chowdhury *et al*., 2022). The persistence of *E. histolytica* was only observed in final effluents of WWTP3 which may be due to the presence of the biofilm. Chowdhury *et al*., (2022) stated that while *Entamoeba* species are particularly resistant to low chlorine dosages, the effectiveness of chlorination is highly dependent on both temperature and pH.

This study focussed on ascertaining the presence of intracellular bacteria harbored within trophozoite via DAPI staining. DAPI allowed the visualization and localization of the intracellular bacteria within amoeba host cells (Fig 5.2). Moreover, DAPI fluorescent endosymbiont cells may be suggestive of possible viability. In a previous study conducted by Johnson and Criss (2013) DAPI, in combination with SYTOX Green, to assess viability, DAPI stained cells, were considered viable while dead cells stained with SYTOX Green (Johnson and Criss 2013).

Following the positive confirmation of the presence of endosymbionts, axenic trophozoites were co-cultured with heat killed *E. coli* in TSY broth in accordance with Muchesa *et al.,* (2014). Morphology of the FLA host cells was assessed on day one of the co-culturing, showed the presence of intact FLAs (Fig 5.3 a and b). Futhermore after five days incubation period, raptured FLA cells were observed (Fig. 5.3 c and d) strongly suggesting the occurrence of host cell lysis. According to Jacquier *et al*., (2013) intracellular bacteria are capable of lysing their amoeba hosts under amoeba co-culture conditions (Jacquier *et al.,* 2013). Selected human pathogens viz.*L. pneumophila, P. aeruginosa, S. enterica*, also identified as amoeba resistant bacteria have been documented for their ability to lyse host amoeba and exit into the environment (Maschio *et al*., 2015, Van der Henst et al., 2016, Riquelme *et al*., 2016; Van der Henst *et al.,* 2016; Cui *et al*., 2017). In line with this, the presence of common FLA endosymbionts was characterized using metagenomic analysis. Results made apparent the presence of the top most dominant bacterial pathogens, including *P. aeruginosa, S. maltophilia, A. spanius, C. testosteroni, and E. cloacae.*

(Fig 5.4) among bacterial genera present from the retrieved DNA after amoeba cell lysis. According to Methvin (2009) the growth of pathogenic endosymbionts within *Acanthamoeba* may physically change the bacteria and make it more virulent. Furthermore, the survival of pathogenic bacteria ingested by FLA is an evolutionary precursor to surviving in humans and higher animals (Methvin, 2009). Moreover, pathogens surviving and proliferating within FLAs have been associated with antibiotic resistance.

Consequently, the presence and interaction of pathogens and non-pathogens within amoeba hosts in wastewater may significantly contribute to the sharing of genes between genetically different bacteria via MGEs which are effective carriers of ARGs (Cacace *et al.,*2019). This, therefore suggests that bacteria internalized and surviving within amoeba may significantly contribute to ARGs that exit WWTPs with treated effluents. Mobile elements are the drivers of ARG persistence in wastewater, particularly plasmid-ARG association which are the most predominant mediators antibiotic resistance (Che *et al*., 2019). Wastewater treatment can disrupt bacterial enzymatic systems, but does little to no damage to the ARGs inserted in MGEs (Yuan *et al*., 2015). This, therefore is of great concern, particularly where bacteria are capable of amoeba lysis, and exit with their more ARG's into the environment.

To evaluate this, the presence of ARGs from within amoeba was investigated in this study. Genomic DNA from all samples was screened using metagenomics for the presence of ARG's. We restricted our selection of ARGs for this portion of the study to those detected in the

metagenome and transcript datasets, as described in chapter 3. It is necessary, however, to conduct further analysis to get a better understanding of the complete ARGs profile of the metagenomes. The results have shown the presenece of ARGs which conferred resistance to aminoglycosides, sulfonamides, trimethoprim, quinolone, rifamycin, macrolides and tetracyclines within FLA. *aadA* gene conferring resistance to aminoglycosides was detected in secondary and post chlorinated final effluent of WWTP1 and WWTP3, respectively (Table 5.2). In a previous study by Cameron *et al*. (2018) *aadA* encoding resistance to aminoglycoside group has been detected on transmissible MGEs, such as plasmid in effluents (Cameron *et al*. 2018). The *qnr* gene conferring resistance to fluoroquinolones was detected in post chlorinated effluent of WWTP3. This observation was in line with that of (Kaplan *et al*., 2018), who detected *qnr* genes inserted on plasmids in treated final effluents (Kaplan *et al*., 2018). Yuan *et al.,* (2015) studied the fate of ARG's during wastewater chlorination and concluded that chlorination was ineffective in the reduction and elimination of ARG's (Yuan *et al.,* 2015). Furthermore, Shi *et al.,* (2013) stated that chlorination caused the enrichment of the *TEM* gene as well as the concentration of ARG's and plasmids (Shi *et al*., 2013). *SulI* and *SulII* conferring resistance to sulfonamide were also detected (Table 5.3). Sulfonamide gene, *SulI,* was detected in the secondary effluents of WWTP3 and final chlorinated effluents of WWTP2. The presence of *SulI* has been previously linked to the discharge of treated effluents (Paul *et al*., 2018). WWTP1 effluents and WWTP2 final effluent samples were positive for the *SulI* and *SulII* genes, respectively. Recent studies have detected sulfonamide genes *SulI* and *SulII*

on conjugative plasmids at high concentrations in treated wastewater effluents (Hamidian *et al*., 2016; Makowska *et al*., 2016).

Similarly, tetracycline resistance genes, *TetA* and *TetG* were detected from FLA isolated from WWTP1 effluent samples. This observation was in line with that of previous studies where the tetracyclines resistance genes were detected in effluents (Szczepanowski et al., 2009, Huang *et al*.,2015, Pazda *et al*., 2020).

FLA together with their endosymbionts and ARGs were present in the post chlorinated effluents of wastewater treatment plants. Hence, FLA in WWTPS significantly contributes to the dissemination of antibiotic resistant pathogens and genes in the environment.

5. Conclusions

FLA were successfully isolated and profiled from pre-chlorinated post chlorinated effluent samples from three different WWTPs. The presence of endosymbionts was detected in all FLA isolated from treated effluents. Interestingly ARB detected from within the FLA were able to lyse their amoeba hosts under favorable conditions. Their ability to lyse amoeba hosts validated their viability. Secondary and final effluent metagenomes showcased dominant bacterial endosymbionts detected within FLA included *P aeruginosa*, *S. maltophilia, A. spanius, C. testosteroni,* and *E. cloacae*. *S. maltophilia* and *P. stutzeri* were detected with varying abundance in final effluents of WWTP2 and WWTP3 which are the only two treatment plants that shared a similar influent source. Further analysis showed that these organisms harbored ARGs which conferred resistance to 6 antibiotic classes namely, among the detectable genes were genes conferring resistance to aminoglycosides, trimethoprim, sulfonamides, macrolide, rifamycin, quinolone and tetracyclines. Aminoglycoside gene *AadA*, trimethoprim gene *dfrA15,* quinolone *qnrE1* and sulfonamide gene *SulII* were among the genes detected in final treated effluents samples, suggesting FLA are trojan horses aiding the passage of ARGs. Based on the observations of this study, it can be concluded that FLA harbored bacterial pathogens that possessed ARGs. The in-effectivity of the disinfection method employed by the plants under investigation warrants the need for exploration of alternative measures to mitigate the release of ARBs and ARGs harbored by FLA.

CHAPTER SIX: CONCLUSION AND FUTURE PERSPECTIVES

Conclusion

This study determined and compared the diversity, distribution and removal of pathogenicbacteria, ARGs and MGEs in three full-scale WWTPs, using both metagenomic and transcriptomics approaches.

Metagenomic and transcriptomic profiling from influents to final effluents of the three WWTPs made apparent the presence of the top most dominant phyla including Proteobacteria, Terrabacteria group and FCB group. Proteobacteria exhibited the highest abundance in both genomes and transcripts of all samples across the three WWTPs. Influent genomes showed a shared dominance of *A. hydrophila* and *A. baumanii,* across all three treatment plants. Transcripts however, showcased a different dominance in influence where *E. coli* and *A. baumanii* were more dominant among the viable pathogens profiled in the WWTPs investigated.

Genes conferring resistance to aminoglycoside, broad class of beta-lactams, chloramphenicol, macrolides, quinolone, rifamycin (*arr*), sulfonamide, and tetracycline were profiled in both genomes and transcripts from influent to final effluents samples. Based on genomics and transcript datasets significant reduction of ARGs occurred in all three WWTPs. However, certain ARGs were persistent to final effluents samples these included gene conferring resistance to aminoglycosides and sulfonamides. The persistence of specific genes such sulfonamides gene *SulI*,

aminoglycoside (*AadA5*, *AadA*) and tetracyclines (*Tet X*) was facilitated by ARG-MGE associations where plasmids were the most prominent MGE linked to persistence in both genomes and transcripts. Overall reduction of ARBs, ARGs and MGEs was apparent in all three plants. However, the presence of all three groups of biological contaminants post the disinfection stage strongly suggested the current of disinfection is not efficient in the elimination of these contaminants.

Additionally, the fate of selected opportunistic bacterial pathogens viz. *Aeromonas* spp*.* *Acinetobacter* spp., *P. aeruginosa* and *K. pneumoniae* were influenced by operational process parameters (DO, temperature, TSS and pH). *Acinetobacter* spp showcased thehighest abundance in all influent samples, followed by *Aeromonas* spp. A shift in abundance wasobserved in aerations samples were *Aeromonas* spp. assumed highest abundance, while *Acinetobacter* spp. were top most abundant in trickling filter samples. Similarly, *Acinetobacter* spp., *Aeromonas* spp., and *P. aeruginosa* dominated effluent samples with varying abundances. While *Acinetobacter* spp., *Aeromonas* spp., and *Aeromonas* spp. dominated final effluents. The impact of process parameters varied across the treatment stages in all three WWTPs.The abundance of *Acinetobacter* spp., across the study period showcased significant correlation with high DO levels, while *P. aeruginosa* abundance was correlated to both temperature and pH. Both *K. pneumoniae* and *Aeromonas* spp*.,* correlated to the increase of TSS levels.

Metagnome profiles of the isolated FLAs showcased genera *Acanthamoeba* and *Entamoeba* spp. which were widely distributed across the three WWTPs. *A. castellini* was the most prevalent dominant species detected in all samples. While *A. culbertsoni*, *A. astronyxis* and *E. histolytica* which have been identified as causative agents of human diseases, were detected only in treatment plants receiving hospital effluents, namely WWTP2 and WWTP3. Profiling of bacterial endosymbionts made apparent the presence of opportunistic pathogens such as *P. aeruginosa*, *S. maltophilia*, *A. spanius*, *C. testosteroni*, and *E. cloacae* harbored by FLA in secondary and final effluent samples of the WWTPs. *P. aeruginosa* and *S. stutzeri* were seen to persist to final effluents. Further the presence of ARGs within the FLA hosts showcased the presence of genes conferring resistance to aminoglyscosides ((*aadA)*, trimethoprim (*dfrA15* and *dfrA5*); sulfonamides (*Sul1* and *SulII*), macrolide (*msr A, mphC*); rifamycin (*Arr*); quinolone (*qnrE1*) and tetracyclines (*TetA and TetG*). Subtypes belonging to aminoglycosides, sulfonamides and quinolones were seen to persist to final effluents. These findings therefore suggest FLA play a crucial role in the protection of both ARBs and ARGs against the rigor of wastewater treatment, furthermore they shelter biological contaminants from the toxicity of disinfection. The overall findings of this study show great potential to pave a path for development of risk assessment models for WWTPs and further influence the scope of community health care.

6.1 Significant Findings

- This study profiled ARBs, ARGs and MGEs using a combined metagenomics and transcriptomics approach this made apparent the reduction of all emerging contaminants, the persistence of selected contaminants particularly those profiled in transcripts is noteworthy.
- ARG-MGE associations were observed to be the prominent drivers of antibiotic resistance.
- This study also made apparent the effect of selected process parameters viz. TSS, DO and pH on the fate of bacterial pathogens.
- This study may be considered as novel in its investigation and confirmation of the presence of ARGs associated with endosymbionts within FLA.
- Additionally, the profiling of bacterial endosymbionts within FLA in wastewater treatment using metagenomics is novel.
- Presence of ARG within FLA hosts may suggest the possibility of gene exchange between different bacteria within FLA.

6.2 Future Perspectives

The use of methods such as metatranscriptomics and metabolomics for profiling of bacterial functional genes would be beneficial in understanding the propagation and persistence of biological contaminants. Additionally, the effects of FLA colonization by endosymbionts is still in need of in-depth investigation. This need may be addressed by use of bacterial Quorum Sensing. This approach may be capable of bringing to our understanding the extent of interaction between the FLA hosts and endosymbiont counterparts, furthermore revealing the functional genes that facilitate this relationship.

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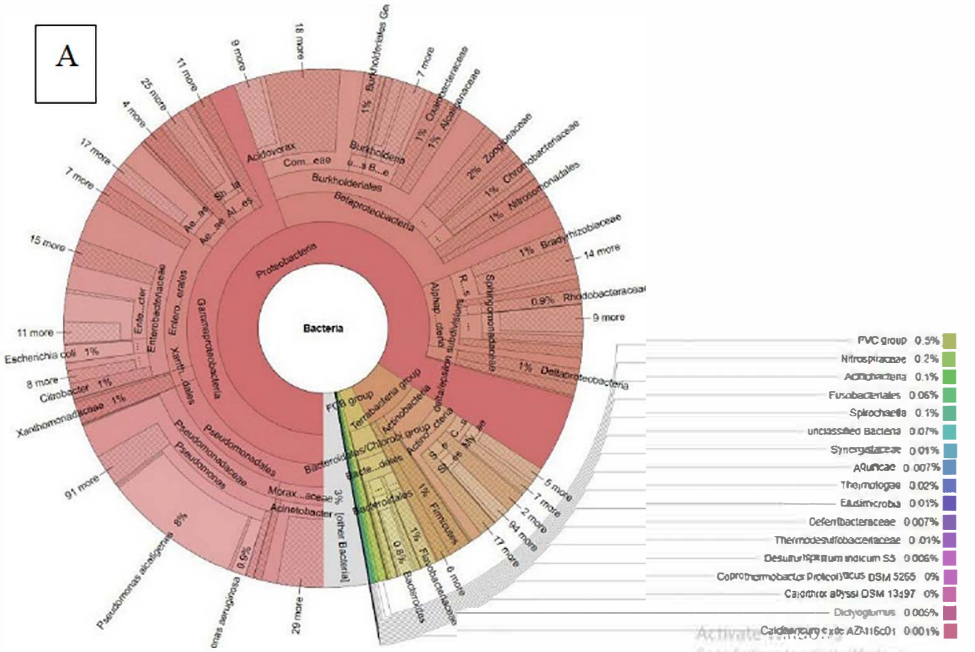
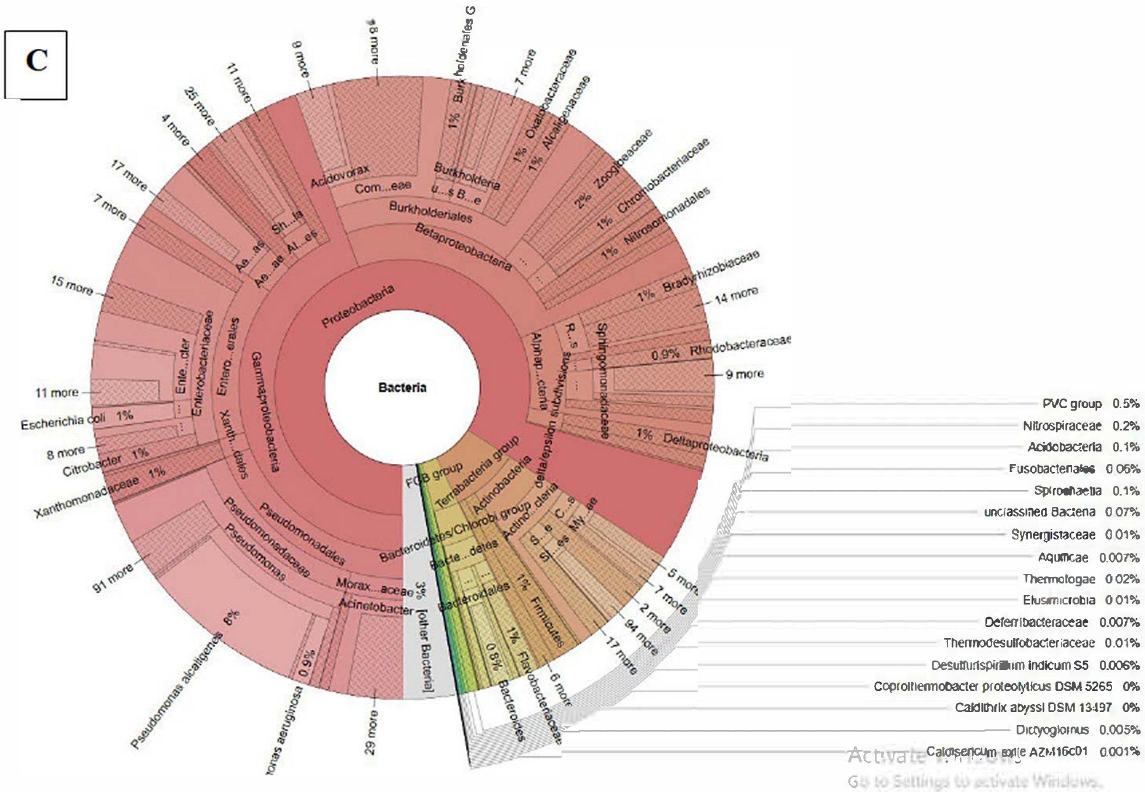
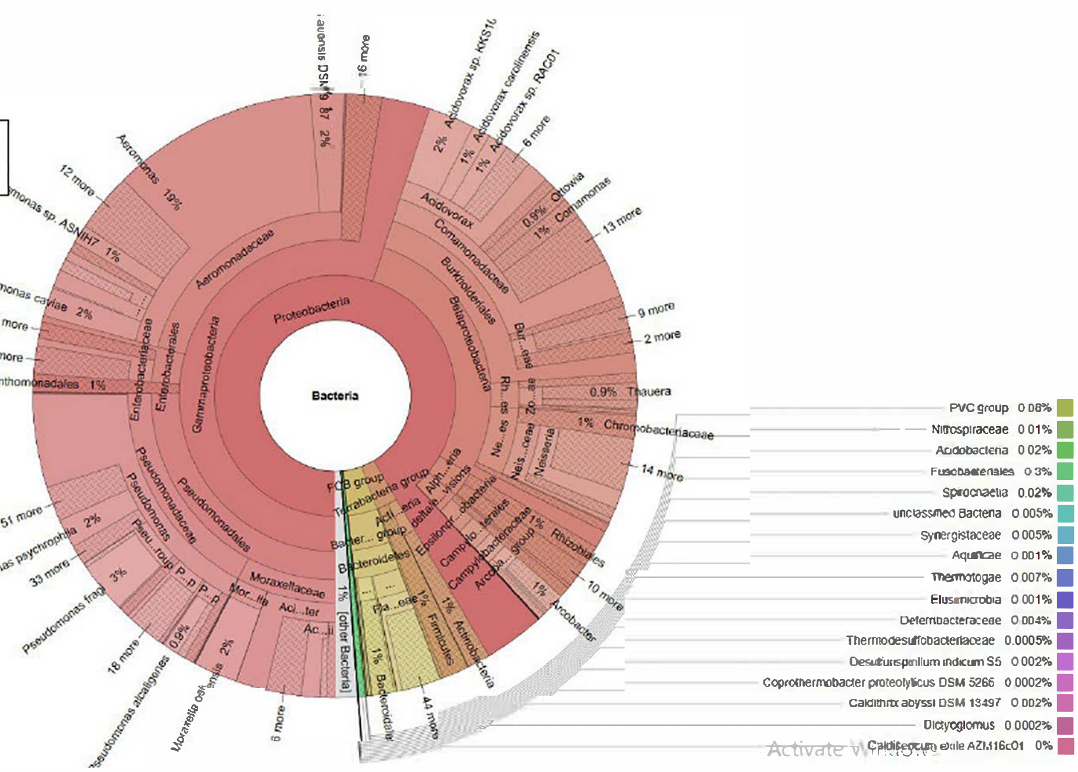
APPENDIX A. SUPPLEMENTARY DATA FOR CHAPTER 3



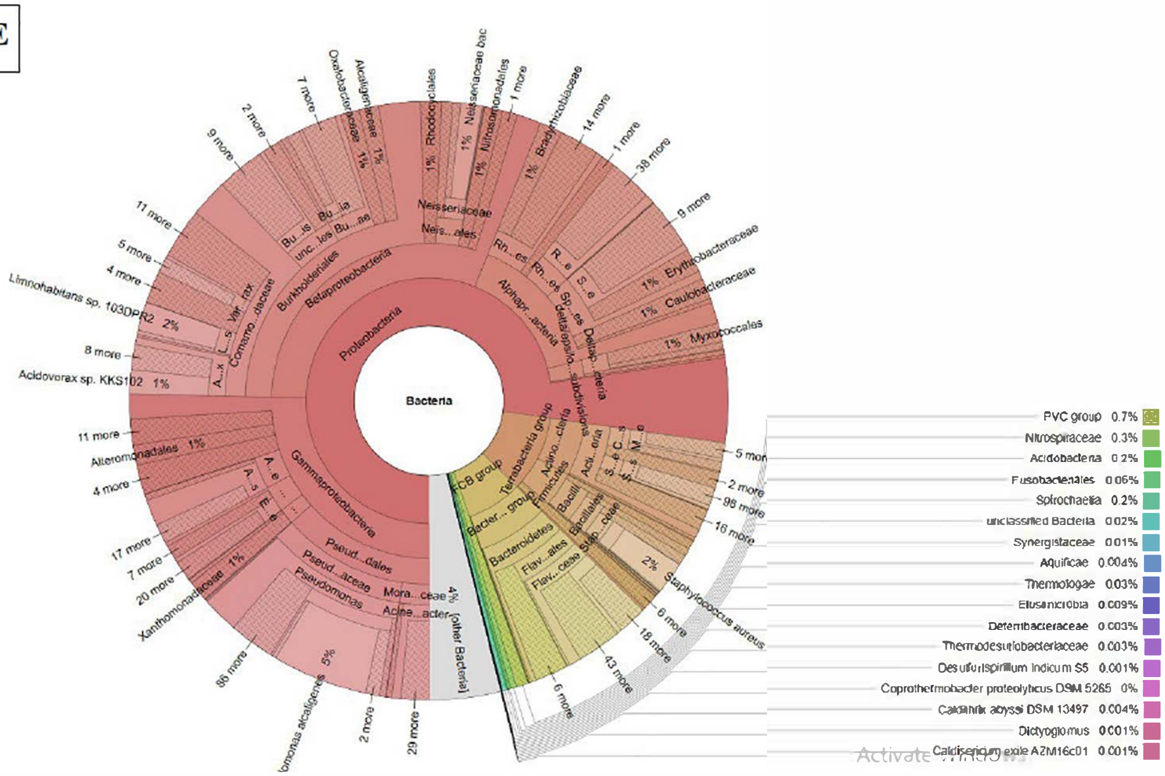
C


Bacteria
Proteobacteria
Gammaproteobacteria
Betaproteobacteria
Burkholderiales
Pseudomonadales
Pseudomonas alcaligenes 8%
PVC group 0.5%
Nitrospiraceae 0.2%
Acidobacteria 0.1%
Fusobacteriales 0.06%
Spirochaetia 0.1%
unclassified Bacteria 0.07%
Synergistaceae 0.01%
Aquificae 0.007%
Thermotogae 0.02%
Elusimicrobia 0.01%
Deferribacteraceae 0.007%
Thermodesulfobacteriaceae 0.01%
Desulfurispirillum indicum S5 0.006%
Coprothermobacter proteolyticus DSM 5265 0%
Caldithrix abyssi DSM 13497 0%
Dictyoglomus 0.005%
Caldisericum exile AZM16c01 0.001%

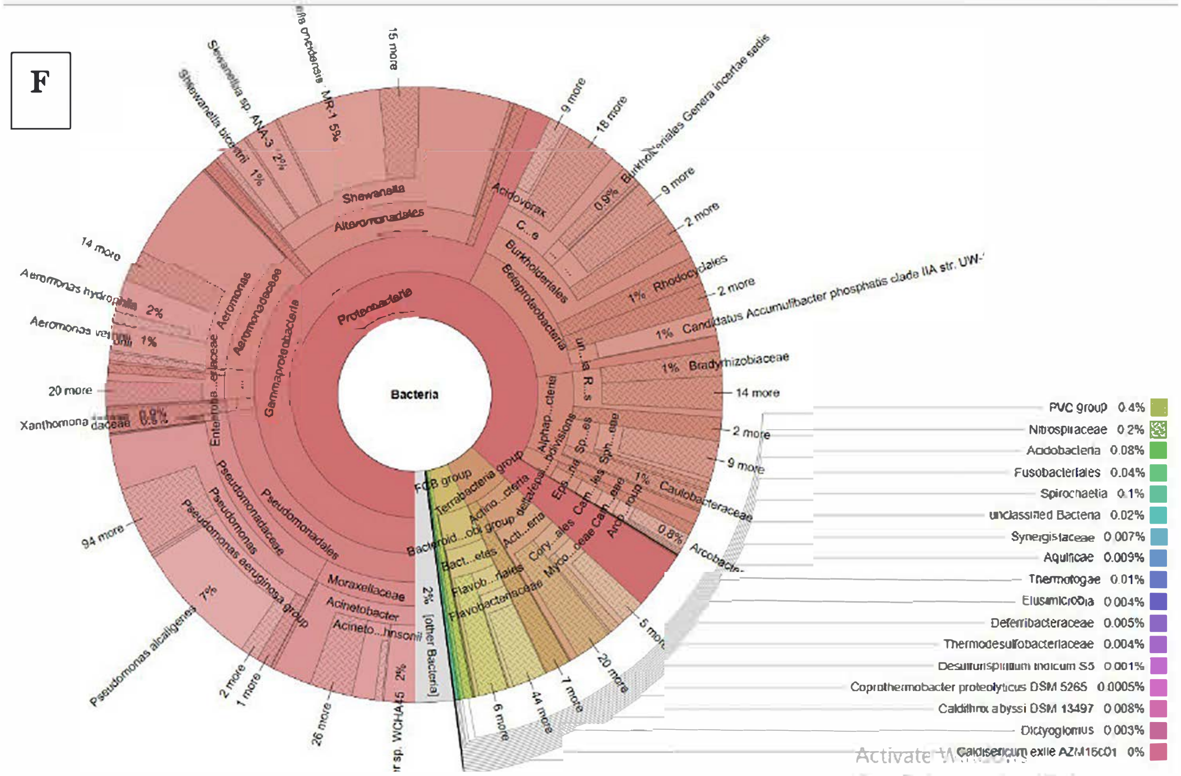
D

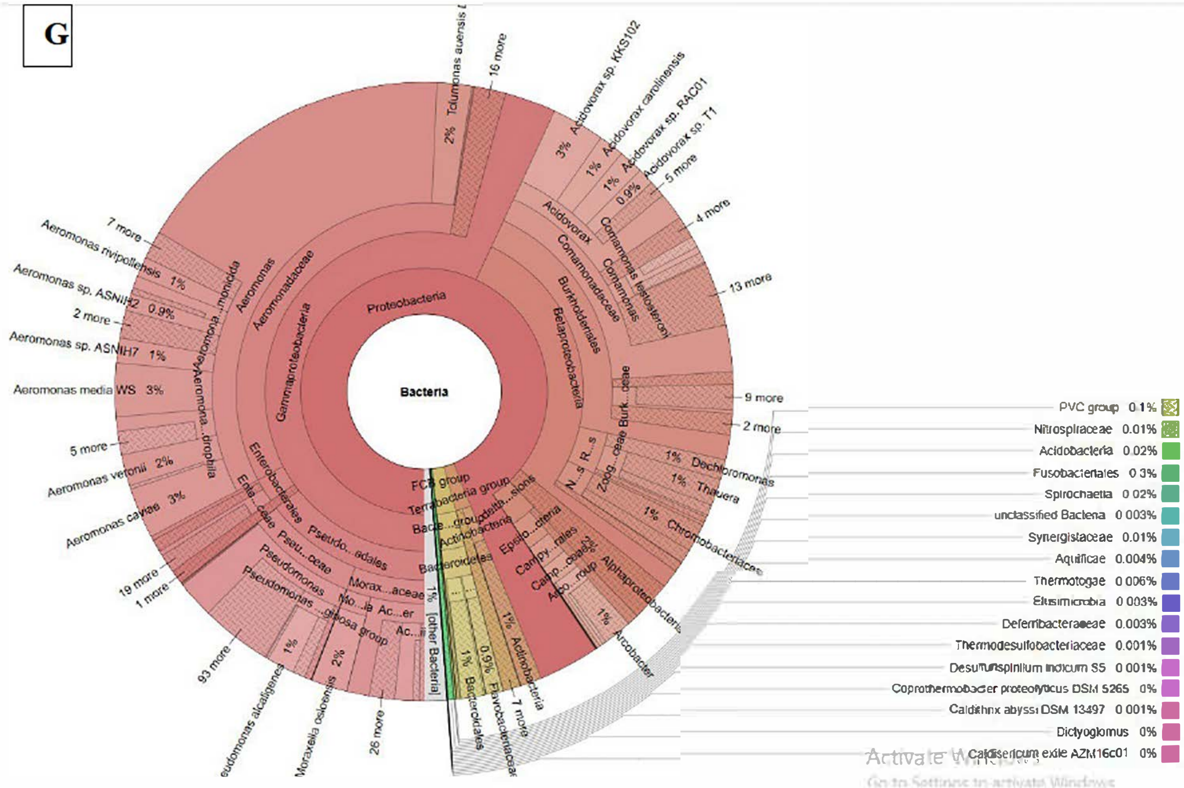

Bacteria
Proteobacteria
Gammaproteobacteria
Aeromonadaceae
Aeromonas 19%
Betaproteobacteria
Pseudomonadales
PVC group 0.08%
Nitrospiraceae 0.01%
Acidobacteria 0.02%
Fusobacteriales 0.3%
Spirochaetia 0.02%
unclassified Bacteria 0.005%
Synergistaceae 0.005%
Aquificae 0.001%
Thermotogae 0.007%
Elusimicrobia 0.001%
Deferribacteraceae 0.004%
Thermodesulfobacteriaceae 0.0005%
Desulfurispirillum indicum S5 0.002%
Coprothermobacter proteolyticus DSM 5265 0.0002%
Caldithrix abyssi DSM 13497 0.002%
Dictyoglomus 0.0002%
Caldisericum exile AZM16c01 0%

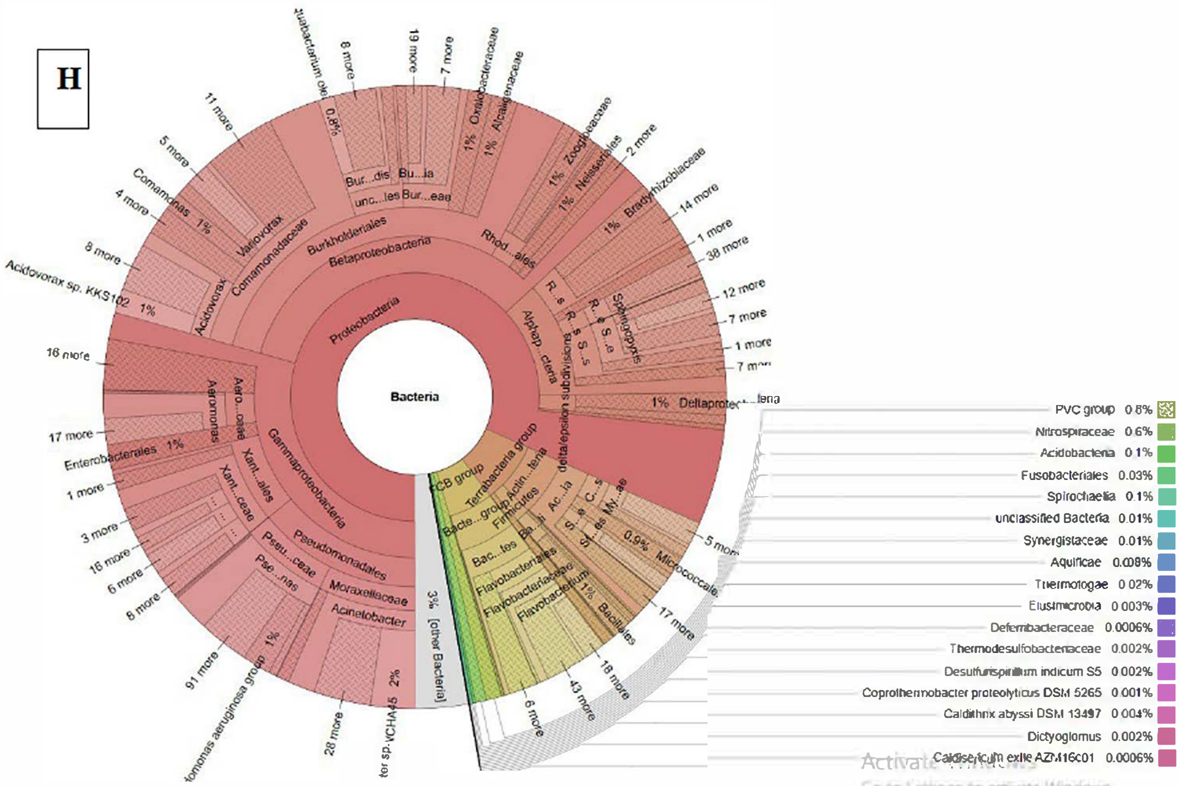
E

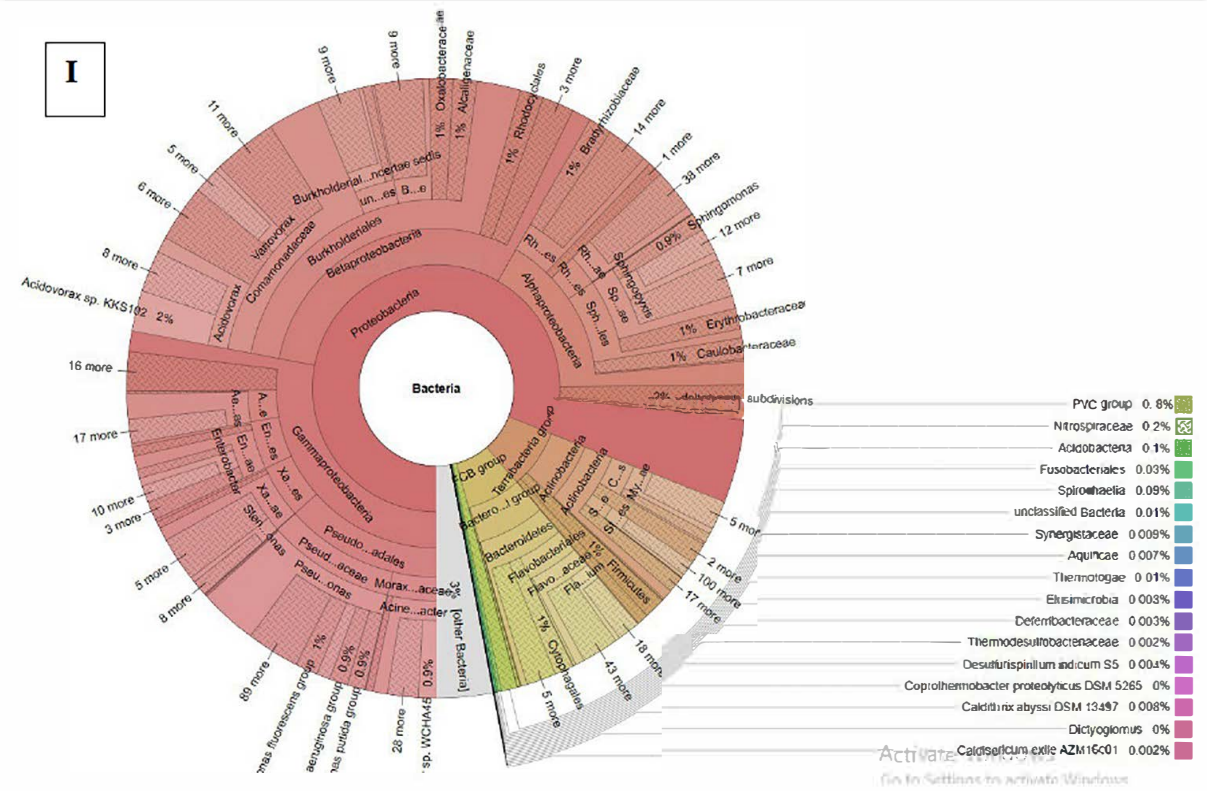

Bacteria
Proteobacteria
Betaproteobacteria
Burkholderiales
Gammaproteobacteria
Pseudomonas
[other Bacteria]
FCB group
Terrabacteria group
Bacteroidetes
Firmicutes
Staphylococcus aureus
Limnohabitans sp. 103DPR2 2%
Acidovorax sp. KKS102 1%
Alteromonadales 1%
Xanthomonadaceae 1%
Pseudomonas alcaligenes 5%
PVC group 0.7%
Nitrospiraceae 0.3%
Acidobacteria 0.2%
Fusobacteriales 0.06%
Spirochaetia 0.2%
unclassified Bacteria 0.02%
Synergistaceae 0.01%
Aquificae 0.004%
Thermotogae 0.03%
Elusimicrobia 0.009%
Deferribacteraceae 0.003%
Thermodesulfobacteriaceae 0.003%
Desulfurispirillum indicum S5 0.001%
Coprothermobacter proteolyticus DSM 5265 0%
Caldithrix abyssi DSM 13497 0.004%
Dictyoglomus 0.001%
Caldisericum exile AZM16c01 0.001%

F


Bacteria
Proteobacteria
Gammaproteobacteria
Alteromonadales
Shewanella
Aeromonas hydrophila 2%
Aeromonas veronii 1%
Pseudomonadales
Pseudomonas aeruginosa group
Pseudomonas alcaligenes 7%
Moraxellaceae
Acinetobacter
Betaproteobacteria
Burkholderiales
Rhodocyclales 1%
Candidatus Accumulibacter phosphatis clade IIA str. UW-
Bradyrhizobiaceae 1%
Caulobacteraceae
[other Bacteria] 2%
FCB group
Terrabacteria group
PVC group 0.4%
Nitrospiraceae 0.2%
Acidobacteria 0.08%
Fusobacteriales 0.04%
Spirochaetia 0.1%
unclassified Bacteria 0.02%
Synergistaceae 0.007%
Aquificae 0.009%
Thermotogae 0.01%
Elusimicrobia 0.004%
Deferribacteraceae 0.005%
Thermodesulfobacteriaceae 0.004%
Desulfurispirillum indicum S5 0.001%
Coprothermobacter proteolyticus DSM 5265 0.0005%
Caldithrix abyssi DSM 13497 0.008%
Dictyoglomus 0.003%
Caldisericum exile AZM16c01 0%


G
Bacteria
Proteobacteria
Gammaproteobacteria
Betaproteobacteria
Aeromonadaceae
Aeromonas
Comamonadaceae
Burkholderiales
Acidovorax
Pseudomonas
Enterobacteriales
Alphaproteobacteria
Actinobacteria
Chromobacteriaceae
Arcobacter
[other Bacteria]
Aeromonas media WS 3%
Aeromonas veronii 2%
Aeromonas caviae 3%
Acidovorax sp. KKS102
Acidovorax carolinensis
Acidovorax sp. RAC01
Acidovorax sp. T1
PVC group 0.1%
Nitrospiraceae 0.01%
Acidobacteria 0.02%
Fusobacteriales 0.3%
Spirochaetia 0.02%
unclassified Bacteria 0.003%
Synergistaceae 0.01%
Aquificae 0.004%
Thermotogae 0.006%
Elusimicrobia 0.003%
Deferribacteraceae 0.003%
Thermodesulfobacteriaceae 0.001%
Desulfurispirillum indicum S5 0.001%
Coprothermobacter proteolyticus DSM 5265 0%
Caldithrix abyssi DSM 13497 0.001%
Dictyoglomus 0%
Caldisericum exile AZM16c01 0%


H
Bacteria
Proteobacteria
Betaproteobacteria
Gammaproteobacteria
Burkholderiales
Comamonadaceae
Acidovorax
Variovorax
Pseudomonadales
Moraxellaceae
Acinetobacter
Aeromonas
Enterobacterales 1%
Acidovorax sp. KKS102 1%
Flavobacteriales
Flavobacteriaceae
Flavobacterium
Micrococcales
Bacillales
Bradyrhizobiaceae
Deltaproteobacteria
3% [other Bacteria]
PVC group 0.8%
Nitrospiraceae 0.6%
Acidobacteria 0.1%
Fusobacteriales 0.03%
Spirochaetia 0.1%
unclassified Bacteria 0.01%
Synergistaceae 0.01%
Aquificae 0.008%
Thermotogae 0.02%
Elusimicrobia 0.003%
Deferribacteraceae 0.0006%
Thermodesulfobacteriaceae 0.002%
Desulfurispirillum indicum S5 0.002%
Coprothermobacter proteolyticus DSM 5265 0.001%
Caldithrix abyssi DSM 13497 0.004%
Dictyoglomus 0.002%
Caldisericum exile AZM16c01 0.0006%



Supplementary figure 1. Krona plots displaying distribution of whole bacterial communities detected in metagenomes from influents to final effluents of the three WWTPs. WWTP1 A) Influent, B) Effluent and C) Final Effluent. WWTP2 D) Influent, E) Effluents, and F) Final Effluents. WWTP3 G) Influent, H) Effluent and I) Final Effluents.

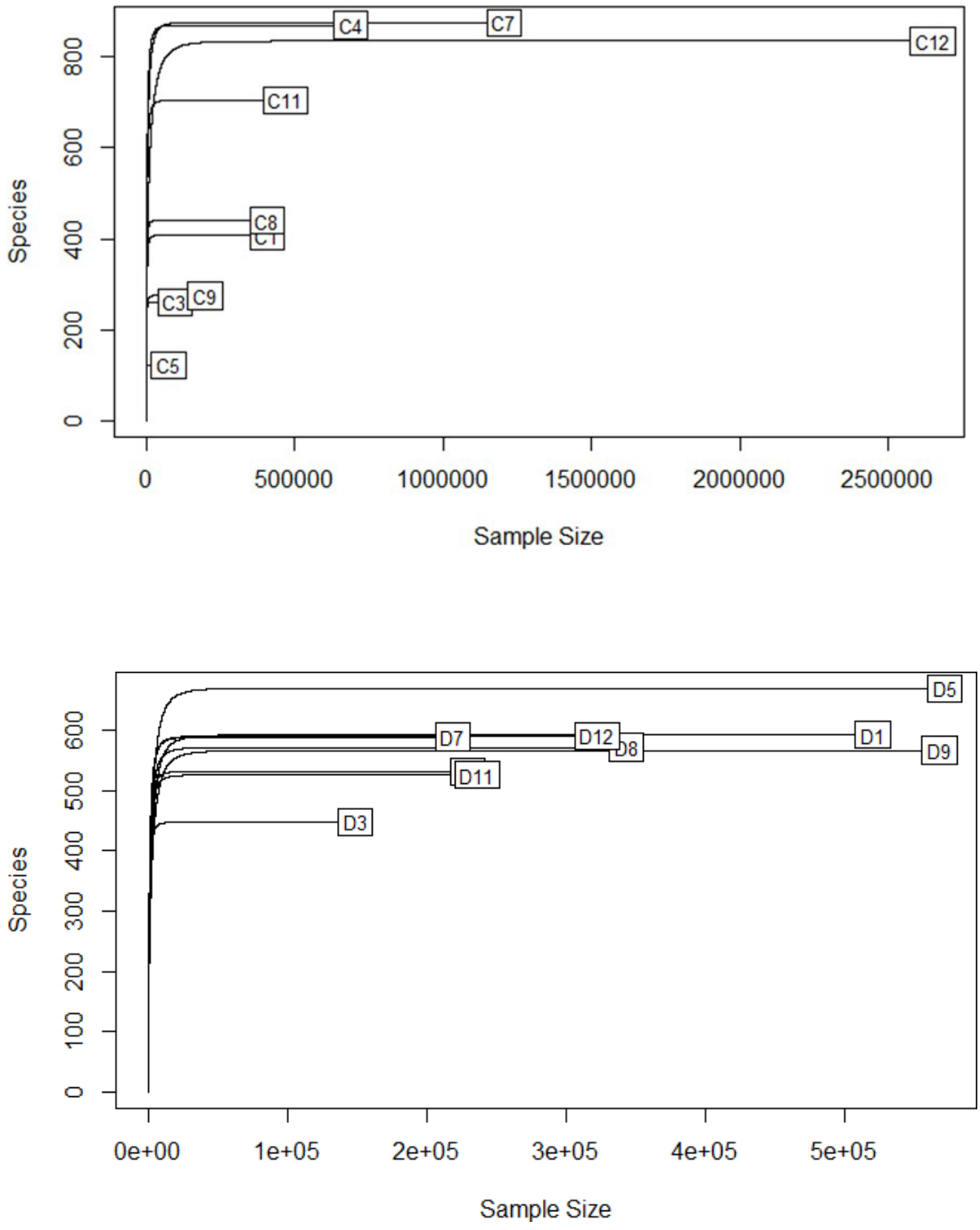


Figure 2. Rarefaction curves depicting the diversity of bacterial communities in influent to final effluent samples. A) Bacterial communities in metagenomes. B). Bacterial communities in transcripts. In metagenomes D1,3 and D4 denote WWTP1 influent, effluent and final effluent

samples respectively. D5,7 and D8 denote WWTP2 influent, effluent and final effluent samples. D9,11 and D12 denote WWTP3 influent, effluent and final effluent samples. In transcripts C1,3 and 4 denote WWTP1 influent, effluent and final effluent samples. C5, 7 and C8 denotes WWP2 influent, effluent and final effluent samples. C9, 11 and C12 denotes influent, effluent and final effluent samples.

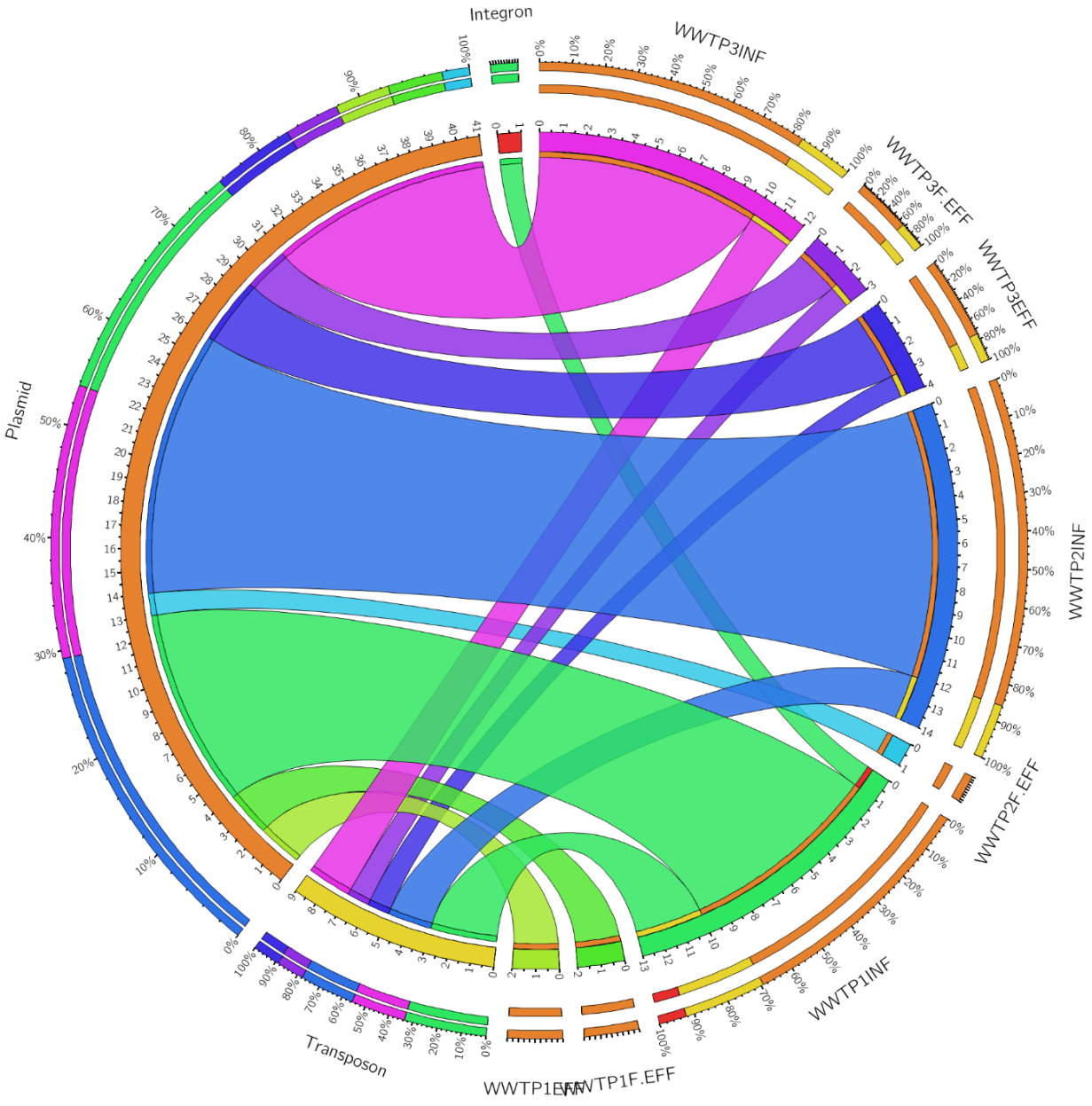


Figure 3. Distribution of various mobile genetic elements in the three WWTP's metagenomes. Length of the outer ring represents the abundance of the of MGE across the 3 treatment plants.

Supplementary Table. 1 Metagenome datasets used in this study

Name	Number of reads	Avg.length	Number of reads after trim	% trimmed	Avg.length after trim	Database matches	Unclassified reads
WWTP1 INF	1,981,488	216.4	1,978,722	99.9%	197.8	520,068	1,454,140
WWTP1 EFF	2,125,478	217.2	2,122,676	99.9%	199.7	149,848	1,967,658
WWTP1 F.EFF	2,194,448	182.6	2,191,748	99.9%	172.9	230,638	1,950,728
WWTP2 INF	2,168,580	206.3	2,166,008	99.9%	194.8	572,036	1,589,424
WWTP2 EFF	2,337,384	194.2	2,334,378	99.9%	183.8	220,040	2,106,658
WWTP2 F.EFF	2,132,874	182.6	2,128,980	99.8%	172.4	345,198	1,772,694
WWTP3 INF	2,003,756	217.0	2,001,230	99.9%	202.1	569,284	1,427,492
WWTP3 EFF	1,949,826	219.3	1,947,722	99.9%	203.5	237,088	1,705,590
WWTP3 F.EF	2,227,726	193.0	2,225,200	99.9%	181.7	323,578	1,896,164

Supplementary Table. 2 Metatranscriptome datasets used in this study

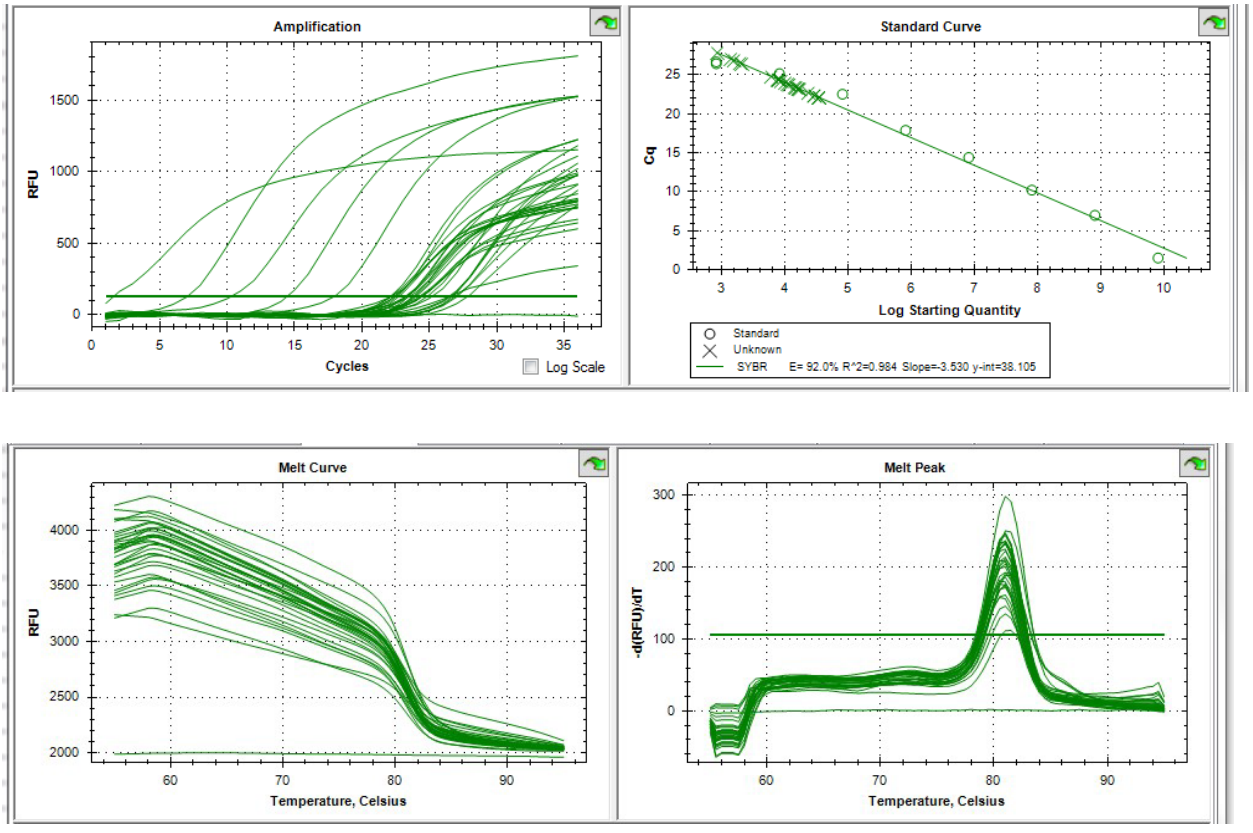
Name	Number of reads	Avg.length	Number of reads after trim	% trimmed	Avg.length after trim	Database matches	Unclassified reads
WWTP1 INF	1,386,284	220.5	1,385,612	100.0%	204.1	425,578	957,868
WWTP1 EFF	1,628,146	279.3	1,626,598	100.0%	243.9	111,726	1,511,956
WWTP1 F.EFF	1,831,802	242.0	1,830,598	100.0%	221.0	819,238	1,010,432
WWTP2 INF	236,604	207.2	236,304	100.0%	193.9	84,584	148,490
WWTP2 EFF	2,180,890	204.2	2,178,076	100.0%	192.6	1,555,386	619,184
WWTP2 F.EFF	2,530,454	229.8	2,529,160	100.0%	209.1	436,780	2,080,332
WWTP3 INF	1,425,926	259.4	1,424,118	100.0%	229.9	569,222	853,994
WWTP3 EFF	1,652,850	244.9	1,651,916	100.%	222.9	585,918	1,065,672
WWTP3 F.EFF	2,929,616	251.4	2,926,170	100.0%	226.8	1,672,672	1,251,636

APPENDIX B. SUPPLEMENTARY DATA FOR CHAPTER 4

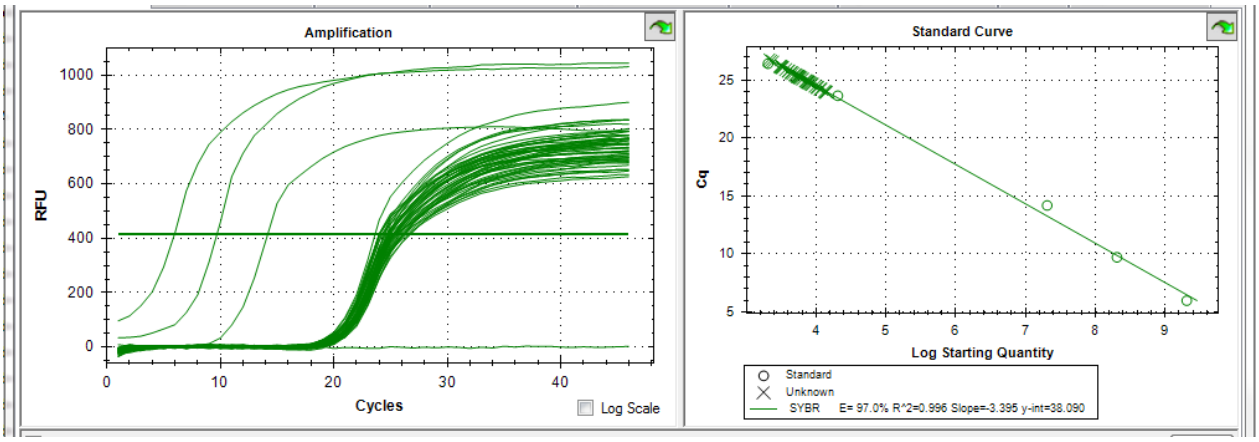
1.QPCR Quality parameter data

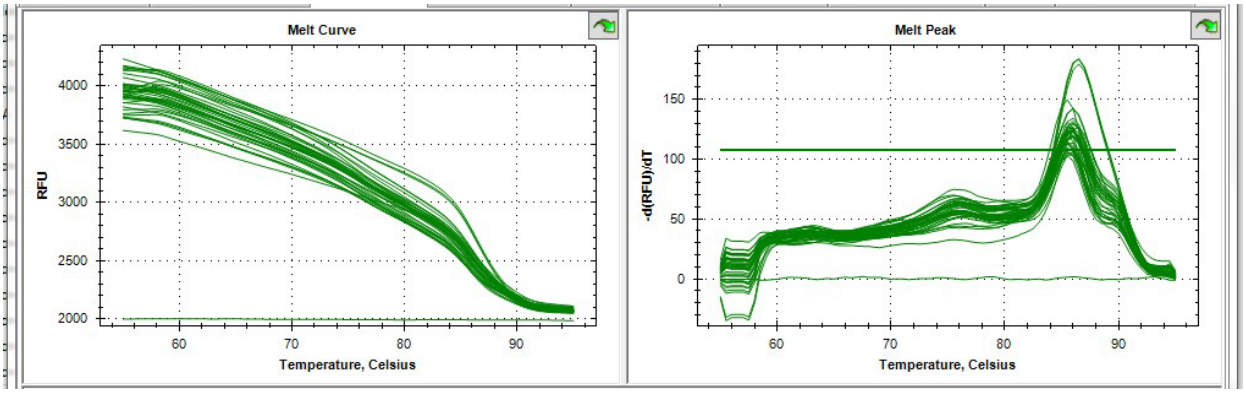
Parameters for the qPCR standard curve, obtained after optimization is shown below. The qPCR efficiencies were between 92 and 100 % and the standard curves were linear over six order of magnitude (R2>0.99).

Acinetobacter spp:

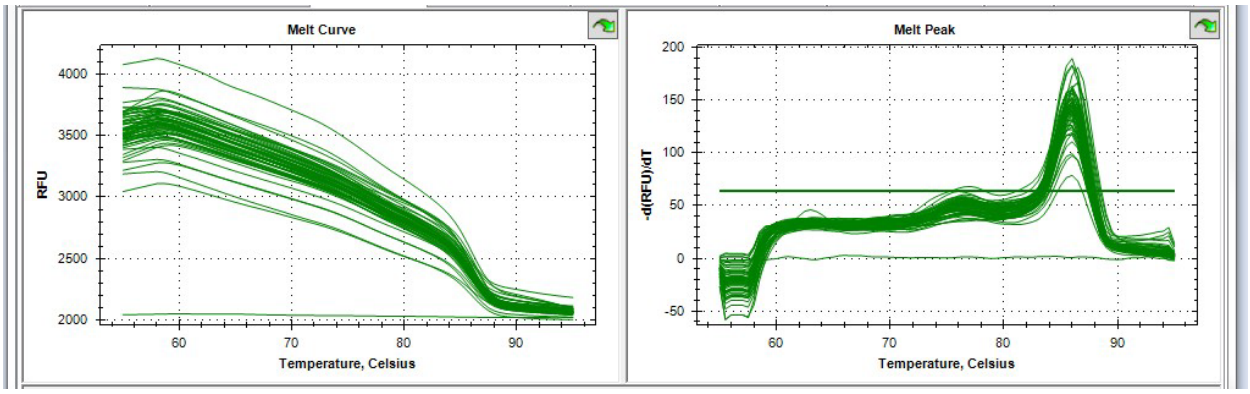


Aeromonas spp:

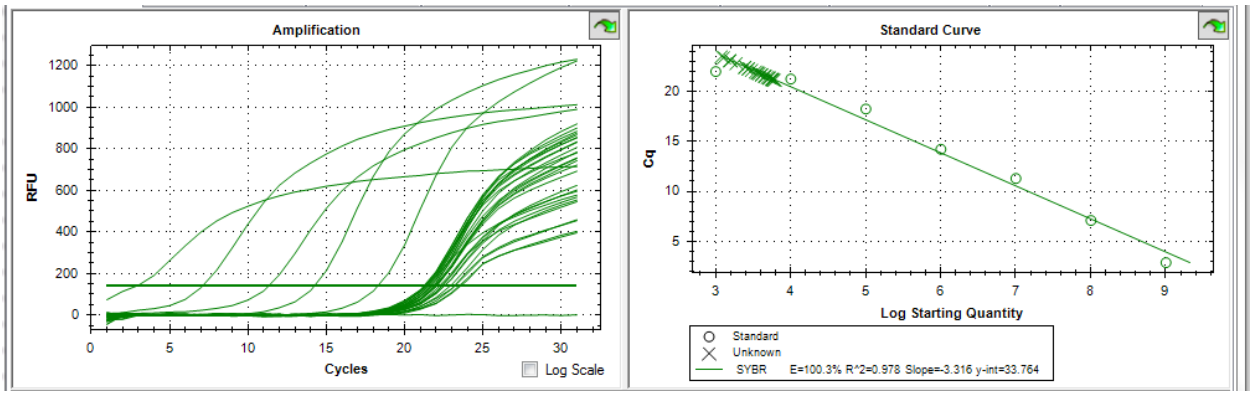


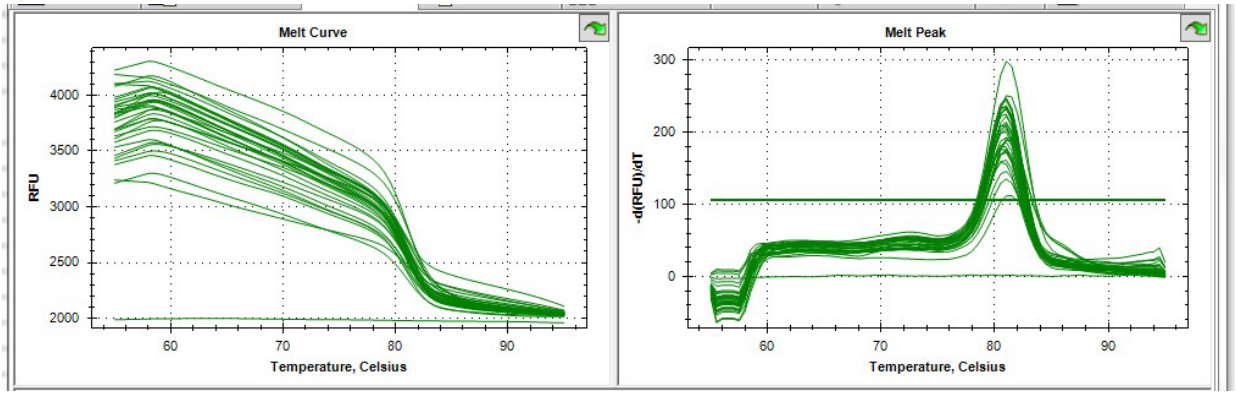


Psuedomonas aeruginosa:



Klebsiella pneumoniae:





APPENDIX C: SUPPLEMENTARY DATA FOR CHAPTER 4

DAPI 4′,6-diamidino-2-phenylindole

Reagents

DAPI (10 mg/mL in H2O stock solution; Invitrogen D1306)

Store stock solution at 4°C, protected from light.

Formaldehyde (3.7%), freshly prepared

Phosphate-buffered saline (PBS)

Prepare PBS with added $CaCl_2$ and $MgCl_2$ (PBS+). This solution allows cells to adhere to each other and to the substrate. If cells are in medium containing no Ca^{2+} or Mg^{++}, they will round up and detach from the substrate.

Triton X-100 (0.2%)

Equipment

Cell culture dishes, sterile

Microscope,
fluorescence, equipped with an ultraviolet (UV) filter set

For Epifluorescence laser excitation,

Method:

1. Dilute the DAPI stock solution 1:5000 in PBS+.

2. Aspirate the cell medium from cells grown on coverslips. Rinse the cells three times with PBS+.

3. Fix the cells for 10 min in 3.7% formaldehyde.

4. Aspirate the fixative. Rinse the cells three times, 5 min each, in PBS+.

5. Permeabilize the cells by immersion in 0.2% Triton X-100 for 5 min.

6. Aspirate the Triton. Rinse the cells three times, 5 min each, in PBS+.

7. Incubate the cells for 1-5 min at room temperature in DAPI labeling solution (from Step 1).

8. Aspirate the labeling solution. Rinse the cells three times in PBS+.

9. Mount the coverslips as described in Mounting Live Cells onto Microscope Slides (Chazotte 2011).

10. Image the cells.

APPENDIX D: SUPPLEMENTARY DATA FOR CHAPTER 4

Table 4.2: Plant operating conditions of the selected WWTP indicating of operational parameters.

		KWWTP				SWWTP				IWWTP		
		INF	AER	EFF	F.EFF	INF	AER	EFF	F.EFF	INF	EFF	F.EFF
JUN	Temperature	23.4 ±0.6	24.5 ±0.5	23.3 ±0.4	22.9 ±0.02	21.75 ±0.4	25.88 ±0.3	23.14 ±0.2	21.34 ±2.1	24.06 ±0.03	19.92 ±0.61	20.17 ±0.60
	DO(mg/L)	1.25 ±0.01	0.83 ±0.03	1.88 ±0.07	2.25 ±0.21	1.30 ±0.09	0.67 ±0.17	2.02 ±0.06	2.64 ±0.13	1.51 ±0.09	1.69 ±0.4	2.14 ±0.01
	pH	7.48 ±0.14	7.41 ±0.21	7.52 ±0.15	7.60 ±0.04	7.42 ±0.04	7.38 ±0.07	7.28 ±0.08	7.17 ±0.14	7.48 ±0.14	7.37 ±0.10	7.24 ±0.10
	TSS (mg/L)	572 ±24.74	4804 ±124.45	-	21 ±1.41	-	4110 ±42.42	-	-	337 ±12.02	-	49 ±2.83
JUL	Temperature	22.77 ±0.5	24.19 ±0.13	22.32 ±0.5	23.16 ±0.08	21.65 ±0.40	25.93 ±0.09	22.75 ±0.22	23.16 ±0.05	25.05 ±0.18	20.9 ±0.14	18.64 ±0.47
	DO(mgO_2/L)	0.90 ±0.08	0.82 ±0.12	1.16 ±0.06	1.58 ±0.12	0.70 ±0.12	1.03 ±0.06	1.57 ±0.10	1.18 ±0.22	1.40± 0.01	1.67 ±0.06	1.67 ±0.02
	pH	7.42 ±0.14	7.54 ±0.04	7.45 ±0.17	7.33 ±0.16	7.20 ±0.07	7.08 ±0.03	7.19 ±0.03	7.19 ±0.03	7.42 ±0.14	7.33 ±0.12	7.26 ±0.09
	TSS (mg/L)	379 ±9.9	4080.5 ± 129.4	-	48 ±5.65	-	4207 ±114.55	-	-	189 ±4.24		63 ±2.84
A	Temperature	24.53 ±0.24	25.91 ±0.1	22.64 ±0.22	22.92 ±0.17	23.86 ±0.12	25.14 ±0.06	22.52 ±0.18	23.64 ±0.55	24.68 ±0.44	20.95 ±0.11	17.17 ±0.57
	DO(mgO_2/L)	1.62 ±0.02	0.98 ±0.06	1.64 ±0.20	2.08 ±0.04	1.59 ±0.07	0.89 ±0.10	2.36 ±0.32	1.71 ±0.04	1.82 ±0.17	2.68 ±0.04	1.47 ±0.01

UG	pH	7.34 ±0.01	7.45 ±0.3	7.40 ±0.07	7.66 ±0.02	7.35 ±0.08	7.49 ±0.08	7.28 ±0.25	7.44 ±0.01	7.34 ±0.01	7.27 ±0.04	7.30 ±0.09
	TSS (mg/L)	434 ±14.10	5500.5 ±101.11	-	17 ±1.41	-	5742 ±291.32	-	-	276 ±2.83	-	43 ±2.12
	Temperature	24.43 ±0.05	25.55 ±0.38	23.55 ±0.33	24.01 ±0.06	23.79 ±0.05	23.80 ±0.15	23.16 ±0.45	24.25 ±0.25	24.55 ±0.35	20.85 ±0.04	17.50 ±0.80
SEPT	DO(mgO_2/L)	1.42 ±0.13	0.85 ±0.01	1.79 ±0.06	2.19 ±0.04	2.60± 0.35	1.19± 0.15	1.70± 0.89	3.14± 0.77	1.55 ±0.22	1.82 ±0.02	1.63 ±0.26
	pH	7.29 ±0.02	6.91 ±0.01	7.06 ±0.03	7.41 ±0.11	7.03 ±0.20	7.19 ±0.01	7.27 ±0.23	7.40 ±0.04	7.29 ±0.02	7.43 ±0.02	7.18 ±0.01
	TSS (mg/L)	320 ±43.13	4879.5 ±85.55	-	76 ±2.83	-	5311 ±86.26	-	-	-	-	-

APPENDIX E. PUBLICATION

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Profiling of emerging pathogens, antibiotic resistance genes and mobile genetic elements in different biological wastewater treatment plants

Thobela Conco [a], Sheena Kumari [a] ✉, Oluyemi Olatunji Awolusi [a], Mushal Allam [b, c], Arshad Ismail [b], Thor A. Stenström [a], Faizal Bux [a]

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