Profiling of selected filamentous actinomycetes isolated from activated sludge plants

This work is submitted in complete fulfilment of the academic requirements for the degree of Doctor of Philosophy (Biotechnology) in the Department of Biotechnology and Food Sciences, Faculty of Applied Sciences at the Durban University of Technology, Durban, South Africa

Zinhle Gugulethu Marrengane

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SUPERVISOR: Professor Faizal Bux

CO-SUPERVISOR: Prof Sheena Kumari Kuttan Pillai

CO-SUPERVISOR: Dr Oluyemi Olatunji Awolusi

DECLARATION

I declare that the thesis herewith submitted for the degree Doctor of Philosophy: Biotechnology at the Durban University of Technology is my original work and has not been previously submitted for a degree at any other institution of higher education, and that its only prior publication was in the form of conference papers, and journal articles. I further declare that all the sources cited or quoted are acknowledged and indicated by means of a comprehensive list of references."

Zinhle Gugulethu Marrengane

I hereby approve the final submission of the following thesis.

Prof. F. Bux D. Tech	Prof S. Kumari PhD	Dr O. O. Awolusi
(DUT)	(Mangalore University)	(DUT)

ABSTRACT

The formation of stable foams on the surface of aeration tanks has been observed worldwide in activated sludge systems. As a result of foam formation, wastewater treatment works face operational difficulties since foam seeps into effluent, adversely affecting the concentration of suspended solids, which reduces disinfection efficiency. Several efforts have been channelled towards a better comprehension of the microbial ecology of foaming. High abundance of *Candidatus* Microthrix parvicella and branched filaments of mycolic acid-containing actinomycetes have been reported frequently in foaming samples. The proliferation of these microorganisms has been reported from domestic to industrial treatment plants with different process configurations and throughout the different seasons. For process optimisation and troubleshooting, a greater comprehension of the structure and function of microbial community within a wastewater treatment plant is a prerequisite. Excessive growth of filamentous bacteria is promoted by the presence of a variety of physicochemical factors, and changes in process conditions. Though various factors can induce foaming, it is imperative to identify and quantify the indigenous organisms implicated in foaming to pre-empt forthcoming episodes. To date, no metagenomics data have been generated specifically from foaming samples in South African wastewater treatment works. Additionally, the fastidious nature of filamentous bacteria has limited our knowledge of pure culture studies, globally. Almost all of the available literature on pure culture studies in South Africa is more than a decade old and used conventional methods for isolation and purification. Therefore, this study aimed to profile microbial communities that are prevalent in foam samples in selected wastewater treatment works in KwaZulu Natal using a metagenomics approach. Additionally, the study explored the use of micromanipulation techniques for the isolation and cultivation of selected actinomycetes (*Gordonia* spp.) from foam samples. Two wastewater treatment works treating domestic and industrial wastewater were selected for the study.

Microscopic examination of foam samples using wet mount technique indicated the prevalence of right-angled branched filamentous actinomycetes in both wastewater treatment works. The branched filamentous bacteria were selectively isolated using the micromanipulation technique from pre-treated mixed liquor and foam samples. Among the filamentous morphotypes that predominated in mixed liquor were Eikelboom Type 0041, *Thiothrix*, *Gordonia* spp., Eikelboom Type 021N and Eikelboom Type 0092 dominated in wastewater treatment works

A and in wastewater treatment works B, Eikelboom Type 0041, Eikelboom Type 021N and *Gordonia* spp. dominated throughout the sampling period while Eikelboom Type 1851 and *Thiothrix* spp. were identified as transients.

A total of forty-four isolates were obtained from the two wastewater treatment works using the micromanipulation technique. Out of these, nine isolates were further selected for physiological and molecular characterisation. The media that supported most isolates was Reasoner's 2A agar and casitone glucose yeast agar during initial isolation stage. Upon continuous subculturing during preservation, filamentous morphology was permanently lost as the isolate from wastewater treatment works B shifted to single-celled morphology. The selected isolates were further grown in different media containing various carbon substrates such as cholesterol, benzoic acid, glucose, galactose and glycerol and were grown both aerobically and anaerobically. Aerodynamism varied amongst isolates, some displayed no growth under anaerobic conditions whilst only one isolate from wastewater treatment works B utilised all substrates aerobically and anaerobically. Isolates grew optimally at 30^{o}C. Isolates from wastewater treatment works A mixed liquor and foam were fastidious and did not survive the process of isolation and profiling. Phylogenetic analysis of the 16S rRNA sequences indicated that the isolates were close relatives of *Gordonia* spp. However, the similarity index was lower than 97% indicating that the isolates may be novel or represent divergent variants to existing *Gordonia* spp.

Additionally, quantitative polymerase chain reaction was performed to assess the dominance of selected actinomycetes in foaming samples. *Gordonia* spp. were successfully quantified and their abundance was related to selected plant operation parameters to establish trends that induce *Gordonia* spp. proliferation. The ambient temperature of the two respective plants, wastewater treatment works A and B was (24.8 ±5.5 and 28.0^{o}C ± 5.1 respectively) observed to favour the growth of mycolic acid containing actinomycetes. The amount of *Gordonia* spp. in foams was significantly higher in foam samples than mixed liquor in both the plants investigated. It was also observed that *Gordonia* copy numbers of 3.7 X 10^{9} ± 0.1 copies/ng were sufficient to induce foaming in these plants.

Furthermore, the application of next generation sequencing provided further insight into the role of other actinomycetes in foam formation in this study. Three mixed liquor samples and three foam samples were subjected to next generation sequencing from each wastewater

treatment works. Based on the next generation sequencing approach, the microbial community did not vary significantly in mixed liquor and foam, however, abundance changed significantly amongst mycolic acid containing Actinobacteria. An average increase of 17% was observed from *Nocardiaceae*, *Mycobacteriaceae* and *Gordoniaceae* in foams than in mixed liquor. The dominant foam formers in both the wastewater treatment were *Gordonia* spp., *Rhodococcus* spp. and *Mycobacterium* spp. irrespective of the influent characteristics. *Gordonia amarae* which has been implicated and greatly studied as a foam inducer were not found to be predominant in these wastewater treatment works highlighting the contribution of other potential *Gordonia* spp. (*Gordonia alkanivorans, Gordonia insulae, Gordonia phthalatica, Gordonia polyisoprenivorans, Gordonia rubripertincta*) in foaming. *Mycobacterium doricum* and *Rhodococcus coprophilus* were also detected from foam samples. The abundance of *Mycobacterium intracellulare*, *Mycobacterium africanum* and *Mycobacterium avium* in wastewater treatment works A and B raises serious health implications. *M. intracellulare* has been implicated in human pulmonary infections even in immunocompetent individuals. The detection of *Mycobacterium tuberculosis* in foams also raises health concerns considering the prevalence of HIV in South Africa and tuberculosis co-infections that emanate from immunosuppression. The prevalence of mycobacterial pathogens in foams necessitates deeper interrogation to circumvent occupational hazards. By contributing to the current knowledge base, this study has made a significant contribution towards understanding the organisms responsible for foam formation and stabilization in the subtropical region of KwaZulu-Natal.

DEDICATION

This thesis is a dedication to all the people who have contributed positively to my life's journey. While some encounters were challenging and unpleasant most were not, I appreciate the lessons learnt and the support received. I am grateful to each one of you especially the ones who believed in me more than I believed in myself.

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ABBREVIATIONS

ASP	Activated sludge process
BLAST	Basic Local Alignment search tool
BNR	Biological nutrient removal
BOD	Biological oxygen demand
CD	methyl -ß - cyclodextrin
COD	Chemical oxygen demand
DGGE	Denaturation gradient gel electrophoresis
DNA	Deoxyribonucleic acid
DNTPs	Deoxy-nucleotide triphosphates
DO	Dissolved oxygen
DSVI	Diluted sludge volume index
FISH	Fluorescent *in situ* hybridization
FISH-MAR	Fluorescent *in situ* hybridization – Microautoradiography
F/M	Food to microorganism ratio
GALOs	*Gordonia amarae* like organisms
LB	Luria Bertani
MAR	Microautoradiography
MLSS	Mixed liquor suspended solids
MLVSS	Mixed liquor volatile suspended solids

Mycolata	Mycolic acid containing actinomycetes
NCBI	Centre for Biotechnology Information
NGS	Next generation sequencing
OTU	Operational taxonomic units
PAO	Phosphorus accumulating organisms
PCR	Polymerase chain reaction
PCR – DGGE	Polymerase chain reaction – denaturation gradient gel electrophoresis
qPCR	quantitative PCR
RAS	Return activated sludge
RFLP	Restriction fragment length polymorphisms
SVI	Sludge volume index
TEFL	Total extended filament length
TGYA	Tryptone glucose yeast extract agar
WWTW	Wastewater treatment works

CHAPTER ONE: INTRODUCTION

1.1 BACKGROUND

The activated sludge process (ASP) is a biological wastewater treatment method that is utilised for the treatment of domestic and industrial wastewater to an acceptable dischargeable level. This simple process involves the removal of nutrients and organic matter through aeration, followed by subsequent settling and removal of flocculated sludge. To ensure optimal operation and troubleshooting, it is crucial to understand the plant configuration and functions of the various microbial populations within the ASP. Influent composition, plant operating parameters and environmental conditions determine the type of microbial community that will be present in WWTWs (McIlroy *et al*., 2015). In ASP, microorganisms play a significant role in reducing pollutants and contaminants (Saunders *et al*., 2016). The ASP microbial consortium is quite diverse with bacteria constituting 95% of the total microbial community. This includes floc forming bacteria that are responsible for organic degradation and filamentous bacteria which forms the skeletal matrix for the formation of stable flocs which are responsible for sludge compaction. To form a compact floc, which is also known as a typical activated sludge structure, there must be an optimal balance between these two groups (Fryer and Gray, 2012). The settling properties of sludge however are largely influenced by filamentous bacteria. The proliferation of filamentous bacteria, such as *Gordonia* spp., may result in poor sludge settling, which could result in ASP discharging high levels of solid particles into the environment (Petrovski *et al*., 2011). Bulking and foaming are the two biological phenomena reported commonly in the ASP in relation to the excess growth of filamentous bacteria (Fryer and Gray, 2012; Jiang *et al*., 2016). Biological wastewater treatment malfunction has been observed in aeration basins and secondary settling tanks of ASP leading to foaming and bulking leading problems in WWTWs (Pal *et al*., 2014).

It has been widely accepted that biological foams are caused by the presence and subsequent stabilisation of gas bubbles due to the occurrence of hydrophobic particles and surfactants. The bubbles then assemble at the surface and accumulate. The hydrophobicity is provided by bacteria particularly filamentous mycolic acid containing actinomycetes (Collivignarelli *et al*., 2020). Microscopic examination of foams has revealed the abundance of branched mycolata

and unbranched *Candidatus* Microthrix parvicella (Kragelund *et al*., 2007; Rossetti *et al*., 2005). *Ca.* Microthrix calida has also been reported from industrial WWTW (Levantesi *et al*., 2006a). An unidentified *Ca*. Microthrix subdominans spp. has been identified that seems to coexist with *Ca.* M. parvicella although in lesser concentrations (Nierychlo *et al*., 2021). Mycolata have a wider association with foaming, which has implicated genera such as *Gordonia*, *Skermania*, *Tsukamurella*, *Rhodococcus* and *Nocardia* (Pal *et al*., 2014; Ju and Zhang, 2015; Guo *et al*., 2015). *Gordonia* has been frequently reported as the predominant genus within mycolata associated with foaming (Guo *et al*., 2015; Kallistova *et al*., 2014). Wastewater treatment system performance continued to be hindered by frequent proliferation of foam-formers globally (Collivignarelli *et al*., 2020).

Plant operating parameters, environmental conditions and influent composition can lead to populations shifts among floc formers and filamentous bacteria. Among other things, it has been reported that, under optimum environmental and operating conditions, fatty acids in the influent can promote filamentous bacteria, especially mycolata, capable of metabolizing lipids (Asvapathanagul *et al*., 2012). Similarly, temperature has been attributed to mycolata dominance, leading to their proliferation in countries with warmer climates (Marrengane *et al*., 2011; Asvapathanagul *et al*., 2012; Khan and Faheem, 2012; Ahansazan *et al*., 2014). Several other factors have also been described as inducers of filamentous bulking and foaming such as oxygen and nutrient deficiencies, septic conditions, low Food to Microorganism (F/M) ratios and influent composition (Pal *et al*., 2014). Gaval *et al*. (2002) found that when ASP is subjected to environmental or operational stress, it was not an individual filamentous bacterium that would dominate, but it was more a family succession to the same stimuli. The underlying mechanism that enables mycolata proliferation still therefore requires further investigation.

Traditionally, the observation of filamentous bacteria in activated sludge has been conducted using light microscopy as per guidelines by descriptions stipulated by Jenkins *et al*., (2004). The challenge posed by traditional microscopic identification is the inability to distinguish amongst mycolata.

Similarly, isolation of filamentous bacteria has been conducted using conventional methods (Khan and Faheem, 2012). However, these methods are marred by contamination and failure to maintain isolates in pure culture (Ramothokang *et al*., 2003). The cited historical isolation of *Ca.* Microthrix parvicella was documented in 1973 based on morphological similarities with the *Ca.* M. parvicella that was later isolated and maintained in pure culture (Van Veen, 1973

as cited by Rossetti *et al* 1994; Rossetti *et al*., 2005). The isolates were successfully grown under microaerophilic conditions on medium containing glucose as a carbon source (Van Veen *et al*., 1973 as cited by Rossetti *et al*., 2005). However, these isolates failed to successfully maintain in pure culture for long. Later, successful isolation and maintenance axenically were conducted on Reasoner's 2A agar (R2A agar) by Blackall *et al*. (1994) and Rossetti *et al*. (1997). Other media that have been used to cultivate filamentous bacteria include Casitone Glycerol yeast agar (CGYA) and Tryptone glucose yeast extract agar (TGYA) (Ramothokang *et al*., 2003 and Faheem *et al*., 2012). None of these isolates from the study conducted by Ramothokang *et al* (2003) were subjected to molecular characterization. Micromanipulation is a microscopic isolation technique that facilitates selective isolation of filamentous bacteria from mixed liquor and foams (Rossetti *et al*., 1997). Filamentous actinomycetes that resembled the right-angled branching pattern of *Gordonia* spp. and pine-tree-like morphology of *Skermania piniformis* from Australia were isolated successfully using micromanipulation (Soddell and Seviour, 1998; Soddell *et al*., 2006). Although micromanipulation had been successful, physiological studies from one geographical location cannot be adopted as a solution to circumvent foaming episodes worldwide. Efforts to isolate filamentous nocadioforms using traditional direct plating methods have been largely unsuccessful due to overgrowth by floc-foaming contaminants (Khan and Faheem, 2012). Although micromanipulation has successfully led to isolation of filamentous bacteria (Fan *et al*., 2019), pure culture studies continue to be derailed due to difficulty to maintain organisms in pure culture. Studies on pre-treatment of mixed liquor and foams prior to micromanipulation have not been well documented, globally. This study has contributed to the available practices that can be adopted to eliminate contamination by fast growing foam -formers.

The advancements in molecular biology techniques have led to major improvements in identification and quantification of foaming microbial communities without the need for isolation (Asvapathanagul *et al*., 2016; Guo *et al*., 2015; Ju and Zhang 2015). For instance, comprehensive chemotaxonomic studies combined with almost complete 16S rRNA gene sequencing and conventional phenotypic description led to the reclassification of *Norcadia pinensis* as *Skermania piniformis* (Chun *et al*., 1997). The data presented in those studies highlighted the need for this member of mycolata to have a separate genus within mycolata. Additionally, application of techniques such as fluorescent *in-situ* hybridisation (FISH) has achieved qualitative and quantitative clarity and causative organisms and concentration in

mixed liquor and foam samples (Kallistova *et al*., 2014). Davenport *et al*. (2000) used mycolata specific oligonucleotide probes to quantify mycolata in mixed liquor and foam samples irrespective of morphology and found that non-filamentous mycolata were abundant in foams.

In another study, Carr *et al*., (2006) carried out FISH using *Gordonia* spp. specific probe (GOR 596). The findings were flawed due to the inability of probe GOR 596 to hybridise with pure cultures of *Gordonia* and some *Gordonia* spp. found in foam. Similarly, a PCR based detection for *Gordonia* spp. was developed by Shen and Young (2005). Subsequent studies by Shen *at al*. (2007) coupled nested PCR with denaturation gradient gel electrophoresis (DGGE) to profile different species of *Gordonia* in foaming samples based on banding pattern. However, it was observed that some DGGE banding patterns were not attributed to their melting behaviour, but bias caused by complex interactions among different DNA structures and some bands were inseparable due to DGGE limitations (Guo *et al*., 2015).

The application of qPCR in determining microbial composition and quantification in WWTW has also been employed by researchers (Asvapathanagul *et al*., 2012; Houari *et al*., 2020; Asvapathanagul and Olson, 2016). This technique is reported to be highly accurate in quantifying specific microbial population from mixed microbial communities. In a study, Asvapathanagul *et al*., 2016 used a primer set coupled with *Nocardia* spp. probe to capture a broad group of mycolata for qPCR analysis. However, it found that regardless of the specificity of the primer, quantification of target organisms was inefficient (Asvapathanagul *et al*., 2016). Most recent advances to circumvent this limitation have utilised high throughput platforms such as 16S rRNA amplicon sequencing and shotgun sequencing to identify filamentous bacteria from full scale WWTWs. Such advances provide in depth information on the microbial consortia in WWTWs as observed in amplicon based global study MIDas 4 (Dueholm *et al*., 2022). Earlier studies had alluded to ecological variation as one of main factors that determine the microbial composition in WWTWs (Zhang *et al*., 2021). The advances in metagenomics also seek to provide greater insight on population dynamics and abundance in foams. However, the foaming threshold may vary for each organism with increases of 0.1% sufficient to induce foaming (Guo *et al*., 2015).

1.2 SCOPE OF THE STUDY

Biological foaming in ASP is a widespread problem and has been reported as a common operational problem across the globe. The ASP in the province of KwaZulu-Natal have not been exempted from experiencing foaming. Excessive growth of mycolata has been observed sporadically in the Durban metro and surrounding areas in both domestic and industrial WWTWs throughout the different seasons. The need for process optimisation and troubleshooting necessitates greater comprehension of the structure and function of microbial communities under different geographical conditions.

Although there is research that has been conducted on foaming, microbial consortia and conditions that induce foaming vary considerably due to geographical locations, environmental conditions, and influent composition. Knowledge gaps exist on the dominant foam formers in this region which hinder the ability to pre-empt foaming. Thus, this study sought to identify principal foam formers from two WWTWs treating domestic and industrial wastewater whilst investigating environmental and operational factors that promote their excessive growth. Findings from this study may be useful in understanding the metagenomic diversity of microbes in foaming samples, threshold that induces foaming, influent composition and the operational parameters that promote foaming in the selected industrial and domestic WWTWs.

Successful micromanipulation of *Gordonia* spp. from this study can be applied for the isolation of other slow growing filamentous bacteria from ASP. Advances in molecular biology have led to the characterization of bacteria without culture-based information that renders their classification incomplete. Pretreatment practices, selective isolation and monitoring of filamentous bacterial isolates during incubation can elucidate candidatus status of bacteria.

1.3 AIMS AND OBJECTIVES

AIM

This study aims to isolate and characterize *Gordonia* spp. by micromanipulation and to profile the dominant Actinomycetes involved in foaming incidents in industrial and domestic activated sludge processes within KwaZulu Natal, South Africa, using advanced molecular methods.

OBJECTIVES

- To isolate and characterise *Gordonia* spp. from foaming samples collected from two WWTW in KwaZulu Natal using micromanipulation.
- To determine the biochemical and physiological properties of the isolates, as well as examine the genetic and physiological variations between isolates obtained from domestic and industrial WWTW.
- To monitor the operational and environmental parameters of the WWTW and correlate them with the *Gordonia* spp. population density using quantitative polymerase chain reaction.
- To characterize the microbial communities associated with mixed liquor and foam samples from the selected WWTWs using next generation sequencing methods.

1.4 THESIS OUTLINE

This thesis comprises of two broad sections, isolation, and characterization of *Gordonia* spp. followed by microbiological characterization and identification of isolates. Additionally, advanced molecular techniques such as qPCR and NGS methods were employed to quantify and characterize the dominant *Actinomycetes* in mixed liquor and foam samples. The outline of the thesis structure is provided below:

Chapter 1 introduces the research conducted as well as the motivation to study, a general introduction to foaming, causative organisms, physiological traits of foam formers and identification and quantitative methods. The aims for this research study and the specific objectives to achieve the aim are stipulated. Lastly, the summary for each chapter is provided.

Chapter 2 presents the background information on ASP, microbial functional groups in ASP, foaming, qualitative and quantitative monitoring of mycolata using molecular techniques and wastewater characterization and impact on the ecophysiology of mycolata.

Chapter 3 outlines the first technical chapter that addresses the first two objectives of this study. This chapter focuses on the description of the two WWTWs chosen, micromanipulation for selective isolation of filamentous mycolata from foam and mixed liquor samples, biochemical fingerprinting by focusing on growth kinetics of isolates on minimal media aerobically and anaerobically.

Chapter 4 presents the results from monitoring and quantification of *Gordonia* spp. from two WWTWs over a period of three months using qPCR to establish the plant operational and wastewater characteristics under which foaming occurred.

Chapter 5 addresses metagenomic insight into the distribution of Actinomycetes in mixed liquor and foam of the two WWTW investigated. Next generation sequencing was conducted and provided qualitative and quantitative comparison on the microbial community between mixed liquor and foam samples.

Chapter 6 provides the summary and conclusion. It summarizes the major findings from the technical chapters. Also includes recommendations for possible future research**.**

CHAPTER 2

LITERATURE REVIEW

2.1 The activated sludge process

Domestic and industrial wastewaters contain a variety of organic and inorganic matter, xenobiotic compounds, and surfactants. Wastewater treatment systems ultimately need to reduce or remove these contaminants from the influent before discharge into receiving environment (Garrido-Cardenas *et al*., 2017). The use of the ASP is widespread to remove pollutants and contaminants from a wide variety of wastewaters (Xia *et al*., 2018).

Conventional ASPs are designed to primarily remove carbonaceous organic matter. The efficiency of the process relies on the removal of organic material in the influent which is expressed as chemical oxygen demand (COD). The removal of COD is facilitated by chemoorganoheterotrophic microorganisms (Wells *et al*., 2011). Process modifications were later introduced that competitively select for biological nutrient removal (BNR) by incorporating sequential anoxic/aerobic or aerobic/anoxic zones (Bunce *et al*., 2018; Sam *et al*., 2022). During BNR, nitrification process is favoured by enriching the nitrifying community (Wang *et al*., 2012). The nitrification process involves the biological conversion of ammonia/ammonium to nitrite by ammonia-oxidizing bacteria (AOB) and ammonia-oxidizing archaea subsequently followed by the conversion of nitrite to nitrate by nitrite-oxidizing bacteria (NOB) (Bae *et al*., 2013; Mukherjee, 2013). Nitrifiers are deemed as fastidious with characteristic slow growth rate (Wei *et al*., 2013). Denitrification involves the biological reduction of nitrate to nitric oxide, nitrous oxide, and nitrogen gas (Metcalf and Eddy, 2003). The process of denitrification is facilitated by both heterotrophic and autotrophic bacteria.

The removal of phosphate in wastewater, can be achieved biologically or chemically. In enhanced biological phosphorus systems (EBPR), the removal is facilitated by anaerobic heterotrophs capable of storing orthophosphate intracellularly. Phosphate accumulating organisms (PAO) are responsible for biological phosphate removal (Seviour, 2010, Valverde-Perez *et al*., 2016). Under anaerobic conditions, PAOs convert readily available organic matter such as volatile fatty acids to carbon compounds called polyhydroxyalkanoates (PHA). PAOs

use energy generated through the breakdown of polyphosphate molecules to create PHA. This breakdown results in the anaerobic release of orthophosphates which can be utilised under aerobic or anoxic conditions for microbial growth. EBPR facilitates the removal of total phosphorus through enriching the presence of PAOs, enabling P removal through sludge wastage that contains PAOs (Bunce *et al*., 2018). Such modifications ensure that the microbial consortia that are required for nutrient removal are enriched as outlined in Table 2.1 (Wang *et al*., 2012, Bunce *et al*., 2018). There is a fine balance between these different groups removal that must be maintained for optimal functioning of these processes (McIlroy *et al*., 2015).

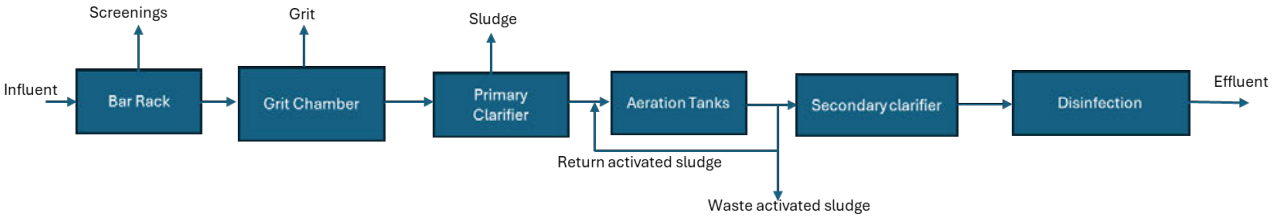


Fig. 2.1: The schematic representation of a conventional activated sludge process inclusive of primary and secondary treatment.

The most critical steps in the ASP ensure biochemical conversion of soluble and particulate organics to biomass in the aeration tanks and settling and compaction of the biomass in the secondary clarifier, as illustrated in Fig. 2.1. The separation of biosolids from liquids is the fundamental goal of the ASP. Therefore, interactions between the aeration tank and the secondary clarifier cannot be neglected (Jin *et al*., 2003). The presence of high concentrations of nitrogen and phosphorus in municipal WWTW influent necessitated the modification of the ASP to incorporate anaerobic and/or anoxic reactors.

Table 2.1: The role of microbial functional groups in biological nutrient removal bioreactors (Thobejane, 2023; Ugwuanyi *et al*., 2024)

Reactor	Biochemical transformation	Enriched functional group and function
Anaerobic	Utilisation of stored polyphosphate by PAO Fermentation of organic matter	Selection of PAO COD removal
Anoxic	Denitrification - conversion of NO_2^- and NO_3^- to N_2 Oxidation of organic matter Alkalinity generation	Selection of denitrifying bacteria and N removal COD removal
Aerobic	Nitrification – conversion of NH_4^+ to NO_2^- and NO_3^- P uptake and formation of polyphosphates by PAO Oxidation of organic matter Alkalinity consumption	NH_4^+ removal P removal COD removal

PAO – phosphate accumulating bacteria COD – chemical oxygen demand.

However, metabolic versatility of filamentous bacteria in BNR plants led to observations in proliferation of filamentous bacteria over floc-forming bacteria. Such imbalances between filamentous bacteria and floc-forming bacteria promote bulking and foaming (Wells *et al*., 2011; Jiang *et al*., 2016).

Although the ASP was developed in 1914, operational problems and poor treatment efficiency due to bulking and foaming continue to be observed sporadically, globally (Jiang *et al*., 2016). Although these modifications are essential, they are also marred by overgrowth of filamentous bacteria (Noutsoupolos *et al*., 2010; Wang *et al*., 2016). Episodes of bulking resulted in microbial shifts as dominance shifted from Proteobacteria to Actinobacteria which was

characterised by increased concentration of *Ca*. M. parvicella (Wang *et al*., 2016). Dominance of *M.parvicella*, *Gordonia amarae* like organisms (GALOs), Eikelboom Type 0092 and *N.limicola* from BNR processes were also observed in sludge samples that exhibited filament proliferation (Noutsoupolos *et al*., 2010). Seasonal fluctuations and failures in nutrients removal due to the operational and environmental changes have been widely reported in engineered wastewater treatment systems (Wang *et al.*, 2012).

2.2 Activated sludge floc

The efficiency of the ASP depends on the formation of large, dense and strong flocs that have the ability to withstand shearing during mechanical aeration. Suitable floc formation ensures settling and compaction of the sludge, which is crucial to process efficiency. Activated sludge flocs are comprised of biological and non-biological components (Jenkins *et al*., 2004, Minnie *et al*., 2022).

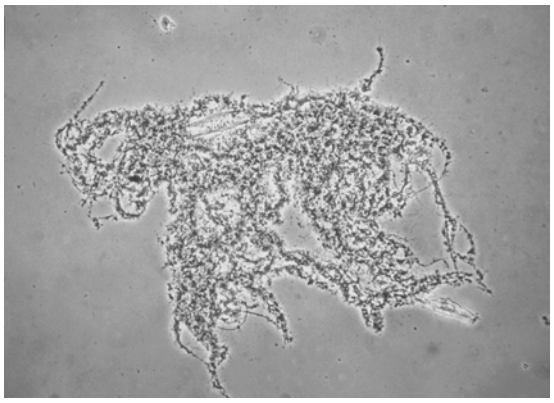


Fig. 2.2: Phase contrast micrograph showing a compact, activated sludge floc depicting an irregularly shaped floc containing filamentous organisms (Jenkins *et al*., 2004).

The biological component includes bacteria, fungi, protozoa and metazoans. Heterotrophic bacteria such as *Pseudomonas*, *Achromobacter*, *Flavobacterium*, *Alcaligenes Arthrobacter*, *Citromonas, Zooglea* as well as the autotrophic bacteria such as nitrifiers form the microstructure of the floc. The microbial physiologies of autotrophic bacteria in biological nutrient removal plants facilitate nitrification. The macrostructure comprises of filamentous bacteria bound by extra polymeric substances as observed in Figure 2.2 (Jenkins *et al*., 2004; Dogan *et al.*, 2014, Li *et al*., 2020). The floc structure determines settling properties,

dewatering, thickening and clarity of the final effluent; hence it directly affects process efficiency (Li *et al*., 2020). Floc-forming bacteria are responsible for organic degradation, whilst filamentous bacteria have a fundamental role in providing the skeletal matrix for the formation of stable flocs (Burger *et al*., 2017).

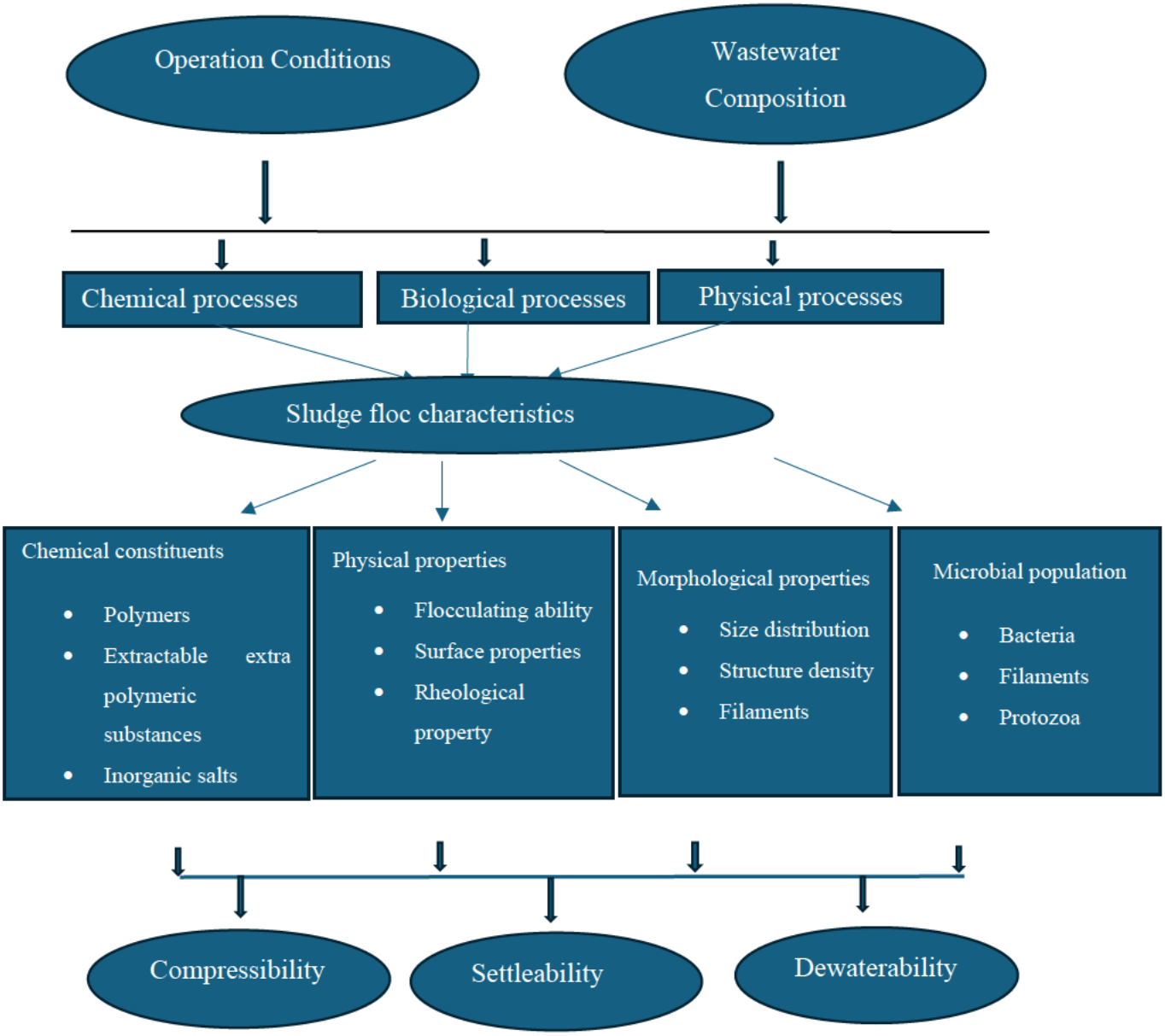


Fig. 2.3: Floc characteristics and activated sludge properties adapted from Jin *et al*., 2003.

Activated sludge separation problems, to a greater degree can be attributed to the nature of the activated sludge floc due to operational conditions and wastewater composition as illustrated in Fig 2.3. Several phenomena lead to activated sludge solids separation complications (Burger *et al*., 2017). Deviations in process parameters such as wastewater composition, temperature, dissolved oxygen concentration, sludge age and organic loading rate can affect the formation of compact flocs (Fryer and Gray, 2012). Colloidal and floc structure are essential for flocculation and settling properties of the ASP (Wilme *et al*., 2010). The classification of solids separation problems is generally named after the effects they induce in the treatment process:

- **Dispersed growth** – results from the disruption of exopolymer bridging such that microorganisms do not agglomerate. Microorganisms are therefore dispersed forming only small conglomerates or remaining as single cells (Figure 2.4a) which results in turbid effluent due to poor settling properties (Jenkins *et al*., 2004; Wilme *et al*., 2010).
- **Viscous bulking** – or zoogleal bulking, is caused by excessive amounts of exocellular material that may not be associated with zoogleal growths. Flocculent microbial cells are surrounded by large amounts of water-retentive, exocellular biopolymers (Wanner, 2014).
- **Pin-point flocs** – When flocculation has not sufficiently developed due to a lack of filamentous bacteria, such flocs may be sheared during aeration. They cannot survive the turbulence caused by mechanical aeration or coarse bubbling from diffused aeration due to the minute size of the 20 – 80 μm floc structure. This results in turbid supernatant. Pin-point flocs can also result when flocs are formed in the absence of filamentous bacteria due to low F/M ratio, long sludge age and occasionally chronic toxicity (Wanner, 1994; Jenkins et al., 2003; Minnie et al., 2020).
- **Filamentous bulking** – This phenomenon is caused by the proliferation of filamentous organisms over floc forming bacteria. This results in internal profusion in flocs resulting in open floc structures impacting effluent quality (Jenkins *et al*., 2004; Li *et al*., 2020). Filamentous bulking generally occurs in BNR AS plants when operational conditions favour the growth of filamentous bacteria over floc forming bacteria creating an imbalance (Henriet *et al*., 2017).
- **Filamentous foaming** - Foaming can be observed as thick surface scum on the surface of aeration basins and secondary settling tanks. Wastewater composition is amongst

the significant factors that can induce foam formation. It has been found that slowly degradable organic material and lipids favours the growth of floc-forming bacteria (Rossetti *et al*., 2005; Pal *et al*., 2014). Sludge ages of greater than 10 days can stimulate filamentous foaming complications (Raskin 2002; Pal *et al*., 2014). Foaming reduces effluent quality and causes loss of biomass (Jiang *et al*., 2016).

Microscopic examination of the activated sludge is imperative to determine the floc structure and the abundance of filamentous bacteria. The physical information that is derived from microscopic examination provides useful information on solid separation problems that may persist in the activated sludge (Jenkins *et al*., 2004; Wanner, 1994).

2. 3 Biological Bulking and Foaming

The excessive growth of filamentous bacteria that lead to poor flocculation and sludge compaction is a common problem in WWTW (Pal *et al*., 2014; Wanner, 2017). Filamentous bulking sludge results in extensive growth of filaments that extend from the floc resulting in interfloc bridging. Bulking sludge is observed as poor settling sludge in the secondary settling basins (Minnie *et al*., 2020). Several factors contribute towards bulking, including substrate or nutrient deficiency and low F/M in the case of readily degradable wastewater as outlined in Table 2.2.

Table 2.2: Filament types as indicators of conditions that induce activated sludge bulking (Jenkins *et al*., 2004)

Causative condition	**Filament types**
Low dissolved oxygen (for the applied organic loading)	*S. natans*, Eikelboom Type 1701 and *H. hydrossis*
Low organic loading rate (low F/M)	*Ca.* M. parvicella, *Norcardia* spp., and Eikelboom Type 0041, Eikelboom Type 0675, Eikelboom Type 1851 and Eikelboom Type 0803.
Septic waste / sulphides (high organic acids)	*Thiothrix I* and *II*, *Beggiatoa* spp., *N. limicola II* and Eikelboom Type 021N
Nutrient deficiency- N and /P	*Thiothrix I* and *II* and Eikelboom Type 021N
Low pH (<pH 6.0)	Fungi
High grease/ oil	*Gordonia* spp., *Ca.* M. parvicella and Eikelboom Type 1863

F/M -food to microorganism ratio N- Nitrogen P - Phosphorus

The growth of filamentous bacteria or total extended filament length (TEFL) has been related to the sludge volume index (SVI). Sludge settleability is grossly determined by measuring the SVI, which is the volume of settled sludge after thirty minutes (mL/L) based on mixed liquor suspended solids (MLSS) levels (Jenkins et al., 2004). Although SVI is popular with plant operators, the accuracy of defining bulking sludge with an SVI of 150-200 mL/g has been deemed to be a visual artefact (Schuler and Jassby, 2007). The thirty-minute settling test and SVI have received scrutiny and were subsequently considered unreliable as bulking indicators. The diluted sludge volume index (DSVI) has been adopted as a preferred method in various countries (Seviour, 2010). Eikelboom and van Buijsen (1983) and Jenkins *et al*. (2004) quantified filaments implicated in bulking by determining filament abundance through microscopic techniques. The fundamental difference between SVI and DSVI is that DSVI incorporates an additional dilution step prior to settling. The advantage of the DSVI lies in its insensitivity to sludge concentration allowing for consistent comparison of sludge settleability between different ASP (Bakos *et al*., 2019). The Foaming Scum Index (FSI) is another tool that was established to rank and characterise foams which are already present on the surface of

aeration basins. This index has been established using physical foam characteristics of colour, bubble size and solids content. The characterization of FSI also considered the information generated from several individual tests normally employed to investigate foaming such as foam potential, stability, and filament abundance (Fryer and Gray, 2012).

Very common and excessive scores of filaments were correlated to DSVI values for bulking detection. TEFL refers to filament extension outside the floc structure and it was correlated to parameters such as DSVI to predict the onset of bulking. This correlation was not widely adopted, and most plant facilities utilise the simpler biased filament abundance scale (Jenkins *et al*., 2004). Table 2.2 highlights guidelines that have been adopted for the microscopic examination of flocs and the influence of filaments on floc structure.

Although the ASP is a well biologically engineered process, it sporadically experiences bulking which can be attributed to the overgrowth filamentous bacteria and fungi (Zhang *et al*., 2017). Bulking due to filamentous bacteria can result in poor settleability, sludge washout leading to degradation of the quality of the effluent. In severe cases, it can lead to complete system failure (Lu et al., 2023). The extension of filamentous bacteria from flocs into the bulk solution can result in elevated SVI. Influent consisting of high carbohydrate wastes, low F/M, nutrient deficiency are amongst the causes of bulking (Nittami *et al*., 2022). Through metagenomic analysis, *Sphaerotilus*, *Thiothrix*, *Chloroflexi* and Ca. M. parvicella have been identified as the most prevalent filamentous bacteria implicated in bulking (Fan *et al*., 2020; Ravin *et al*., 2021; Sam *et al*., 2022; Dueholm *et al*., 2022, Speirs *et al*., 2022). Bulking sludge is less detectable visually in its inception than foaming episodes; hence molecular biology quantitative methods provide better insights into bulking. Biological foams also contain greater amounts of foam-inducing filaments, whilst the bulking sludge microbial population is similar to mixed liquor. Therefore, sole reliance on microscopic examination can be misleading (Wanner, 2017).

Table 2.3: Subjective scheme of Jenkins *et al*. (2004) for determination of filament abundance in mixed liquor microscopically

Score value	**Assessment of filament abundance**	**Comments**
0	None seen	
1	Few present	Only seen in an occasional floc
2	Some present	Commonly seen but not in all flocs
3	Common	Seen in all flocs but only at low frequencies (1-5 per floc)
4	Very common	Seen in all flocs at frequencies of 5 – 20 per floc
5	Abundant presence	Seen in all flocs, at frequencies of ≥ 20 per floc
6	Excessive numbers	Seen in all flocs. Biomass mostly filaments which are plentiful in bulk liquids

2.3.1 Biological foaming

The filamentous bacteria implicated in biological foaming include GALOs, *Ca*. M. parvicella, *N. limicola* (*Tetrasphaera*) and Eikelboom Types such as Eikelboom Type 1863, Eikelboom Type 0092 and Eikelboom Type 0675 (Madoni *et al*., 2000; Dunkel *et al*., 2018; Sam *et al*., 2022). Typical foam is visualised as bubbling, stable foam on the surface of the aeration basin and it is detrimental to process efficiency as it leads to a series of operational issues, including deterioration of effluent quality and loss of biomass (Guo *et al*., 2015; Collivignarelli *et al*., 2020). Foaming can be attributed to biological and chemical sources resulting in various types of foam, as listed in Table 2.4.

Table 2.4: Activated sludge foam types from the aeration basin and their causes (Richard *et al*., 2003; D' Antonio *et al*., 2017)

Foam description	Causes
1. White, frothy, billowing foam	Due to non-biodegradable detergents
2. Pumice-like grey foam	Excessive fines recycle from other processes.
3. Thick, pasty or slimy, greyish foam	Nutrient-deficit foam
4. Thick, brown, stable foam enriched in filaments.	Caused by overgrowth of foam foaming organisms such as *Gordonia*, *Ca*. M. parvicella, *Rhodococcus*, *Skermania*, *Tsukamurella*, *Mycobacterium*; Eikelboom Type 1863; Eikelboom Type 0041, Eikelboom Type 0675.

Several factors induce foaming such as through surfactants in the liquid, proliferation of floc-forming bacteria and/or the presence of hydrophobic-high molecular weight compounds (Petrovski *et al*., 2011; Collivignarelli *et al*., 2020). Previous studies have shown that foaming causative organisms are mycolic acid containing *Actinomycetales* whose cell walls exhibit hydrophobicity. The right angled, branched filamentous *Gordonia amarae* has been the most studied foam former although *Mycobacterium*, *Rhodococcus*, *Skermania* and *Tsukamurella* have also been implicated in foaming (Davenport *et al*., 2000; Stainsby *et al*., 2002; Nam *et al*., 2004; Kragelund *et al*., 2007). The implication of other organisms that displayed the right-angled filamentous morphology of *Gordonia amarae* led to an encompassing term of this shared morphotype called GALOs (Carr *et al*., 2006). The phylum *Actinomycetota* encompasses Gram-positive bacteria with a high guanine-cytosine (GC) content. Within *Actinomycetota*, are Corynebacterianeae which possess mycolic acids and other lipids in their outer membrane hence they are referred to as mycolata. The Gram positive bacterial group containing mycolic acids are termed mycolata. Table 2.5 outlines the genera that constitute mycolata. No information was found that implicated *Segniliparus* in foaming, whilst all the other genera have been implicated in foaming (Arenskötter *et al*., 2004; Soddell and Seviour *et al*., 2008; Guo *et al*., 2015).

Table 2.5: Genera in the suborder *Corynebacterineae* (Soddell and Seviour, 2008)

Family	Genus
Gordoniaceae	*Gordonia*
	Skermania
Corynebacteriaceae	*Corynebacterium*
	Turicella
Dietziaceae	*Dietzia*
Mycobacteriaceae	*Mycobacterium*
Norcadiaceae	*Norcadia*
	Rhodococcus
Tsukamurellaceae	*Tsukamurella*
Williamsiaceae	*Willimsia*
Segniliparaceae	*Segniliparus*

Stainsby *et al*. (2002) isolated and characterised non-filamentous mycolata in foam, highlighting the inadequacy of microscopic examination in elucidating foam forming organisms. Traditionally mycolata were identified by their morphology using the guidelines by Jenkins *et al*. (2004). A seven-point based system of 0 – 6 is also used to quantify filamentous bacteria by determining their abundance level from mixed liquor, as shown in Table 2.3. The level that induces foaming is level 4 when filaments are regarded as dominant ranging from 5 – 20 filaments per floc (Jenkins *et al*., 2004, Chen *et al*., 2016).

2.4 Micromanipulation of filamentous bacteria from activated sludge mixed liquor and foam

Pure culture studies of filamentous bacteria are generally halted by the overgrowth by floc forming bacteria (Khan and Faheem, 2012). Therefore, selective isolation through micromanipulation has been developed for the isolation of filamentous bacteria from mixed liquor samples (Rossetti *et al*., 2005).

Fig. 2.4: Illustration of micromanipulator used for cell isolation

Several filamentous morphotypes have been isolated using micromanipulation (Fig. 2.4), and their biochemical and phylogenetic characteristics have been investigated in pure culture (Hornsby and Horan, 1994; Bradford *et al*., 1996, Rosetti *et al*., 2005; Kragelund *et al*., 2008). In a study by Snaider *et al*., 2002, filamentous bacteria that resembled "*Nostocoida limicola*" like filamentous bacteria from WWTW were micromanipulated which led to the development of FISH probes for their *in-situ* detection (Snaidr *et al*., 2002). Similarly, micromanipulation and subsequent molecular analysis led to the discovery of a novel species within the genus Microthrix called *Ca*. Microthrix calida, that was isolated from ASP that treats industrial effluent (Levantesi *et al*., 2006b). In another study, mycolata were micromanipulated from activated sludge and foam and subsequent phylogenetic and phenotypic characteristics determined (Stainsby *et al*., 2002). The study implicated *Mycobacterium*, *Rhodococcus* and *Gordonia* spp. dispelling the myth that *G. amarae* is the primary foam former. This study supported earlier findings by Davenport *et al*., (2000) that foam formers are diverse.

Furthermore, micromanipulation has also enabled holistic investigations that examined identity, abundance, and ecophysiology of *Haliscomenobacter hydrossis* from ASP (Kragelund *et al*., 2008). Their study led to the detection of *Bacteroidetes* and their contribution to bulking episodes whilst investigating ecophysiological traits through FISH-microautoradiography (Kragelund *et al*., 2008). *Gordonia* spp. have generally displayed versatility when it comes to their physiology (Arenskotter *et al*., 2004; Drzyzga *et al*., 2009; Matsui *et al*., 2014). These foams occur in all types of plants with no dependence on modes of aeration or operation (Asvapathanagul *et al*., 2012; Guo *et al*., 2015). GALOs proliferate equally in domestic, industrial, and mixed waste ASP (Tsang *et al*., 2008; Kragelund *et al*., 2007; Guo *et al*., 2017). It is evident that filamentous bacteria possess vast physiological and ecological traits, therefore studies that can combine ecophysiology with process conditions can provide more efficient control strategies (Minnie *et al*., 2022).

To date, only a few filamentous bacteria exist in axenic cultures. *Ca.* M. parvicella has been micromanipulated and maintained in a viable state enabling physiological studies that determine its prevalence in bulking sludge (Rossetti *et al*., 2005). The availability of pure cultures allows extensive research into conditions that promote bulking and foaming by the respective organism. In a recent study, the application of micromanipulation technology led to the isolation of 545 isolates of *Ca*. M. parvicella from bulking ASP using six different types of media from a wastewater treatment plant (WWTP) in Beijing (Fan *et al*., 2019). However, due to the paucity of *M. parvicella*, only 1% of the isolates survived until enrichment and preservation was concluded. This successful isolation however, led to correlation of filament abundance to key plant operating parameters that prevailed during bulking episodes. They found that the proliferation of *M. parvicella* was linked to low temperatures that ranged between 14 to 18.8^{o}C and temperature was considered a limiting growth factor (Fan *et al*., 2019). Laborious conventional isolation and purification led to successful isolation of *Gordonia caeni*, *Gordonia cholesterolivorans* from WWTWs (Srinivasan *et al*., 2012; Drzyzga *et al*., 2009), however micromanipulated *Gordonia* spp. have also been isolated from ASP foams (Soddell *et al*., 2006).

2.5 Pure culture studies of Gordonia spp. isolated from activated sludge plants

Gordonia spp. has generally displayed versatility when it comes to their physiology. *Gordonia amarae* has been the most studied species because it is commonly observed in foaming aeration

basins worldwide (Guo *et al*., 2015). Carr *et al*. (2006) conducted physiological studies on *G. amarae* and showed that *G. amarae* was unable to assimilate glucose, oleic acid and estradiol under aerobic, anaerobic and anoxic conditions. Moreover, they observed that *G. amarae* are highly selective for hydrophobic substrates. Failure to grow on oleic acid was attributed to the unsaturated form of oleic acid (Carr *et al*., 2006). The assimilation of hydrophobic substrates was further supported by Drzyzga *et al*., (2009) when a cholesterol degrading organism was isolated.

Pagilla *et al*. (2002) conducted an experiment on *G. amarae* foaming investigating its biosurfactant production ability. Using laboratory-scale batch reactor, acetate and sparingly soluble hexadecane were used as carbon sources for growth and biosurfactant production. Growth was observed on acetate as a carbon source, which supported earlier findings, but biosurfactant production was minimal. When hexadecane and acetate were provided as carbon sources, biosurfactant production was observed, and it was later demonstrated that the biosurfactant produced can be used as a carbon source. This clearly demonstrates that *G. amarae* can solubilise hydrophobic substrates by stimulating the production of biosurfactants, thereby inducing *Gordonia* related foams (Pagilla *et al*., 2002). Although pure culture studies formed the basis of physiological studies, validating the findings *in situ* remains vital. The lack of sufficient data generated through pure-culture studies led to application FISH – MAR which has been applied successfully in environmental biota, particularly AS samples, to investigate the physiology of bacteria under conditions that mimic *in situ* conditions (Andreasen and Nielsen, 1997, Lee *et al*., 1999). However, studies by Kragelund *et al*. (2007) using FISH-MAR found discrepancies with earlier published data on pure culture studies as substrate uptake patterns did not support earlier findings of assimilation of long chain fatty acids. In their study, *Gordonia* spp. that were identified *in situ* using Gor 596 probe specific for *Gordonia* spp. had failed to assimilate oleic acid and palmitate. Further findings by Carr *et al*. (2006) on hydrophilic and hydrophobic substrates *in situ* using FISH-MAR revealed that *G. amarae* could utilize benzoic acid under anaerobic conditions. Just like Ca. *M. parvicella*, *G. amarae* has been reported as capable of metabolic activity under aerobic, anaerobic and anoxic conditions though *Gordonia* spp. are deemed aerobic actinomycetes (Carr *et al* 2006; Eales *et al*., 2006). Kragelund *et al*., (2007) found no metabolic activity under anaerobic conditions when monitoring substrate uptake by *Gordonia* spp. and *Skermania piniformis*.

The undesirable foams have been implicated in ASP of different configurations and influent types. However, industrial influent with high grease, fats and oils, such as abattoirs, food processing and pharmaceutical industry, has been considered the main cause of mycolata overgrowth (Drzyzga *et al*., 2009; Delegan *et al*., 2020). Such foams lead to operational problems when foams seep into the effluent, adversely affecting the suspended solids concentration in the effluent, thus reducing subsequent disinfection effectiveness (Richard, 2003; Collivignarelli *et al*., 2020). The presence of fatty acids has proven to promote the growth of *G. amarae*. In a study conducted by Tsang *et al*., (2008) has shown that an increase in fatty acid concentration led to an increased foaming threshold, and the stability of the foam was observed even after aeration was halted (Tsang *et al*., 2008). A prior study by Frigon *et al*., (2006) also regarded *Gordonia* spp. as specialised lipid consumers with an increased ability to foam formation and stabilisation in increasing temperatures. Foam stabilization is attributed to the presence of mycolic acids with very long aliphatic chains that render the cells hydrophobic which is one of the key components required for foam stabilization (Soddell, 1999).

Pitt and Jenkins (1990) stated that there is a consensus that growth of *Gordonia* spp. and foaming is associated with higher temperatures of above 14ºC with maximum growth between 23ºC and 37ºC. A study by Soddell and Seviour (1995) found that certain GALOs can grow at 5ºC. Yet some GALOs can grow above ambient temperatures between 40º to 50ºC. Earlier studies by Eikelboom (1991), as cited by Soddell and Seviour (1995) revealed that actinomycete foams were more likely to occur in higher warmer climates whilst *M. parvicella* was more likely in cooler climates.

2.6 Ecophysiology of mycolata implicated in foaming episodes.

Globally, the vast majority of ASP are affected by foaming phenomenon, however, not much has been achieved to pre-empt it due to insufficient knowledge of the physiology and ecology of causative organisms. Biological foaming is rooted in microbial physiology that is strongly influenced by environmental and operational conditions such as Temperature, pH, MLSS, COD, influent composition, and sludge age (Nielsen *et al*., 2002; Gaval and Pernelle, 2003; Pal *et al*., 2014). The prevention of foam formation due to mycolata requires absolute prevention of conditions that favour their proliferation. Various conditions have been linked to

the overgrowth of actinomycetes, such as aeration rates, reduced MLSS, F:M ratio, and seasonal patterns, amongst others (Gerhardi and Frank, 1990, Jiang *et al*., 2016).

The proliferation of actinomycetes is not only confined to wastewater characteristics, but environmental conditions could also impact their dominance. Certain temperature ranges have been linked to the overgrowth of filamentous bacteria (Che *et al*., 2016; Jiang *et al*., 2016). Laboratory scale studies that investigated the impact of temperature and sludge loading of *M. parvicella* found that it grew optimally at temperature ranges of 12 to 15^{o}C (Knoop and Kunst, 1998). Their proliferation in winter was also supported by other studies globally (Lienen *et al*., 2014 and Deepnarain *et al*., 2020).

A study conducted by Gaval and Pernelle (2003) investigated the impact of different oxygen deficiencies in the induction of filamentous bulking. Two pilot studies were conducted, pilot A had DO values dropped from 3 to 0.7 mg/L and pilot B had DO concentrations dropped from 1.9 to 0 mg/L. After each stress period, pilot plants were returned to normal operating conditions. *Haliscomenobacter hydrossis* revealed significant increase in pilot B from 1.6 X 10^4 filaments/mg of MLSS to a maximum of 8.2 X 10^4 filaments/mg of MLSS.

A study conducted in Hong Hong, Shatin WWTW examined the abundance of bulking and foaming bacteria and correlated their abundance with environmental parameters and biotic factors. A novel *Gordonia* sp. was found to be a dominant foam former that had been observed in a previous study (Guo *et al*., 2015; Jiang *et al*., 2016). Mycobacteria were also observed as a secondary foam former, accounting for 3% ± 1.62 and its abundance was not affected by seasonal variations (Jiang *et al*., 2016). This finding contradicted an Italian survey that implicated *M. parvicella* as a dominant foaming and bulking organism (Madoni *et al*., 2000). The abundance of *Gordonia* spp. in Shatin was deemed to be favoured by temperature and was mostly observed during the winter-spring climate. However, Ju and Zhang (2015) from the same WWTP, Shatin, stated that population dynamics were not affected by a climatic variation which contradicted most findings. Earlier findings that quantified filamentous bacteria using FISH from Danish WWTPs had contradictory findings implicating *Microthrix* and *Chloroflexi* Type 0092 and 0803 (Mielczarek *et al*., 2012). The variation could be attributed to differences

in influent composition, operational parameters and climatic conditions (Pal *et al*., 2013; Jiang *et al*., 2016). Such variations hamper universal solutions to elucidate foaming and bulking. The influence of operational parameters on the population dynamics of bulking and foaming bacteria led to a positive correlation of *Gordonia* spp. with nitrite – nitrogen (NO_2 – N) in the aeration tank (Jiang *et al*., 2016). Earlier reports have implicated *Gordonia* spp. in nitrate reduction (Kim *et al*., 2003; Romanowska *et al*., 2010). Studies on statistical correlation of *Gordonia* spp. with operational parameters to identify the sensitive parameters remain elusive due to a lack of supporting experimentation to support these relationships.

Advances in molecular biology applications have led to reclassification of filamentous bacteria. *Thiothrix* spp. have been observed in ASP experiencing bulking. In the past two decades, the taxonomy of *Thiothrix* has been revised several times (Howard *et al*., 1998, 1999; Aruga *et al*., 2002). Nearly complete 16S rRNA sequences were determined from axenic strains of Eikelboom Type 021N, Type 1701 that were originally isolated from bulking ASP. Eikelboom Type 021N strains revealed a sufficiently strong relationship to the *Thiothrix* group (Howard *et al*., 1998). Eikelboom Type 1701 contained within its sequence the target sequence of oligonucleotide probe for *Sphaerothilus natans*. A subsequent study revealed that *Thiothrix* spp. and Eikelboom Type 021N formed a monophyletic group. The study proposed new *Thiothrix* species, namely *Thiothrix eikelboomii* sp, *Thiothrix unzii* and *Thiothrix fructosivorans* and *Thiothrix defluvii* sp. (Howard *et al*., 1999). Another study investigated 15 strains of Eikelboom Type 021 N bacteria and found that the strains shared many characteristics with *Thiothrix* species. However, they only had 88.3 to 92% 16S rRNA similarity to *T. nivea* group including *T. ramova*, *T. unzii* and *T. fructosivorans*. That suggested that the isolates belong to species distinct from *T. nivea* group. The isolates were further divided into three distinct groups based on namely, groups I, II and III based on genotypic and phenotypic characterisation. They further proposed that two novel species for strains and *Thiothrix disciformis* for group I and *Thiothrix flexilis* sp. for group II (Aruga, 2002). *Thiothrix* were formerly known as members of the *Thiotrichaceae* family, and are now classified into three different families *Thiolineaceae*, *Thiofilaceae*, and *Thiotrichaceae* (Boden and Scott, 2018). Metagenomic studies conducted from an EBPR led to the discovery of two novel species referred to as *Ca*. T. moscowensis and *Ca*. T. singaporensis (Mardanov *et al*., 2020). Another study discovered three new *Thiothrix* spp. namely, *Thiothrix subterranea*., *Thiothrix litoralis* and Ca. *Thiothrix anitrata* (Ravin *et al*., 2021). Therefore, obtaining new isolates and

metagenome assembled genomes, especially from atypical habitats, could expand our knowledge of the diversity of *Thiothrix*.

The phylum *Chloroflexi* is highly abundant in a wide range of wastewater treatment bioreactors. *Chloroflexi* participate in organic matter degradation, nitrogen removal and can also provide structural backbone for floc-formation (Bovio-Winkler *et al*., 2023). However, *Chloroflexi* have been implicated in bulking episodes affecting WWTW plant performance (Björnsson *et al*., 2002). Advances of more discerning molecular biology methods elucidated the classification of Eikelboom Type 0914, Eikelboom Type 0092, Eikelboom Type 1851 and Type *Nostocoida limicola*-like filaments into phylum *Chloroflexi* (Spiers *et al*, 2022). The *Chloroflexi* filament *Ca*. Kouleothrix aurantiaca has been grown and characterised phenotypically (Kragelund *et al*., 2007) and *Chloroflexi* filament isolated from Korea has been named *Ca.* Defluvithrix (Yoon *et al*., 2010). Eikelboom Type 1851 was reclassified as *Ca.* Kouleothrix, Eikelboom Type 0092 as *Ca.* Promineofilum and *Ca*. Amorolinea and Eikelboom Type 0914 as *Ca.* Amarolinea (Speirs *et al*., 2022). Nomenclature of filamentous bacteria has been greatly improved by molecular biology applications that could not be resolved using basic microscopic techniques. Continuous efforts to obtain filamentous bacteria in pure culture would lead to full characterization, thus resolving the canditatus status of genotypically identified filamentous bacteria.

2.7 Qualitative and quantitative monitoring of mycolic acid-containing Actinomycetes using molecular techniques

The application of molecular techniques has provided rapid identification and quantification of individual microbial cells from various habitats. However, tentative identification achieved by traditional microscopic methods is inconclusive due to limited morphological distinction through visual identification. Conventional cultivation methods are also time-consuming and cannot recover all organisms as non-culturable organisms remain unaccountable (Jenkins *et al*., 2004; Hennessy, 2021).

The presence of right angled, branched, Gram positive filamentous bacteria led to ambiguous terms such as GALOs as numerous other genera share morphology within mycolata (Soddell,

1999; Seviour *et al*., 2008). Advances in molecular biology, including FISH led to unequivocal identification of causative organisms in foams regardless of morphology, as some filaments display pleomorphism (Liao *et al*., 2004; de los Reyes, 2010; Nielsen *et al*., 2009; Seviour *et al*., 2008). This was made possible by progression in the development of target-specific probes and primers, permeabilization protocols for FISH analysis and quantification methods (Davenport *et al*., 2000).

Fluorescently labelled, rRNA targeted oligonucleotide probes have become a common tool for direct, cultivation-independent identification of bacterial cells. Comparative sequence analysis and 16S Rrna gene sequences that are directly retrieved from the natural microbial communities represent a well-established method for describing species composition *in situ* (Amann *et al*., 2001; Schuppler *et al*., 1998). Sequence information can be used to design rRNA-targeted oligonucleotide probes which enable quantification and spatial distribution of these microorganisms in ecosystems by FISH (Amann, 1995). The presence of large databases and rRNA gene fragments has been retrieved from various ecosystems, including ASP. This has enabled the successful detection and quantification of microbial community structures ranging from phyla to individual species level (Wagner *et al*., 1993; 1994; Liao *et al*., 2004; Mielzarek *et al*., 2012). FISH has enabled progressions in taxonomic diversity among morphologically indistinguishable filamentous bacteria (Levantesi *et al*., 2006). Generally, Gram negative bacteria do not require further enzymatic permeabilization for whole-cell hybridization, while Gram positive bacteria, such as mycolata, require enzyme pretreatment as their cell walls are not readily permeabilized. This can be attributed to rigid cell walls containing mycolic acids with long aliphatic chains (Schuppler *et al*., 1998). This hinders hybridization due to cell inaccessibility. Enzymatic treatment permeabilizes the cell wall. Various enzymes have been used successfully for pretreatment (Beimfohr *et al*., 1993; Davenport *et al*., 2000). Lysozyme, mutanolysin, lipase and proteinase K were the most effective enzymes for the permeabilization of mycolata (Carr *et al*., 2005).

The application of FISH also detected non filamentous mycolata highlighting the inadequacy of purely morphological methods for the identification of mycolata (Davenport *et al*., 2000). This supported earlier findings by Soddell and Seviour (1990) that there is no fundamental

physical reason why the contribution of non-filamentous mycolata should be negated in foam stabilisation. A subsequent study investigated the fundamental role of filamentous mycolata in foaming by quantifying the different mycolata morphotypes in foaming and non-foaming ASP. Single celled rod and coccoidal forms were observed and accounted for 79% of the mycolata population at the onset of foaming using probe Myc657, which targets mycolata. This further supports the notion that it is the growth of mycolata populations, irrespective of morphology that is responsible for the onset of foaming (Davenport and Curtis, 2002). However, the identity of all mycolata present was not accomplished using available FISH probes, suggesting the presence of undetectable and unexplored populations. Thus, the application of other molecular biology techniques not reliant on known 16S or 23S rRNA gene-targeted oligonucleotide probes is mandatory to overcome this limitation.

2.7.1 Polymerase chain reaction-based techniques

The application of molecular techniques such as polymerase chain reaction (PCR) ensures amplification of targeted genes, yielding less unprejudiced information about microbial communities than culture-based approaches (LaMontagne *et al*., 2002). Shen and Young (2005) designed G268F and G1096R primers for rapid detection and identification of *Gordonia* spp. from wastewater samples. The primers produced no amplification with closely related species from other genera, although amplification was unsuccessful with two strains of *Gordonia,* viz., *Gordonia nitida* and *Gordonia rubripertinctus*. In a subsequent study (Shen *et al*., 2007), semi - nested PCR amplification coupled with DGGE was conducted to detect *Gordonia* spp. from foam samples. The identification relied on the application of specifically designed primers G699F and G1096R that target the hypervariable region of *Gordonia* 16S rRNA gene sequence (Table 2.6). This approach used the 829 base pair PCR amplicon from primer sets G268F and G699 that had been designed during an earlier study as a template (Shen *et al*., 2007).

Table 2 6: *Gordonia* spp. specific primers used in PCR reported in the literature.

Primer name	Sequence (5' to 3')	References
G268F	CGACCTGAGAGGGTGATCG	Shen and Young, 2005
G699F	ATAACCCGCTGGCAATACAG	Shen *et al*., 2007
G1096R	AGGCGGGTCTCTGGGTAGTA	Shen and Young, 2005

2.7.1.1 The application of quantitative PCR for the detection of Gordonia spp. in wastewater

Quantitative PCR is a modification of conventional PCR that relies on end-point detection and quantifies the concentration of specific nucleic acids by measuring the intensity of fluorescence emitted at each cycle. The fluorescent signal is directly proportional to the number of amplified PCR amplicons generated by qPCR sprints. Hence, the data generated during exponential amplification phase in real time allows users to generate quantitative information on the initial quantity of the amplified quantity with great precision (Gokul *et al*., 2016). Asvapathanagul *et al*., (2016) designed qPCR primer set coupled with a *Nocardia* genus-specific probe for a wide range of bacteria within the subclass Actinobacteridae. The primer set 915F and 1004R was designed to target a wide range of genera amongst mycolata, highlighting overall efficacy and mispriming challenges. The study produced varying efficacy amongst *Nocardia* spp. Amongst the organisms quantified, *Nocardia breviatena*, *N. carnea* and *N. flavorosea*, compared well to copies generated by qPCR assays and produced values within regular qPCR variation. However, species like *N. jejuensis* produced qPCR reactions below the minimum detection limit. Previous studies by Wang *et al*., (2010) combined primers and probes targeting the rRNA spacer region to enable more precise and simultaneous *Nocardia* spp. identification. Xiao *et al*., (2010) found that the first 500 bp of the 16S rRNA gene of the genus *Norcadia* contained variable homology that could be used to conduct differentiation at the species level. The studies conducted by Wang *et al*., (2010) and Xiao *et al*., (2010) were non quantitative but may be adopted for quantitative trials due to specificity of the probes and primers used. Such specificity can prevent amplification from highly similar organisms such as *Corynebacteriacea* (Asvapathanagul *et al*., 2016).

2.7.2 Next generation sequencing methods

The dense and diverse microbial communities in ASP require the application of techniques such as shotgun sequencing to detect and quantify specific genes and genera. Several researchers have generated extensive metagenomic data to determine functional groups and antibiotic-resistant genes within wastewater treatment plants of various configurations (Miller *et al*., 2013; Yang *et al*., 2013; Guo *et al*., 2017; Gupta *et al*., 2018). There are various methods employed to generate metagenomic information. Amongst them is shot-gun sequencing which locates randomly located short fragments called reads. A great number of reads comprising of 100 – 1000 base pairs (bp) are generated from long DNA strands. Shotgun metagenomics sequences all the nucleic acid material that has been extracted from a sample, providing identity of all organisms potentially present in a sample (Marits *et al*., 2019).

Extracted material is sequenced on a NGS platform, and the resulting reads compared to a reference database. These databases are much larger than those used in deep amplicon sequencing (DAS), as they contain all known sequences from all organisms rather than a set of sequences from a single gene family (Miller *et al*., 2013; Wu *et al*., 2019). Although this makes the analytical part of a shotgun study computationally intensive, the advantages over DAS are numerous. Shotgun methods are less biased and generate data that better reflect the sample's true population structure, Furthermore, only shotgun methods can interrogate the accessory genome, that is, the non-core set of genes (Miller *et al*., 2013). Thus, in recent years large-scale microbial communities profiling with high throughput sequencing of 16S rRNA gene amplicons and whole environmental DNA have become powerful tools to investigate microbes in environmental samples (Jiang *et al*., 2016). Guo *et al*., (2017) investigated dominant classes in AS BNR plants.

A long term metagenomic study was conducted in Hong Kong to monitor the microbial consortium and abundance from foam samples and mixed liquor. The study was particularly aimed at monitoring microbial shifts among foaming AS and non-foaming AS prior, during and post foaming incidents (Guo *et al*., 2015). The abundance of *Gordonia* spp. in foams supported earlier findings though conducted through FISH (de los Reyes *et al*., 1998). An unknown *Gordonia* spp. displayed close homology to *G. amarae* was found during foaming

episodes, reduced levels of *Gordonia* spp. were observed before and after foaming incidents (Guo *et al*., 2015). Furthermore, foaming events were not solely attributed to *Gordonia* spp. The abundance and prevalence of *Clostridium* XI, *Acrobacter* and *Flavobacterium* were noted in foam samples than in AS mixed liquor. All these genera possess foam stabilisation characteristics, *Clostridium* and *Flavobacterium* are known biosurfactant producers and *Acrobacter* an oil degrading genus with high-cell hydrophobicity (Guo *et al*., 2015).

Advances in metagenomic studies have led to studies that simultaneously identify and quantify functional groups within a single sample. Bulking and foaming bacteria were determined from Shatin, Hong Kong and it revealed significant diversity of bulking and foaming bacteria that averaged 8.52 ± 7.3% of total bacteria in the activated sludge system that displayed lower abundance in summer-autumn months (Jiang *et al*., 2016). This finding contradicted findings from a Danish survey which found insignificant changes in filamentous bacteria in BNR plants. Such variations are imminent due to variations in influent composition, operational conditions, and climatic conditions vary considerably between Hong Kong and Denmark (Mielczarek, 2012). In another study that investigated bulking and foaming microbial community prevalence through high throughput sequencing, *Gordonia* and *Mycobacterium* spp. were observed in all sample points, with *Gordonia* spp. present in higher abundance during the five-year study (Jiang *et al*., 2016). Metagenomic analysis serves to elucidate shortcomings in molecular biology such as limited availability of primers for PCR and quantitative PCR approaches (Yang *et al*., 2013, Ravin *et al*., 2021).

CHAPTER 3

MICROMANIPULATION OF *GORDONIA* SPP. FROM ACTIVATED SLUDGE SAMPLES AND ITS BIOCHEMICAL CHARACTERIZATION

3.1 INTRODUCTION

The ability of ASP to separate sludge from the treated wastewater during the settling phase is the measure of process efficiency. However, as a result of filamentous bacteria overgrowth, the ASP is occasionally affected by foaming and bulking incidents (Kampfer, 1997, Davenport *et al*., 2000; Shen *et al*., 2007; Fan *et al*., 2019). This challenging phenomenon can be visualized with the presence of stable foams in the aeration tank and rising sludge in the secondary sedimentation tank. Filamentous bacteria in foams have been observed in ASP worldwide (Guo *et al*., 2015; Wanner, 2017). Foam formation could pose process operation failure and lead to effluent quality deterioration and biomass loss from the aeration tank (Soler *et al*., 2018). Despite the advancements in wastewater treatment technologies, this remains a major challenge to several WWTPs across the globe (Soddell *et al*., 2006; Soler *et al*., 2018).

Filamentous foaming has been attributed to the copious growth of mycolic acid containing actinomycetes particularly, branched mycolic acid containing actinomycetes. Filamentous bacterial species viz., *Nocardia* spp., *Rhodococcus* spp., *Gordonia* spp., *Skermania* spp. and *Tsukamurella* spp. have been identified and isolated from foam samples (Mori *et al*., 1988; Soddell, 1990; Soddell and Seviour, 1990; Goodfellow *et al*., 1995; Soddell and Seviour 1998; Davenport, 2000; Stratton *et al*., 2002 and Soddell *et al*., 2006). Although biological foaming has been attributed to branched mycolata, single celled mycolata have also been implicated in foaming. The application of *FISH* technique has revealed that mycolata display significant pleomorphism as coccoidal and rod shaped mycolata have been observed in foams which may have been overlooked upon isolation due to the focus on branched mycolata (Davenport and Curtis, 2002; Stainsby *et al*., 2002).

Wastewater composition is among the significant factors affecting foam formation. It has been revealed that slowly degradable organic material and lipids favour the growth of filamentous actinomycetes, Ca. *M. parvicella* and *Gordonia* spp. (Rossetti *et al*., 2005; Pal *et al*., 2014). The ability to utilise steroid compounds has been established for *Rhodococcus*, *Mycobacterium* and *Gordonia* (Wilmanska *et al*., 1995; Lashkarian *et al*., 2010, Drzyzga *et al*., 2011). Some

of these organisms, such as *Gordonia cholesterolivorans* have the inherent ability to synthesize cholesterol oxidase, an enzyme which catalyses the oxidation of cholesterol into cholesten-3-one. This enables *Gordonia* spp. to predominate in the presence of hydrophobic compounds in wastewater influent. Although these mycolic acids containing actinomycetes are considered aerobic, they have been observed to utilise long chain fatty acids anaerobically (Drzyga *et al*., 2011). The extremely slow growth of *Ca. M. parvicella* isolates and difficulty with culture maintenance have hampered its physiological characterisation (Fan *et al*., 2019). The richness of metabolic activities of *Gordonia* spp. needs to be explored as in-depth understanding of its ecophysiology in activated sludge can prevent *Gordonia* spp. related bulking and foaming that is experienced in this region. The lack of physiological knowledge from actinomycete-morphotypes due to insufficient information generated from pure culture studies hinders plant personnel from pre-empting foaming episodes (Guo *et al*., 2015).

Traditional isolation methods, namely direct plating on selective and enriched media for filamentous bacteria, have been conducted in the past successfully, although the recovery rate is quite low (Hozzein *et al*., 2012; Khan and Faheem, 2012). However, it is tedious and often results in fast growing floc-forming bacteria out-competing slow growing filamentous bacteria (Ramothokang *et al*., 2003). Although recovery is quite slow using traditional methods, novel *Gordonia phthalatica* sp. has been successfully isolated using conventional isolation techniques on nutrient agar medium from an ASP in Beijing recently (Jin *et al*., 2017). Further limitations of traditional cultivations are brought by the inability of defined media to maintain the viability of these fastidious filamentous bacteria (Khan and Faheem, 2012; Fan *et al*., 2019). Studies have also explored other improved techniques, such as the micromanipulation technique to selectively isolate filamentous bacteria from the complex samples as opposed to the conventional plating technique as described in chapter 2. Micromanipulation technique uses specialised inverted microscopes to selectively isolate the desired filamentous organism from mixed liquor and foam samples, thus excluding contamination from fast growing organisms (Blackall *et al*., 1989; Tandoi *et al*., 1992; Soddell and Seviour, 1999 and Levantesi *et al*., 2006; Fan *et al*., 2019). Among the foam formers that have been identified globally in bulking and foaming samples, *Microthrix parvicella* and *Microthrix calida* have been successfully isolated through micromanipulation after numerous failed attempts (Rossetti *et al*., 1997; Fan *et al*., 2019 and Levantesi *et al*., 2006). The failure to isolate and maintain pure cultures of *M. parvicella* stemmed from its fastidious nature and slow growth (Rossetti *et al*., 1997). A

successful attempt to isolate *Ca*. *M. parvicella* also highlighted extensive enrichment and purification required even though the isolates had been acquired through micromanipulation (Rossetti *et al*., 1997). Successful isolation is also often marred by the inability of isolates to maintain viability (Rossetti *et al*., 2005; Fan *et al*., 2019). This selective isolation method enables axenic physiological characterisation to provide an insight into the conditions that induce proliferation of filamentous mycolata and its' growth kinetics. Efforts have also been conducted to isolate mycolata using micromanipulation from WWTW foam and mixed liquor. That study led to putative identification of novel species belonging to *Gordonia*, *Mycobacterium* and *Rhodococcus*. However, difficulty was also encountered as only 4% of the micromanipulated isolates were maintained in pure culture (Stainsby *et al*., 2002).

Although several efforts to isolate *Gordonia* spp. from WWTW using micromanipulation have been successfully conducted (Stainsby *et al*., 2002; Soddell *et al*., 2006; Khan and Faheem, 2012), there is still a need to understand their physiological traits under different geographical locations or conditions. The versatility of filamentous actinomycetes requires that solutions to circumvent foaming be examined in all geographical locations that experience bulking and foaming. Thus, the need to control the formation and persistence of foaming requires that the functional roles of causal organisms be further investigated in all regions. Although *Gordonia* spp. have been implicated in foaming in this warm coastal region (Ramothokang *et al*., 2003, Deepnarain *et al*., 2020), no documented physiological studies have been conducted from South African isolates from the southeast and east coast of KwaZulu-Natal. The isolation of filamentous bacteria is marred by challenges. The challenges range from accidental isolation of floc-formers which outgrow the slow growing filamentous bacteria, inability to provide growth factors that limit growth and physical damage during isolation. Successful isolation also does not guarantee viability during purification and culture preservation (Rossetti *et al*., 1997; Fan *et al*., 2019). Thus, the aim of this study was to optimise micromanipulation technique for the isolation of *Gordonia* spp. from full-scale ASP and to determine their growth kinetics axenically.

3.2 MATERIALS AND METHODS

3.2.1 Description of wastewater treatment works considered under this study

Two full scale WWTW (domestic and industrial) were selected for this study which showed occasional (WWTW A) and continuous (WWTW B) foaming. The WWTW A is situated in the Eastern boundary of Pietermaritzburg and receives an influent of 75 ML/day (average dry weather flow) and is operated using a modified Johannesburg (JHB) configuration which has a sequential primary settling, pre-anoxic, anaerobic, anoxic, and aerobic zones as highlighted in Fig. 3.1. The configuration of WWTW A is that of a BNR plant. A return activated sludge (RAS) was recycled from the clarifiers to the preanoxic zone to allow for phosphate removal and denitrification. The influent is primarily domestic (90%), with 10% industrial wastewater with textile dyes, waxes and greases.

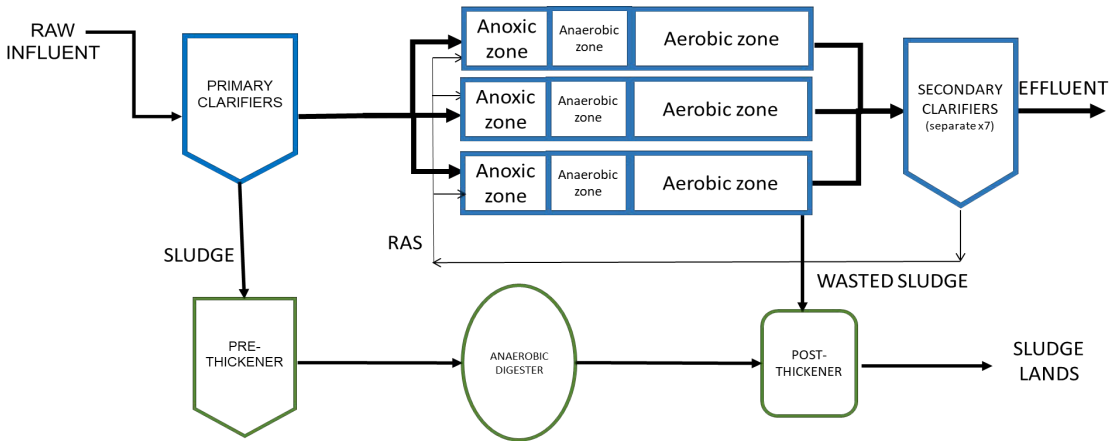


Fig. 3.1: The modified Johannesburg configuration of WWTW A.

The WWTW B is situated in the West of Durban towards Pietermaritzburg, KwaZulu-Natal in South Africa and receives an average of 27 Ml/day of influent. The plant is a 5 – step Bardenpho process consisting of sequential anaerobic, anoxic, aerobic, anoxic and aerobic reactors, as highlighted in Figure 3.2, which is configured for EBPR. The influent is primarily 90% industrial from the textile and poultry industries.

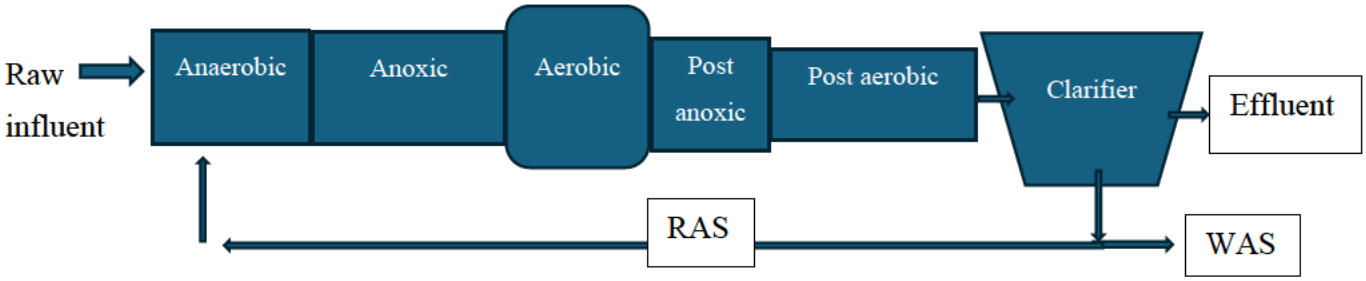


Fig. 3.2: The five-step Bardenpho process configuration of WWTW B

3.2.2 Sample collection

Grab samples were taken from the aeration tank, including the entry point, middle portion, and end portion of the aeration basin, to obtain a representative sample. A volume of 1 L was collected from each point and upon arrival at the lab, a composite sample was prepared. Both mixed liquor and foam samples were collected forthnightly from March to August (6 Months). Additionally, approximately 100 mL of foam was collected from the surfaces of the reactors. The samples were transported to the laboratory in cooler boxes packed with ice packs. Wet mount analysis of foam and composite mixed liquor samples was conducted immediately upon arrival. The remaining samples were stored for further use in the refrigerator at 4°C prior to isolation which was conducted within 24 hours of collection. The foam sample was used directly for isolation without any pre-treatment.

3.2.3 Presumptive identification of filamentous bacteria

3.2.3.1 Wet mount procedure

The observation of characteristic traits of filamentous bacteria was conducted by performing wet mounts. A drop of the sample was placed on a cleaned glass slide and covered with a cover slip. The observation was conducted under phase contrast at 400X magnification using Nikon Eclipse E100 (Tokyo, Japan). Further, the filament index was rated on a scale ranging from 1 to 7, where 1 denoted no filamentous organisms, and 7 represented excess growth of filamentous organisms (Table 2.2) (Eikelboom, 2003; Jenkins *et al*., 2004). The morphotype and staining characteristics were conducted according to Eikelboom *et al*. (1998).

3.2.3.2 Gram staining technique

Gram staining was prepared by introducing a dropful of sample onto the slide and smearing it across the slide. The smear was air dried, and heat fixed. Thereafter, the slides were subjected to Gram staining using standard protocol and was visualized under 1000 X magnification using brightfield illumination Nikon Eclipse E100 microscope (Tokyo, Japan) (Jenkins *et al*., 2004).

3.2.4 Optimization of micromanipulation techniques for isolation of filamentous bacteria from mixed liquor and foam samples

3.2.4.1 Preparation of the samples

Approximately 100 mL of mixed liquor samples were filtered through 300, 150 and 100 µm mechanical sieves and rinsed with sterile distilled water. Filtration was conducted to remove larger suspended particles from the samples. The resulting filtrate was used for the isolation of filamentous bacteria. Foam samples did not undergo filtration prior to micromanipulation. Briefly, approximately 100 µL of the filtrate was inoculated along the centre of the respective medium and allowed to dry at ambient temperature for 5 to 10 min. Four different types of media were used for isolation in this study viz., R2A, Actinomycete isolation agar, CGYA and minimal media containing 1% cholesterol as a carbon source (Stratton *et al*., 2003; Khan and Faheem, 2012). The plates used for micromanipulation were prepared very thin by using 8 mL of media to enable better light penetration during micromanipulation. The foam samples were not pre-treated prior to isolation. The media used for isolation of isolates and its composition is listed in Table 3.1.

Table 3.1: Medium composition of the various media used in this study

Type of Medium	Medium composition
Actinomycete isolation agar	L-asparagine, dipotassium phosphate, ferrous sulphate, magnesium sulphate, sodium caseinate, sodium propionate, agar bacteriological
Cholesterol minimal media (1%)	M9 salts, $MgSO_4$, $FeCl_3$, $CaCl_2$, Cholesterol, Histidine, Leucine, Yeast extract.
TGYA	Casitone, yeast extract, $MgSO_4$. 7 H_2O, Agar bacteriological
R2A	Yeast extract, Proteose peptone, Casein hydrolysate, glucose, starch soluble, sodium pyruvate, di-Potassium hydrogen phosphate, magnesium sulfate anhydrous, agar bacteriological.

3.2.4.2 MICROMANIPULATION

The prepared slides thereafter subjected to micromanipulation using using Nikon Eclipse TS 100. Different glass microneedles (10, 20 and 40 μL tip sizes) were evaluated to select the optimum needle diameter for filament isolation. The microglass needles used for this study were obtained from Tygerberg Reproduction biology unit, Cape Town, South Africa. Based on the preliminary results, glass microneedles of 20 μL tip size were selected as the optimum microneedle size for successful filament isolation.

Using the 20 μL tip size needle, the desired single filament was dragged towards a clean, uninoculated part of the plate. This was followed by aseptic excision of the filament onto a respective medium CGYA, Cholesterol minimal agar, R2A and Actinomycete isolation agar. The freshly inoculated plates with micromanipulated filaments were incubated at 30°C for 10 days. The isolates were monitored daily using microscope to ensure that the observed colony emanated from the desired single organism. In addition, Gram staining was routinely performed to ensure that cultures remained axenic after isolation. The principle of

micromanipulation used in this study was adopted from Rossetti *et al*. (1997) and Stainsby *et al*. (2002). Purified cultures were maintained at 4ºC and sub-cultured bi-weekly for short-term storage. In addition, glycerol stock cultures as a cell suspension in 50% (v/v) glycerol–Luria Bertani (LB) broth were prepared and kept at -80ºC for long term storage (Drzyzga *et al*., 2009). Pure cultures were subjected to DNA extraction and amplification using *Gordonia* spp. specific primers prior to biochemical analysis.

3.2.5 DNA extraction, amplification and sequencing

Bacterial biomass for PCR studies was obtained by growing colonies in LB broth in shake flasks for 48 h at 30ºC on a rotary shaker at 150 rpm. The cells were harvested by centrifugation at 10000 rpm for 5 min. The harvested cells were washed twice in distilled water (Drzyzga *et al*., 2009). Genomic DNA was extracted from pure cultures using a modified, Cetyltrimethylammonium Bromide (CTAB) method (You and Sue, 2009). Pure culture (1 g) was initially washed with 800 µl of 1 X phosphate buffered saline (PBS) and dissolved in Tris ethylenediaminetetraacetic acid (EDTA) buffer (10 mM Tris-HCl, 1 mM sodium EDTA, pH 8.0). To this, 800 µl of Lysozyme (10 mg/ml) was added and incubated at 37°C for 1 h. After incubation, 1000 µl of pre-warmed CTAB buffer (100 mM Tris-HCl, 10 mM EDTA, 100 mM NaCl, 1% [w/v] CTAB, pH 8.0) and 1000 µl of 50 mg/ml Proteinase K was added and incubated at 60°C for 45 min to remove all protein inhibitors. The DNA was then purified using phenol:chloroform:isoamyl alcohol (25:24:1) followed by precipitation using 600 µl Isopropanol. The precipitated DNA was subjected to two cycles of cold 70% ethanol wash and dissolved in 10 µl of TE buffer (10 mM Tris-HCl, 1 mM sodium ethylenediaminetetraacetic acid (EDTA), pH 8.0) and kept at –20°C. The purity and concentration of the extracted DNA was determined using the Nanodrop ND –1000 spectrophotometer (Nanodrop Technologies Inc., Wilmington, USA).

3.2.6 Polymerase Chain Reaction

Two sets of *Gordonia* spp. specific primers were used for PCR, as indicated in Table 3.1. The PCR reactions in this study contained 2.5 µl (10X) Dream Taq buffer (Thermo Scientific, Massachusetts, USA), 200 µM dNTPs, 50 pmol of the forward and reverse primers and 0.5 Unit Dream Taq DNA polymerase to a final volume of 25 µl. Amplification condition followed

include: initial denaturation for 4 min at 94°C, followed by 35 cycles of denaturation for 1 min at 94°C, annealing for 1 min at 52°C, extension for 1 min at 72°C and a final extension of 7 min at 72°C using Veriti Thermal cycler (Applied Biosciences, California, USA). The amplicons were viewed on 1.6% agarose pre-stained with ethidium bromide. The obtained PCR products were thereafter subjected to a nested PCR using specific primers using primer sets G268F and G1096R as described by (Shen and Young, 2005). In addition, the universal bacterial primers 27F and 1494R were also used to confirm the identity of the isolates (Table 3.2). The purified PCR amplicons were submitted to Inqaba Biotechnical Industries (Pty), Pretoria, South Africa, for further confirmation using sequencing and analysis. The sequences were obtained and subjected to the Basic Local Alignment Search Tool (BLAST) to compare the query sequences against a database of known sequences to identify regions of similarity. Only isolates that were identified as belonging to *Gordonia* spp. were thereafter subjected to biochemical fingerprinting.

Table 3.2: Oligonucleotide primers and sequences that were used in this study

Primer	Sequence (5'-3')	Target organism
G268F	CGACCTGAGAGGTGATCG	*Gordonia* spp. (Shen and Young, 2005)
G699F	CGCGCGGCGGGGCGGGGGCCG GGC	*Gordonia* spp. (Shen and Young, 2005)
G1096R	ATAACCCGCTGGCAATACAG	*Gordonia* spp. (Shen and Young, 2005)
27F	GAGTTTGATCCGGCTCAG	All bacteria (Pujol *et al*., 1991)
1492R	TACGGCTACCTTCTTACGACTT	All bacteria (Pujol *et al*., 1991)

3.2.7 Growth kinetics on various solid minimal media supplemented with different carbon sources

The purified isolates were subjected to various carbon compounds on 1% minimal media viz., acetate, glucose, galactose, glycerol, cholesterol, cholestenone and benzoic acid aerobically and anaerobically (Parekh and Desai, 2013 and Drzyzga *et al*., 2011). Continuous streaking was conducted on isolates onto relevant media in duplicates. Due to the limitations of solubility of cholesterol, cholestenone and benzoic acid, they were dissolved in 16.4 mM methyl-ß-cyclodextrin (CD) (Sigma-Aldrich, Missouri, USA) to increase their solubility. Minimal media with CD only served as a negative control (Lashkarian *et al*., 2010).

The ability of the isolates to grow anaerobically was investigated by placing the various inoculated plates in AnaeroGen sachets (AnaeroGen, Oxoid, ThermoFisher Scientific, Massachusetts, USA) in 2.5 L anaerobic jars. The effect of temperature on the growth was assessed by incubating under different temperatures of 20, 30 and 40^{o}C for ten days under different carbon sources and oxic conditions as described above for each carbon source stated above. Observation on minimal agar plates was expressed as no growth (-), scanty growth (+), moderate growth (++) and abundant growth (+++).

3.3 RESULTS

3.3.1 Microscopic examination of foam and mixed liquor samples

Microscopic examination of the activated sludge mixed liquor revealed the presence of different filamentous morphotypes in all the samples. Among these, Type 0041, *Thiothrix*, *Gordonia* spp., Type 021N and Type 0092 were the predominant morphotypes observed in WWTW A as outlined in Fig 3.3 from mixed liquor, which receives primarily domestic influent. Filamentous morphotypes Type 0041, *Gordonia* spp. and Type 021N were observed as dominant filaments throughout the sampling period whilst Type 1851 and *Thiothrix* were observed as transient filamentous bacteria in WWTW B which receives industrial effluent. The foam samples from both WWTWs were dominated by GALOs.

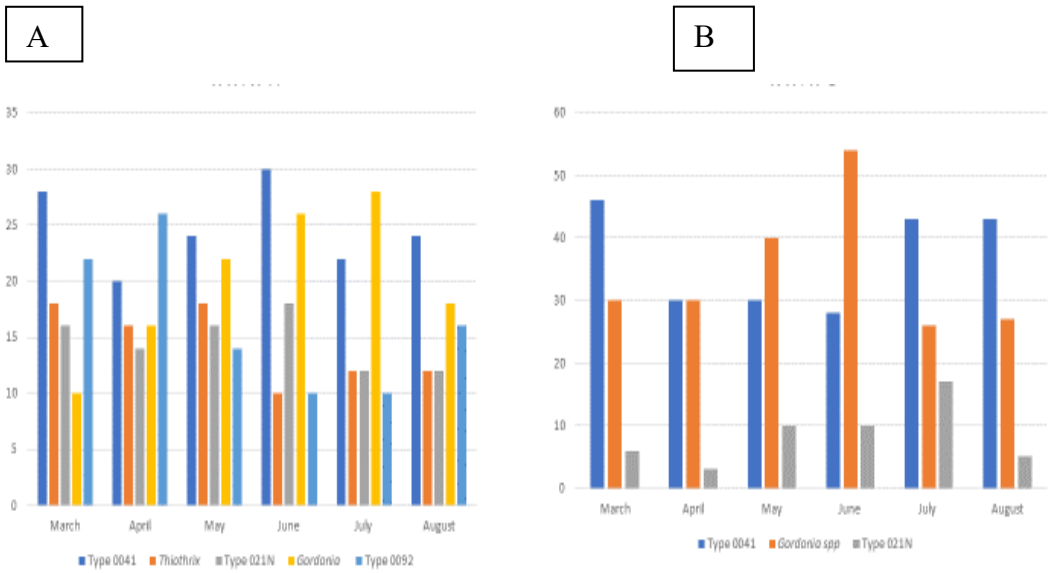


Fig. 3.3: Filament abundance from WWTW A (A) and WWTW B (B) mixed liquor based on microscopic analysis on wet mount and Gram staining.

3.3.2 Optimization of micromanipulation technique

A micromanipulation technique was used in this study to isolate *Gordonia* spp. selectively from mixed liquor samples. Microneedle ranging from 10 to 40 mm was evaluated for its efficacy in isolating a single filament under the microscope. A size of 20 µm microneedle was observed to be the optimum size to visualise under the microscope as well as to drag the filament to a cleaner section of the plate. The large tip size of 40 mm of the microneedles however blocked the visibility of the micromanipulated filament under a microscope, while, small sized needles (10 µm microneedle) was too small to visualise under the microscope. However, using 20 µm size microneedle, the tip of the needle and filament could be visualised simultaneously during isolation and found to be optimum for the isolation of filamentous bacteria.

Using the above optimized method, a total of 27 filamentous bacteria were successfully micromanipulated and transferred to a fresh medium, where eleven of those isolates were

isolated from foam. The filtration of mixed liquor samples allowed for a better separation of filaments as most samples did not contain suspended particles. Contamination was monitored microscopically during incubation and excised if present. During the subsequent incubation period, 33% of the isolates were lost due to contamination post micromanipulation as highlighted in Table 3.2. Amongst the isolates that survived initial isolation, 14 stopped growing during short term preservation and loss of viability was observed after two to four months of isolation. It was also observed that most of the isolates that survived isolation and preservation during this study were obtained from WWTW B. Among the different media tested, the medium that supported most isolates was TGYA and produced substantial cell growth (Fig 3.4), followed by R2A agar. Actinomycete isolation agar and cholesterol minimal agar had the lowest recovery rate as observed in Table 3.3.

Table 3.3: Survival of isolates on various isolation media

Medium	Micromanipulated filaments	No of isolates that survived initial isolation and preservation	Final number of viable isolates
TGYA	14	7	2
R2A	6	4	2
Cholesterol minimal agar	5	5	0
Actinomycete isolation agar	2	2	0

3.3.3 Growth morphology of isolates

Growth monitoring during isolation revealed several growth stages. Initially, a) cells enlarged as observed after 24 hours in Fig. 3.4 A, B) thickening was followed by elongation and branching in Fig. 3.4 B and C) colony formation in Fig 3.4 C and visibility from the desired organism. Visible colony formation was observed between day four to six of incubation.

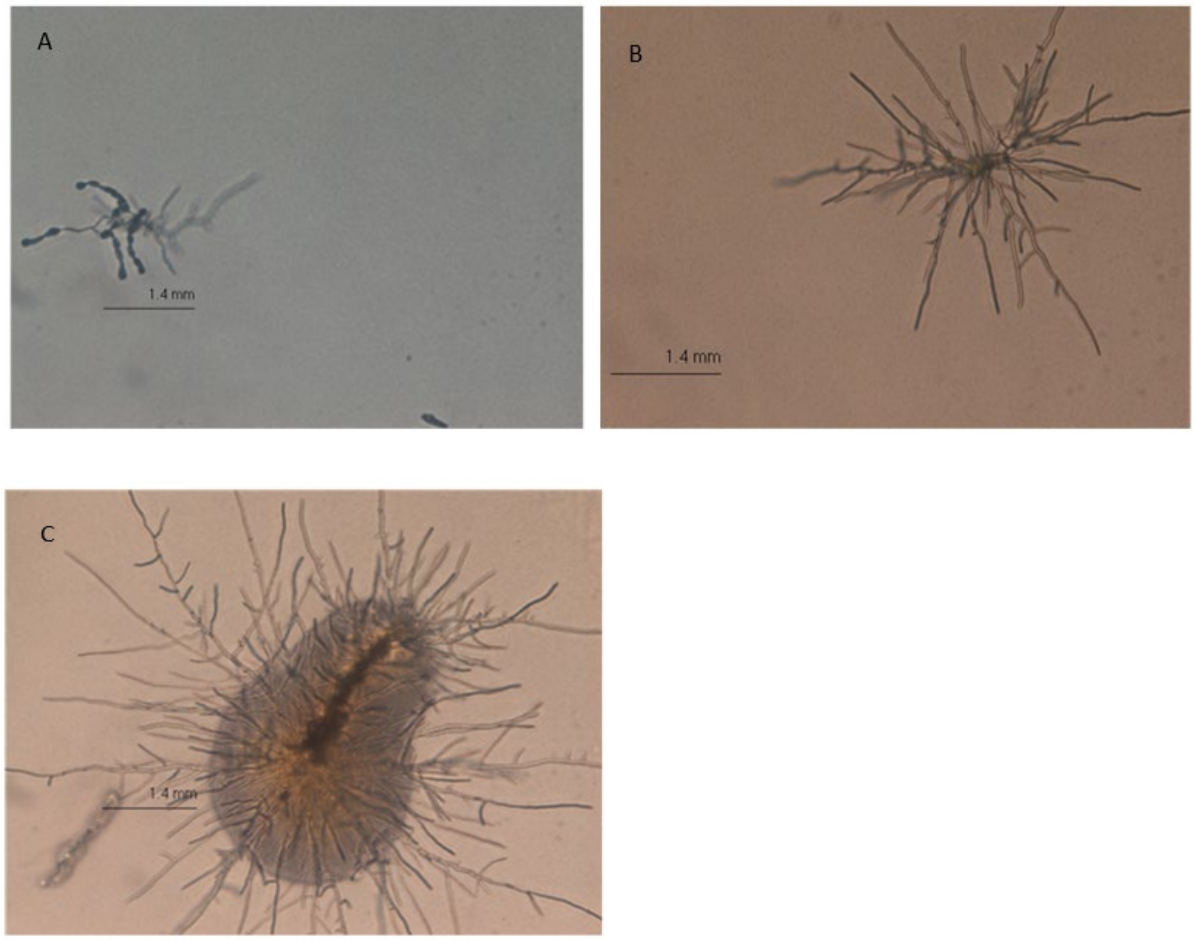


Fig. 3.4: A) Isolate R2AHS1 observed under the micromanipulator after 24 hr of incubation illustrating cell enlargement B) Elongation and branching of the isolate after 72 hr of incubation C) The colony was visible at this point and filamentous morphology displayed. All observations made under 400 X magnification.

Table 3.4: Microscopic and phenotypic characteristics of isolates

Isolate name	Gram reaction	Colony morphology	Isolate origin
R2AHS1	Positive, branched filaments	Very small colonies with an entire margin, cream white, raised with sticky consistency	WWTW B - foam
TGYHS708	Positive branched filaments	Small, cream white, filamentous margin, clumped colonies.	WWTW B - foam
TGYHF1B	Positive filaments and rods	Beige, large, matt, filamentous margin, oval shaped colonies	WWTW B - foam
R2AKAcolB	Positive rods, short, branched fil observed	Small, cream white, smooth, entire margin, dry and flat colonies.	WWTA – mixed liquor

Isolates R2AHS1 (Fig 3.4 A) and TGYHF1B (Fig 3.4 C) had similar phenotypic characteristics. The colonies were very small, cream white, with entire margins. Colonial growth was conglomerated when grown on minimal media containing cholesterol. The isolate was observed shifting to single-celled morphology. Isolate A, B and C were isolated from WWTW *B* foam. R2AKAcol B produced flaky, rough, and dry colonies with a slight pink pigment from the 07th day of incubation. TGYHF1B produced rather large, mucoid colonies unlike the other three cultures. TGYHF1B changed from filamentous morphology to single celled morphology during preservation. None of the isolates produced any pigmentation.

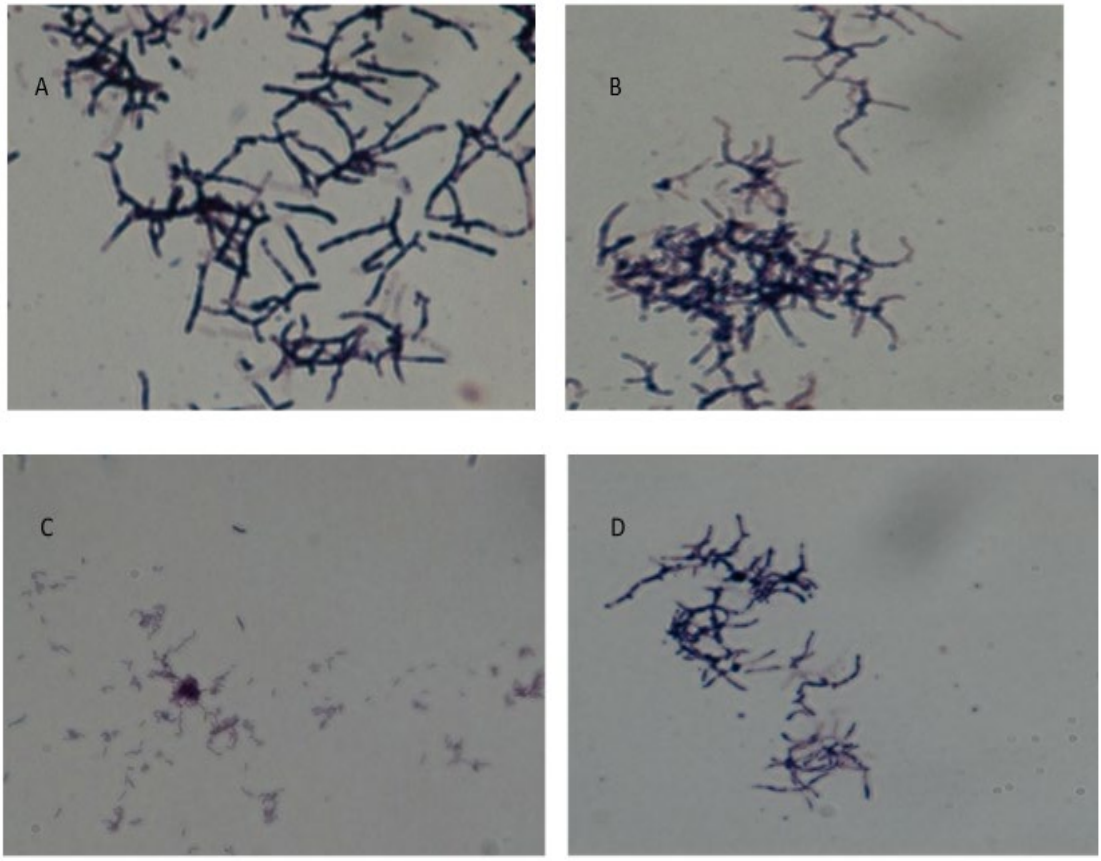


Fig. 3.5: Gram stain micrograph of isolate R2AHS1. B) Gram stain micrograph of isolate TGYHS708. C) Gram stain micrograph of TGYHF1B. D) Gram stain micrograph of R2AKAcolB.

3.3.4 Polymerase chain reaction and phylogenetic identification

The four isolates obtained in pure culture were further subjected to a nested PCR using *Gordonia* spp. primers as described elsewhere to confirm the identity of the isolates. All four isolates produced the expected PCR band size of 397 bp using nested PCR. The PCR confirmed isolates were thereafter sent to a commercial facility for Sanger sequencing and analysis of the 16S rRNA gene. The obtained sequences were thereafter subjected to phylogenetic analysis and the closest relative of the isolate was determined. All isolates showed *Gordonia* spp. as its closest relative, however, the similarities were less than 97% to known *Gordonia* species in the National Centre for Biotechnology Information (NCBI) database as observed in Table 3.5.

Table 3.5: Database matches of closest relative of isolates as obtained from NCBI nucleotide BLAST search tool

Isolate	**Closest relative**	**Similarity (%) (Query cover)**	**E-value**	**% identification**
TGYHF1B	*Gordonia cholesterolivorans*	100	1e-40	94.57
R2AHS1	*Gordonia alkanivorans*	91.67	1e-40	89.81
TGYHS708	*Gordonia terrae*	100	1e-46	89.61
R2AKAcolB	*Gordonia rubripertincta*	97	5e-27	95.18

3.3.5 Biochemical profile of the isolates

The ability of isolates to grow on various substrates was investigated on minimal agar plates at different temperatures and oxic conditions. Substrate utilisation differed amongst organisms and a temperature of 20^{o}C was observed to be not conducive to the growth of most isolates except for one isolate (TGYHF1B).

Isolate TGYHS708 displayed an inability to grow at 20^{o}C, as no growth was observed from all substrates. Cholesterol was not utilised under both conditions and scanty growth was observed aerobically at 30^{o}C. Acetate utilisation was similar under aerobic and anaerobic conditions. Glucose, galactose and glycerol were abundantly assimilated, but only under oxic conditions, as observed in Table 3.6.

Table 3.6: Growth of isolate TGYHS708 on various minimal media under aerobic and anaerobic conditions at 20°C and 30°C

Oxic/Anaerobic condition	***Cholesterol***		***Benzoic acid***		***Acetate***		***Glucose***		***Galactose***		***Glycerol***	
	20°C	30°C	20°C	30°C	20°C	30°C	20°C	30°C	20°C	30°C	20°C	30°C
Oxic	-	-	-	+	-	+	-	+++	-	+++	-	+++
Anaerobic	-	-	-	-	-	+	-	-	-	-	-	-

- no growth + scanty growth ++ moderate growth +++ abundant growth

Table 3.7: Growth of isolate TGYHF1B on various minimal media under aerobic and anaerobic conditions at 20°C and 30°C

Oxic/Anaerobic condition	***Cholesterol***		***Benzoic acid***		***Acetate***		***Glucose***		***Galactose***		***Glycerol***	
	20°C	30°C	20°C	30°C	20°C	30°C	20°C	30°C	20°C	30°C	20°C	30°C
Oxic	++	+++	-	+++	++	+++	+	+++	++	+++	++	+++
Anaerobic	++	+++	++	+++	++	+++	++	+++	++	+++	++	+++

- no growth + scanty growth ++ moderate growth +++ abundant growth

Isolate TGYHF1B displayed the most versatility based on substrate utilisation, temperature, and oxygen conditions. Among the different carbon sources used, Cholesterol was utilised abundantly by isolate TGYHF1B and displayed an ability to grow moderately at 20°C on all substrates under aerobic conditions. This growth pattern was only displayed by this isolate. TGYHF1B displayed aerodynamism as growth was observed aerobically and anaerobically as observed in Table 3.7. The morphology of the colonies displayed by isolate TGYHF1B on cholesterol minimal media produced small, clustered colonies.

Minimal growth was observed from isolate R2AHS1. Growth was only observed at 30°C. The isolate was unable to utilise any substrate at 20°C under oxic and anaerobic conditions. Benzoic acid was utilised though sparingly at 30°C. Cholesterol did not support any growth under all

conditions. Abundant growth was observed on glucose, galactose and glycerol at 30ºC as observed in Table 3.8.

Table 3.8: Growth of isolate R2AHS1 on various minimal media under aerobic and anaerobic conditions at 20ºC and 30ºC

Oxic/Anaerobic condition	***Cholesterol***		***Benzoic acid***		***Acetate***		***Glucose***		***Galactose***		***Glycerol***	
	20ºC	30ºC	20ºC	30ºC	20ºC	30ºC	20ºC	30ºC	20ºC	30ºC	20ºC	30ºC
Oxic	-	-	-	+	-	+	-	+++	-	+++	-	+++
Anaerobic	-	-	-	-	-	+	-	-	-	-	-	-

- no growth + scanty growth ++ moderate growth +++ abundant growth

Table 3.9: Growth of isolate R2AKAcolB on various minimal media under aerobic and anaerobic conditions at 20ºC and 30ºC

Oxic/Anaerobic condition	***Cholesterol***		***Benzoic acid***		***Acetate***		***Glucose***		***Galactose***		***Glycerol***	
	20ºC	30ºC	20ºC	30ºC	20ºC	30ºC	20ºC	30ºC	20ºC	30ºC	20ºC	30ºC
Oxic	-	+	-	+	-	++	-	++	-	++	-	++
Anaerobic	-	-	-	-	-	++	-	++	-	++	-	++

- no growth + scanty growth ++ moderate growth +++ abundant growth

R2AKAcolB grew moderately at 30ºC on acetic acid, glucose, galactose and glycerol and the amount of growth produced were similar under oxic and anaerobic conditions. At 30°C, cholesterol and benzoic acids were sparingly assimilated under oxic conditions as observed in Table 3.9.

3.4 *DISCUSSION*

3.4.1 Distribution of filaments in the mixed liquor and foam samples

Excessive growth of certain filamentous bacteria sporadically results in the formation of viscous thick stable foam as well as sludge settling problems (bulking), causing solid separation challenges in wastewater treatment plants. Overgrowth of certain filamentous bacteria in the aeration basin and clarifier can result in foaming and bulking. *Ca*. M. parvicella and GALOs are cited as the most common filamentous bacteria implicated in bulking and foaming in WWTW worldwide (Wang *et al*., 2016; Fan *et al*., 2019). Among the filamentous bacteria identified from full scale activated sludge plants, *Gordonia* spp. has been frequently reported from foam samples from different parts of the world, including South Africa (Guo *et al*., 2015; Deepnarain *et al*., 2020).The proliferation of actinomycetes-mycolata morphotypes has been linked to influent with high oil and grease concentration, textile influent and seasonal variations with preference for winter-spring months (Jiang *et al*., 2016; Silva *et al*., 2019; Hennessy, 2021). The phylum '*Chloroflexi*, embraces an ecologically and physiologically diverse group of bacteria, which have been detected in an increasingly wide range of anaerobic habitats including sediments, hot springs, methanogenic anaerobic sludge digesters and activated sludge communities (Speirs *et al*., 2019). Amplicon sequencing data of Australian EBPR plant biomass containing Eikelboom Types 0041 and Eikelboom 0675, and phylogenetic analysis have revealed that both, are members of the phylum *Chloroflexi* and probably representatives of two different genera (Speirs *et al*., 2017). In this study, Eikelboom Type 0041 was observed throughout the sampling period in both WWTW investigated in mixed liquor samples. This observation is in accordance with previous reports. For instance, Thomsen *et al*. (2002) reported that the Gram variable, sheathed, curved, and unbranched filament Eikelboom Type 0041 was deemed the most abundant filamentous bacterium in activated sludge as it appeared in 88% of WWTWs investigated in their study. In a previous study conducted in South Africa, filament Eikelboom Type 0041 was also found to be among the dominant filaments in mixed liquor from domestic influent (Blackbeard *et al.* 1986; Brand *et al*., 1987). Another study by Welz *et al*., 2014, at a different geographical location within South Africa, reported Eikelboom Type 0041 as a secondary filament in the mixed liquor samples (Welz *et al*., 2014). A more recent study by Deepnarain *et al*., 2020, has reported their dominance in various geographical locations within South Africa highlighting its ability to grow under various environmental conditions (Deepnarain *et al*., 2020). In this study its ubiquitous nature was observed from

WWTW B that treats industrial influent which displayed F/M ratios of 0.19 ± 0.03 (Table 4.3) which favours their abundance in mixed liquor. An earlier study focusing on WWTW in Australia had regarded Eikelboom Type 0041 as an 'all zone grower' due to its ability to induce foaming and bulking in ASP (Seviour *et al*., 1994), which also supports the prevalence of this organism in this study. Similarly, Eikelboom Type 0041 was observed from WWTW of varying configurations highlighting their heightened versatility (Speirs, 2017).

The prevalence of *Thiothrix* morphotypes was observed in Fig. 3.3 B namely Eikelboom Type 012N and *Thiothrix* spp. Over the past decades, the taxonomy of *Thiothrix* spp. has been revised several times. *Thiothrix* spp. that exhibited similar morphotype to Eikelboom Type 012N, *Thiothrix* I and *Thiothrix* II (Eikelboom, 2000; Jenkins *et al*., 2003). However, Aruga *et al*., (2002) found inconsistencies with that classification as depicted in those classification manuals. Subsequently, it was proposed that *Thiothrix disciformis* and *Thiothrix eikelboomii*, previously included in Type 021N, should be classified as the genus *Thiolinea*, while *Thiothrix flexilis* should be classified as the genus *Thiofilum (*Aruga *et al.,* 2002*). Thiothrix* morphotypes have been noted to proliferate in WWTW that treat industrial wastewater from textile and food industries (Zou *et al*., 2022; Henriet *et al*., 2017). Therefore, the prevalence of two *Thiothrix* morphotypes in WWTW B may be attributed to influent composition because WWTW B receives influent from poultry processing plant and textile industry.

Low F/M ratios were observed as the most selective parameter for Eikelboom Type 021N and *Thiothrix* dominance during a national South African survey (Water Research Commission Report, 2022). In addition, the dominance of Eikelboom Type 021N was also detected in both WWTW as secondary filamentous bacteria. The prevalence of Eikelboom Type 021N in the same geographical location as this study was detected as few to common, rarely contributing towards filamentous bulking (Deepnarain *et al*., 2019), which supports the findings from this study.

The distribution of *Gordonia* spp. showed that it was the second dominant filament in WWTW B mixed liquor, which receives mainly industrial wastewater from poultry processing and the textile industry. *Gordonia* spp. have been documented to display a strong affinity for non-readily biodegradable fatty acids (Tsang *et al*., 2008) which may have favoured their dominance in WWTW B compared to WWTW A that treats primarily domestic influent. The prevalence of *Gordonia* spp. in South Africa has been observed previously (Water Research

Commission Report, 2022). In addition, the foaming episodes that were observed in May and June were attributed to increased levels of GALOs in mixed liquor that exhibited true right-angled branching morphology. A study conducted by Dunkel *et al*, (2018) also found a similar increase of GALOs in foams from industrial WWTW. The abundance of *Gordonia* spp. also increased during June and July from WWTW A, with foaming observed in July when *Gordonia* spp. were the most abundant filament. This was also observed in Shatin, China, when foaming episodes during winter were attributed to *Gordonia* spp. (Jiang *et al*., 2016). The optimum temperature during pure culture studies for *Gordonia* spp. have been documented to be within 15 to 30^{o}C (Soddell *et al*., 2006; Khan and Faheem, 2012). It can be speculated that the prevalence of *Gordonia* spp. throughout the year in this region can be attributed to conducive temperature ranges of 22.80 ± 5.06 in WWTW A and 24.77 ± 5.47 in WWTW B (Table 4.2).

In a national survey conducted across three provinces in South Africa, namely in Johannesburg, Cape Town and Durban, GALOs were the fifth most abundant filament in mixed liquor (Water Research Commission, 2022). High prevalence of *Chloroflexi*, *Ca*. M. parvicella and *Thiothrix* spp. were found in Cape Town and Gauteng WWTWs whilst in KwaZulu-Natal, Eikelboom Type 1851 was significantly higher in Durban than in Cape Town and Ekurhuleni. Filament abundance analysis depicted that the most abundant bulking filaments were *Chloroflexi,* Eikelboom Type 1851 and GALOs in Cape Town (Welz *et al*., 2014) whilst Durban reported GALOs, Eikelboom Type 0041 and Eikelboom Type 0675 (Lacko *et al.*, 1999). Therefore, even within the same country, geographical location and prevailing climatic conditions may influence the prevalence of specific filamentous bacteria. Such variations were also observed in three provinces within China in Hong Kong, Hangzhou and Zengzhou where primary and secondary filamentous bacteria in foaming sludges were completely different Table 5.1 (Guo *et al*., 2015, Wang *et al*., 2016 and Zhang *et al*., 2021).

3.4.2 Micromanipulation and biochemical fingerprinting of isolates

Filamentous bacteria are generally slow-growing organisms thus, their growth is hindered if floc formers are not excluded upon isolation (Rossetti *et al*., 2005; Fan *et al*., 2019). Conventional spread plating techniques are marred by low recovery rates of desired organisms (Ramothokang *et al*., 2006; Hozzein *et al*., 2012; Khan and Faheem, 2012); hence other

selective methods were adopted to minimise contamination and monitor growth during incubation. Efforts to exclude contaminants have been achieved through various pre-treatment procedures, such as dilution and centrifugation prior to isolation (Khan and Faheem, 2012). This study exercised filtration as a pre-treatment method, which decreased the number of isolates lost due to contamination (Table 3.4).

Micromanipulation is a preferred method for the isolation of filamentous bacteria as it enables simultaneous isolation and identification of the desired organism from a diverse community (Soddell and Seviour, 1999; Levantesi *et al*., 2006; Seviour, 2010). As a result of the application of a micromanipulator during this study, *Gordonia* spp. were successfully isolated from mixed liquor and foam with minimal contamination. Few other studies have successfully isolated and studied growth kinetics of actinomycetes from ASP using micromanipulation technique. For instance, *M. parvicella* has been isolated through micromanipulation from various countries such as Australia, Denmark, Italy and China (Tandoi *et al*., 1998; Soddell *et al*., 2006, Fan *et al*., 2019). Using micromanipulation technique, Stainsby *et al*., 2002 found a high diversity of actinomycetes-mycolata morphotypes in foams in WWTWS in the United Kingdom. Their findings also showed non-filamentous mycolic acid-containing rhodococci strains in foam (Stainsby *et al*., 2002). Higher cell surface hydrophobicity found in mycolata cells in the water film is essential for the stability of foam, therefore the role of non-filamentous rhodococci must not be negated. Fragmentation can also be attributed age of filamentous bacteria, as old cultures were shown to form athrospores (Hao *et al*., 1988). In this study, the initial isolation was conducted on the previously reported isolation media viz., Actinomycete isolation agar, CGYA, cholesterol minimal agar and R2A (Rossetti *et al*., 1997; Seviour *et al*., 2002; Stainsby *et al*., 2002; Drzyzga *et al*., 2009; Khan *et al*., 2012). Among these, CGYA and R2A showed better support than Actinomycete isolation agar and cholesterol minimal agar. Successful isolation and maintenance of unbranched actinomycete *M. parvicella* has previously been conducted on R2A medium (Rossetti *et al*., 1997; 2002). Similarly, Khan and Faheem (2012) used R2A, CGYA, I-media and TGYA for the successful isolation of mycolata. Recently, the use of R2A also led to the successful isolation of *M. parvicella* from a Chinese WWTW (Fan *et al*., 2019). Other media such as glucose yeast-extract agar, tryptone yeast extract agar, Münz paraffin agar have also been used for the successful isolation of *Gordonia* and *Rhodococcus* spp. (Stainsby *et al*., 2002) with visible colonies observed between 5 and 13 days, whereas in this study visible colonies were observed between 4 to 6 days using R2A and

CGYA. In another study, Jin *et al* (2011) reported visible colonies within 2-3 days when incubated aerobically on nutrient agar. According to this study, growth on R2A agar was slightly delayed compared to TGYA, resulting in R2A producing visible colonies on the 5th day of incubation, as opposed to *M. parvicella* growth which took two weeks to be observed (Fan *et al*., 2019). It is possible that the initial loss of isolates during isolation and purification was due to conditions that did not favour their growth. Isolates that were subjected to excision of contaminants during isolation did not survive as well. Moreover, the maximal recovery of isolates was observed in WWTW B foam used to treat industrial wastewater, indicating their ability to survive under unfavorable conditions. In another study, Type 1851 has also been successfully micromanipulated and characterised from bulking activated sludge samples receiving industrial wastewater in Japan (Kohno *et al*., 2002). Difficulty during isolation and loss of viability during isolation of filamentous bacteria has also been reported during the isolation of *Nostocoida limicola* III (Seviour *et al*., 2002)

Fragmentation into rod shaped or coccoid shaped cells was observed in Fig. 3.5 C. The isolates upon sub-culturing retained *in-situ* morphological traits except isolate TGYHF1B which assumed single cell morphology. Short coccoidal rods were observed after two incidents of sub-culturing as observed in Fig. 3.5 C. Morphological shifts have been documented in pure culture studies from *Gordonia westfalica*, *Gordonia* spp., *Thiothrix nivea* and *Acinetobacter* spp. (Stainsby *et al*., 2002; Linos *et al*., 2002; Ramothokang *et al*., 2006). Pleomorphism was also detected by other researchers after continuous sub-culturing (Davenport and Curtis, 2002; Arenskötter *et al*., 2004; Khan and Faheem, 2012) when a significant number of mycolata morphotypes implicated in foaming displayed coccoidal and rod-shaped morphology. Stable foams are generated by a selective enrichment of hydrophobic bacteria in them by a process of flotation. Regardless of morphology, all hydrophobic bacteria, regardless of their morphology have the potential to stabilise foams (Petrovski *et al*., 2011).

Members of *Gordonia* spp. are Gram positive to Gram variable organisms, slightly acid fast nocardioform actinomycetes (Arenskötter *et al*., 2004). In this study, all isolates exhibited Gram positive reactions, as listed in Table 3.2. The phenotypic morphology of isolates resembled that of previously reported morphology of *Gordonia* spp. The colony morphology of isolate R2AHS1 and TGYHS708 shared characteristics that were displayed by *Gordonia* spp. as reported by Soddell *et al*. (2006).

The isolate, TGYHF1B shared characteristics with the *Gordonia cholesterolivorans* isolate reported by Drzyzga *et al*. (2009), including beige and matt colonies. Similarly, the isolate, R2AKAcolB exhibited morphology comparable to *Gordonia westfalica* colonies, as reported by Linos *et al*. (2002). However, the isolates from this study did not produce pigmentation, which has also been reported in a few previous studies (Stainsby *et al*., 2002; Soddell *et al*., 2006). Not all *Gordonia* spp. produce pigments whilst some *Gordonia* spp. produce pigmented colonies with colours of orange to orange-red and shiny surfaces (Arenskötter, 2004; Goodfellow *et al*. 2012). However, culture conditions may have hindered production of pigmentation from this study from all isolates. The phylogenetic analysis of the 16s rRNA gene sequences indicated that the isolates were close relatives of *Gordonia* spp. (Table 3.4). Isolate R2AHS1 showed close similarity to *Gordonia alkanivorans*; TGYHS708 had close similarity to *Gordonia terrae*; TGYHF1B had close similarity to *Gordonia cholesterolivorans* and R2AKAcolB had close similarity to *Gordonia rubripertincta*. *Gordonia cholesterolivorans* was previously detected and isolated from foam samples (Drzyzga *et al*., 2009), whilst *Gordonia terrae* and *Gordonia rubripertincta* was isolated from soils that was contaminated with oil (Shen *et al*., 2007). A similarity index lower than 97% may indicate that the strains might belong to some novel species that are yet to be described or some divergent variant of an existing species (Gomilla *et al*., 2015). Silvia *et al* (2023) reported diverse 16S-haplotypes with the *Gordonia* spp. Hence, there is a need for whole genome sequencing for more in-depth sequencing for a more in-depth characterisation of these isolates, since 16S rRNA sequencing may not be enough for differentiating them. However, 16S rRNA sequences of *Gordonia* species reveal similarities ranging from 94.8% to 99.9% (Arenskötter *et al*., 2004).

Isolate TGYHF1B used all substrates in abundance at 30ºC aerobically and anaerobically as evident in Tables 3.5 and 3.6. At 20ºC, the monosaccharides were used sparingly whilst glycerol, acetic acid, LB medium and cholesterol minimal agar yielded moderate growth compared to the scanty growth from glucose and galactose. The incorporation of cholesterol as a hydrophobic compound resulted in a colonial morphology that displayed clumped and dry colonies that stuck to the media. This was previously observed by Soddell *et al*., (2006) when isolates were subjected to olive oil resulted clumped colonial arrangement. This indicated that their cell hydrophobicity increased in the presence of hydrophobic substrates thus increasing their ability to use hydrophobic compounds for carbon and energy source. The morphology of

Gordonia spp. has been found to vary depending on the medium used for cultivation even within the same organism (Arenskötter *et al*., 2004). This was also observed by isolate TGYHF1B which had clumped colonies on cholesterol minimal media which differed from colonial morphology on TGYA.

Spermidine, putrescine, phospholipids and cholesterol are abundant in municipal wastewaters (Song *et al*., 2020). Carbon source utilisation pattern by the isolates also highlighted the ability of actinomycetes to utilise a vast range of substrates as carbon sources, a trait shared by most actinomycetes (Goodfellow *et al*., 2002). In this study, the isolates displayed versatility regarding the utilisation of various carbon substrates under different oxic conditions and temperature range. Benzoic acid was not utilised aerobically at 20^{o}C but was utilised abundantly under anaerobic conditions by TGYHF1B. These findings were supported by previous study based on FISH-MAR findings that certain *Gordonia* spp. can metabolise benzoic acid under anaerobic conditions although it is deemed an aerobic organism (Carr *et al*., 2006). Some isolates did not utilise glucose in this study. The availability of glucose in AS plants could be minimal compared to readily available acetate and glycerol as observed in Tables 3.6 and 3.7. The inability to utilise glucose under aerobic, anoxic and anaerobic conditions for selected *Gordonia* spp. was also previously demonstrated using FISH-MAR analysis by Carr *et al*., (2006) and Eales *et al*., 2006. However, few other studies have also revealed a strong uptake of glucose by some *Gordonia* spp. *in situ* through FISH-MAR under aerobic conditions (Kragelund *et al*., 2007). These developments highlighted the discrepancy that exists in physiological traits obtained from pure culture and *in situ* studies (Carr *et al*., 2006; Kragelund *et al*., 2007). Although pure culture studies are marred by the paucity of filamentous bacteria, physiological studies have been conducted on micromanipulated *Ca*. M. parvicella RN1 which highlighted its ability to grow under aerobic and microaerophilic conditions whilst exhibiting limited nitrification capabilities. This strain further displayed an ability to grow on a wide variety of substrates such as organic acids, complex substrates and fatty acids utilising the substrates as energy and carbon sources (Tandoi *et al*., 1998, Rossetti *et al*., 2002). It was also observed that substrate utilization varied among isolates at different temperatures as shown in Tables 3.6 - 3.9. Of the isolates, only TGYHF1B displayed the ability to grow at 20°C, while most organisms grew at 30°C. Isolate R2AHS1 and R2AKAcolB also grew only at 30^{o}C as shown in Table 3.8 Table 3.9. Isolate R2AKAcolB managed to grow anaerobically on acetate, glucose and galactose as shown in Table 3.9. Previous studies have

shown 27ºC as the optimum for *Gordonia* spp. (Khan and Faheem, 2012). Some other studies have also isolated GALOs that grew between 20 to 30ºC (Stainsby *et al*., 2002) and 15 to 30ºC (Soddell *et al*., 2006). An isolate from WWTW in Spain, *Gordonia cholesterolivorans* was successfully maintained at 30ºC (Drzyzga *et al*., 2009). However, in the same study it was reported that, *Gordonia defluvii* displayed reduced growth at 30ºC (Drzyzga *et al*., 2009). This variation could be attributed to geographical locations with ambient temperature that seldom reaches 30ºC even during summer. It was however noted that the abundance of actinomycete morphotypes in mixed liquor from both WWTWs was observed during colder months at 17ºC and 18.5ºC for WWTW A and B respectively (Table 4.3 and Table 4.4) whilst pure culture studies yielded satisfactory growth at 30ºC from this study. No growth was observed at 40ºC for any of the isolates studied. Nevertheless, a study conducted in China isolated novel species of *Gordonia* from municipal WWTW. The novel isolate *Gordonia phthalatica* was capable of degradation of dibutyl phthalate at a temperature of 40°C (Jin *et al*., 2017).

Gordonia spp. have been reported to possess broad capabilities to degrade recalcitrant organic compounds, including hydrocarbons from sewage sludge (Drzyzga *et al*., 2009; Drzyzga, 2012). Sparse growth was observed on benzoic acid aerobic conditions. Isolate TGYHF1B could not utilise benzoic acid as a carbon source at 20ºC, but utilised all other substrates at 20ºC and 30ºC, though 30ºC yielded abundant growth compared to moderate growth at 20ºC. The ability of isolate TGYHF1B to utilise cholesterol and benzoate has also been demonstrated by *Gordonia cholesterolivorans* which was isolated from sewage sludge at 30ºC (Drzyzga *et al*., 2009). It was reported that *Actinomycetes* such as *Rhodococcus jostii* possess a significant number of genes encoding cytochrome P 450 which catalyse the terminal hydroxylation of C - 27 sterol. Drzyzga *et al*. (2009) identified novel *Gordonia cholesterolivorans* as an *Actinomycete* that can degrade cholesterol. Further studies revealed its' ability to utilise other long chain fatty acids such as cholestenone and stigmasterol. Isolate TGYHF1B unlike *Gordonia cholesterolivorans* displayed an ability to grow well on C-21 steroid (Drzyzga *et al*., 2011). Isolate TGYHF1B displayed versatility that is characteristic with its' closest relative *Gordonia cholesterolivorans* (Table 3.4) to use a variety of organic acids, saccharides and lipids (Drzyzga 2009, 2011).

Unlike *Gordonia cholesterolivorans*, *Norcadia amarae* was found to possess an ability to utilise C8, C9 and C14 fatty acids. Prior studies have revealed that fatty acid concentrations of

1 g/L could lead to its proliferation (Tsang *et al*., 2008). The gas chromatography-mass spectrometry (GC-MS) analysis conducted by Silva *et al*. (2019) expressed n-hexadecane degradation rates at 86% and 100% for *Gordonia paraffinivorans* and *Gordonia sihwensis* respectively. This highlights the ability of certain *Gordonia* spp. to biotransform hydrocarbons which gives *Gordonia* spp. an opportunity to outgrow foam formers particularly from industrial WWTW. Although isolates TGYHF1B, TGYHS708 and R2AHS1 were isolated from the same WWTW, only isolate TGYHF1B was capable of utilizing cholesterol and benzoic acid, whereas isolates TGYHS708 and R2AHS1 displayed similar patterns of substrate utilization (Tables 3.5 – 3.7). When grown under aerobic conditions at 30°C, isolate R2AHS1 was unable to utilize cholesterol, but used benzoic acid and acetate sparingly. This isolate also utilized glucose, galactose, and glycerol abundantly under oxic conditions. This isolate was closely related to *G. alkanivorans* as stated in Table 3.4. In a previous study, *G. alkanivorans* isolated from a WWTW in Russia failed to utilize benzene as its sole carbon source (Delegan *et al*., 2021). In light of these observations, it is evident that this organism is highly versatile and adaptable to a wide range of environmental conditions. Table 3.10 highlights microbial metabolic variations that exists amongst foaming sludges.

Table 3.10: Comparative data on growth of *Gordonia* spp. from previous studies

Organism name	Acetate	Glucose	Galactose	Glycerol	Glycogen	Phenol	Benzoic acid
G. rubropertincta[1]	++	++	+	+	++	+	+
G. terrae[1]	+	+	+	+	+	+	+
G. hydrophobica[2,3*]	+	-	+	+	+	+	+

- no growth + scanty growth ++ moderate growth [1]Goodfellow *et al*., 1996; [2, 3] Drzyzga *et al*., 2009, 2011) * ergosterol, cholesterol, and cholestenone positive

It is worthwhile to state that the information reviewed although supportive, contradictory information on physiological studies of *Gordonia* spp. have been observed as stated in Table 3.10 (Kurane *et al*., 1986; Goodfellow *et al*., 2009; Drzyzga *et al*., 2009; 2011; Khan and Faheem, 2012; Silva *et al*., 2019). With this rationale, the supposition that has been reached is that *Gordonia* spp. possess broad metabolic capabilities, and not all species possess the capabilities even when isolated from the same WWTW. This would provide an advantage for *Gordonia* spp. to thrive under different wastewater characteristics without competing for substrates.

Table 3.11: Overview of described *Gordonia* spp. and characteristic source

Type strain	Characteristic/Source	Reference
G. alkanivorans DSM 44369^T	Alkane-degrading bacterium - tar contaminated soil.	Kummer *et al*., 1999
G. westfalica	Cis-1,4-polyisoprene-degrading bacterium (strain Kb2T) – water from deteriorated automobile tyre	Linos *et al*., 2002
G. sinesedis DSM 44455^T	Isolated from soil	Maldonado *et al*., 2003
G. paraffinivorans DSM44064^T	Hydrocarbon-degrading bacterium – oil producing well	Xue *et al*., 2003
G. cholesterolivorans	Isolated from sewage sludge – cholesterol degrading bacterium	Drzyzga *et al*., 2009
G. phthalatica	Di-n-butyl phthalate-degrading bacterium - activated sludge	Jin *et al*., 2017

Table 3.11 describes *Gordonia* spp. that have been isolated or characterised from different ecological habitats ranging from ASP to soils contaminated with hydrocarbons. Arenskötter *et al*., (2004) stated that many *Gordonia* spp. can degrade recalcitrant compounds. *G. alkanivorans* was isolated from tar-contaminated soil and displayed an ability to use alkanes

as a carbon source (Kummer *et al*., 1999). *G. westfalica* is capable of Cis-1,4-polyisoprene-degradation (Linos *et al*., 2002).

Matabolic capabilities of *Gordonia* spp. in WWTW require further investigations that will extend to provide knowledge on the co-metabollic interactions that contribute to foam formation. Currently, there is limited research on how to regulate foam formation through microbial co-metabollic pathways.

3.5 CONCLUSIONS

- The dominant filamentous organisms observed in mixed liquor varied between WWTW A which treats primarily domestic influent and WWTW B which receives industrial effluent. The dominant filaments in WWTW A were Eikelboom Type 0041, *Thiothrix*, *Gordonia* and Eikelboom Type 0092 whilst WWTW B was dominated by Eikelboom Type 0041, *Gordonia* spp. and Eikelboom Type 021N.

- The study revealed that micromanipulation proved to be an efficient method for selective isolation of filamentous mycolata as it produced isolates that were not heavily laden with contaminants, and it allowed growth monitoring during incubation. Twenty-seven isolates survived micromanipulation process, however culture maintenance proved to be challenging as most isolates lost viability. Morphological shifts from filamentous to single-celled morphology were also observed.

- The isolated mycolata from mixed liquor and foam samples displayed an ability to utilise various substrates showing physiological diversity and biochemical characteristics. Isolates from domestic WWTW A did not possess the ability to utilise long chain fatty acids however, an isolate from WWTW B showed the ability to utilise cholesterol and benzoic acid.

- Metabolic capabilities of isolates from industrial WWTW must be further investigated to determine their functional roles in foaming in order to minimise conditions that lead to their proliferation. Co-metabolic interactions must also be investigated for their contribution, if any, to foam formation and stabilisation.

- The findings of this study suggest that these isolates differ significantly in their substrate uptake patterns hence there is no general control to their proliferation in WWTW. Based on the findings from this study, remedial strategies require site-specific solutions.

CHAPTER 4: DETECTION AND QUANTIFICATION OF *GORDONIA* SPP. FROM MIXED LIQUOR AND FOAM USING QUANTITATIVE POLYMERASE CHAIN REACTION

4.1 INTRODUCTION

Biological foams in WWTWs are highly correlated with the proliferation of mycolata such as *Gordonia* spp., *Mycobacterium*, *Rhodococcus*, and *Tsukamurella* (Guo *et al*., 2015). Wastewater characteristics such as Low F/M, DO concentration, sludge age, and environmental factors viz., temperature and pH are all known to promote the proliferation of different types of foam-forming filamentous bacteria (Jenkins *et al*., 1993; Oerther *et al*., 2001; Wanner, 1994; Frigon *et al*., 2006). Proliferation of certain foam-forming bacteria can only be controlled by specific control strategies that selectively target specific organisms thus preventing its proliferation. Non-specific control methods will only improve the symptoms associated with the filamentous overgrowth without addressing the actual cause, which tends to provide temporary solutions (Mamais *et al*., 1998). Conventional microscopic methods are, to date, widely used techniques by most water professionals as an affordable method for the tentative identification and quantification of filaments (Jenkins *et al*., 2004). It, however, possesses limitations and is prone to discrepancies especially if morphological differences are limited (Hennessy, 2021). In light of this, DNA based detection and quantification techniques are increasingly prevalent because of the availability of genetic databases which facilitate the design of specific primers for the development of speedy molecular assays (Karst *et al*., 2016). The application of qPCR in determining microbial composition and quantification in ASP has also been employed by previous researchers (Davenport and Curtis, 2002; Asvapathanagul and Olson, 2016). This technique is reported to be highly accurate in quantifying specific microbial population from a mixed biocenosis or quantifying bacteria belonging to specific bacterial taxonomic groups (Karst *et al*., 2016). However, efforts by other researchers to design a primer set to capture broad group within mycolata for qPCR analysis found that accuracy was negatively affected regardless of the specificity of the primer (Asvapathanagul *et al*., 2016). Hence *Gordonia* specific primers (Shen *et al*., 2007) were used to investigate their prevalence in mixed liquor and foam samples to establish a threshold that is sufficient to induce foaming. It is vital to establish the prevalent foam formers in specific WWTWs and to establish rapid

methods to quantify them. Such information can strengthen measures to pre-empt filamentous species-specific foaming.

Thus, the aim of this study was to adopt qPCR method for the detection and quantification of *Gordonia* spp. from foam and mixed liquor samples of two elected WWTWs considered under this study over a period of three months. The application of qPCR was adopted to provide a rapid quantitative tool to detect foaming incidents in WWTWs and their relation to the dominance of *Gordonia* spp. as indicator organisms in mixed liquor and foam samples. In addition, plant operational and wastewater characteristics were determined to establish the circumstances that prevailed when foaming was observed. The WWTWs were monitored for six months (January to June 2021) and qPCR was conducted on samples over three months when the WWTWs in question displayed biological foaming.

4.2 MATERIALS AND METHODS

4.2.1 Samples collection

Mixed liquor and foam samples were collected from the aeration basins and foam, as described in sections 3.2.1 and 3.2.2. Both foam and mixed liquor samples were collected from the aeration basin monthly over a period of 3 months. A volume of 1 L was collected from three different locations in the aeration basin. The three grab samples were mixed to form one composite sample for molecular analysis. Grab samples obtained from three different locations within the aeration basins were analyzed individually for chemical analysis. The data generated were used to calculate the mean and standard deviation.

Influent and effluent wastewater samples were collected in triplicate for COD analysis. A sample volume of 2.5 mL was filtered through a Whatman 0.45 μm filter prior to analysis. The analysis was conducted on the day that sampling was conducted.

4.2.1 Conventional staining and microscopic methods

Initially, the morphotype and staining reactions of filamentous bacteria were performed using the classification system of Eikelboom *et al*. (2000) as outlined in section 3.2.3.1 and 3.2.3.2.

4.2.2 Chemical analysis

The wastewater characteristics were analysed using standard methods. The samples were filtered using 0.45 μm filter prior to chemical analysis which were conducted within 24h hours of samples collection as outlined in Appendix 1 and 2.

4.2.2.1 Chemical oxygen demand

The soluble COD concentration in wastewater (influent and effluent) was determined according to the standard method, 5220D (APHA–AWWA–WPCF, 1998), using microwave digestion. A volume of 1.5 mL of digestion solution and 3.5 mL of sulphuric acid were carefully added to the 2.5 mL sample, carefully mixed, tightly capped and digested at 150^{o}C for 2 h in COD vials using the microwave digestor (Milestone Start D, Sorisole, Italy). Standards and blank reagents were prepared and digested with the samples as controls. After cooling, the COD concentration was measured using a spectrophotometer at 600 nm and the results were recorded in mg/L.

COD was calculated using the formula below:

$$\text{COD as mg } O_2/L = \frac{\text{mg } O_2 \text{ in final volume X } 1000}{\text{mL sample}}$$

The COD % removal efficiency was calculated as follows:

$$= \frac{C_{in} - C_{eff}}{C_{in}} \text{ X } 100$$

4.2.2.2 Temperature, dissolved oxygen and pH

Temperature, dissolved oxygen (DO) concentrations and pH measurements were done on-site using a portable YSI meter (YSI 556 Multiprobe System, USA).

4.2.2.3 Mixed liquor suspended solids and mixed liquor volatile suspended solids

For the MLSS measurement, 25 mL of the mixed samples were added to a clean, pre-weighed ceramic crucible. The crucibles were heated at 105°C for 2 h after which they were cooled in

a desiccator containing silica gel. The crucible was weighed before the sample was introduced (M_o) and after drying (M_1) using a Mettler-Toledo ME204 analytical balance (Mettler-Toledo International Inc., USA) to gravimetrically MLSS was determined using the following formula:

$$MLSS = \frac{(M_1 - M_2)x\ 1000}{V}$$

The MLVSS fractions were determined gravimetrically by incineration of the dehydrated sample (M_1) in a muffle furnace at 550°C for 1 h. The crucible was cooled and weighed after incineration (M_2). MLVSS was calculated using the following formula:

$$MLVSS = \frac{(M_1 - M_2)x\ 1000}{V}$$

MLSS and MLVSS was done according to standard methods for examination of water and wastewater (APHA, 2012).

4.2.2.4 Sludge volume index

A well-mixed AS mixed liquor sample was poured into a graduated Imhoff cone to 1 L to determine the sludge volume index. The contents were allowed to settle for 30 min. The settled sludge volume occupied was measured and reported in mL (APHA–AWWA–WPCF, 1998).

The sludge volume index was calculated as follows:

$$SVI = \frac{Settled\ sludge\ \left(\frac{ml}{L}\right)\ x\ 100}{Suspended\ solids\ \left(\frac{mg}{L}\right)}$$

All the analyses were carried out in triplicate and Microsoft Excel 2010 was used in calculating standard deviation.

4.2.2.5 Food to microorganism ratio

The F/M ratio was determined using the equation:

$$= \frac{Q \; x \; COD}{MLVSS \; x \; V}$$

4.2.3 Genomic DNA extraction

A volume of 20 mL mixed liquor and foam samples were centrifuged at 2600 x g for 4 min at 4°C and the supernatant was discarded. A volume of 2 mL was obtained from the pelleted biomass and washed with 1 X PBS prior to DNA extraction (Enitan *et al*., 2014). The DNA was extracted from foam and mixed liquor samples using DNeasy Powersoil kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The quantity and purity of the genomic DNA were assessed using Spectrophotometer (Implen N80 NanoPhotometer Muenchen, Germany). The extracted DNA was kept at -20°C until further use.

4.2.3.1 Standard curve preparation for quantitative real-time polymerase chain reaction analysis

The standard curve was prepared for the *Gordonia* spp. using purified 16S rRNA gene fragments (target DNA) obtained from PCR-amplified products using *Gordonia* specific primer pairs (G699F and G1096R) (Shen *et al*., 2007). The concentrations (µg/µl) of the purified DNA (purified 16S rRNA gene fragments) used as templates for the standard were determined using Implen N80 Nanospectrophotometer (Implen, Muenchen, Germany). This was used in calculating their copy numbers, which were based on their molecular weight and Avogadro's number (Trivedi *et al.*, 2009).

The formula used in estimating the copy number is as shown below:

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

The average weight of a bp is 650 Daltons and Avogadro's number is 6.022×1023. The Ten-fold serial dilutions of the target DNA were prepared from 10^8 to 10^2 copy numbers.

4.2.3.2 Quantitative real-time polymerase chain reaction analysis

The real time qPCR was carried out with the primer set targeting genus *Gordonia* (Table 4.1) according to the method described by Nishiguchi and Winkler (2020) using the Applied Biosystems™ QuantStudio™ 3 Real-Time PCR System (ThermoFISHer Scientific, USA). The qPCR reaction mixture was made up of 8 µl of PowerUp™ SYBR™ Green master mix (Applied Biosystems), 1 µl of each primer (final concentration of 0.4 µM), 4 µl of template DNA (final concentration of 1 ng), and molecular grade water to a final volume of 20 µl. The amplification protocol used was according to modified Nishiguchi and Winkler (2020): denaturation was conducted at 95ºC for 10 min, followed by 40 cycles of three-step amplification at 95ºC for 10 sec, annealing at 60ºC for 10 sec and extension at 72ºC for 50 sec and 81ºC for 3 sec. This was followed by 1 cycle of melt curve analysis at 95ºC for 10 sec, 65ºC at 1 min, 97ºC at 1 sec.

For each experimental set-up, no template control containing no genomic DNA was subjected to the same amplification condition.

Table 4.1: *Gordonia* spp. specific primers used for qPCR in this study

Primers	**Sequence (**5'-3')
G699F	C GGG GCG GGG GCC GGG C (Shen *et al*., 2007)
G1096R	ATA ACC CGC TGG CAA TAC AG (Shen and Young., 2005)

In each assay, a plot of the threshold cycle (Cq) against the logarithmic starting quantity value of every 16S rRNA gene fragment (DNA standard) was made, and the standard curve with linear range having regression analysis correlation co-efficient (R^2) value that is greater than 0.98 was considered suitable (Yapsakli *et al.*, 2011; Awolusi *et al.,* 2018). The qPCR assay with a standard curve having an efficiency range between 90 – 110 %, and a slope ranged –

3.58 to –3.1 were used for the quantification. To quantify the gene copies of *Gordonia* spp. in the unknown DNA sample, the Cq values of each sample were interpolated into the respective standard curve.

4.3 RESULTS

4.3.1 Microscopic monitoring of mixed liquor and foam samples

The prevalence of foam in WWTW A was observed occasionally. Formations of stable foams on the surface of the aeration basins were during two sampling events (February and June) (Table 4.4). It was also noted that WWTW A (domestic WWTW) was less prone to foaming than WWTW B (industrial WWTW). The prevalence of foam on the surface of WWTW B that treats industrial influent was observed frequently during sampling. Thick brown foam was often observed as observed in Fig. 4.1. Occasionally the foam would be dark grey due to the dyes from the textile industry influent. The presence of stable foam was observed mainly during summer period and less foams were observed during winter. Consequently, thin though stable foam was observed in June as stated in Table 4.5. The prevalence and abundance of right-angled branched, filamentous actinomycetes was more prevalent in WWTW B than WWTW A that treats mostly domestic influent as observed in Fig 4.2 (A) and (B).

Fig. 4.1: Foaming sludge observed from WWTW B during sampling. This sampling episode was observed in June.

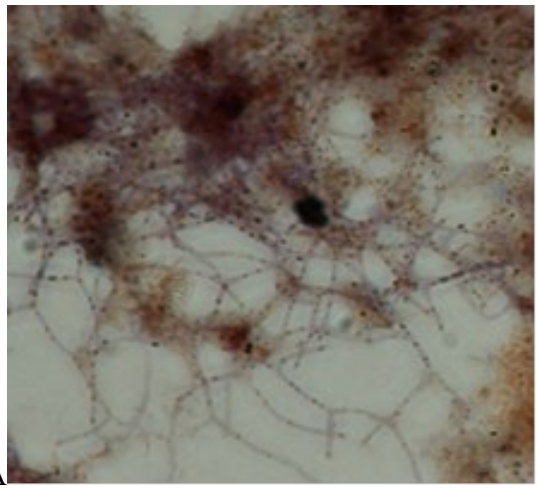
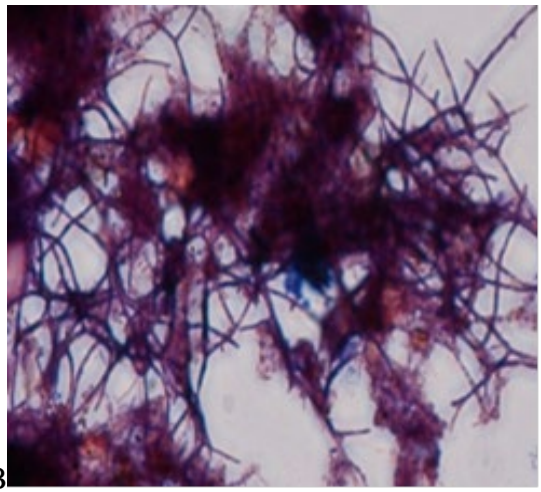
A  B 

Fig. 4.2: Images of filamentous bacteria observed from WWTW B mixed liquor: (A) Neisser positive granulated GALOs and (B) branched, Gram positive branched GALOs

4.3.2 Subjective scoring of filamentous bacteria abundance from mixed liquor

Wet mount observations of floc structural characteristics during sampling periods revealed that during the months presented in this study from WWTW A, filaments formed bridging among flocs which extended towards the surface area. This led to an observation of less compact floc structure observed in January with a subjective score of 'common' filamentous bacteria. February displayed 'dominant' filament bacterial scoring, with June exhibiting a score of 6 which is indicative of excessive filamentous bacterial presence. The prevalence of filamentous bacteria particularly mycolata dominated in WWTW B. In the months of sampling presented in this study, there was an excessive presence of filamentous bacteria in WWTW B.

4.3.3 Wastewater and physico-chemical characteristics of the respective wastewater treatment works

The results of the physico-chemical analyses are presented in Tables 4.2 to 4.4. The water temperature within the aeration basin ranged from 25.4 to 28.6ºC in summer for WWTW A and 17.0 to 23.2ºC during winter (Table 4.3). The temperature experienced during summer months for WWTW B ranged from 27.2 to 30.2ºC in summer and 18.5 to 22.6ºC in winter

(Table 4.4). The average DO concentration in the aeration tank was 2.2 ± 0. 1 mg/L for WWTW A and 2.3±0.3 for WWTW B. The influent COD range in WWTW A was 565 ± 236 and 797 ± 143 in WWTW B. WWTW A showed COD removal rate of 84 ± 8% whilst WWTW B had lower COD removal rate (76 ± 1%). The average mixed liquor suspended solids (MLSS) of 7381 ± 1160.14 mg/l was recorded for WWTW A and 7974.66 ± 2753.75mg/L for WWTW B. The pH was relatively stable and constant with a mean of 6.53 ± 0.19 pH units. There was no settleability observed for SVI in WWTW B during two sample periods in January and February, SVI was 118.85 in June and SVI ranged between 109.08 ± 10.09 for WWTW A.

Table 4.2: Average values of wastewater characteristics and operational parameters of the selected WWTWs

WWTW	**T°**	**F/M**	**pH**	**COD**		**DO**	**MLSS**	**MLVSS**	**SVI**
				Influent	**Effluent**				
WWTW A	22.8 ± 5.	0.29 ± 0.1	6.6 ± 0.2	565 ±236	84 ± 35	2.10±0.1	7381 ± 1160	5504 ± 922	109 ± 10
WWTW B	24.8 ± 6	0.19 ± 0.1	6.8 ± 0.4	797 ±143	195 ± 40	2.30 ± 0.3	7974.67±2753	3757 ± 822	ND

Key: ND – not done T – temperature F/M – food to microorganism COD – chemical oxygen demand DO – dissolved oxygen MLSS – mixed liquor suspended solids MLVSS – mixed liquor volatile suspended solids SVI – sludge volume index

Table 4.3: Wastewater characteristics and operational parameters in WWTW A

Month	T°C	F/M ratio	pH	COD		DO	MLSS	MLVSS	SVI	Visual foam observation
				Influent	Effluent					
Jan	25.4	0.4	6.8	408	75	2.1	7360	5405	105	No
Feb	26.1	0.2	6.7	851	91	2.2	6232	4636	120	Yes
June	17.0	0.3	6.4	836	87	2.1	8552	6472	101	Yes

T – temperature F/M – food to microorganism COD – chemical oxygen demand DO – dissolved oxygen MLSS – mixed liquor suspended solids MLVSS – mixed liquor volatile suspended solids SVI – sludge volume index

Table 4.4: Wastewater characteristics and operational parameters in WWTW B

Month	TºC	F/M ratio	pH	COD		DO	MLSS	MLVSS	SVI	Visual foam observation
				Influent	Effluent					
Jan	28.6	0.1	7.1	926	236	2.0	9492	4602	NS	Yes
Feb	27.2	0.2	6.4	820	193	2.6	9636	3709	NS	Yes
June	18.5	0.3	6.8	644	157	2.3	4796	2960	118.9	Yes

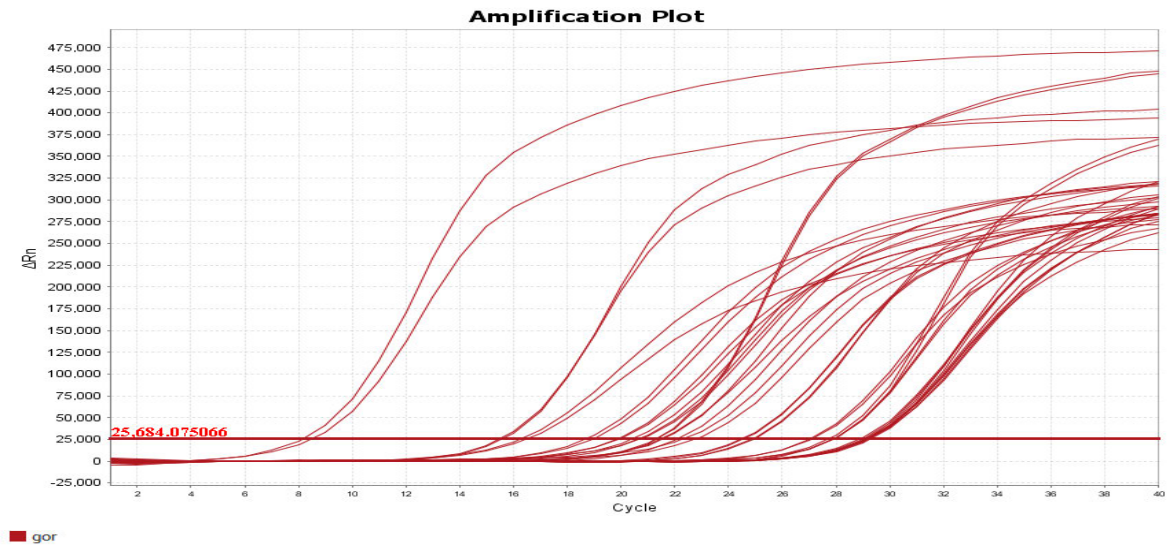
NS – no settleability observed after 30 min T – temperature F/M – food to microorganism COD – chemical oxygen demand DO – dissolved oxygen MLSS – mixed liquor suspended solids MLVSS – mixed liquor volatile suspended solids SVI – sludge volume index

4.3.4 Quantification of Gordonia spp. in mixed liquor and foam using quantitative polymerase chain reaction

The abundance of *Gordonia* spp. as determined by qPCR was reported as DNA copy numbers of 16S rRNA genes per nanoGram of genomic DNA isolated from foam and mixed liquor samples. The qPCR efficiencies were between 90 and 110% and the standard curves were linear over six orders of magnitude (R^2>0.98). The melt curve analysis expressed a single curve showing specificity of the primer selected (Appendix 3).

The concentration of *Gordonia* spp. was significantly higher in foam than in mixed liquor samples. The WWTW A mixed liquor ranged from 3.7 X 10^9 to 2.5 X 10^{11} copies/ng DNA *Gordonia* spp. whilst WWTW B ranged from 1.0 X 10^9 to 1.1 X 10^{11}copies/ng DNA. The *Gordonia* spp. population in stable foams was within the range of 1.4 X 10^{10} to 3.1 X 10^{12} copies/ng DNA in WWTW B and 1.4 X 10^{10} to 4.5 X 10^{11} copies/ng DNA from WWTW A. The higher concentration of *Gordonia* spp. was observed in foam from WWTW B, which supported the traditional microscopic examination.

A



B

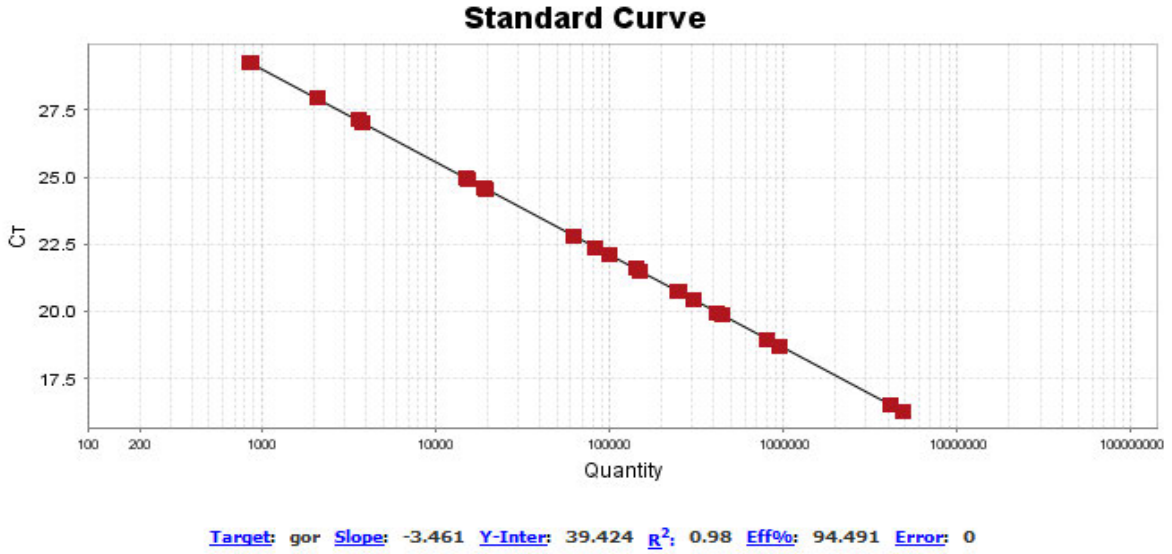


Fig. 4.3: qPCR data for the purified DNA used in generating standard curve indicating linearity (R^2>0.99). (a) - The qPCR amplification plot using *Gordonia* specific primers (b) The standard curve was constructed from 1 X 10^3 to 10^7 copies of DNA quantification standard per reaction.

The pattern, however, was different in WWTW B as excessive foam was observed in summer than in winter. The temperature of the mixed liquor on the day of sampling was 28.6°C when exceedingly higher copy numbers detected were 4.5X10^5 copies/µg from foam. Thick foam was also observed in February, with no settling observed during the settleability test and 8.8 X 10^4 copies/ng quantified. The concentration of *Gordonia* spp. in mixed liquor was relatively consistent throughout the sampling period.

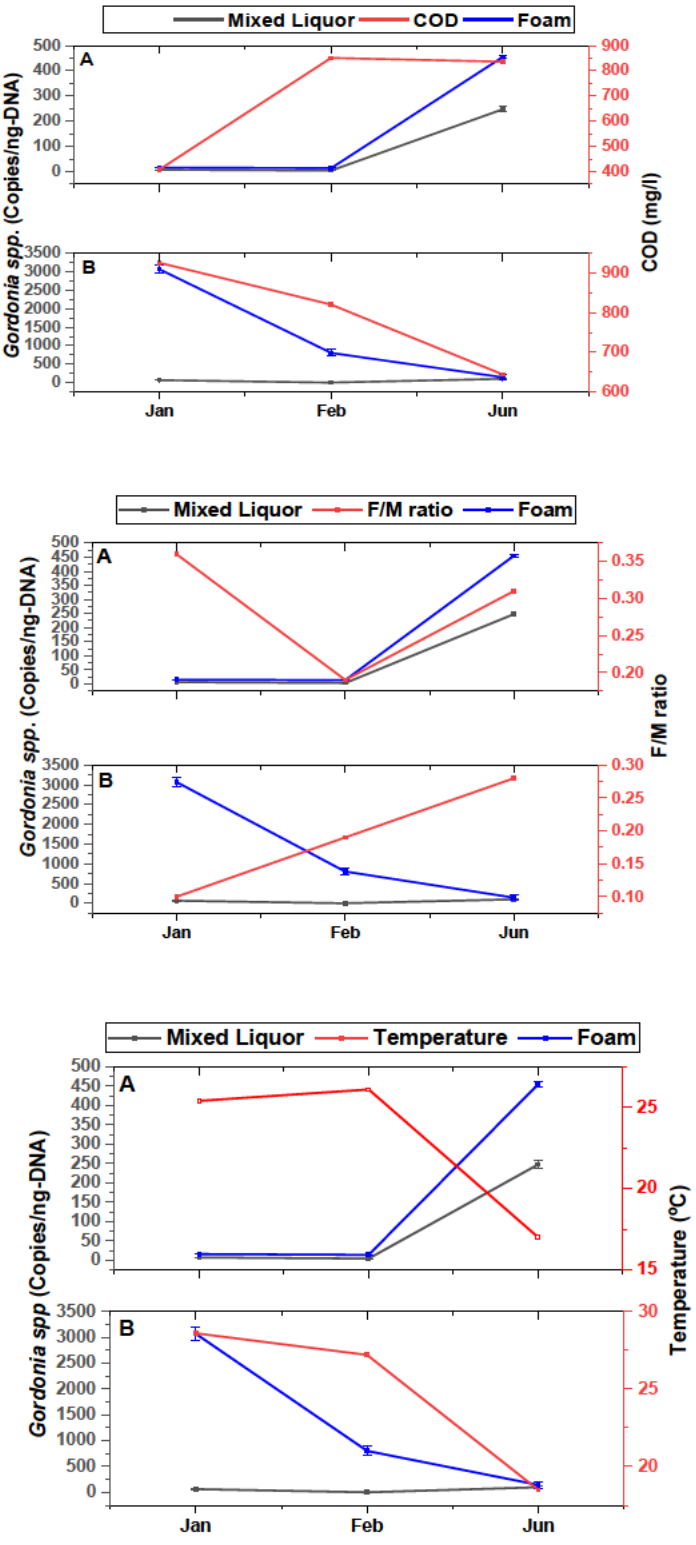


Fig. 4.4: Abundance of *Gordonia* spp. expressed as DNA copy numbers of 16S rRNA genes per nanogram of genomic DNA extracted from mixed liquor and foam samples plotted against COD, F/M ratio and temperature from WWTW A (A) and WWTW B (B).

The impact of F/M ratio, COD and temperature on the abundance of *Gordonia* spp. was investigated in Fig. 4.4. When the F/M ratio was low at 0.1, a higher abundance of *Gordonia* spp. was observed in WWTW B. The F/M ratio in WWTW A was within the acceptable range, except in February marked by reduced concentration of *Gordonia* spp. The removal of organic pollutants was more efficient in WWTW A, although foam presence was observed during high organic pollutant presence observed in February and June (851.1 and 836.1 respectively). Similarly, a higher copy number for *Gordonia* spp. was observed during organic pollutant concentration in January and February (926.2 and 820.4) which decreased in June with a decrease in COD rate to 643.8 in WWTW B. A higher abundance of *Gordonia* spp. (4.5×10^{11} copies/ng) was observed when temperature dropped to 17ºC in WWTW A whilst summer temperature ranges (28.6ºC in January and 27.2ºC in February) from WWTW B resulted in more prevalence of *Gordonia* spp. than in June. Correlative analysis was not conducted in this study due to insufficient data, at least six months data points would suffice for correlation studies. To further understand the contribution of other foam-forming bacteria, metagenomic analysis was conducted which is discussed further in chapter 5.

4.4 DISCUSSION

The application of qPCR is amongst the molecular biology techniques that have provided great insight due to accurate and robust quantitative information generated (Karst *et al*., 2016). Although techniques such as FISH have been used successfully for qualitative and quantitative purposes, its adoption for routine monitoring poses challenges as it is time consuming and requires exceptional skill (Fukushima *et al*., 2007). Partial permeability and low rRNA levels attributed to reduced metabolic activity have resulted in erroneous FISH quantification results (Kaetzke *et al*., 2005). The application of qPCR requires the production of target specific primers that display a high level of specificity and PCR efficiency (Guo *et al*., 2015). The application of mycolic acid containing Actinomycetales (mycolata) specific primers has been used previously for quantitative analysis (Petrovski *et al*., 2011). However, not all genera within mycolata are implicated in foaming. It was also observed that broad group primers affect the accuracy and precision of quantification (Asvapathanagul and Olson, 2016). In this study, two *Gordonia* specific primers previously reported in the literature (Shen *et al*., 2007) have been successfully used to quantify the abundance of *Gordonia* spp. in foam and mixed liquor samples. Earlier studies in this region had implicated *Gordonia* spp. amongst the dominant organisms in bulking and foaming (Marrengane *et al*., 2010; Deepnarain *et al*., 2020).

A high foaming potential has been generally linked to species in the genus of *Ca.* Microthrix (Jiang *et al*., 2016) and mycolata (Guo *et al*., 2015). However, the prevalence of *Ca.* M. parvicella has not been detected in this study based on microscopic examination. This can be attributed to elevated temperatures observed in this region as outlined in Tables 4.2; 4.3 and 4.4. This observation has been observed by Kumari *et al*., (2009); Lienen *et al*., (2014) and Deepnarain *et al*., (2020) that attributed it to *Ca*. M. parvicella's low temperature optimum. The presence of mycolata above a certain threshold is sufficient to render the ASP conducive to biological foam-formation (Davenport *et al*., 2000; Frigon *et al*., 2006). Hence, it was imperative to establish the foaming threshold and other underlying conditions that induce biological foaming. It is evident from Figures 4.4 A that the temperature in both WWTWs favoured the foam formation particularly due to *Gordonia* spp. It has been reported previously in many studies that *Gordonia* spp. prefer higher temperatures. Frigon *et al*., (2006) found that temperatures of 10 – 15ºC provided minimum growth temperature requirement for *Gordonia* spp. with an optimum of 20 to 25ºC. The temperature ranges (17.2ºC to 27.2ºC) of the two respective WWTWs in this study were within the optimum growth range for *Gordonia* spp.

The excess growth of *Gordonia* spp. had also been observed to occur during warmer seasons (de los Reyes III *et a*l., 2002; Oerther *et al*., 2001). In addition, WWTW B receives influent from a poultry processing industry coupled with temperature range of 18.5 – 28.6ºC would possess high lipid loading which encourages the accumulation of hydrophobic foam-formers. Thus, amongst the two plants surveyed, WWTW B showed a higher prevalence of *Gordonia* spp. than WWTW A particularly in summer. Fig. 4.4 highlighted a decrease in *Gordonia* spp. when the temperature of wastewater in the aeration basin decreased. Furthermore, the pure culture examination in this study (Chapter 3) also found that most isolates could not grow at 20ºC. The preferred growth temperature of isolates was 30ºC (Tables 3.5 – 3.8). The prevalence of *Gordonia* spp. was also reported previously from industrial WWTW as the causative organism of foaming and bulking incidents also indicating a preference to flotation (Dunkel *et al*., 2018).

Acceptable F/M ratios in the range of 0.25-0.6 g DBO_5/g MLVSS/day produce values of SVI representative of good settleability of the sludge. Filamentous bacteria that tend to induce foaming can attach to surfaces of oils and fats and utilize it as a substrate particularly when substrate availability is low (Tsang *et al*., 2008) which was encountered in WWTW B with F/M ratios ranging from 0.10 to 0.28 during the sampling period as stated in Tables 4.3 and 4.5. Thus, influent containing fats and greases and the prevailing temperature in this region can lead to an increase in the fat hydrolysis rate resulting in an increase of *Gordonia* spp. as speculated in this study. This finding is supported by pure culture studies in section 3.3 that found growth from *Gordonia* isolates on cholesterol minimal media in the range of 25 to 30ºC from WWTW B. The industrial influent received from WWTW B is also from a commercial scale poultry processing plant. The hydrophobic cell wall of foam-forming bacteria imparts a competitive advantage in the assimilation of hydrophobic substrates. The ability of foam-forming bacteria to assimilate hydrophobic substrates has been demonstrated in laboratory scale studies (Iwahori *et al*., 2001; Frigon *et al*., 2006). These foam formers, however, are not just confined to *Gordonia* spp., other mycolata within the sub-order of *Corynebacteriaceae* have also been implicated (Khodabakhshi *et al*., 2015). Furthermore, temperature also influences oxygen transfer by impacting on oxygen diffusion. Temperature experienced in this region could limit oxygen transfer by creating low DO concentrations, enabling hydrophobic

foam formers to partition the air – bubble interface and gain a selective advantage enabling their agglomeration (Pal *et al*., 2014).

The F/M ratio in WWTW A in January was low but did not support excessive growth of *Gordonia* spp. that was observed in June as highlighted in Figs 4.3 A and B and 4.4 when influent COD values were higher. WWTW B on the other hand persistently had low F/M ratios, this was also supported by lack of settling observed during the settleability test. During the same period, microscopic examination also revealed an abundance of filamentous bacteria which denotes that there was an imbalance between floc-formers and filamentous bacteria as observed in Figs 4.2 A and B. Foaming due to *Gordonia* spp. which is promoted by low F/M ratios was observed during feast-fasting operation that was investigated to control foaming (Tsang *et al*., 2008), thus F/M ratio may not be the inducer of foaming observed in June from WWTW A in Fig. 4.4. Findings by Zhang *et al*. (2022) provided evidence that AS microbial community can resist environmental disturbances when the F/M ratio is maintained between 0.2 and 0.5 $kg(kg.d)^{-1}$ as evidenced in WWTW A in Fig. 4.4. In addition, elevated MLSS concentrations increase the probability that mycolata levels may reach the foam-formation threshold as observed in Table 4.2 and 4.4 from WWTW B which experienced frequent foaming episodes. Furthermore, such elevated MLSS concentrations impact DO unfavourably by lowering it, creating an environment conducive to foam formation.

The pH in this study seemed to have a negligible effect on the accumulation of foam-formers as it was maintained close to neutrality as shown in Table 4.2 to 4.4. A previous study found that pH levels that are maintained at a range of 6.5 - 8.5 units were successful in eliminating foaming which can be triggered by acidic pH ranges (Khodabakhshi *et al*., 2015). However, maintaining the pH in that range in this region was insufficient to eliminate foaming.

The quantitative results revealed a higher copy number of *Gordonia* spp. in foams than in mixed liquor as observed in Figs 4.4. This result supported microscopic observations in Fig 4.2 A and B. This observation was supported by earlier studies through the application of FISH (Davenport *et al*., 2000) and recent findings that monitored the abundance of mycolata before, during and after foaming episodes (Guo *et al*., 2015).

It is pragmatic to determine the threshold that induces foaming upon identification of the prevalent foam formers (Asvapathanagun and Olson, 2016). Fig 4.4 depicts the copy

numbers/ng from the two respective WWTWs. Both WWTWs had visible foam when *Gordonia* spp. displayed two-fold increases. Research conducted by Petrovski *et al*., (2011) found that the foaming threshold varies for mycolata ranging from 10^7 to 10^9 cells/ng, however their findings cannot be used as a blanket universal threshold applicable to all foaming mycolata. Guo *et al*., (2015) further concluded that only 0.1% cell abundance of foam formers is sufficient to induce stable foam formation.

The increase in copy numbers/ng of *Gordonia* spp. in foams can be attributed to the production of active, amphiphilic compounds (Franzetti *et al*., 2008). The biosurfactants enable them to adhere to hydrophobic phase thereby decreasing the interfacial tension between the two phases. It also allows them to disperse the hydrophobic compounds leading to an increased surface area for microbial invasion (Iwahori *et al*., 2001; Arenskotter *et al*., 2004). A study conducted by Pagilla *et al*., (2002) confirmed that foaming episodes in ASP were due to biosurfactant production when hydrophobic compounds were present in the effluent. The influent received from WWTW B receives domestic influent and industrial influent from a poultry processing farm which may release influent with high lipid contents.

The findings of this study express that it is possible to elucidate foaming by manipulating operational parameters that can retard the overgrowth of foam formers whilst monitoring their concentration in mixed liquor. It is imperative to monitor key foam formers as reported in literature to assess the impact of operational conditions on their proliferation. Species specific targeted studies could lead to false information and hence a comprehensive study such as metagenomic analysis is imperative to understand the role of different species in foaming incidents and their prevalence under varying operational and environmental conditions. The climatic conditions that prevail under WWTW A and B, create a temperature that allows *Gordonia* spp. to thrive. Hence, foaming mitigation practices should consider measures that can eliminate mycolata foaming behaviour.

4.5 CONCLUSION

The influent composition, geographical location and plant operational conditions render the WWTWs prone to biological foaming.

- The operational parameter that seems to favour foaming episodes due to *Gordonia* spp. in this region is temperature. The temperature range in the region during the study period was within the range for *Gordonia* spp.
- The F/M ratio that prevailed in WWTW B was found conducive to foaming by *Gordonia* spp. particularly during the summer season.
- The relationship between the occurrence of foam, influent composition and favourable temperature may be responsible for the perpetual foaming incidents that are experienced in WWTW B that favour the growth of *Gordonia* spp.
- The quantification of *Gordonia* spp. although successful and applicable, it is necessary to determine other role players in foam formation. Two-fold increases of *Gordonia* population were found sufficient to induce foam. However, the role of other key players needs to be investigated.

CHAPTER 5: METAGENOMIC INSIGHTS INTO THE DISTRIBUTION OF *GORDONIA* SPP. DURING FOAMING AND NON-FOAMING EPISODES

5.1 INTRODUCTION

Advancements in high-throughput sequencing techniques have enabled large-scale microbial community profiling in complex environmental samples to study microbial community dynamics (Jankowski *et al*., 2022). High-throughput sequencing platforms such as shotgun sequencing methods have been applied as promising methods to investigate the genes and gene expression levels of microbial communities in different habitats (Wu *et al*., 2019; Vasudeva *et al*., 2022).

Metagenomics refers to a culture-independent method that allows for the identification and characterization of organisms from various types of samples. Thus, its application ensures total genomic content analysis within a complex sample (Perez-Cobas *et al*., 2020). The metagenomic approach circumvents the limitations of targeted methods such as Sanger or dideoxy-sequencing which has been shown to possess gross limitations (Gokul *et al*., 2016). Several DNA based high throughput sequencing metagenomics have been applied to reveal diverse microbial communities from marine water, soil, human guts and WWTP (Gilbert *et al*., 2008; Urich *et al*., 2008; Qin *et al*., 2010; Lazarevic *et al*., 2009; Frias-Lopez*et al*., 2008; Shi *et al*., 2011; Sun *et al*., 2023; Ríos-Castro *et al*., 2023; Nguyen *et al*., 2023; Zhang *et al*., 2024).

The ASP also displays similar vastness in terms of microbial biocenosis like soil and marine water habitats comprising of bacteria, eubacteria, eukaryotes and viruses. Hence, NGS is more advantageous as it can generate extensive data from ASP without limitations encountered in traditional Sanger sequencing. The taxonomic profile from various studies have shown that the ASP was dominated by organisms belonging to phyla of *Proteobacteria, Actinobacteria, Bacteriodetes, Firmicutes* and *Verrucomicrobia* (Yu and Zhang, 2012; Guo *et al*., 2015; Gokul *et al*., 2016; Guo *et al*., 2017; Wang *et al*., 2022; Sharma *et al*., 2023). Advances in metagenomic studies have led to global campaigns to systematically analyse ASP microbiomes from various countries (McIlroy *et al*., 2015; Wu *et al*., 2019). The study by Wu *et al*. (2019) found significant taxonomic and phylogenetic variation between any two continents amongst the six continents that formed part of their study. Species abundant diversity analysis found that bacterial composition appears to be driven most probably by stochastic processes, such as

dispersal and drift, although temperature, sludge retention time and organic C loading play important roles in regulating the structure of the activated sludge community. A prior metagenomic global study termed MiDAS 4 identified abundant and critical microorganisms in relation to their functional importance (Dueholm *et al*., 2022). Such applications can provide more precise information on microbial ecology and distribution of key microbial groups that are implicated in process efficiency.

Foaming in WWTW causes problems, leading to less efficiency, higher costs, and possible environmental harm. Whilst several efforts have been made in the past using traditional microbiology techniques and PCR-based approaches to understand the microbial causes of foaming, there is still gap in knowledge regarding the microbial basis of foaming in WWTWs and the specific role of *Gordonia* spp., a filamentous bacterium that have been implicated in foam formation, globally. Moreover, the literature search reveals that there are limited studies that have employed metagenomics for investigating foaming and non-foaming episodes in ASP. The metagenomic approach is capable of simultaneously giving a more holistic and detailed microbial resolution of an environment or sample. So far, studies from Germany, China and the United States of America have provided insight into foaming mechanism and the microbial groups involved (Guo *et al*., 2015; Wang *et al*., 2016; Dunkel *et al*., 2018; Li *et al*., 2020; Kang *et al*., 2021, Rosso *et al*., 2018). A detailed study conducted in Hong Kong discovered the prevalence of *Clostridium* XI, *Mycobacterium*, *Arcobacter*, *Flavobacterium* in foaming activated sludge Guo *et al*., 2015). Another study in North Carolina found that *Mycobacterium* spp. were responsible for foaming incidents experienced (Rosso *et al*., 2018). However, there is dearth of research focusing on role of abundant microbial populations during foaming events, especially in South Africa. Understanding the prevalence of mycolata during foaming and non-foaming episodes at WWTW in South Africa is imperative for the implementation of mitigation strategies that are specific to local conditions.

In this chapter, the aim was to employ the shotgun metagenomics sequencing approach to profile the microbial community structure especially particularly actinomycetes population in two South African WWTW, thus providing more substantial evidence of the richness, abundance, and possible role of these bacteria during these foaming events. Samples from both WWTW A and WWTW B were monitored over a period of three months.

5.2 MATERIALS AND METHODS

5.2.1 Wastewater treatment plants description

The WWTWs and characteristics of the plants used in this chapter are described in section 3.2.1

5.2.2 Genomic DNA extraction

Genomic DNA was extracted from mixed liquor and foam samples as indicated in Section 4.2.3. Foam and mixed liquor samples subjected to Illumina sequencing in this chapter are the same as those subjected to qPCR in chapter 4.

5.2.3 Genomic DNA purification

The extracted DNA was purified using GeneJet PCR Purification Kit (Thermo scientific™) according to the manufacturer's instructions. The purified DNA was quantified using Nanophotometer NP 80 (Implen, Muenchen, Germany) and sent to National Centre of Communicable Diseases (NICD) Sequencing Core Facility for high throughput sequencing using Illumina NextSeq 550 platform (Pretoria, South Africa).

5.2.4 Library preparation and Illumina sequencing

The genomic library was prepared using the Nextera DNA Flex library preparation kit (Illumina, San Diego, CA), following standard Illumina sample-preparation protocol. The DNA was bound to beads and fragmented following the Illumina DNA Prep Reference Guide 1000000025416 v09 protocol. Libraries were then amplified according to the Nexflex PCR proGramme with a reaction volume of 12.5 µL as follows: 68°C for 3 min, then 98°C for 3 min, followed by (X) Cycles of (X was based on the initial DNA amount, according to Illumina DNA Prep Reference Guide 1000000025416 v09): 98°C for 45 s, then 62°C for 30 s, 68°C for 2 min and 68°C for 1 min at 10°C. Afterwards, the DNA library clean-up was carried out following the Illumina DNA Prep Reference Guide 1000000025416 v09 protocol. Libraries were quantified with Qubit 2.0 fluorometer (Life Technologies, Carlsbad, CA) and an Agilent

2100 Bioanalyzer (Agilent Technologies) to determine the size distribution and, subsequently, sequenced using 2 × 150-bp paired-end library on a NextSeq550 platform (Illumina) at the National Institute for Communicable Diseases (Pretoria, South Africa).

5.2.4.1 Computational methods

The ASP metagenomic sequencing reads were trimmed using TrimGalore v0.5.0 (Krueger), a wrapper for CutAdapt v1.18, with a minimum quality score of 30 for trimming (--q 30) and a minimum read length of 60 (--length 60). Trimmed reads were deduplicated to remove PCR and optical duplicates using seqtk rmdup v1.3-r106 with default parameters. To assess the microbial composition of the short-read sequencing samples, the Kraken v2.0.8-beta taxonomic sequence classifier with default parameters and a comprehensive custom reference database containing all bacterial and archaeal genomes in GenBank assembled to "complete genome," "chromosome," or "scaffold" quality as of January 2020. Bracken v2.0.0 was then used to re-estimate abundance at each taxonomic rank.

5.2.4.2 K-mer sketches

K-mer sketches were computed using sourmash v2.0.0a11. Low abundance k-mers were trimmed using the "trim-low-abund.py" script from the khmer package with a k-mer abundance cutoff of 3 (-C 3) and trimming coverage of 18 (-Z 18). Signatures were computed for each sample using the command "sourmash compute" with a compression ratio of 1000 (scaled 1000) and k-mer lengths of 21, 31, and 51 (-k 21,31,51). Signatures at each length of k were compared using "sourmash compare" with default parameters and the correct length of k specified with the -k flag.

5.2.4.3 Statistical analysis and plotting

Statistical analyses were performed using R v4.0.0 (R Core Team, 2019) with packages MASS v7.3-51.5 (Venables and Ripley, 2002), stats (R Core Team, 2019), ggsignif v0.6.0 (Ahlmann-Eltze, 2019), and ggpubr v0.2.5. Alpha diversity was calculated using the vegan package v2.5-6. Wilcoxon rank-sum tests were used to compare alpha and beta diversity between cohorts. Data separation in multidimensional scaling was assessed via PERMANOVA (permutation test

with pseudo F ratios) using the adonis function from the vegan package. Differential microbial features between mixed liquor and foam were identified from non-normalized count data output from kraken2 classification and bracken abundance re-estimation and filtered for 20% prevalence and at least 1000 sequencing reads using DESeq2. Plots were generated in R using the following packages: cowplot v1.0.0 (Wilke, 2019), DESeq2 v1.24.0, dplyr v0.8.5, genefilter v1.66.0, ggplot2 v3.3.0, ggpubr v0.2.5, ggrepel v0.8.2, ggsignif v0.6.0, gtools v3.8.2, harrietr v0.2.3, MASS v7.3-51.5, reshape2 v1.4.3 (Wickham, 2007), and vegan v2.5-6. After sequencing, the unique tags obtained were aligned with the 16S rRNA gene database with the aid of the BLASTN proGramme. The tag redundancy was eliminated, and sequences were assigned into operational taxonomic units (OTU) based on similarities of greater than 90%. With the aid of MEGA6 software, representative sequences were aligned using the ClustalW proGramme. The neighbour-joining method was employed for the phylogenetic analysis (Tamura *et al.*, 2013).

5.2.5 Short-read archive accession numbers

The raw reads for the Illumina have been deposited into NCBI Sequence Read Archive under the accession numbers PRJNA719031.

5.3 RESULTS

5.3.1. Microbial richness and diversity measure from foam and mixed liquor

Taxonomic richness, abundance and evenness were investigated using Chao 1 and Shannon Alpha diversity measures for both WWTWs. The p value of 0.93 in Chao 1 denotes that the microbial richness differences between mixed liquor and foam are statistically insignificant. There was greater diversity and evenness in mixed liquor than in foam. Shannon diversity ranged from 5.72 to 5.86 in mixed liquor and 5.06 to 5.87 for foam, indicative of lesser diversity in foam than in mixed liquor. Conversely, the Shannon index revealed near marginal significance with a p value of 0.18. There were fewer genera in foams with high relative abundance (Figs 5.1 and 5.2). Genus abundance in mixed liquor and foam was reported at 17, 606.10 and 900 534.5 reads, respectively, indicating that the actual taxonomic diversity was well covered after filtering low quality reads.

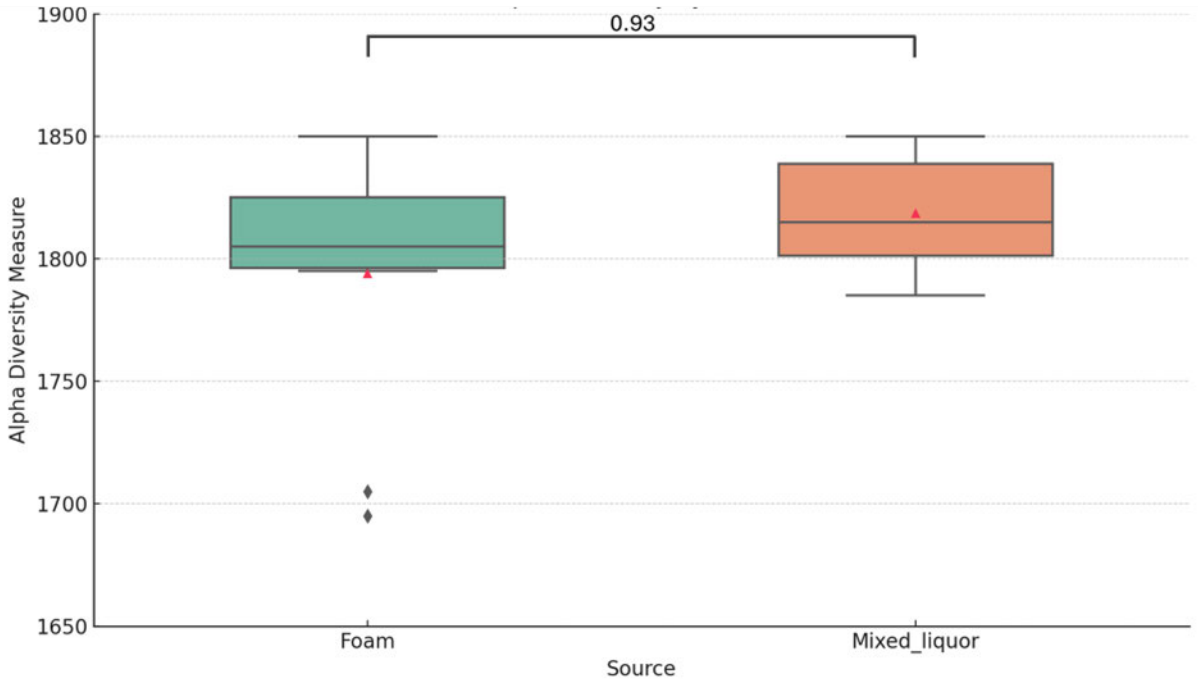


Fig. 5.1: Chao 1 Box plot analysis of Chao 1 depicting measurement of richness between foam and mixed liquor samples based on OTUs at genus level. Taxonomic classification with P values presented at 0.93 were identified.

Box plots highlight variations in richness between foam and mixed liquor. Outliers were detected in the upper and lower quartiles of foam. Outliers in the lower and upper fences in foam share similar values indicating similar abundance in outlying OTUs. The median in both foam and mixed liquor was not symmetrical, more OTUs were located above the second quartile in mixed liquor and more OTUs were in the third and fourth quartile in the foam, indicating that 50% OTUs in foam had OTUs above the upper fence. The median in the mixed liquor is closer to the bottom quartile demonstrating that there are more OTUs that had high value scores which highlight abundance in the third quartile. No outliers were detected in the mixed liquor.

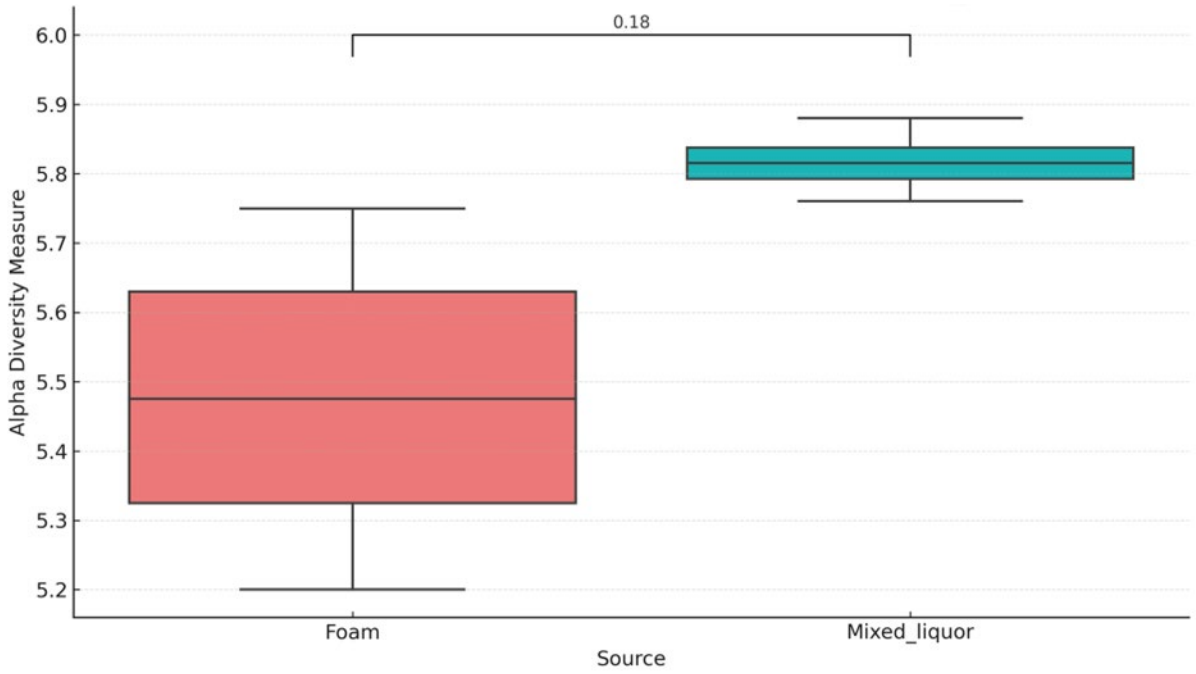


Fig. 5.2: Box plot analysis of Shannon diversity classification from foam and mixed liquor samples. Wilcoxon rank sum tests were used to compare population richness and evenness between foam and mixed liquor.

Mixed liquor has higher measure indicating that mixed liquor has more diversity and evenness depicted by the symmetrical box plot as expected. The upper whiskers and outliers in foam indicate scores that have fallen outside of the interquantile range.

5.3.2 Taxonomic composition of foam and mixed liquor from WWTW A and WWTW B

The five most prevalent phyla included in both foam and mixed liquor sample reads were *Actinobacteria, Bacteriodetes, Firmicutes, Proteobacteria* and *Streptophyta* and they followed the similar trends in both WWTW as shown in Fig 5.3 A and B. Phylum profiling revealed a significant reduction of *Streptophyta*, *Proteobacteria*, *Firmicutes* and *Bacteriodetes* with a significant increase in the relative abundance of *Actinobacteria*, as evident in Fig 5.3 in foam samples from both WWTWs. There was not much variation observed in mixed liquor and in foam in terms of the dominance of the top five phyla, although in the foam microbiome, there was a decrease in the abundance of *Proteobacteria* and an increase in *Actinobacteria*.

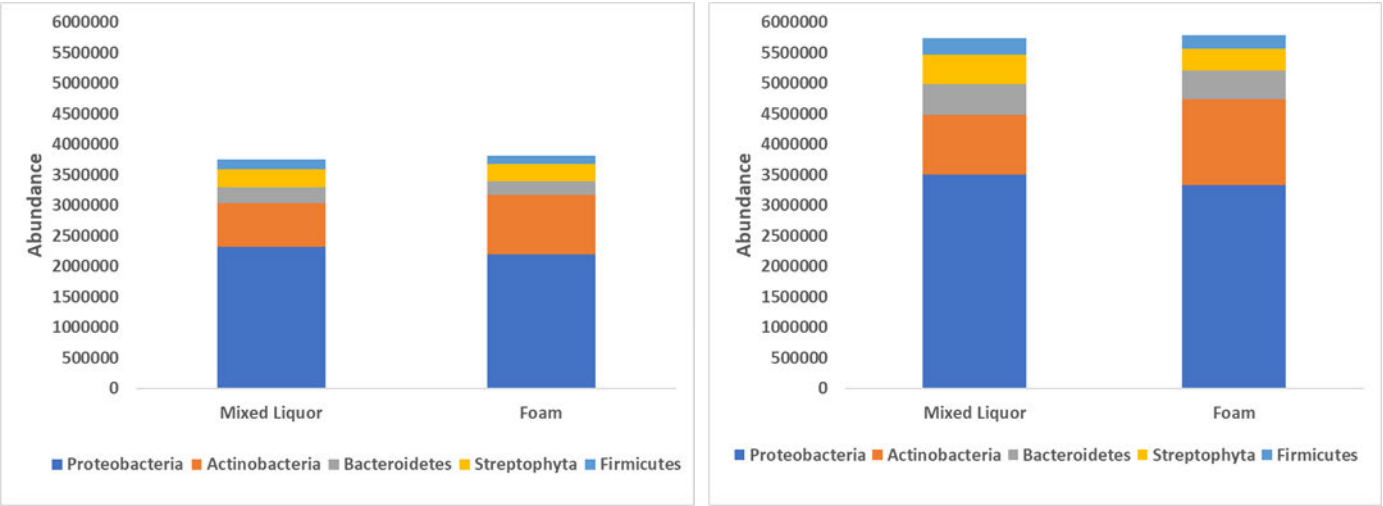


Fig. 5.3: Five most abundant taxonomic phyla by mean relative abundance in foam and mixed liquor samples from WWTW A (A) and B (B).

At family level, the most dominant fifteen families in foam and mixed liquor were relatively the same, however, there was a significant increase of *Norcadiaceae* in foam samples. In-depth comparison at the family level revealed a higher prevalence of known foam formers, *Gordoniaceae*, *Mycobacteriaceae* and *Nocardiaceae* in foam than in mixed liquor observed colorimetrically by the reduced intensity in foam than in mixed liquor (Fig 5.4). This observation was evident in both WWTWs.

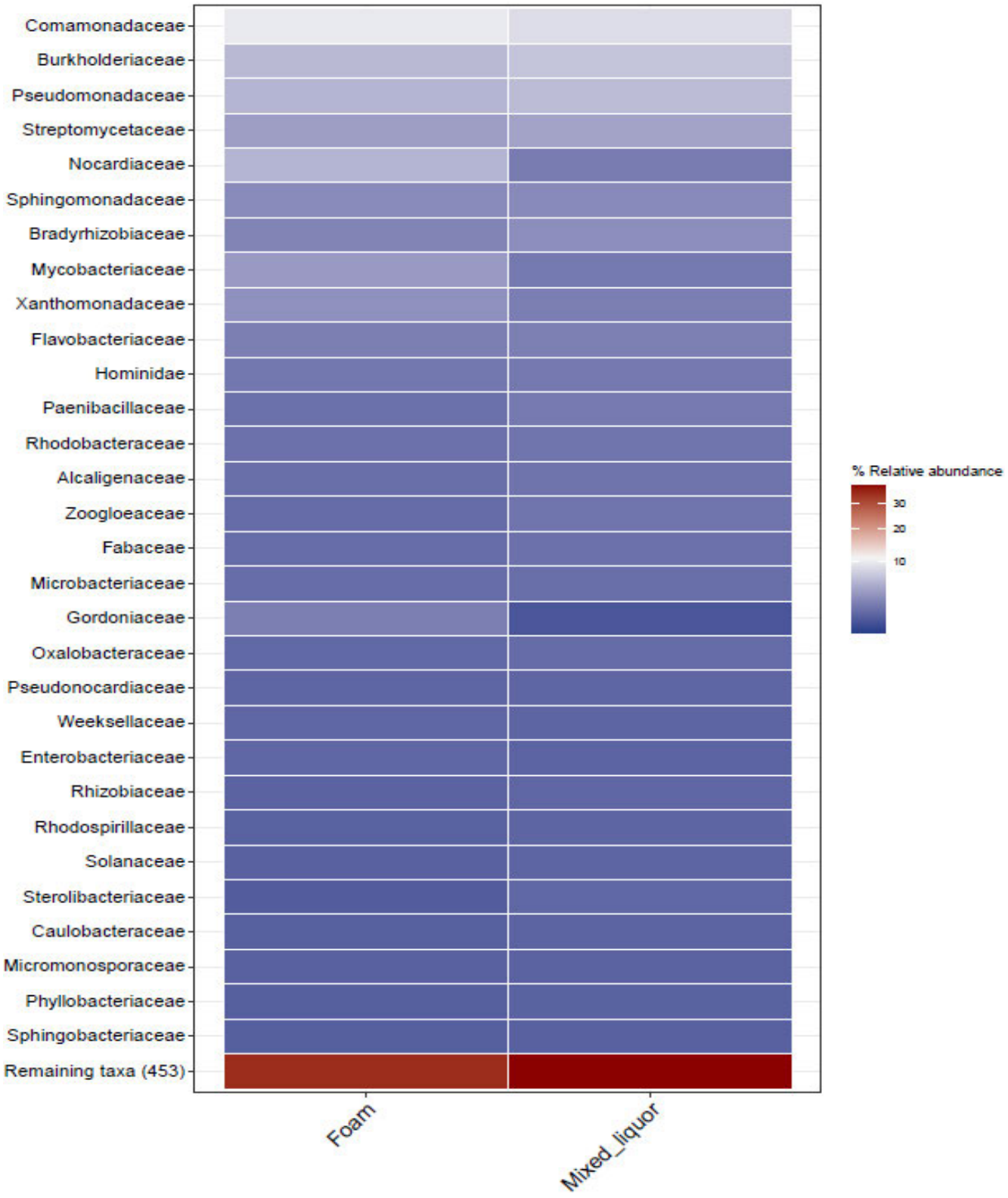


Fig. 5.4: Heatmap of relative abundance variation of the top thirty families in foam and mixed liquor from both WWTWs

Differential abundance was determined by investigating the microbial family that was the most differently abundant in abundance between mixed liquor and foam. Log2fold change value of -2.23 depicts that the *Gordoniaceae* family was significantly more abundant in foam than in mixed liquor. The relative abundance of *Gordonia* spp. in mixed liquor was 0.6% and 2.4% in foam, resulting in a four-fold increase (Fig. 5.5). Differential microbial features between mixed liquor and foam were identified from non-normalized count data output from kraken2 classification and bracken abundance re-estimation and filtered for 20% prevalence and at least 1000 sequencing reads using DESeq2 (Love *et al*, 2014; Lu *et al*., 2022).

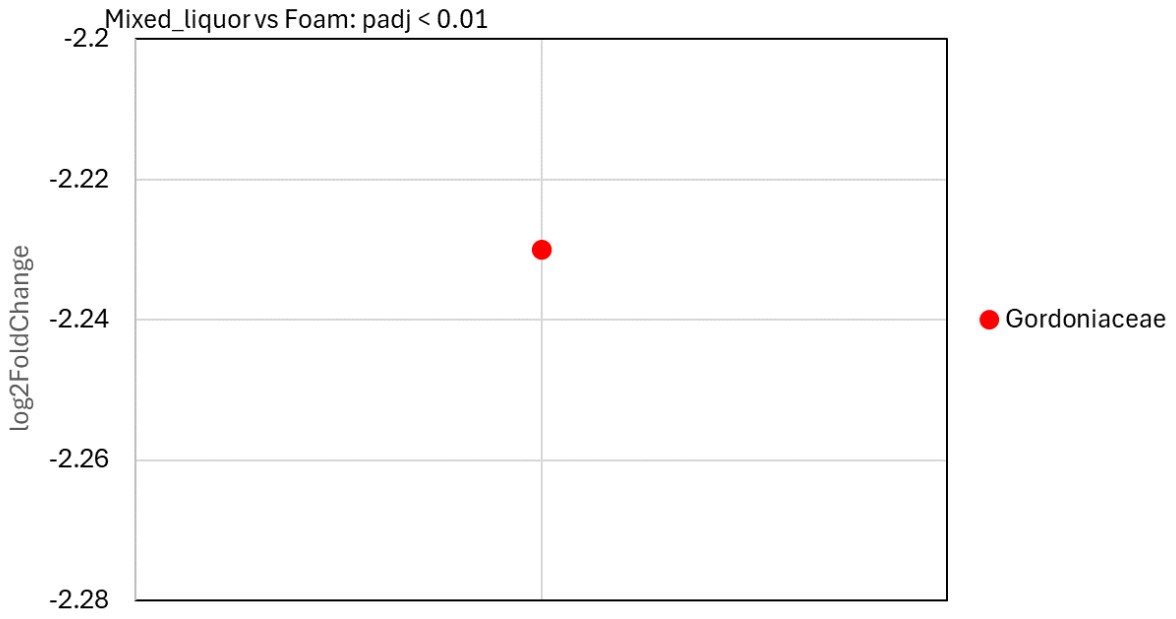


Fig. 5.5: Differentially abundant microbial family between mixed liquor versus foam identified by DESeq2. Identifies microbial family that is differentially abundant in foam versus mixed liquor.

Log2Fold changes in mixed liquor and foam investigated microbial species that were significantly less in mixed liquor than in foam, as observed in Fig 5.6 from both WWTW. All log2Fold change figures were negative, demonstrating substantial decreases of these species in the mixed liquor. *Mycolicibacterium doricum* and *Rhodococcus coprophilus* had a log2 Fold change of -1.2074 and -1.24644 respectively indicating an equivalent decrease in mixed liquor

than in foam. Thirteen *Gordonia* spp. collectively had four-fold increases in foam as their log2Fold changes ranged from -1.8676 to -2.4786.

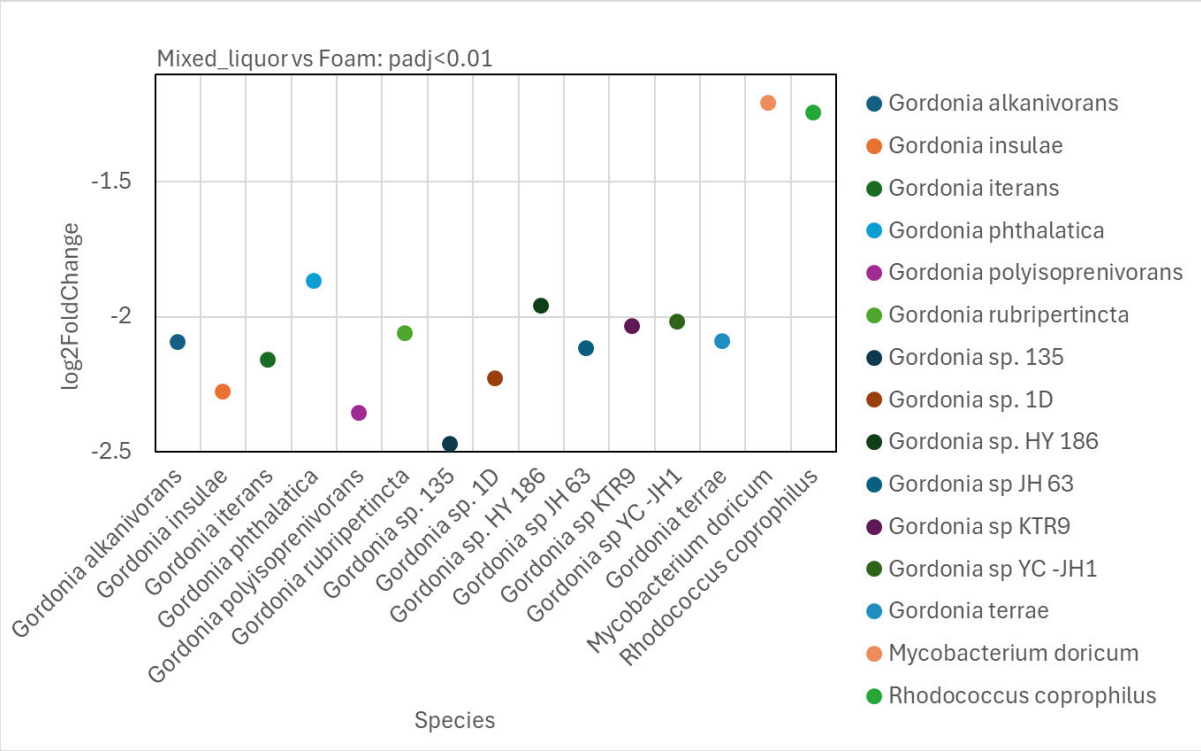


Fig. 5.6: DESeq2 identifies microbial species that are differentially abundant in foam compared to mixed liquor using log2 fold change. Identifies microbial species that are differentially abundant in foam than in mixed liquor.

The analysis based on diversity and relative abundance of different genera indicated a strong variation in relative abundance among the bacterial communities in foam and mixed liquor. Four genera amongst the fifteen dominant genera belonged to Actinomycetes. *Rhodococcus* had a relative abundance of 1.9% in mixed liquor and rose by 0.1% to 2.0% in foam, *Mycolicibacterium* was much higher in foams at 2.1% from 1.1% in mixed liquor. *Mycobacterium* increased by 0.4% in foam and *Gordonia* spp. by 400% from 0.6% in mixed liquor to 2.4% in foam (Fig. 5.6).

5.3.3 Taxonomic composition and variation of foam and mixed liquor from WWTW A and WWTW B across the months

The total microbial composition in WWTW A and WWTW B in mixed liquor and foam is outlined in Figs. 5.7 to Fig 5.12. A variation in species diversity and richness was evident in both mixed liquor and foam samples across the three months. The genera in mixed liquor varied slightly between WWTW A and WWTW B. For instance, species such as *Candidatus Accumulibacter*, *Flavobacterium* and *Sphingobium* predominated only in WWTW B and not detected in WWTW A (Fig 5.8 and 5.11) whilst *Massilia, Methylobacterium* and *Lysobacter*, were only detected in WWTW A and not in WWTW B mixed liquor (Fig. 5.8). There was more diversity and abundance observed in mixed liquor in January samples than February and June in WWTW A. The microbial diversity and abundance in WWTW A mixed liquor displayed less diversity and abundance in June when compared to February as observed in Fig 5.8.

Both WWTWs displayed similar microbial communities in foams showing significant decreases of *Proteobacteria*, *Bacteriodetes*, *Firmicutes* and *Streptophyta*, with significant increases of *Actinobacteria*. However, the genera that predominated in WWTW A and WWTW B foam varied. WWTW A had foam that was attributed to an increase in *Gordonia*, *Nocardia* and *Nocardiodes* spp. Other than *Actinobacteria*, *Thermomonas* and *Thauera* spp. also increased in foam as observed in Figs. 5.7 and 5.9. *Mycobacterium* and *Mycolicibacterium* spp. reported slight decreases in foam than in mixed liquor in WWTW A. The findings in WWTW B had a similar observation to WWTW A. The microbial consortium in WWTW B foam displayed significant increases in *Gordonia* spp., *Mycobacterium*, *Mycolicibacterium*, *Rhodococcus* and *Streptomyces* spp. and *Thermamonas* spp. had also shown significant increases in WWTW B as observed in Figs 5.10 and 5.12. The most abundant genera selection is based on the raw abundances mapped to those genera. The most abundant are there those with the highest raw abundances.

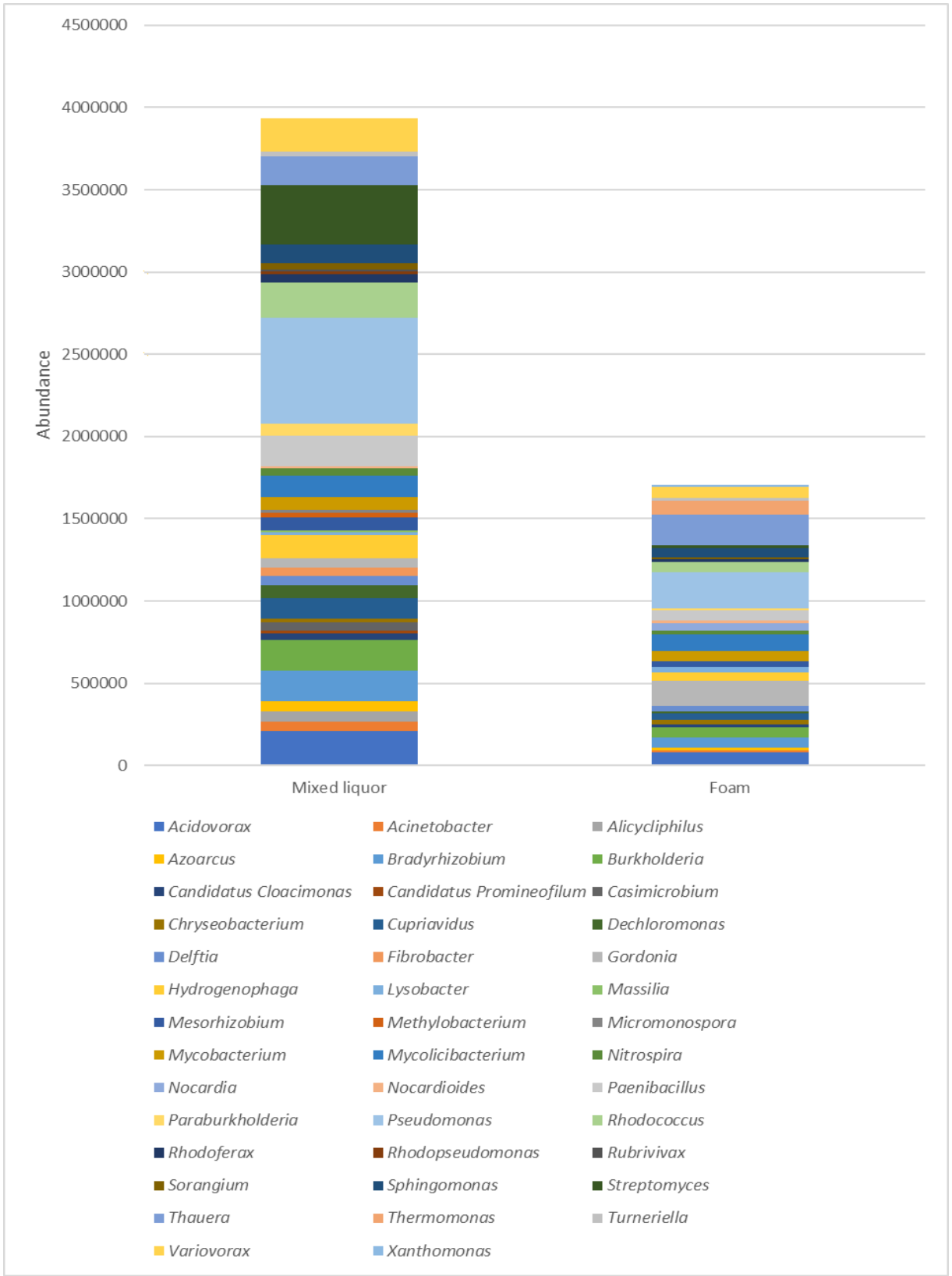


Fig. 5.7: Taxonomic distribution of the most abundant bacterial genera in foam and mixed liquor from WWTW A.

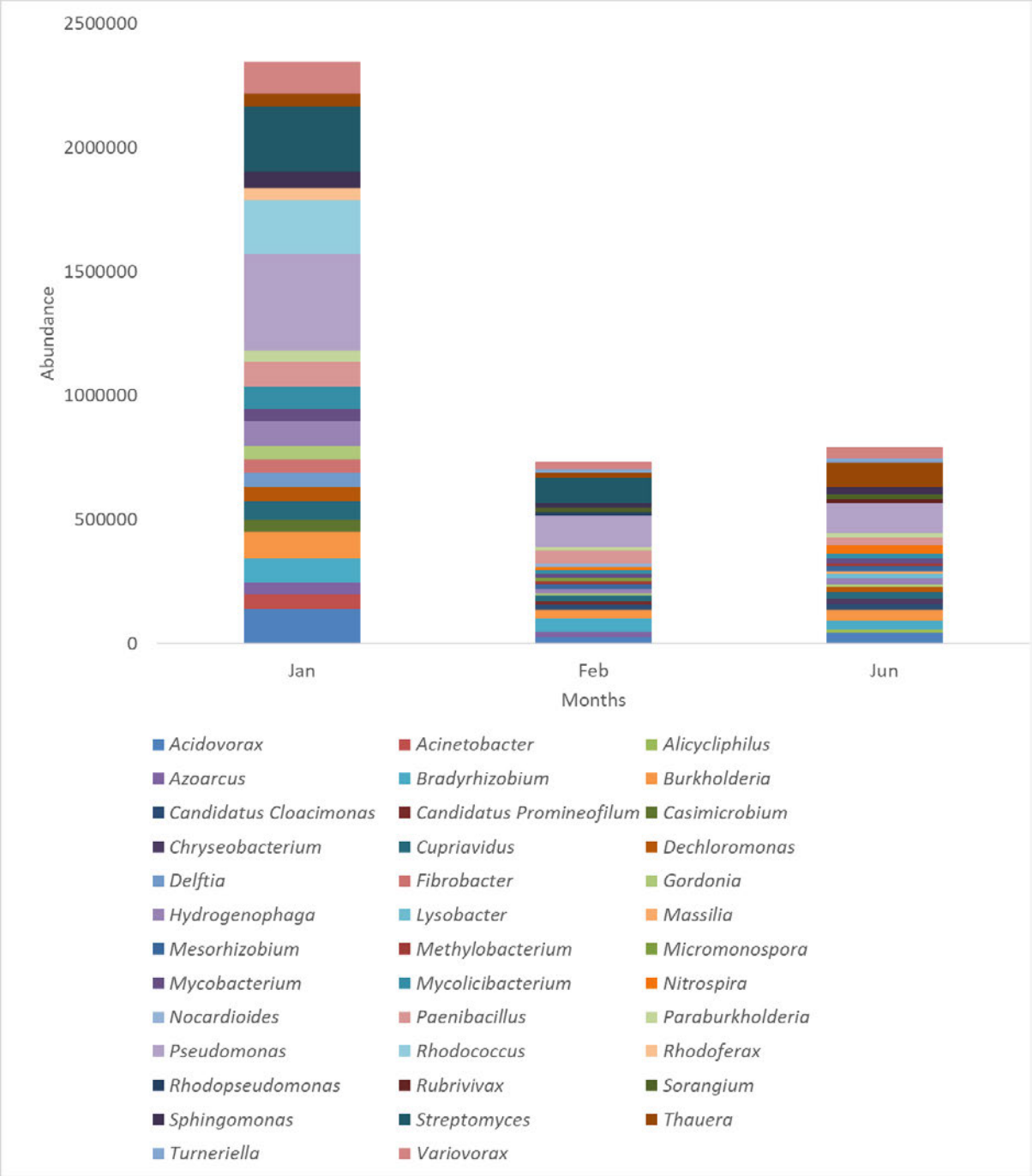


Fig. 5.8: Taxonomic distribution of the most abundant bacterial genera from WWTW A mixed liquor

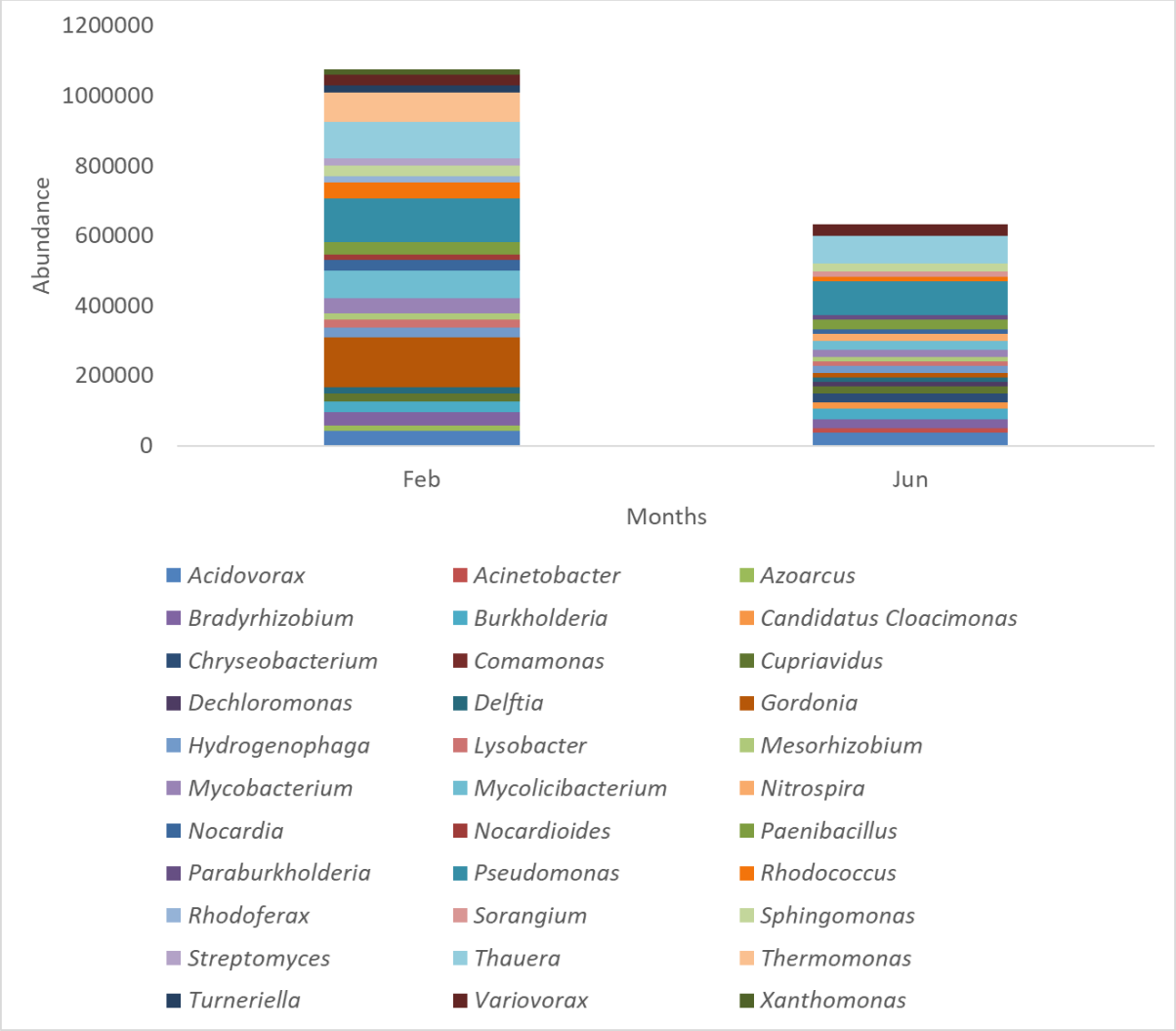


Fig. 5.9: Taxonomic distribution of the most abundant bacterial genera from WWTW A foam

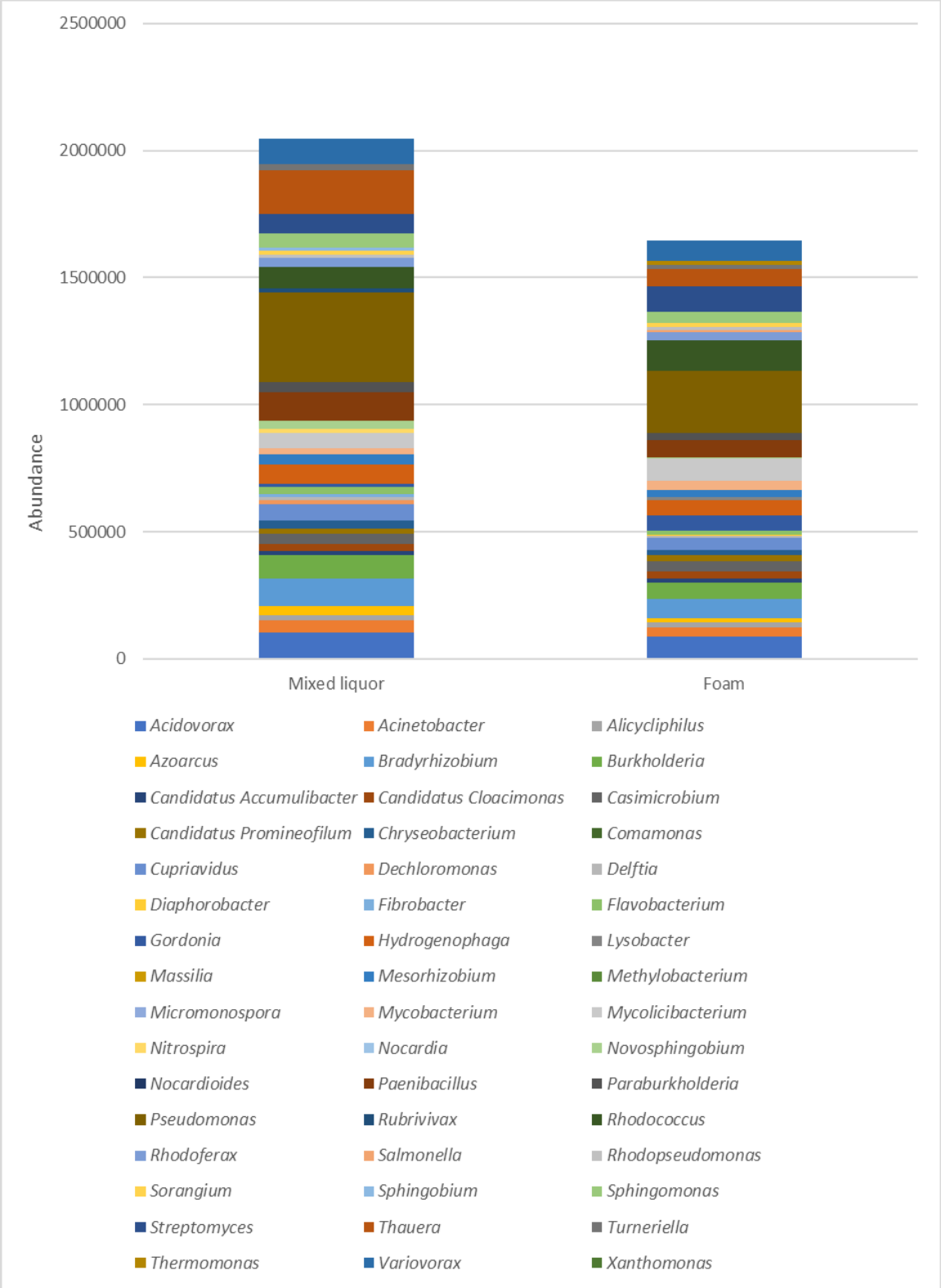


Fig. 5.10: Taxonomic distribution of the most abundant bacterial genera in foam and mixed liquor from WWTW B.

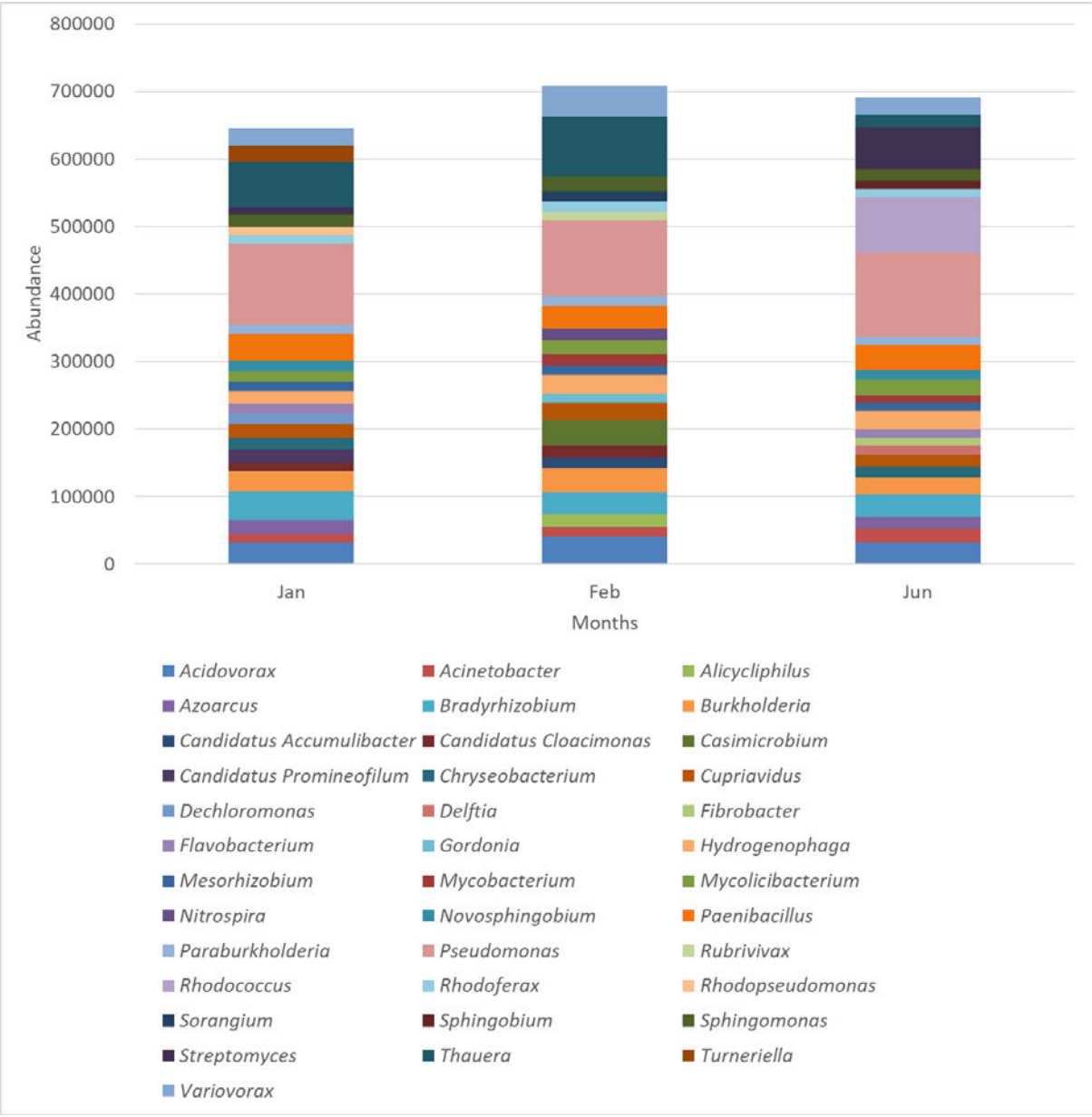


Fig. 5.11: Taxonomic distribution of the most abundant bacterial genera from WWTW B mixed liquor.

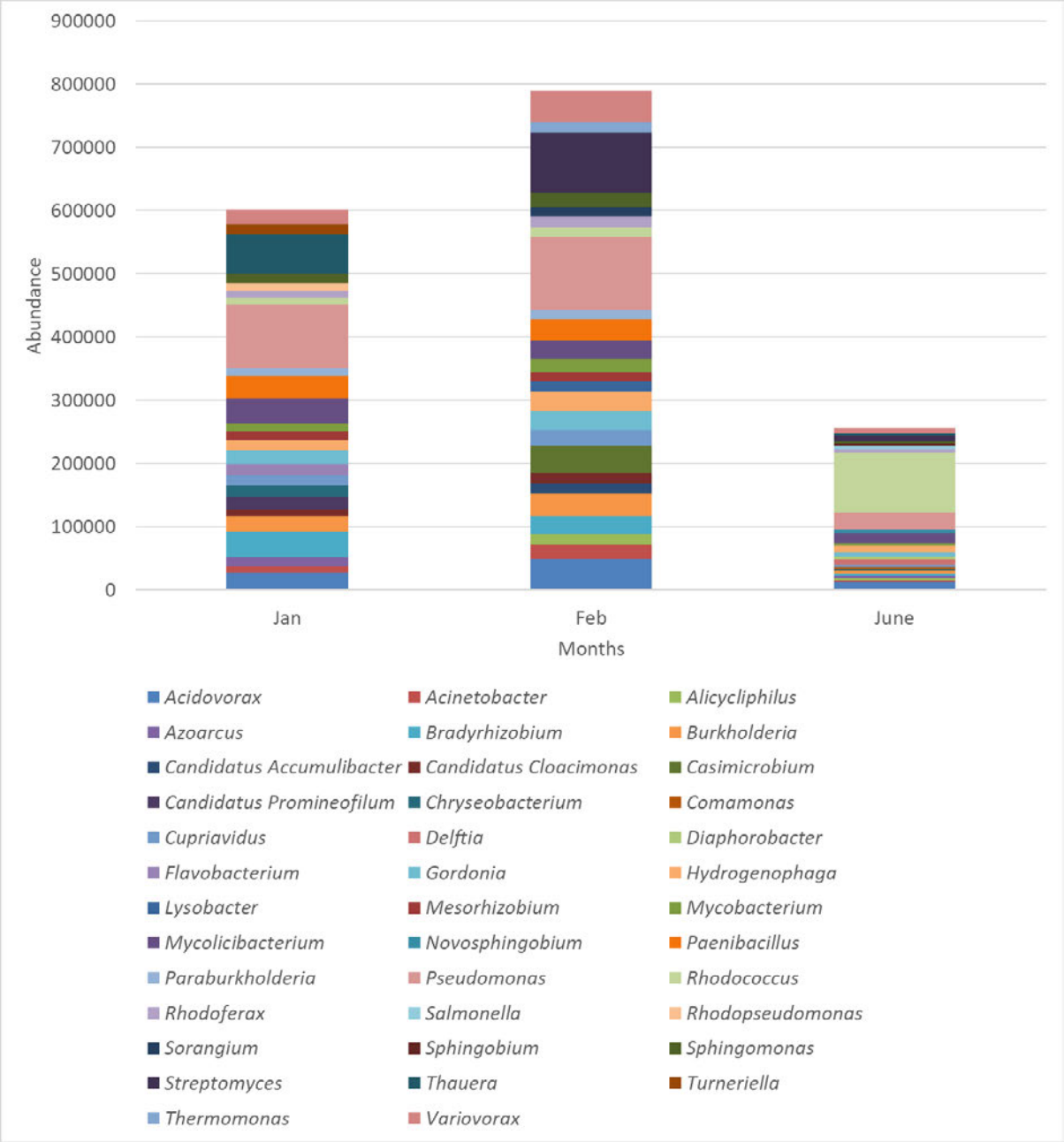


Fig. 5.12: Taxonomic distribution of the most abundant bacterial genera from WWTW B foam

5.3.4 The distribution of key mycolata in foam from WWTW A and WWTW B

The distribution of *Rhodococcus*, *Gordonia* and *Mycobacterium* spp. was investigated in WWTW A and B foam in Figs 5.13 to Fig 5.15. *Rhodococcus* and *Mycobacterium* had the greatest diversity although *Gordonia* spp. had overall richness in foam from both WWTW.

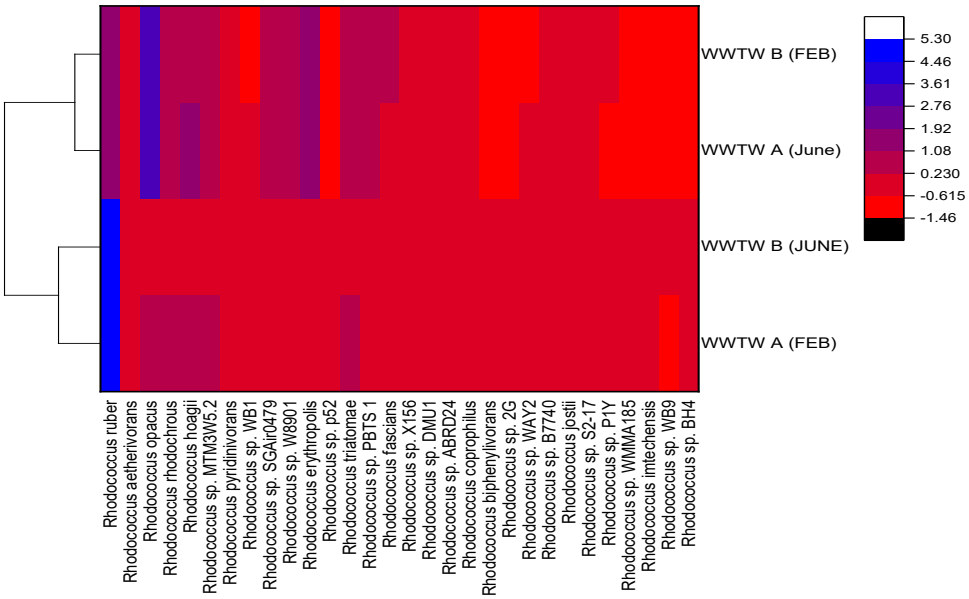


Fig. 5.13: Variation of *Rhodococcus* spp. from foam in WWTW A and WWTW B

Rhodococcus ruber was more abundant in February than June in WWTW A while it was more abundant in June in WWTW B. Other *Rhodococcus* spp. such as *Rhodococcus opacus* and *R. erythropodis* were also observed from both WWTW throughout the sampling period. Abundance varied between the two WWTW and *Rhodococcus hoagi* was more abundant in WWTW A than in B, during both months (Fig. 5.13). The prevalence of *Rhodococcus* spp. in foam from both WWTW highlights their persistence as core population since negligible temporal variation was observed from both WWTWs.

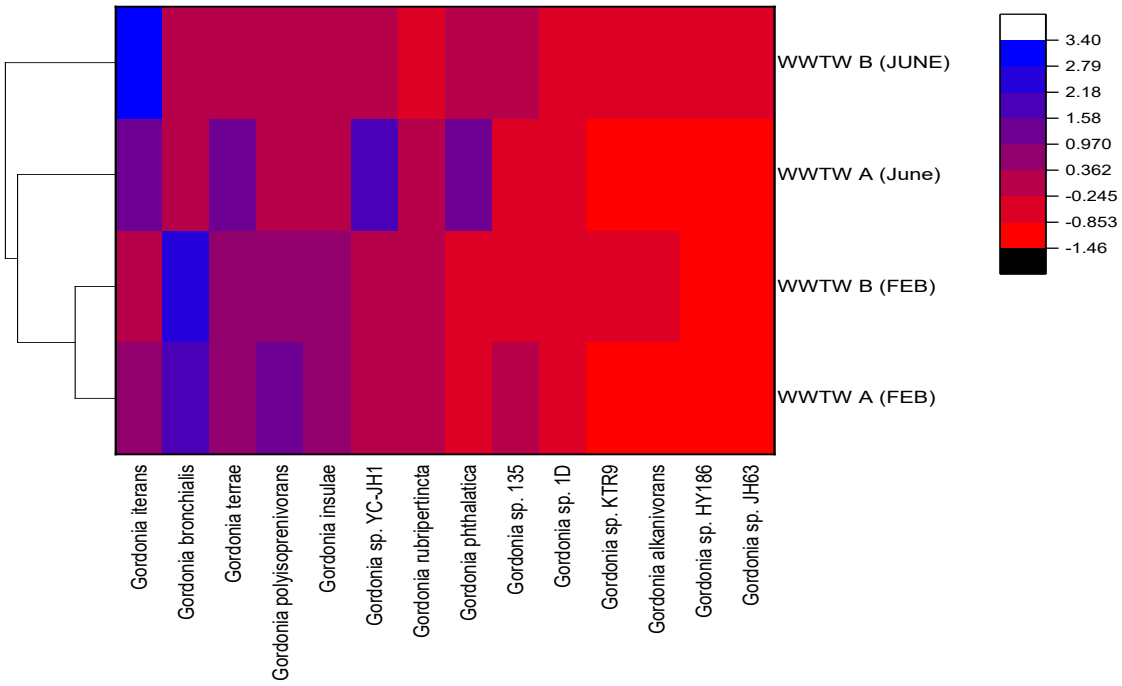


Fig. 5.14: Variation of *Gordonia* spp. from foam in WWTW A and WWTW B

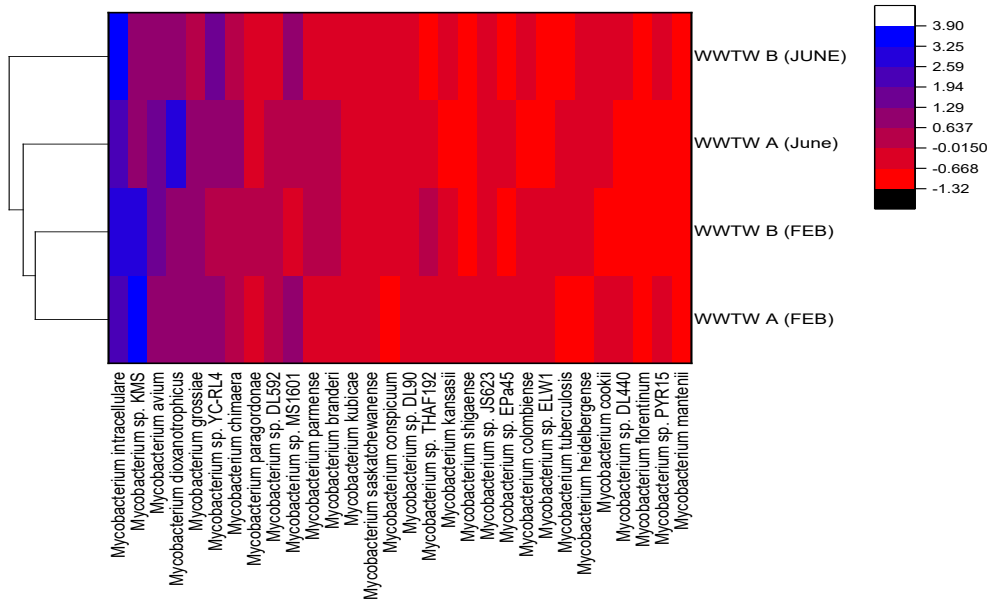


Fig. 5.15: Variation of *Mycobacterium* spp. from foam in WWTW A and WWTW B

The core genera observed in foam from WWTW A and WWTW B were *Gordonia*, *Rhodococcus* and *Mycobacteria* as observed in Figures 5.6, 5.13, 5.14 and 5.15.

Dominant *Gordonia* spp. population in both WWTW foam was similar as highlighted in Fig 5.6 and 5.14. Temporal variation in richness varied during the sampling period as observed in Fig 5.14. The core *Gordonia* spp. that were present throughout the study from both WWTW as observed in Figure 5.6 are *Gordonia alkanivorans*, *G. insulae*, *Gordonia iterans*, *G. phthalatica*. *G. polyisoprenivorans G. neurotica*, *Gordonia* spp. 135, *Gordonia* spp. 1D, *Gordonia* HY186, *Gordonia* JH63 *Gordonia* spp. HY 186, *Gordonia* spp. KTR 3, *Gordonia* spp. JH 63 and *G. terrae*. *G. iterans* was more abundant in June from both plants with most richness observed in WWTW B. *Gordonia* spp. overall richness was observed in June from WWTW A (Fig 5.14) whilst *Mycobacterium* spp. abundance in WWTW A was observed in February (Fig. 5.15).

5.4 DISCUSSION

Population dynamics in mixed liquor and foam samples of the two WWTWs treating domestic and industrial wastewater were investigated in this study. Based on alpha diversity estimates using Chao1 and Shannon indices, foam and mixed liquor microbiomes were found to differ significantly in their diversity and richness. Fig 5.1 highlights Chao 1 estimator, which indicated a decrease in estimated richness in foam than in mixed liquor, which indicates that greater OTUs were detected in mixed liquor than in foam as expected. Similarly, Shannon diversity index analysis revealed a higher species diversity in mixed liquor than in foam (Fig. 5.2), indicating more richness and evenness of microbial community in the mixed liquor. Indications by rarefaction analyses with the number of OTUs determined by Chao1 estimators revealed that an average of 70% of the estimated taxonomic richness was covered hence, a significant fraction of the bacterial diversity within the mixed liquor and foam samples was assessed at species and genus level. The uneven distribution of bacterial population and species richness between mixed liquor and foam may result from different physiological properties that existed between mixed liquor and foam affecting specific growth rates (Park *et al*., 2015 & de los Reyes, 2010). The reduced microbial richness in foam than mixed liquor has been reported by prior studies in ASP. For instance, results from a metagenomic study conducted from eight WWTWs in China showed that alpha diversities of total bacterial communities in foams were lower than those in ASP samples (Li *et al*., 2020) which supports the findings from this study.

The phyla that dominated in the mixed liquor and foam were *Proteobacteria, Actinobacteria, Bacteroidetes, Streptophyta* and *Firmicutes* as evident in Figure 5.3 A and B. Metagenomic analysis by Guo *et al*., (2015) found similar phylum level pattern of *Proteobacteria, Bacteroidetes, Actinobacteria, Nitrospira and Firmicutes*. A study that conducted seasonal fluctuations of microbial populations in foaming and non-foaming WWTWs found that Proteobacteria were the most dominant phylum in mixed liquor with *Actinobacteria* dominating in foams (Wang *et al*., 2016; Jiang *et al*., 2015). A significant reduction in *Proteobacteria* was also observed in this study in foam, with a notable increase in *Actinobacteria*. The Actinomycetes, particularly *Nocardiaceae*, *Mycobacteriaceae* and *Gordonia*ceae were more abundant in foams than mixed liquor, as observed in Fig. 5.5. *Microthrix, Nostocoida limicola II, Tetrasphaera, Rhodococcus, Tsukamurella, Mycobacterium, Skermania and Gordonia* spp. were identified as dominant genera in a bulking and foaming sludge in China (Jiang *et al*., 2016). In this study, the known bulking filamentous bacteria such as *Microthrix, Nostocoida limicola* II and *Skermania* were not detected both in mixed liquor and foam. This is supported by findings from chapter 3.3 using microscopic techniques. Foam formers that have dominated seem to be influenced by wastewater composition and operational parameters (Pal *et al*., 2014). This observation was also observed in this study as wastewater composition WWTW A and B had different populations of foam forming bacteria as observed in Figs. 5.8 – 5.15.

The microbial diversity in foam samples resulted in increased OTU levels of *Actinobacteria*, as evident in Figure 5.6. An average increase of 8.32% of *Actinobacteria* was detected in WWTW A and WWTW B foams, which was similar to the observation by Jiang *et al*. (2016). Figure 5.6 highlights the significantly differential abundant families in mixed liquor versus foam. The results revealed that *Gordonia*ceae were significantly enriched in foam than mixed liquor with a log2Fold reduction of -2.23. In previous studies, the metagenomic analysis of WWTWs in China also found *Gordonia* spp. as the most abundant species in foam samples (Jiang *et al*., 2016; Wang *et al*., 2016). Results in Fig 5.6 highlighted various *Gordonia* spp., *Mycolicibacterium doricum* and *Rhodococcus coprophilus* that were significantly enriched in foam than in mixed liquor. *Mycolicibacterium* was demarcated from *Mycobacterium* based on phylogenetic trees constructed using conserved genome sequences, comparative genomic analyses and average amino acid diversities (Gupta *et al*., 2018). The dominance of *Gordonia* spp. and *Mycobacterium* have also been reported as the most prevalent foaming bacteria by

Jiang *et al*., (2016). The species that were significantly higher in foams in this study belong to the families of *Gordonia*ceae, *Mycobacteriaceae* and *Norcadiaceae* as highlighted in Fig 5.5 from both WWTW. All these three families were previously observed from foaming episodes (Guo *et al*., 2015). *Gordonia* spp. that were most abundant in both WWTW are *Gordonia alkanivorans*, *G. insulae*, *Gordonia iterans*, *G. phthalatica*. *G. polyisoprenivorans G. neurotica*, *Gordonia* spp. 135, *Gordonia* spp. 1D, *Gordonia* HY186, *Gordonia* JH63 *Gordonia* spp. HY 186, *Gordonia* spp. KTR 3, *Gordonia* spp. JH 63 and *G. terrae*. However, the mostly studied foam former *Gordonia amarae* was not prevalent in this study and was not amongst the fourteen abundant *Gordonia* spp. in Fig 5.14. A prior metagenomic study on foam and mixed liquor samples also did not attribute foaming to *G. amarae* but found novel foam formers including an unidentified *Gordonia* spp. foam former (Guo *et al*., 2015). Foaming episodes attributed to *Gordonia* spp. other than *G. amarae* have been observed world-wide. Culture independent approaches have attributed foaming episodes to other *Actinomycetes*. Foam forming bacteria representing the genera *Millisia* (Soddell *et al*., 2006), *Skermania* (Chun *et al*., 1997) and *Tsukamurella* (Nam *et al*., 2004) have been recovered as dominant foam formers from ASP foams. *Gordonia* spp. such as *G. defluvii* have also been isolated from activated sludge foam (Soddell *et al*., 2006). Metagenomic analysis have attributed foaming episodes to *Mycobacterium* spp. (Rosso *et al*., 2018). *G. pseudamarae* was isolated as dominant formers that are closely related to *G. amarae* (Riesco *et al*., 2022), hence the presence of other *Gordonia* spp. from this study was not deemed as an anomaly.

In this study, attention has been given to mycolata for their contribution to foam stabilization. Among the different genera identified, *Mycobacterium* has also been implicated in foaming incidents (de los Reyes, 2010). This study also found that mycobacteria were significantly enriched in foam compared to mixed liquor in both WWTWs. The findings of Guo *et al*. (2015) were in accordance with this finding, which indicated that the role of *Mycobacterium* in foam stabilization. The persistence of mycobacteria in foam has been documented due to the presence of mycolic acids in their cell walls, imparting increased hydrophobicity (de los Reyes *et al*., 1997). The prevalence of *Mycobacterium* in AS was found to contribute significantly to the degradation of cholesterol present in municipal sewage (Guo *et al*., 2019). Thus, influent composition and ambient temperature of the aeration basin could be selective parameters that contributed to their dominance and prevalence in this study. The five species that were most

abundant were *M. brumae, M. crocinum, M. sphagni, M. vanbaalenii and M. aromaticivorans* (Guo *et al*., 2019) which were not amongst those detected in this study. *Mycobacterium doricum* was detected as a differentially enriched organism in foam than in mixed liquor from both WWTWs as observed in Fig 5.6. *Mycobacterium doricum* has been documented as an organism amongst bacterial consortia that have bioremedial application in petrochemical refineries (Ugya *et al*., 2019). As observed in this study (Fig. 5.15) the presence of opportunistic pathogenic species such as *Mycobacterium avium* complex poses a potential health risk (Guo *et al*., 2019). The presence of pathogenic mycobacteria in foam can cause health implications to WWTW personnel (Guo *et al*., 2015; Zhang *et al*., 2021). The presence of *Mycobacterium tuberculosis* from both WWTWs was attributed to excretory matter of infected people. The enrichment of influent with cholesterol has been shown to promote the growth of certain mycobacterial species (Guo *et al*., 2019), hence influent composition from WWTW B could possibly enrich mycobacterial growth since WWTW B also receives influent from a poultry processing plant as described in Chapter 3. A study conducted by Guo *et al*. (2015) found mycobacterial prevalence in foam samples isolated from Hong Kong. However, the prevalent strains were not pathogenic but reported as polycyclic aromatic hydrocarbon degraders (Guo *et al*., 2015). However, a recent South African study has detected pathogenic mycobacteria from wastewater using conventional and droplet digital PCR. The prevalence of *M. tuberculosis* in ASP has been documented in KwaZulu-Natal, South Africa (Mtetwa, 2021). Findings were made on the prevalence of *M. africanum* and *M. tuberculosis* from mixed liquor samples from wastewater obtained from surrounding areas in Durban, South Africa (Mtetwa *et al*., 2022). Earlier studies from Western Cape and Eastern Cape had also detected the presence of *M. africanum* as a causative organism of tuberculosis (Demers *et al*., 2010 and Bhembe and Green, 2020). The ability of mycobacteria to participate in foam stabilisation (Guo *et al*., 2019) requires that health risks associated with presence of mycobacteria in foams be further investigated due to transmission via aerosols. This information should be disseminated to the respective WWTW so that AS personnel can use appropriate personal protective equipment. Furthermore, understanding population dynamics in a specific WWTW can allow site specific remedial strategies. Therefore, understanding the infectivity and viability of human pathogenic mycobacteria is essential.

The settleability test of mixed liquor from WWTW B showed no settleability after thirty minutes, as shown in section 4.4 from all samples. However, the qPCR investigation using *Gordonia* specific primers yielded less than 10 000 copies/ng although foaming was observed in June. Metagenomic analyses also did not attribute foaming in June sample to *Gordonia* spp. as only 1% increase of *Gordonia* spp. was observed. However, *Rhodococcus* accounted for 56% of Actinobacteria in the foam sample as compared to 23% in the mixed liquor sample as observed in Figure 5.7 and 5.13. That amounts to a more than double fold increase in the foam sample. The dominant *Rhodococcus* spp. in the foam samples are outlined in Fig. 5.13. WWTW B receives influent from the textile and poultry industry as described in section 3.2.1 had a significant number of *Rhodococcus* spp., particularly *Rhodococcus ruber,* in mixed liquor and foam, compared to WWTW A. *Rhodococcus ruber* has also been detected amongst dominant foam formers, in a five-year study from a metagenomic investigation in China into bulking and foaming bacteria that their abundance level was 0.6 – 36% (averaging 8.52 ± 7.3) of total bacteria (Jiang *et al*., 2015). Earlier studies had reported bulking and foaming bacteria to range from 1.86 to 8.99% (Guo and Zhang, 2012) which was significantly lower than the findings from this study. That clarified low qPCR gene copies observed in 4.3 A and B in June from WWTW B using *Gordonia* specific primers. The application of Illumina sequencing provided clarity on other foam formers from WWTW A and B, such as *Rhodococcus, Mycolicibacterium and Mycobacterium*. Foaming due to more than one organism has been reported in previous studies. For example, the occurrence of *Gordonia* spp., *Rhodococcus* and *Mycobacterium* as co-dominant mycolata in foams has been reported by Stainsby *et al*., (2002). *Gordonia amarae*-like organisms and *Skermania piniformis* were observed using FISH coexisting from Danish WWTPs treating domestic and industrial phenomenon (Kragelund *et al*., 2007). Traditionally, various foam forming bacteria were implicated in foaming in previous studies (Blackall., 1994; Stratton 2003). However, quantification was not conducted to determine whether rhodococci were principal or secondary foam formers. *Gordonia* spp. was found to be the primary foam former from AS foam, as well as other organisms that contributed to foaming from the same location which included *Skermania* spp., *Mycobacterium*, *Tsukamurella*, *Tetrasphaera*, Nostocoida limicolla II and *Microthrix* (Jiang *et al*., 2015). This finding supports results from this study that foaming incidents can be attributed to more than one type of organisms. Studies have also shown that *Gordonia* spp. even if present as secondary filaments, can still induce foaming (Guo *et al*., 2015). Furthermore, foaming has been observed due to

biosurfactant production by mycolata, and not their enrichment in foam, this was observed when levels of mycolata rRNA in mixed liquor and foam did not have significant variation (de los Reyes and Raskin, 2002).

Traditional microscopic examination is inadequate as rhodococci in filamentous form exhibit a similar morphology as *Gordonia* spp. The relative abundance of *Gordonia* spp., *Rhodococcus*, *Mycobacterium* and *Norcadia* were investigated closely. Some mycolata such as *Skermania* and *Ca*. Microthrix were undetected during this study. *Skermania* has been observed in foams from various countries such as Australia, Denmark and China (Eales *et al*., 2006; Kragelund *et al*., 2007 and Li *et al*., 2020; Dueholm *et al*., 2022). Although *Skermania* are favoured by summer conditions and displayed metabolic versatility under Australian climatic conditions (Eales *et al*., 2006), they were undetected in South Africa (Deepnarain *et al*., 2020; Welz *et al*., 2022). The absence of *M. parvicella* which are commonly detected from foaming samples along with *Gordonia* could be attributed to high temperatures experienced during sampling period. Foaming and bulking due to *M. parvicella* is largely observed under low temperatures (12- 15ºC) (Wang *et al*., 2016; Fan *et al*., 2019). *Tsukamurella* had an average abundance of less than 1% in all samples rendering the genus insignificant as a potential foam former. Although this study focused on *Gordonia* spp., the findings suggest that further analysis is imperative to identify biological foam formers and stabilisers across all seasonal patterns.

Gammaproteobacteria are prevalent in activated sludge as fundamental organisms for compact floc formation (Rosso *et al*., 2018; Zhang *et al*., 2021). The enrichment of *Pseudomonas* and *Acinetobacter* in foams was detected North Carolina and China. Another study found an increase in *Clostridiaceae*, *Pseudomonas* and *Staphylococcaceae* in foaming ASP (Shchegolkova *et al*., 2016). *Pseudomonas putida* was detected as a secondary enriched organism from foam as shown in Table 5.1 (Zhang *et al*., 2021). *Acinetobacter* and *Pseudomonas* are amongst the genera that have been most abundant in the aeration basins of ASP in another study (Rosso *et al*., 2018). Some organisms may only secrete foam related compounds during specific metabolic phases or when exposed to certain substrates. Some *Pseudomonas* spp. may only produce biosurfactants in the presence of hydrocarbons or certain waste products, thereby linking foam formation to the co-metabolism of specific organic compounds.

Table 5. 1: Comparison of primary and secondary foam foaming bacteria from activated sludge plants

Country of study	Period of study	No. of samples	Primary foam formers			Secondary foam formers			Reference
			1	2	3	1	2	3	
China (Hong Kong)	January – March June – July 2010	8	*Gordonia spp.*	*Mycobacterium*	*Actinomycetales*	*Clostridium XI*	*Arcobacter*	*Flavobacterium*	Guo *et al*., 2015
China (Zhengzhou)	January – July 2012	10	*Ca M. parvicella*	*Trichococcus*	*Tetrasphaera*	*Beggiatoa*	*Rhodobacter*	*Mycobacterium*	Wang *et al*., 2016
North Carolina	Apr-15	1	*Mycobacterium*	*Gordonia spp.*	*Rhodococcus*	*Acinetobacter*	*Decholomonas*	*Nitrospira*	Rosso *et al*., 2018
China (Hangzhou)	July – October 2015	5	*Myroides spp.*	*Mycobacterium smegmatis*	*Bacteriodes fragilis*	*Pseudomonas putida*	*Stenotrophomonas maltophilia*	*Not stated*	Zhang *et al*., 2021

Human pathogenic bacteria such as *Enterobacteriaceae*, *Staphylococcus aureus* and *Pseudomonas aeruginosa* have been detected as aerosols in WWTW which confirmed the transmission route of human pathogenic bacteria (Zhang *et al*., 2021). Enriched primary and secondary organisms in AS foams have been highlighted in Table 5.1. The detection of non-filamentous, opportunistic human pathogens such as *P. putida*, *Acinetobacter*, *Arcobacter* in foams was also noted in Table 5.1. Earlier findings that *Alphaproteobacteria* increased considerably along with the occurrence of sludge foaming because of the increase in some functional bacteria such as *Rhodobacter*.

Information has been generated on the influence of environmental factors, influent composition and geographical locations on foam formation, but more research is required to understand microbial co-metabolism to better understand how these variables interact to promote or inhibit foam formation. Rosso *et al*., (2018) attributed foaming to recalcitrant organic molecule degradation. *Acinetobacter* and *Dechloromonas* were enriched during foaming episode, and it was attributed to influent composition. However, *Sphingomonas*, *Pseudomonas* and *Sinorhizobium* with similar capabilities were not enriched. Therefore, metabolic activity to recalcitrant metabolism is only suggestive. In the same study, *Acinetobacter* proliferation was not accounted for. Composition of functional groups for nitrogen removal was altered when sludge foaming occurred. *Trichococcus* which possesses denitrifying potential were more abundant in foaming (Table 5.1). Similarly, significant changes in the composition of the phosphorus removal groups were observed when foaming occurred. Findings by Wang *et al*., (2014) found that *Ca*. M. parvicella might have been responsible for phosphorus removal. *Ca*.

M. parvicella and *Tetrasphaera* had high abundance during foaming whilst they were both low in non-foaming sludge. The abundance of functional groups such as *Beggiatoa*, *Nitrospira* and *Acinetobacter* as secondary foam formers ensures that sulphur, nitrogen and phosphorus are optimally removed even during episodes. However, conditions that enable their enrichment must be further investigated. Furthermore, understanding the population of foam formers within a specific site, would enable site specific remedies and interventions.

Site specific remedies that can be adopted by WWTW A and B include skimming systems, pump inlet systems and nanobacteria (Pal *et al*., 2014; Petrovski *et al*., 2022). Nonspecific measures such as skimming baffles and pump inlets systems can be placed in the aeration tanks. Skimming weirs can be placed at strategic positions to encourage movement of scum towards entrapment. Pump inlet systems can remove materials on the liquid surface and pump it to the plant wasting system (Pal *et al*., 2014). Biological foaming control using phages has limitations such as the conversion of non-pathogenic *Gordonia* spp. to pathogenic forms (Dyson *et al*., 2015). However, parasitic nanobacteria such as *Saccharibacteria*, have been proven to be effective against mycolata (Petrovski *et al*., 2022). This technology can be adopted by the two respective WWTWs because they are prone to foaming my mycolata.

5.5 CONCLUSION

The metagenomic analysis provided tangible information on the significance of mycolata on foam formation. Phylum abundance revealed an increase in *Actinobacteria*, with subsequent population shifts in *Gordonia* spp., *Mycobacterium* spp. and *Rhodococcus* spp.

- NGS revealed higher richness in foams as 2-fold decreases were observed in mixed liquor than in foams due to foam formers belonging to *Actinobacteria*.
- The prevalence of specific foam formers is strongly affected by influent composition, WWTW operational parameters and prevailing temperature of the WWTWs. *Ca.* M. parvicella although it has been implicated globally in foaming was not detected by metagenomic analysis likely due to the ambient temperature of this region and season during foaming episodes. The foaming episodes in this study occurred during the warmer summer season when *Ca.* Microthrix is not favoured. This finding supports previous findings by other researchers.
- The microbial composition between mixed liquor and foams was similar throughout the sampling period, however species richness of certain mycolata changed significantly during foaming.
- There was reduced diversity and richness from June foam samples from both WWTW. WWTW A also exhibited reduced diversity in June from mixed liquor.
- Although *Gordonia* spp. were the core foam formers from both WWTW, other foam formers were detected. Metagenomic analysis provided further insight into other foam formers that predominated during foaming episodes particularly in WWTW B treating industrial wastewater.
- Metagenomic studies provided further insight into different foam forming organisms such as *Rhodococcus*, and *Mycobacterium* which predominated in foam samples from both the WWTW.
- The prevalence of pathogenic mycobacteria in foams poses a health risk to WWTW personnel.
- The physiological traits of the prevalent foam foaming bacteria (*Gordonia* spp., *Rhodococcus* and *Mycobacterium* spp.) that were abundant in WWTW can be applied

as a basis of site-specific remedial strategies that can be employed to pre-empt and eliminate foaming incidents.

CHAPTER 6: GENERAL CONCLUSIONS AND RECOMMENDATIONS

6.1 CONCLUSIONS

The study examined the prevalent actinomycetes in the foam and mixed liquor samples of two full-scale ASP using a combination of microbiological and molecular biology techniques.

The major conclusions drawn from this study are:

- Conventional microscopy revealed that the filamentous bacterial populations present in mixed liquor samples from WWTW A differed from those present in WWTW B. Among these, Eikelboom Type 0041, *Thiothrix*, *Gordonia* spp., Eikelboom Type 021N and Eikelboom Type 0092 were the predominant morphotypes observed in WWTW A. Filamentous morphotypes Eikelboom Type 0041, *Gordonia* spp. and Eikelboom Type 021N were observed as dominant filaments throughout the sampling period whilst Eikelboom Type 1851 and *Thiothrix* were observed as transient filamentous bacteria from WWTW B. Microscopic examination of foam samples revealed the abundance of right-angled branched filamentous actinomycetes in both WWTWs, with more prevalence in WWTW B than WWTW A. Filamentous actinomycetes in WWTW A ranged from common to dominant filament index whilst WWTW B had a filament index ranging from dominant to excessiveness.

- The pre-treatment of mixed liquor and foam samples prior to micromanipulation eliminated contamination during isolation. The twenty-seven isolates that survived micromanipulation were monitored for growth and contamination during isolation. However, culture maintenance and preservation were challenging as loss of viability was observed during subculturing. Additionally, the optimum growth temperature of micromanipulated filaments was observed at 30^{o}C under aerobic conditions. Furthermore, the isolates varied in their carbon utilization. The nocadioform morphological traits were maintained by all isolates except one isolate that displayed single-celled morphology after continuous subculturing.

- The abundance of *Gordonia* spp. was determined through qPCR using *Gordonia* spp. specific primers (G699F and G1096R). The amount of *Gordonia* spp. in foams was higher than mixed liquor samples and two-fold increases were sufficient to induce foaming. The

application of qPCR attributed foaming incidents that were experienced to the prevalence of *Gordonia* spp. and favourable temperatures. The low F/M ratios in WWTW B were also experienced during foaming episodes.

- The application of NGS elucidated the active foam formers in the region and established the existence of indigenous *Gordonia* spp., *Rhodococcus*, and *Mycobacterium* in foams. The prevalence of mycobacteria in foam from both WWTWs can potentially pose a health risk to WWTW personnel. Illumina 16S rRNA gene sequencing further revealed heightened abundance of *Gordonia* spp. in foams compared to mixed liquor which supported qPCR findings. The physiological traits of the prevalent foam foaming bacteria (*Gordonia* spp., *Rhodococcus* and *Mycobacterium* spp.) that were abundant in WWTWs can be applied as a basis of site-specific remedial strategies that can be employed to pre-empt and eliminate foaming incidents. *Ca*. M. parvicella was undetected during this study, this can be attributed to environmental conditions that prevailed during the sampling period that eliminated the growth of this psychrotrophic organism.

6.2 SIGNIFICANCE AND NOVELTY OF RESEARCH

- Successful isolation through micromanipulation of *Gordonia* spp. and growth monitoring was done. Furthermore, suitable growth conditions for this region were determined for the isolates. Successful isolation can improve the current situation of filamentous bacteria that have *Candidatus* status.

- Quantification using qPCR and Illumina 16S rRNA gene sequencing provided expansion on the taxonomic width of potential foam formers and their enrichment in foam. The prevalence of other foam formers requires that qPCR be expanded to include primers that are specific to other mycolata that have been implicated in this study and not just limited to *Gordonia* spp. This will avoid limitations of microscopic examination that eliminated organisms such as rhodococci and mycobacteria that do not exhibit filamentous morphology.

- This study has elucidated the microbial community that persists in foams in the two respective plants using metagenomic analysis. Such an in-depth study has not been conducted from mixed liquor and foam samples in South Africa.

- The findings from the current study have set the foundation for future application of these advanced molecular techniques in understanding the diversity and role of mycolata in foaming.

6.3 RECOMMENDATIONS

- It is recommended that the isolates be subjected to whole genome sequencing to further investigate their metabolic characteristics based on their genomic composition. Efforts must be made to examine the prevalence of single-celled rods or coccoid cells in foam. Microscopic examination of organisms that exhibit nocardioform morphology eliminates isolation of rhodococci from foam and mixed liquor.

- The application of *Gordonia* spp. primers in qPCR eliminated the quantification of other possible foam-formers that were present and detected using Illumina sequencing. Therefore, new primers must be investigated that will target all implicated Actinomycetes in foaming from this region. Simultaneous application of group specific primers during qPCR would be more appropriate coupled with Illumina 16S rRNA sequencing to correlate their abundance to metabolic and functional roles.

- Establish foam-management strategies that are specific to the causative organisms as mycobacterial detection in foam may indicate biosafety concerns. It is recommended that foam-forming population during winter season be investigated to monitor seasonal population shifts that may prevail.

- The application of bacteriophages or rotifers that infect foam foaming bacteria has been noted as a promising alternative due to its specificity. This biological application must be investigated for its ability to reduce filamentous bacteria that prevail in this region.

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APPENDICES

Appendix 1: Physico-chemical characterization (Chapter 4)

Physical observations

During sample collection, temperature, pH, dissolved oxygen and oxidation-reduction potential were measured using calibrated electrode (YSI 556MPS, *Yellow Springs*, USA). During sampling, observations for the colour and thickness of the foam in the aeration basin reactors were made.

Chemical analysis

All the samples collected were filtered through the 0.45µm glass fibre filter using the membrane filtration technique and concentration of phosphate, nitrite, total dissolved nitrogen, ammonia, chloride and sulphate were measured using Gallery automated photometric analyzer according to standard methods. Other analyses that were conducted include chemical oxygen demand (COD), biological oxygen demand (BOD_5), mixed liquor suspended solids (MLSS) and mixed liquor volatile suspended solids (MLVSS) according to standard method (APHA-AWWA-WPCF, 1998).

Chemical Oxygen Demand

Culture tubes were washed with sulfuric water before use. Five millitres of each collected samples followed by 0.1 ml of H_2SO_4, 3 ml of the digestion solution and 7 ml of the sulfuric acid reagent were added into the tubes. Tubes were capped and mixed thoroughly. The tubes were placed on the digester preheated to 150 °C for 2 hours. After 2 hours, samples were cooled to room temperature and transferred to COD vials and the samples were ran on the Galley automated photometric analyzer (Thermo Scientific, UK) (APHA-AWWA-WPCF, 1998).

Mixed Liquor Suspended Solids and Mixed Liquor Volatile Suspended Solids

Filter preparation, 0.45 µm glass fiber filter papers were placed on the vacuum flask, using the membrane filtration, three times 20 ml of distilled was filtered for each filter. The filters were then placed on a weighing dish and stored in the drying oven with the temperature of 103°C-105°C for one hour. After an hour, the filters were stored on the desiccator for future use.

Mixed liquor suspended solids analysis: For each set of duplicate samples, samples were mixed thoroughly and poured into 250 ml beakers. Filters were taken from the desiccator and labelled accordingly. Each 0.45 µm glass fiber filter placed on the crucible were individually weighed and the initial weight was recorded. Using a pair of tweezers, filters were placed on the vacuum flask. 250 ml of the sample was filtered, and 10 ml of distilled water was added to rinse of the remaining residues. Filter was placed back on the crucible. Filters were then placed in the 103°C - 105°C drying oven overnight. Filters were then placed on the desiccator to cool for 30 minutes, and the final weight was recorded. The MLSS concentration was calculated using equation 1.

$$\text{MLSS} = \frac{(\text{Initial weight} - \text{final weight}) \times 1000}{\text{sample volume}} \quad (1)$$

Mixed liquor volatile suspended solids analysis: Glass fiber filters (0.45 µm) from the MLSS test were used. The final weight from the MLSS test was recorded as the initial weight for the test. The glass fiber filters were placed on the muffle furnace at 550°C for 20 minutes. After 20 minutes the glass fiber filter papers was placed on the desiccator for 30 minutes to cool, weighed and the final weight was recorded. The mixed liquor suspended solids was calculated using equation 2 (APHA-AWWA-WPCF, 1998).

$$\text{MLVSS} = \frac{(\text{Initial weight} - \text{final weight}) \times 1000}{\text{sample volume}} \quad (2)$$

Sludge Volume Index

Aeration samples were mixed thoroughly and poured onto a 1000 ml beaker. The samples were left to settle for 30 minutes. Thereafter the settled sludge was measured, and the SVI was calculated using equation 3.

$$\text{SVI (mL/g)} = \frac{\text{Settled volume}}{\text{Total suspended solids}} \times 1000 \quad (3)$$

Appendix 2: Determination of Total and volatile solids dried (chapter 4)

Determination of Total Suspended solids

Procedure:

A volume of 100 mL of wastewater mixed liquor was measured into a centrifuge tube. Centrifuged at 4000 rpm for 5 min. Supernatant was discarded and quantitatively scoop the sludge into a pre-weighed crucible. Crucible was placed in a drying oven at 105˚C and leave overnight. Then, removed from oven and allowed to cool in a desiccator. Crucible was re-weighed. The MLSS was determined according to the following calculation:

TS (g/L) = mass of (crucible + sludge) – mass of (crucible) in Grams x 10/100 mL

Appendix 3: Melt curve plot from qPCR using *Gordonia* specific primers producing a single peak at 86.5ºC for all samples.