Development and Optimization of Remedial Measures to Control Filamentous Bacteria in a Full-scale Biological Nutrient Removal Plant

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(B.Tech: Biotechnology)

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DECLARATION

Development and Optimization of Remedial Measures to Control Filamentous Bacteria in a Full-scale Biological Nutrient Removal Plant

Nashia Deepnarain

I hereby declare that the dissertation herewith represents my own work. It has not been submitted before for any diploma/degree or examination at any other Technikon/University

_____ _____

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I hereby approve the final submission of the following dissertation.

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This _____ day of _____ 2014, at the Durban University of Technology.

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DEDICATION

I dedicate this work to my late grandmother whom I miss dearly & to my parents, who have been my strength and guiding light in all obstacles and challenges that crossed my path.

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ABSTRACT

Wastewater treatment plants (WWTPs) frequently experience bulking and foaming episodes, which present operational challenges by affecting sludge settling due to the excessive proliferation of filamentous bacteria. Various control strategies have been implemented over the years to minimize filamentous growth, however, filamentous bulking still remains an unresolved problem in many WWTPs worldwide. The current study focused on developing and optimizing remedial measures *viz.*, specific and non-specific methods to reduce problematic filamentous bacteria in a full-scale WWTP. Specific methods demonstrated the influence of plant operational parameters *viz.* chemical oxygen demand, influent $N\text{-}NH_4^+$, food to microorganism ratio, dissolved oxygen, temperature and pH on the abundance of filamentous bacteria. A cumulative logit model was used to determine the significant relationships between the individual filamentous bacteria at present and the prevailing plant operational parameters. Using the above statistical approach, significant observations and predictions were made with respect to the individual filamentous growth under certain operational parameters. With further validation, this model could be successfully applied to other full-scale WWTPs identifying specific parameters that could contribute to filamentous bulking, thus providing a useful guide for regulating specific filamentous growth. Non-specific control methods such as chlorine, ultraviolet irradiation and ozone treatment were investigated on filamentous bacteria using a live/dead staining technique. To achieve at least 50% reduction of filamentous bacteria, a chlorine dose of 10 mg Cl_2/L was required, all filaments were killed at a dose of 22 mg Cl_2/L. In addition, an effective UV and ozone dose of 4418.91 μw seconds/cm^2 and ±20 mg O_3/L respectively, was required to kill 50% of the filamentous bacterial population. Among the three non-specific methods, ozone treatment seemed to be an effective method in controlling the filamentous population with a low negative impact to the surrounding environment. This study serves as a useful guide on the problems and control of filamentous bulking in activated sludge plants.

PREFACE

Aspects of the work covered in the following dissertation can be found in the following conference presentation:

Nashia Deepnarain, Sheena Kumari, Jordache Ramjith, Kriveshin Pillay, Faizal Bux. A Predictive Model to Minimize Filamentous Bulking in Wastewater Treatment Plants. Oral presentation in: *Microbial Ecology and Water Engineering*: proceedings of the 5th International IWA conference at the University of Michigan, Ann Arbour, USA, 7-10 June 2013.

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Nashia Deepnarain, Sheena Kumari, Faizal Bux. Evaluation of Filamentous Bacteria in a Biological Nutrient Removal Wastewater Treatment Plant. Poster presentation in: 2nd Regional Conference of Southern African Young Water Professionals conference at the CSIR International Convention Centre, Pretoria, S.A, June 2012.

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ABBREVIATIONS

AIC	Akaike Information Criterion
ANN	Artificial Neural Network
AOB	Ammonia Oxidizing Bacteria
AS	Activated Sludge
ASP	Activated Sludge Process
BNR	Biological Nutrient Removal
BOD	Biological Oxygen Demand
Cl_2	Chlorine
CLASI-FISH	Combinatorial Labelling And Spectral Imaging FISH
COD	Chemical Oxygen Demand
DAPI	4′, 6′-Diamidino-2-Phenylindol
DNA	Deoxyribonucleic Acid
DO	Dissolved Oxygen
DSVI	Diluted Sludge Volume Index
EPS	Extracellular Polysaccharide
F/M	Food to Microorganism Ratio
FA	Formamide
FI	Filament Index
FISH	Fluorescent *in situ* hybridization
FISH-MAR	Fluorescent *in situ* hybridization - Microautoradiography
FLUOS	5(6)-carboxyfluorescein-*N*-hydroxy-succinimide
MLSS	Mixed Liquor Suspended Solids
NA	Nitrogen Activity
N_2	Nitrogen gas
NH_3	Ammonia
NOB	Nitrite Oxidizing Bacteria
O_3	Ozone
OUR	Oxygen Uptake Rate
PAO	Polyphosphate Accumulating Organisms
PBS	Phosphate Buffered Saline
PCR	Principal component regression

PHB	Poly-β- Hydroxybutyrate
RAS	Return Activated Sludge
RBCOD	Readily Biodegradable COD
SAS	Statistical Analysis Software
SC	Schwarz Criterion
SSVI	Settled Sludge Volume Index
SVI	Sludge Volume Index
THM	Trihalomethanes
UCT	University of Cape Town
UV	Ultraviolet Light
WWTP	Wastewater Treatment Plant

1. INTRODUCTION

Water is the most crucial of limiting resources to humans and to every natural process on earth. In order to protect and manage the available water reservoirs in our country, the South African government had promulgated the National Water Act in 1998 to focus on improving water and wastewater management systems. Protecting water quality is an imperative concern as it allows for the conservation of a scarce resource as well as the prevention of eutrophication in the receiving water bodies. According to the latest Green Drop Report (2012), more than 50% of the country's 821 wastewater treatment works treating megalitres of effluent received each day were well below acceptable standards. Thus, the impact of wastewater discharged into reservoirs is of primary concern due to current treatment methods failing to address pollution levels in over half of discharges.

The activated sludge (AS) system is the most widely used technology for the treatment of domestic and industrial wastewater (Guo *et al.*, 2012). Bacteria, that make up approximately 95% of the total microbial population in AS, are vital for the removal of nutrients and organic material in the treatment process (Bitton, 1994; Jenkins *et al.*, 1993). Among these, floc formers and filamentous bacteria were found to be the essential bacteria involved in the AS process, where heterotrophic bacteria form the bulk of the floc formers and filamentous bacteria form the backbone of the floc (Jenkins *et al.*, 1993). Each network of bacteria within the floc forms functional groups which play a significant role in the removal of phosphorus, ammonia ($N\text{-}NH_4^+$) and organic carbonaceous compounds. Under optimum conditions, floc-forming bacteria were shown to predominate, resulting in good sludge settling characteristics and excellent settling properties such that there can be adequate separation in the final clarifier, thus resulting in a clear effluent (Bitton, 1994; Seviour, 1999).

Bulking is a phenomenon which describes the inability to separate solids from treated effluent in the clarifiers, and it is caused by the abundance of filamentous bacteria. These organisms were found to extend out of the floc structure and form interlinks between the flocs, subsequently affecting the sludge settling (Contreras *et al.*, 2004; Jenkins *et al.*, 1993). As a result, the final effluent had high levels of suspended solids, and consequently, a reduced sludge was recycled back into system. Thus, the plant efficiency of the treatment process was exacerbated and the plant failed to comply to acceptable discharge standards

(Lou and Zhao, 2012). Some of the common filamentous bacteria detected from bulking samples worldwide included *Haliscomenobacter hydrossis*, *Microthrix parvicella*, Nocardiaforms, *Sphaerotilus natans*, *Thiothrix* spp., Type 0041, Type 0092, Type 0675, Type 1701 and Type 021N (Gerardi, 2006a). "Type" relates to filamentous morphotypes that have been identified by conventional methods, however, these filamentous bacteria were not fully characterised and classified (Jenkins *et al.*, 2004a).

To date, filamentous bulking is a common and serious problem in the operation of AS plants worldwide (Martins *et al.*, 2004; Mielczarek *et al.*, 2012; Sezgin *et al.*, 1978). The overgrowth of these organisms were associated with various operational parameters. Despite much research, the conditions that promoted the abundance of filamentous bacteria still remained unclear. Therefore, there was a need to understand the microbial contribution to this problem and to evaluate current remedial measures in order to remediate overgrowth of filamentous bacteria in the return activated sludge (RAS) stream, hence, reducing the problem of bulking (Jenkins *et al.*, 2004b; Martins *et al.*, 2004).

Various control measures are currently being used to target excessive filamentous bacteria. Some of these methods included the use of coagulants and flocculants (*viz.*, aluminium sulphate, aluminium chloride, ferric chloride, ferrous chloride, polyaluminium chloride, talc), chlorine, hydrogen peroxide and the use of ozone (O_3). However, such methods only temporarily hindered bulking and were very expensive to apply in full-scale wastewater systems. In addition, these methods were not always effective and did not eliminate the cause of the problem (Jenkins *et al.*, 2004a; Leeuwen, 1992). Specific and non-specific control strategies have been widely implemented over the years, however, a suitable method to eliminate bulking had not yet been unravelled. Cost and maintenance of wastewater treatment plants (WWTPs) continued to further increase and the control of filamentous bacteria via specific techniques were still far too complicated (Jenkins *et al.*, 1993; Martins *et al.*, 2004; Mielczarek *et al.*, 2012).

Although the distribution of filamentous bacteria varies from plant to plant, the presence of certain filamentous bacterial types is an indication of certain conditions prevalent within AS systems. Thus, filamentous bacteria can be used to identify the functional condition of the sludge. Identifying filamentous bacteria and monitoring their abundance allowed operators to estimate the potential quality of the effluent based on the condition of the sludge (Jenkins *et*

al., 1993). An assessment of filamentous bacterial density among the biocoenosis in AS has been found to be critically important in evaluating the efficiency of wastewater treatment systems. The appearance of different filamentous bacteria was found to be associated with specific cultivation conditions and operational parameters (Wanner *et al.*, 1998). For instance, Type 1701 indicates low DO, Type 1851 indicates low F/M ratio, *Thiothrix* I and II indicates septicity, *Gordonia amarae* indicated a high grease and oil content (Jenkins *et al.*, 1993; Richard *et al.*, 2003). Once the indicator organism had been identified, conditions such as mixed liquor suspended solids (MLSS), RAS flow, pH and nutrients could be modified or controlled in order to minimise the growth of these organisms. Some of the physical parameters which could very well correlate to filamentous population included; sludge volume index (SVI), biochemical oxygen demand (BOD), DO, MLSS, pH, F/M, temperature, sludge wastage, and sludge retention time (Lou and Zhao, 2012; Martins *et al.*, 2004).

This study focused on understanding the *in situ* behaviour of the dominant filamentous bacteria within a full-scale biological nutrient removal (BNR) plant. In addition, specific operational parameters and non-specific chemical/physical control measures to reduce filamentous bacteria in the return AS stream were identified. A cumulative logit model was applied in this study on specific operational parameters, using statistical analysis software to determine significant relationships and predictions of the filamentous bacterial occurrence over a range of operational parameters. The developed model, after validation across many plants, could be used as a tool to control filamentous bulking. The latter method (i.e. non-specific control), included the use of chemical and physical treatment (chlorine, UV light and ozone) which can be applied to the RAS stream line in full-scale systems to aid in the reduction of filamentous bacteria when these organisms are excessive.

2. LITERATURE REVIEW

2.1. Activated Sludge Process

The activated sludge process (ASP) was initially designed for the removal of biodegradable organic material from industrial and domestic wastewater, during the early 1900s. Edward Arden and William T. Lockett significantly contributed to the preliminary operation of the ASP, they discovered that the addition of sludge into wastewater, together with aeration, caused the organic matter to be removed at a much faster rate than without aeration (Hartmann, 1999; van Haandel and Van der Lubbe, 2012a). By providing oxygen to the mixture of microorganisms in the raw wastewater, the microbes were capable of metabolising the organic material. The mixed liquor in the aeration tank, was found to contain a mixture of microbial biomass and organic matter, and the microbes in the AS were able to degrade about 95% of the organic matter, with the supplementation of oxygen. These organics were found to be broken down via aerobic cellular respiration into simpler compounds predominantly by heterotrophic bacteria, as a result producing carbon dioxide and water (Cheremisinoff, 1996).

A flow diagram for the ASP is illustrated in Figure 2.1, the design structure primarily consists of an aerobic tank, a settling tank (secondary clarifier) and the RAS stream. In the aerobic tank, incoming waste together with the microbial biomass and diffused oxygen supply assisted in the removal of organic material (Cheremisinoff, 1996; Martins *et al.*, 2004).

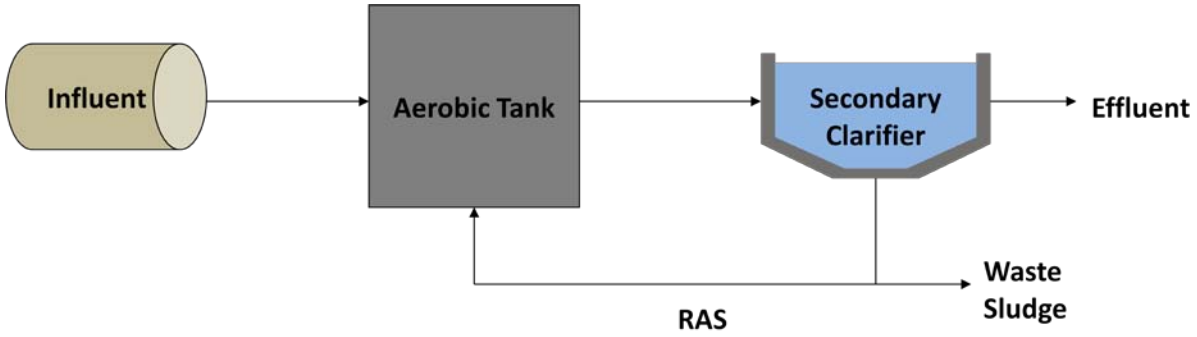

Figure 2.1: Schematic diagram of a typical ASP design with a settling tank (secondary clarifier) (Seviour *et al.*, 2010).

Following aeration (aerobic tank), the organisms were subsequently allowed to settle in the secondary clarifier over a period of time. The secondary clarifier provided an environment which allowed the AS solids to separate from the liquid and thus settle to the bottom of the tank, this separation occurred by flocculation and gravity sedimentation. The primary purpose of the clarifier was to clarify water for discharge by separation from biomass (Jenkins *et al.*, 2004b). The biomass is then recycled via a return activated sludge stream (RAS) transferring settled sludge from the secondary clarifier to the beginning of the process (Figure 2.1). The separation of biomass from the liquid in the secondary clarifier was necessary to achieve an effective, high-quality effluent (Martins *et al.*, 2004).

2.1.1. Modifications of the Initial Activated Sludge Process and its Effects on Sludge Settling and Nutrient Removal

A proficient understanding on the historical overview of the ASP was extensively explored by Jeppson (1996) and further knowledge on the development of the different process configurations, have been explained by researches in great detail (Albertson, 1987; Albertson, 1991; Seviour *et al.*, 2010). Over the years, due to increased human activities and industrialization, the organic loading and the nature of these systems have drastically changed, thus, the process had been subjected to various modifications (Jeppson, 1996). Due to these modifications, several other problems occurred such as, reduced DO level, organic overloading, inadequate sludge settling and excess sludge production (Seviour *et al.*, 2010). The most widespread problem was poor sludge settling and this was primarily due to the overgrowth of filamentous bacteria (Jenkins *et al.*, 2004a; Jeppson, 1996).

With several modifications, plug flow systems and tapered aeration systems developed in the early 1930s, were thought to reduce filamentous bacterial predominance and thus increase the settleability of the sludge. These systems were modified to assist in treating influents with high organic loading and encourage nitrification over longer hydraulic retention times, as well as to further overcome the imbalances of diffused oxygen (Grady *et al.*, 1999; Seviour *et al.*, 2010). The return sludge was mixed with the raw influent at the head of the aeration tank, thus requiring a higher oxygen demand, since the microbial biomass utilises oxygen at a much faster rate for the degradation of organic materials. Towards the end of the reaction tank, the concentration of the organic material was found to be microbially reduced thereby

concomitantly lowering the oxygen demand. Thus, the tapered aeration system was designed to distribute the oxygen across the tank relative to the biomass requirements (Seviour *et al.*, 2010) (Figure 2.2). However, lack of oxygen in some regions (towards the outlet of the primary tank), led to settling of the biomass. Solids which settled at the bottom turns septic and this further resulted in an increased oxygen demand in the aeration reactor, thus, filamentous bacteria that were found to be associated with septic conditions proliferated (*viz.*, *Thiothrix spp.*, Type 021N, Type 1851 and *Nostocoida limicola*). In the mid 1940s, step aeration was introduced by partitioning the diffused air into two portions, with a larger portion towards the inlet and a smaller portion towards the end of the reactor vessel. As a result, sludge settled rapidly in the secondary clarifier, however, the effluent quality was still very poor (Jeppson, 1996). This could possibly have resulted due to the heterotrophic bacteria outcompeting the filamentous bacteria in the presence of increased oxygen and readily available nutrients. Smaller pin-point flocs were thus formed, which remained suspended in the liquid, resulting in a turbid final effluent (Seviour, 2010).

In addition, due to the irregularity of DO supply, as well as the insufficient sludge mixture in the system, the process was further modified into a completely mixed system (Figure 2.2).

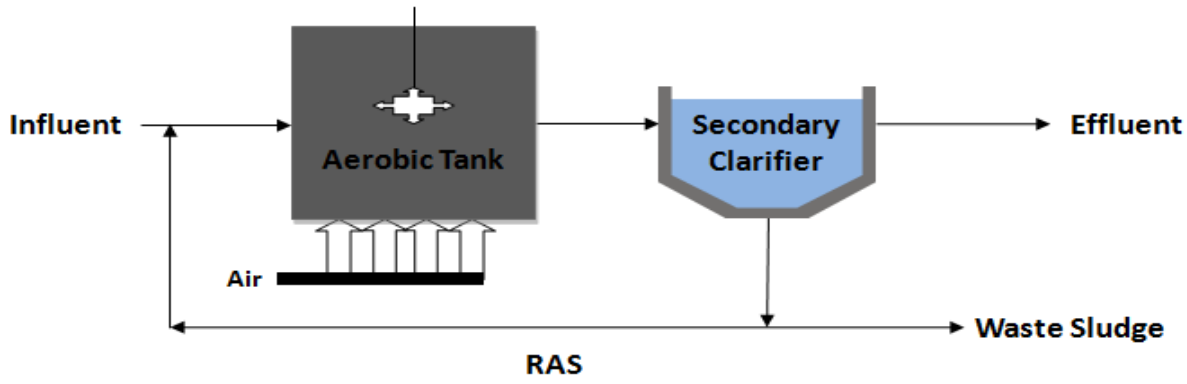

Figure 2.2: Flow diagram illustrating a completely mixed system of the ASP (Seviour *et al.*, 2010).

The system allowed AS to mix with the influent waste as uniformly as possible, therefore, the oxygen demand and the microbial biomass were considered constant throughout the tank. These systems allowed the RAS and the influent to rapidly mix with the microbial biomass (Seviour *et al.*, 2010).

The major disadvantage of the continuous completely mixed systems was improper settling, i.e., sludge bulking. The design modification seemed favourable for the increase in filamentous bacterial abundance and the sludge settling problem was exacerbated (Houtmeyers *et al.*, 1980; Martins *et al.*, 2004; Seviour *et al.*, 2010). Some studies indicated that the growth of filamentous bacteria were more advantageous in completely mixed systems than in plug flow systems (Chudoba *et al.*, 1973; Martins *et al.*, 2004; Seviour *et al.*, 2010). Due to the low substrate concentrations in completely mixed systems, filamentous organisms were found to extend rapidly in search of nutrients, thus, these organisms outcompeted the floc-forming eubacteria (Mangrum, 1998; Martins *et al.*, 2004; Van Loosdrecht *et al.*, 2008). In plug flow systems the substrate concentration and oxygen supply was high at the inlet of the reactor, therefore, the floc formers outcompeted the filamentous bacteria. Nutrients were found to be readily available in the plug flow systems, and for that reason, there was no need for the filamentous bacteria to extend and grow outside the flocs resulting in small, firm, compact flocs and a well settling sludge (Martins *et al.*, 2004; Van Loosdrecht *et al.*, 2008).

2.2. Biological Nutrient Removal Wastewater Treatment Plants

The ASP was initially designed to remove organic compounds, however, over the last three decades, the removal of nutrients (*viz*., nitrogen and phosphorus) from wastewater became more important due to the increase in human population and industrialisation (Jeppson, 1996). High levels of nutrients such as nitrogen and phosphorus in the final effluent was found to cause eutrophication which was harmful to the environment, hence considerable work was carried out to improve the design and operation of WWTPs. This led researchers to the development of the anaerobic and anoxic selectors in combination with an aerobic reactor to facilitate nitrogen and phosphorus removal (Seviour *et al.*, 2010).

The initial development of the BNR process, consisted of the anoxic and aeration tanks. The inclusion of the anoxic reactor in combination with an aerobic reactor was made to further

enhance nitrification and denitrification in both tanks respectively (Chudoba *et al.*, 1973; Grady *et al.*, 1999; Van Loosdrecht *et al.*, 2008). Barnard revealed that the inclusion of the anaerobic tank followed by an aerobic tank, assisted in the removal of phosphorus and this led to the development of a 5-stage Phoredox process. Due to the partial nitrification which occurred in the 5-stage Phoredox process (Barnard, 1983), a modified 3-stage process was later configured (Figure 2.3). This design favoured a decreased concentration of nitrates being recycled back to the beginning of the process, hence improving the nutrient uptake efficiency (Jeppson, 1996; Seviour *et al.*, 2010). To further improve on the nitrification and phosphorus removal, these systems were modified into a Johannesburg process and later, a University of Cape Town (UCT) process (Seviour *et al.*, 2010). However, a recent survey on BNR plants showed that a poor sludge settling, due to the high abundance of filamentous bacteria was found to be the major challenges of these processes (Mielczarek *et al.*, 2012). Among some of the factors contributing to filamentous abundance, the use of alternating selectors (anoxic to aerobic tanks) and low organic loading were proven to favour the undesirable over-growth of filamentous bacteria (Chudoba *et al.*, 1973; Musvoto *et al.*, 1999). Numerous reports also revealed that the exploitation of the selectors did not control the problems of bulking (Jenkins *et al.*, 1993; Lakay *et al.*, 1999).

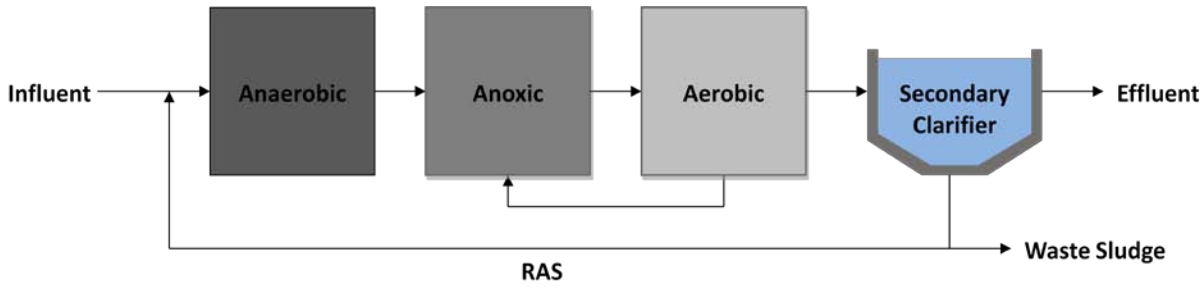

Figure 2.3: Basic conventional process diagram for the removal of nitrogen and phosphorus resembling a 3-stage Phoredox process (Seviour *et al.*, 2010).

To date, research and development continues to improve the wastewater process engineering design, plant operation and treatment optimisation so as to overcome the challenges of sludge settling, carbon removal, nitrogen removal, denitrification and phosphorus removal in one complete system.

2.3. Activated Sludge Flocs

Microorganisms aggregate together forming flocs which are enclosed by a "gel-like" substance also known as the extracellular polymeric substance (EPS) layer (Figure 2.4) (McSwain *et al.*, 2005). Bioflocculation was thought to be mediated in the mixed liquor by microorganisms secreting the EPS. London forces, electrostatic interactions and hydrogen bonds were found to occur between the EPS and the microbial biomass, thus, binding the microorganisms within the EPS layer and allowing flocculation to occur (Tian *et al.*, 2006). Parker *et al.* (1972) postulated the "polymer bridging model" revealing the existence of two levels of structure in AS flocs i.e. the microstructure, consisting of polymer bridges between primary particles, as well as the macrostructure, consisting a filamentous network for the build-up of primary particles (Jenkins *et al.*, 1993; Luque, 2005; Parker *et al.*, 1972).

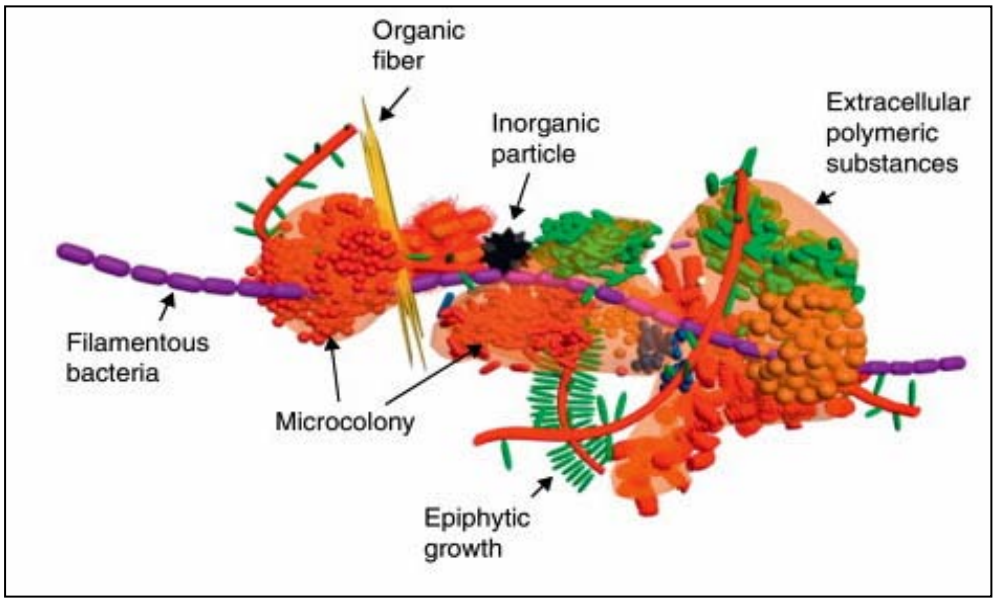

Figure 2.4: Activated sludge floc composition constituting a microbial biocoenosis. Filamentous bacteria serves as the backbone of floc formation (Nielsen *et al.*, 2012).

The bacterial glycocalyx is made up of several polysaccharides and it is found outside the bacterial cell wall facilitating floc formation by "sticking" cells together. Younger bacterial cells were found to produce polysaccharides that were weak bonding whilst the older cells produced polysaccharides that were strong bonding hence at a young sludge age, weak and buoyant flocs were formed and, conversely, at an old sludge age, firm and dense floc particles

formed (Gerardi, 2006c). The floc structure can thus be used as an indicator of process performance e.g. ideal flocs (filamentous bacteria were found to be conserved within floc), pin point flocs (occurring at long sludge ages and very low food to microbes ratios leading to a very low SVI, and turbid effluent) and diffused flocs (seen in filamentous bulking conditions resulting in a high SVI), (Figures 2.5 a, b and d respectively) (Martins *et al.*, 2004; Mesquita *et al.*, 2011).

Zoogloeal bulking has a slime or jelly-like characteristic of the sludge solids (Figure 2.5 c), is caused by the increased bio-polymers in the floc. This type of bulking usually occurred in high loaded systems and rarely occurred in South African WWTPs (Eikelboom, 2000; Saayman *et al.*, 1997).

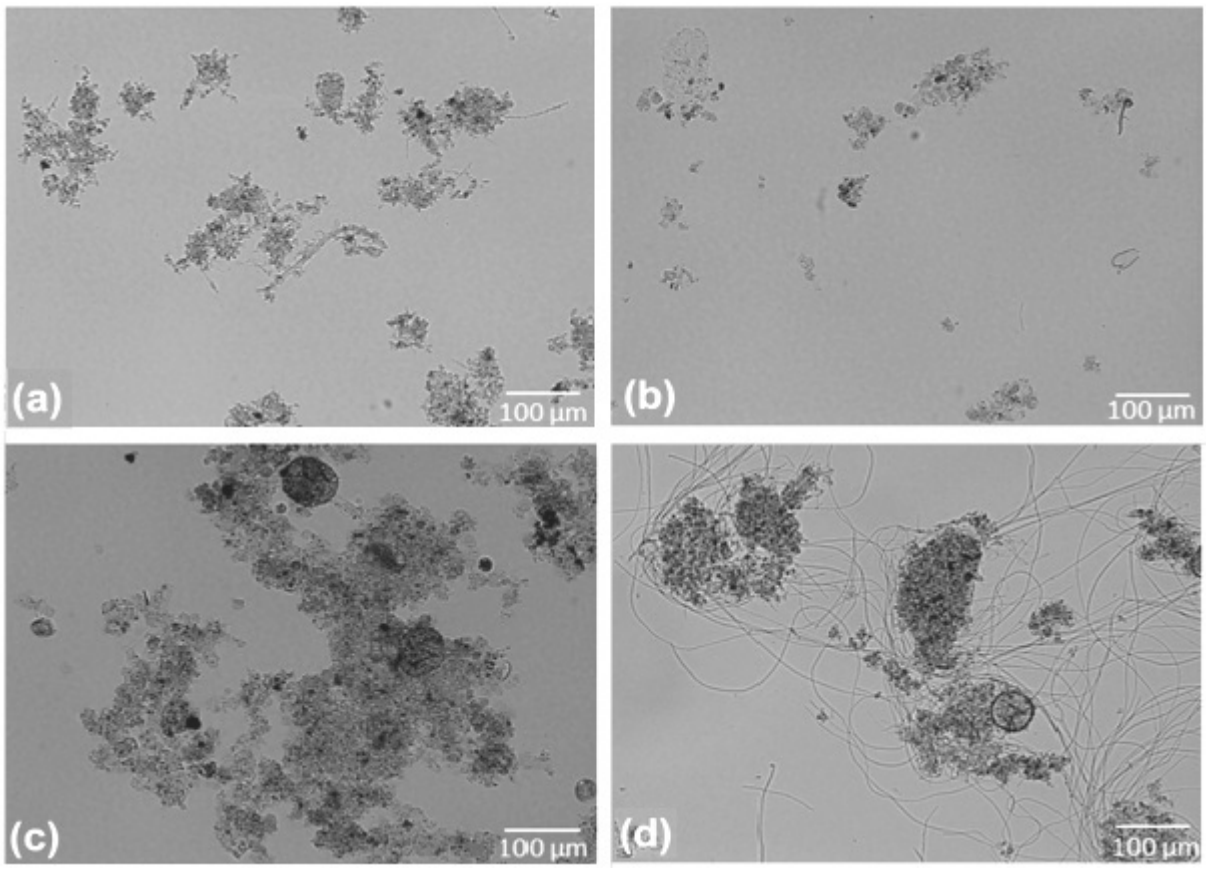

Figure 2.5: (a) Ideal flocs and filamentous bulking caused by (b) pinpoint flocs, (c) zoogleal bacteria (d) irregular and diffuse flocs (Mesquita *et al*, 2011).

During suitable operational parameters, the microbes in the mixed liquor formed a well-balanced functional cluster within the floc, together with the filamentous bacteria. Hence, the flocs settled well and sludge was retained in the system (Govoreanu *et al.*, 2003; Grady *et al.*, 1999). As a positive outcome, these organisms further assisted in the removal of nutrients. Thus, the microbial community within activated sludge has shown to play an integral role in the efficiency of the treatment process; they were shown to form ecological interactions within the floc structure and they also contributed to flocculation (Figure 2.4), allowing sludge to settle effectively. With the support of firm healthy flocs, it was found that organic matter and nutrients (carbon, nitrogen and phosphorous) were more effectively removed, hence, providing a high-quality effluent (Martins *et al.*, 2004).

The microbial populations (Figure 2.4) in wastewater systems forming the biotic community which were found to be involved in the nutrient removal process included:

- Heterotrophic bacteria to facilitate COD removal.
- Nitrifying bacteria encompassing two groups of bacteria facilitating nitrification *viz.*, ammonia oxidising bacteria (AOB) which catalyse the oxidation of ammonia to nitrite and nitrate oxidising bacteria (NOB) which in turn convert nitrite to nitrate (Wagner *et al.*, 2002).
- Denitrifying heterotrophic bacteria further converting nitrate to dinitrogen (N_2) gas by a process called denitrification.
- Phosphate accumulating organisms (PAO) responsible for biological removal of phosphorous (Crocetti *et al.*, 2000; Nielsen *et al.*, 2012).
- Filamentous bacteria responsible the floc formation in the ASP, these organisms formed the core structure of the floc (Figure 2.4; 2.5 a). However, excessive amounts of filamentous bacteria in a WWTP were shown to lead to improper settling of the sludge (bulking), and alternatively, a lack of filamentous bacteria may have led to pinpoint flocs (Figure 2.5 b) and thus a turbid final effluent (Martins *et al.*, 2004).

In theory, good settling depends primarily on the flocs' structural properties and the biocoenosis of the activated sludge. According to Sezgin *et al.* (1978), settling was influenced by the size of the floc, sludge concentration as well as the filamentous abundance. Filamentous bacteria were shown to form the "backbone" for floc forming bacteria to adhere

(Figure 2.4), thus, providing compact flocs (Figure 2.5 a). During optimum plant operating parameters, these organisms formed a fine balance with the floc forming bacteria, thus improving the process efficiency of the AS system (Gerardi, 2006c; Sezgin *et al.*, 1978; Sezgin *et al.*, 1982). A change in the wastewater treatment conditions may favour the increased growth of filamentous bacteria, causing filaments to extend into the bulk solution, leading to irregular, diffuse flocs and bridging between flocs. When the filamentous bacteria were highly abundant, they interfered with the settling and the compaction of the settled sludge causing bulking and foaming (Jenkins *et al.*, 1993; Lee *et al.*, 2003; Martins *et al.*, 2004). For proper operation of the AS system, a balance between floc formers and filamentous bacteria should be maintained. Although the distribution of filamentous bacteria varies from plant to plant, the presence of certain filament types was demonstrative of conditions prevalent within the system.

2.4. Filamentous Bulking and Foaming

Bulking and foaming are among the most frequent problems in activated sludge WWTPs (Mielczarek *et al.*, 2012). The most well-known method to monitor AS bulking was the settleability test, where the mixed liquor of a known volume (usually 1 L) was allowed to settle in a measuring cylinder for 30 minutes. Thereafter, the volume of settled sludge per gram of solids was measured (Jenkins *et al.*, 1993). From this an activated sludge volume index (SVI) can be calculated using the following equation (2.1).

$$SVI = \frac{volume\ ss\ /\ sample\ volume\ (after\ 30min)\ X\ 1000}{MLSS} \qquad (2.1)$$

SVI = Sludge volume index (mL/g).
volume ss = volume of sludge settled after 30 mins (mL). Sample volume = 1 L (1000 mL)
MLSS = Mixed Liquor Suspended Solids (g)

Sludge volume index greater than 150 mL/g is usually an operational measure used to determine sludge bulking. Stirred sludge volume index (SSVI) has also been a popular method used by plant operators (Martins *et al.*, 2004), which had been described similar to the above method with small increments of stirring, however, these methods were considered unsuitable and not a clear representation of settled sludge (van Haandel and van der Lubbe,

2012b). Diluted sludge volume index (DSVI) seemed to be the most reliable method (Seviour, 2010). The DSVI was based on diluting sludge in batches until the diluted suspension reaches ≤ 200 mL/g, this method was developed such that the DSVI value did not depend on the initial sludge concentration. Thus, the sludge concentration was kept constant, thereby, eliminating the influence of the initial sludge concentration to the SVI value (Jenkins *et al.*, 2004a; van Haandel and van der Lubbe, 2012b).

Certain filamentous organisms resulted in sludge bulking, while a few others were found predominantly during foaming incidences. Both conditions were shown to be primarily due to excessive filamentous bacteria in the mixed liquor (Jenkins *et al.*, 1993; Martins *et al.*, 2004). Filamentous bacteria most frequently observed in bulking incidence were *Microthrix parvicella*, Type 0041, Type 0092, *Sphaerotilus natans* and Type 021N (Kappeler and Gujer, 1994; Madoni *et al.*, 2000). Similarly, *Gordonia amarae* and *Microthrix parvicella* have been shown as among the most common filaments found in foaming sludge. *Microthrix parvicella* was found to be one of the dominant organisms found in Europe, South Africa and Australia (Blackbeard *et al.*, 1988; Eikelboom *et al.*, 1998; Rossetti *et al.*, 2005).

Foaming or a thick scum layer may appear on the surface of anoxic and aerobic tanks and it is caused by a reduced level of oxygen making contact with the microorganisms in AS. Foaming has usually been caused by the mycolic acid producing filamentous organisms. These filaments often attached to gas bubbles and floc particles thereby floating to the surface of the tanks (Fryer *et al.*, 2011; Madoni *et al.*, 2000). Mycolata were found to be a group of microbes, essentially foam forming filamentous bacteria (*Gordonia amarae*) which contain mycolic acid within their cell walls (Kragelund *et al.*, 2010). Studies revealed that the presence of mycolata at a certain threshold were associated with foaming (Davenport *et al.*, 2000; Pagilla *et al.*, 2002; Soddell and Seviour, 1990).

2.4.1. Bulking and Foaming Incidence in South Africa

Activated Sludge plants in South Africa were initially established in 1921. A survey conducted by Blackbeard *et al* (1988) in 33 BNR plants in South Africa indicated that filamentous bulking was common in South African WWTPs and was found in approximately 75% of all plants. The most common, dominating filamentous organisms in bulking AS

WWTPs in descending order were found to be Type 0092, Type 0675, Type 0041, *M. parvicella*, Type 0914 and Type 1851 (Blackbeard *et al.*, 1988). Type 0092 and *M. parvicella* were frequently observed in low food to microorganism ratio (F/M ratio) conditions. The F/M ratio was found to be a significant operational parameter and it is defined as the load of food or substrate supplied within the system per unit biomass. A low F/M can mean that the nutrients or substrates in a reactor are lower than the microbes in the system, and in some cases can favor certain filamentous organisms. In WWTPs were the F/M ratios were low, filamentous organisms Type 0675 and Type 0041 were common. The systems with low F/M and nutrient deficiency showing incidence of these type of filamentous bacteria can be made clear by the kinetic selection theory (Chudoba *et al.*, 1973). Clearly, the reduction or elimination of the above mentioned filamentous organisms, was likely to make a significant impact to reducing the problems of bulking in nutrient removal WWTPs.

Type 0092, *M. parvicella* and *Gordonia amarea* were most frequently in foaming samples than in the mixed liquor of the nutrient removal plants, Type 0092 had the highest incidence (Blackbeard and Ekama, 1986). High incidence of *Nocardia amarae* (presently known as *Gordonia amarae*) were reported in the foaming AS samples, in the KwaZulu-Natal (KZN) region of South Africa (Lacko *et al.*, 1999).

2.5. Filamentous Bacteria Identification

Descriptions of a wide range of filamentous bacteria have been documented by Jenkins *et al* (1993, 2004) using conventional methods. Filamentous bacteria are conventionally classified using the identification keys based on morphological traits and staining characteristics (Eikelboom, 1975; Jenkins *et al.*, 1993; Jenkins *et al.*, 2004a; Krhůtková *et al.*, 2005). Microscopic examination of AS has been found to be useful for determining the physical nature of the AS flocs, as well as the abundance and types of filamentous organisms present (Jenkins *et al.*, 1993). Filamentous abundance was proposed by Eikelboom were expressed as a numerical filament index (FI) (Table 2.1).

Table 2.1: Subjective scoring based on filament abundance by the use of microscopic analysis (Eikelboom, 2000; Jenkins *et al.*, 2004a)

Numerical Value/ (FI)	Abundance	Description
0	none	no filaments present
1	few	few filaments observed but not present in all flocs
2	some	Filaments commonly observed, but not present in all flocs
3	common	common filaments observed in all flocs but in low densities between the range of 1 to 5 filaments per floc
4	very common	very common filaments observed in all flocs at medium density between 5 to 20 filaments per floc
5	abundant	indicating abundant filaments in all flocs at high densities greater than 20 per floc
6	excessive	excessive filaments present in all flocs/ appears more filaments tan floc and evidence of filaments growing profusely in bulk solution)

NB: (FI) Filament Index was applied to indicate the quantity of filamentous bacteria population.

The most abundant filamentous bacteria in mixed liquor can be termed as the dominant filaments. These filaments are ranked at the higher end between 4 - 6 on the subjective scoring scale (Table 2.1). There may be up to three dominating filaments in a bulking sludge, all contributing to poor sludge settleability. Secondary filaments in the mixed liquor are usually ranked below 3 on the subjective scoring scale (Eikelboom, 2000; Ekama *et al.*, 1985). Microscopic examination of AS was found to be useful for determining the physical nature of the AS flocs, together with the abundance and types of filamentous organisms present (Jenkins *et al.*, 1993; Krhůtková *et al.*, 2005). The identification of the microbial population of AS was shown to be crucial for proper plant operation. Identification of filamentous bacteria via microscopic examination following Eikelboom keys are based on morphology and staining techniques which are useful but can be misleading based on imprecise filamentous bacteria identified and human biases.

2.5.1. Conventional Staining Techniques for Tentative Identification of Filamentous Bacteria

A suite of tests were used to identify filamentous bacteria, some of them included Gram and Neisser staining. Most filamentous organisms observed in AS are Gram negative, while a few others are Gram positive. Certain filamentous bacteria were shown to stain weakly Gram

positive (eg. Type 1851) (Appendix 1). *Thiothrix* I, *Beggiatoa* spp. and Type 021N stains Gram negative, these organisms may stain Gram positive when they contain large amounts of sulphur within their trichome (Jenkins *et al.*, 1993). A trichome was found to be an arrangement of bacterial cells enclosed by a sheath and they generally differ in diameter and length (Aonofriesei and Petrosanu, 2007).

The presence of polyphosphates granules stored in the cells within the trichome can be detected by Neisser's stain. This test method has been shown to help identify certain strains of filamentous bacteria (Filamentous Type 0092 and Type *N.limicola* stains Neisser positive). Neisser negative cells stain hardly or not at all, showing a slightly brown or yellow colour, whereas Neisser positive bacteria display a dark blackish colour (Jenkins *et al.*, 1993). The poly-*β*-hydroxyl butyrate (PHB) staining test is used for the detection of intracellular storage products (Jenkins *et al.*, 1993). The sulphur oxidation test (S Test) is indicative by the deposition of intracellular sulphur granules within the trichome of the filament after adding sodium sulphide ($Na_2S.9H_20$) solution. Sulphur granules were found to appear as bright, refractive, yellow coloured cell inclusions when viewed using phase-contrast microscopy, either in the shape of spheres as observed for *Thiothrix* spp., *Beggiatoa* spp., and Type 021N (Jenkins *et al.*, 1993). Some of the frequently occurring filamentous bacteria together with their important features (Gram stain, Neisser stain, trichome shape and key characteristic) that assist in filament identification are listed in Appendix 1.

2.5.2. Fluorescent *in situ* Hybridization

Fluorescent *in situ* hybridization (FISH) is a molecular technique that can be used to identify and quantify specific individual microbial cells, using rRNA-targeted oligonucleotide probes (Amann *et al.*, 1995). Oligonucleotide probes are short, synthetic sequences of nucleic acid that are designed to complement the target sequence, to a conserved region of RNA in a particular organism or group of organisms (Coskuner, 2002). The probes can be designed to target different phylogenetic groups ranging from family to species level (Keller and Manak, 1989). These probes are labelled with a fragment of fluorescent dye (*viz*., fluorescein and tetramethyl rhodamine). This allows for the colori-metric detection of hybridized cells with the means of an epifluorescent and confocal scanning electron microscope or by flow cytometry (Keller and Manak, 1989; Wagner *et al.*, 1994b). Some of the most recent rRNA

oligonucleotide probes used for the detection of specific bulking filamentous bacteria are outlined in Table 2.4, these probes have been designed to hybridize to the homologous region of the target organism (Kragelund *et al.*, 2007).

2.5.2.1. Principles of the Fluorescent *in situ* Hybridisation Technique

The fundamental components are the oligonucleotide probe labelled with a flourophore molecule and the rRNA target sequence (Figure 2.6).

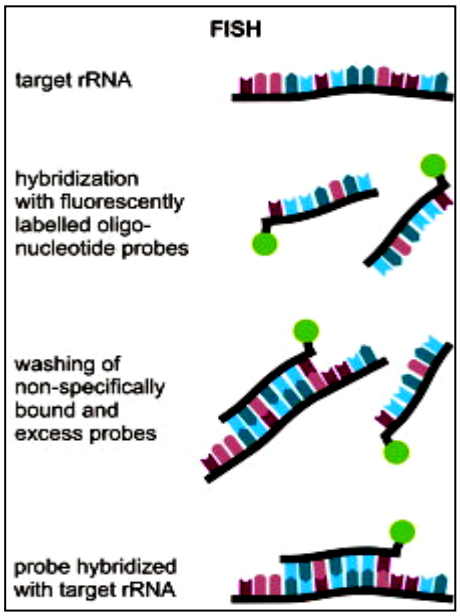

Figure 2.6: Principle steps of the FISH technique (Thilo and Rolf, 2008).

A fluorophores were found to attach or stain cells and these molecules emit light during excitation, Fluorescien (FITC) has been one of the most popular fluorophores. The process requires optimum conditions for the hybridisation and washing stages with high temperatures to allow specificity of the probe binding (Amann *et al.*, 1995). The nucleotide probe binds to the complementary sequence of interest during the annealing stage. The washing step prevents non-specific binding and the excess probe is removed at a slightly higher temperature than the one used during hybridisation.

2.5.2.2. Hybridisation and Washing

The cells were usually pre-treated with aldehydes (formalin, paraformaldehyde, and glutaraldehyde) or alcohols (ethanol, methanol) to permeabilise the cell walls for penetration by the probes (Amann *et al.*, 1995). The hybridization step occurred at high temperatures, usually 45°C, in the presence of the hybridization buffer that requires stringency of the probe. Stringent hybridization conditions for the different oligonucleotide probes were adjusted by different formamide concentrations in the hybridization buffer (Schuppler *et al.*, 1998). This allowed for the probe to bind to the complementary base sequence, if the complementary sequence was absent, the probes were found unable to hybridize and would thus be removed during the washing step (Amann *et al.*, 1995).

The specificity of the probe binding to the target was not only dependent on the sequence of the probe but also the hybridization and washing stringencies. The Formamide concentration in the hybridization buffer and sodium chloride in the wash buffer were inter-dependent and were carefully optimized for each probe (Amann *et al.*, 1995; Hoshino *et al.*, 2007). Cells that have undergone successful hybridization can be visualized using epifluorescence microscopy. It was found that filaments had the ability to undergo morphological changes and convert to a unicellular form, hence, a much more specific and reliable technique had to be used to identify these organisms. (Carr *et al.*, 2005; Ramothokang *et al.*, 2006b).

2.5.2.3. Advantages and Limitations of Fluorescent *in situ* Hybridization

The primary advantage of the technique was shown to be that it provides an effective tool for phylogenetic identification, enumeration and the analysis of spatial distribution of the organisms in the environment without the need for cultivation (Amann *et al.*, 1995; Hugenholtz *et al.*, 2001; Valm *et al.*, 2012). The limitation with the FISH technique was demonstrated to be that it can only be successful if the fluorescently tagged probe can access the rRNA of the specific organism. Due to the high G+C content of the cell wall of certain bacteria, acid or enzyme treatment was found to be usually required prior to hybridization (Carr *et al.*, 2005), as the probes have difficulty in penetrating the cell wall of these bacteria. The foremost limitation was that only a few oligonucleotide are currently available to identify a few species of filamentous bacteria, some of the morphologically identified filamentous bacteria (in both industrial and domestic WWTPs) could not be positively identified by FISH

due to the lack of probes for these specific organisms (Martins *et al.*, 2004). Insufficient probe penetration within the bacterial cells and Autofluorescence have been shown to be common limitations of FISH. Autofluorescence was found to be caused by the natural fluorescent biological or inorganic debris (da Moter and Gobel, 2000).

2.5.3. Current Identification Techniques

Current and more reliable methods of identification are rapidly developing and are presently far more precise for some bacteria in wastewater. These methods include the use of multiple *in situ* identification combining FISH together with live/dead staining. The combined technique determines the physiological properties and monitors viability as well as identifies the target organism. Combinatorial labelling and spectral imaging FISH (CLASI-FISH) is a technique has been used to successfully examine and identify a mixture of organisms in one spectral image using various probes, thus, assessing the special distribution of the different microbes. The FISH technique, can only distinguish a few types of microbes in a single experiment using bandpass filters, however CLASI-FISH enabled researchers to identify numerous microbes in a given fluorescent image (Guo and Zhang, 2012; Valm *et al.*, 2011; Zhang *et al.*, 2010). This can be highly significant in terms of identification of microorganisms together with evaluating significant relationships between microorganisms in an environment.

Another technique employed to examine floc structure was FISH Microautoradiography (MAR), investigating the uptake of substrates, oxygen, nitrogen etc. by the organisms (Martins *et al.*, 2004; Nielsen *et al.*, 2003; Nielsen *et al.*, 1999). With such pivotal information, the function of filamentous bacteria could be determined in activated sludge WWTPs relative to its operational and environmental conditions. In addition, the role of filamentous bacteria on bioflocculation could also be determined (Martins., 2004). Thus far, the *in situ* behaviour of the filamentous organisms (*Candidatus* Meganema perideroedes and *Thiothrix* spp.) have been investigated using MAR and FISH, determining their growth kinetics relative to substrate uptake (Nielsen *et al.*, 2003). However, the technique has some limitations and need further improvement.

2.6.Methods Used to Control Filamentous Bacteria

The two approaches commonly used to control filamentous bulking are: (1) specific control measures which have been aimed at controlling the causes or conditions which favour the growth of filamentous organisms and (2) non-specific control which attempts to treat the symptoms of bulking (i.e. eradicating filamentous bacteria extending out of the floc by the use of chemicals/oxidants). Specific control measures have been deemed more preferable as they target only the organisms of choice (i.e. filamentous bacteria and not the entire population). However, non-specific methods have been shown to be transitory and these methods could be harmful to the entire microbial population (Rossetti *et al.*, 2005). Jenkins *et al.* (1986) identified five conditions that lead to filamentous organism proliferation *viz*., low DO, low F/M, nutrient deficiency, septic influent and low pH (Table 2.2). In contrast, chlorination, ozonation and hydrogen peroxide were found to be some of the common non-specific control methods (Jenkins *et al.*, 2004a; Martins *et al.*, 2004), focused on to eliminate the symptoms of filamentous bulking i.e. eradicate the excessive filamentous bacteria with an appropriate physical/chemical dose. These methods have been l difficult to employ, and an easy, cost effective solution for control of the AS plant has not yet been implemented by plant operators, thus, the filamentous bulking problem remains unresolved (Jenkins *et al.*, 1986; Martins *et al.*, 2004).

2.7. Specific Control Methods

Influent composition and plant operating parameters determine the type of microbial population that will be present in the AS plant. Theoretically, filamentous bacteria predominate under long sludge ages, low DO, low organic matter (food) to microorganism ratio and nutrient deficiency conditions (Table 2.4) (Scruggs and Randall, 1998; Vaiopoulou *et al.*, 2007).

Table 2.2: Dominant filament types indicative of AS operational parameters (Jenkins *et al.*, 1993; Mamais *et al.*, 2011; Martins *et al.*, 2004; Richard *et al.*, 2003)

Filamentous bacteria	Conditions	Possible/Suggested cure methods
H. hydrossis	Low F/M (long sludge age)	Reduce MLSS in the aeration basin and increase the F/M.
Thiothrix spp.		
Types 0041		Increase the substrate concentration available to the AS, introduce batch or plug-flow characteristics to the aeration basin.
Type 0675		
Type 0092		
Type 1851		
Type 1701	Low DO	Increase dissolve oxygen level in the plant (> 1.5 mg/L),
S. natans		
H. hydrossis		Increase sludge retention time (SRT).
Thiothrix I and II.	Nutrient deficiency	Addition of the lacking nutrient,
Type 021N	Septic sludge	chemical oxidation (*viz*., chlorine, hydrogen peroxide, potassium permanganate), or chemical precipitation (ferric chloride),
		addition of sodium nitrate as an “oxygen source”.
M. parvicella	Fatty acids in wastewater	Still unclear but the most recommended solutions are: Removal of lipids, grease and oil content by the use of a primary settling tank.
	low F/M	
	Low temperature	
Gordonia amarae		Increase oxygen level (> 1.5 mg/L) (Note that higher aeration causes more foam formation, due to the physical action of more air present. Many operators reduce aeration when foaming occurs to reduce the foam, but this only causes more filament growth in the long term).
*(*Formally known as *Nocardia amarae)*		
Type 1863		
		Minimise ammonium concentration (< 1mg/L).
		Septicity should be controlled. Reduce sludge age.
		Dosage with chemicals (chlorine), however not always effective due to the high dose required.
		Use of coagulants- polyaluminium chloride (PAX) dosages ranging from 1.5 to 4.5 g Al+3/kg MLSS reported by Roels *et al.* (2002).

2.7.1. The Effects of Operational Parameters on Filamentous Bacteria and Possible Control Measures

The competition and relationship between filamentous bacteria and floc forming bacteria at different substrate concentrations has been fitted to the Monod function and this investigation showed specific growth rate of filamentous bacteria verses soluble readily biodegradable chemical oxygen demand (RBCOD), both filamentous and floc formers differed significantly. For many filamentous organisms their growth rate was higher than floc formers at low COD concentrations and vice versa at higher COD concentrations (Chudoba *et al.*, 1973; Van Loosdrecht *et al.*, 2008). According to this function, in systems where substrate concentration was low, filamentous bacteria proliferated and outcompeted floc formers by growing profusely and extending out of the flocs. These filaments gained nutrients from the bulk liquid rather than within the flocs itself but the growth of floc formers was suppressed due to much lower nutrients diffusing into the flocs. Consequently, the flocs were less compact due to the irregular shape caused by filamentous bacteria (Martins *et al.*, 2004; Van Loosdrecht *et al.*, 2008).

The time in which solids are retained within the systems is referred to as sludge age or solids retention time which occurs between 4 -10 days in a conventional AS plant. However, it was found that the sludge age in many plants often exceeded 10 days generally to sustain the slow growing nitrifying population allowing nitrification and denitrification to occur. Floc forming microbes tend to utilise the nutrients hence forming a relatively high biomass, as a result a low F/M ratio may be formed. These observations have lead to the accumulation of the low F/M filamentous population group (Martins *et al.*, 2004). As described by the Monad function above, the filamentous organisms tend to outgrow the floc forming bacteria, hence, transpiring to bulking conditions.

Bulking has also been related to a DO deficiency in the activated sludge system, commonly a deficiency of oxygen in the aeration tank. *Thiothrix* spp., Type 021N and Type 1851 were the common filamentous organisms observed when DO levels were low (less than 1 mg O_2/L) (Martins *et al.*, 2003). However, some filamentous bacteria (*viz., S. natans, N. limicola,* and *M.parvicella*) were shown to be able to grow over a wide range of DO concentrations (Rossetti *et al.*, 2005). Therefore, bulking in AS systems can also occur to a wide range of

oxygen supply, as a result filamentous bacteria should be monitored with respect to the oxygen availability in the aeration tank together with other plant operational parameters.

Certain operational parameters may stimulate the growth of filamentous bacteria (Table 2.2), and possible selective control measures such as the use of anoxic and/or anaerobic tanks (also known as selectors), increased sludge age and DO concentrations could be applied to reduce specific filamentous bacteria in the treatment process (Jenkins *et al.*, 1993). Theoretically, filamentous bacteria in AS plants could be affected by wastewater composition, nutrient concentrations, and environmental conditions that exist in the biological reactors. Thus, knowing the nutrient requirements and conditions that enhance the activity of filamentous bacteria may also be useful in controlling their growth in the AS system (Gerardi, 2006b). However, despite the above mentioned control measures, filamentous sludge bulking and foaming still remain major problems in many WWTPs and factors triggering the proliferation of specific filamentous bacteria are still not clearly understood (Martins *et al.*, 2004; Tandoi *et al.*, 2006).

2.8. Non-specific Control

Chlorine, ozonation and ultraviolet (UV) disinfection are some of the most commonly used treatment methods in WWTPs. Chlorine in the form of chlorine gas was found to be the most common disinfectant used in municipal wastewater, the chemical agent has been shown to destroy microorganisms by oxidising their cellular material. Ozone is a strong oxidising agent, it is a very unstable gas generated by an electrical discharge through pure oxygen or dry air (EPA, 1999a). It was found that UV radiation was produced by an electrical discharge through mercury vapour, the intensity of light has been shown to penetrate the genetic material (DNA and RNA) of the microbes, thus, disrupting the organisms ability to replicate (Scheible, 1987). For microbial inactivation at an appropriate UV dosage, thymine dimers (i.e. a cytobutyl ring between two adjacent thymine nucleic acids located on the same DNA/RNA strand) were formed along the DNA chain, thus, preventing replication of the organism (EPA, 1999b; Martin, 2004). Low pressure UV-lamps specific to the 254 nm wavelength (optimal range of germicidal activity) are commonly used to inactivate bacteria in wastewater (Scheible, 1987).

Saayman *et al.* (1999) investigated chlorination, hydrogen peroxide (H_2O_2) as well as ozonation to control the growth of filamentous bacteria causing bulking in the BNR plants. From their investigation, it was proposed that chlorination was the most economical non-specific method for controlling filamentous growth in BNR plants, H_2O_2 controlled bulking temporarily and low ozone doses improved sludge settleability consistently. However, due to technical issues they were unable to increase the ozone concentration and further analyze the full extent of ozone on filamentous bulking (Saayman *et al.*, 1999).

2.8.1. Effects of Chlorine on Bulking Control

A great deal of work has been reported on bulking control by chlorination, however, not much has been done in BNR systems (Jenkins *et al.*, 1993; Lakay *et al.*, 1988; Leeuwen, 1992; Salvado *et al.*, 2000). Theoretically, chlorine is one of the first existing technologies to selectively control filamentous bacteria and therefore minimise bulking (Caravelli *et al.*, 2003; Jenkins *et al.*, 1993). A minimum requirement of 10 – 20 mg Cl_2/L was suggested, since doses greater than 20 mg Cl_2/L could possibly cause de-flocculation and the formation of pin-point flocs (Lakay *et al.*, 1988; Nasr, 2010).

2.8.1.1. Case Studies of Bulking Control by Chlorination

To date, chlorine has been the most widespread form of disinfection in wastewater treatment (Caravelli *et al.*, 2003; Jenkins *et al.*, 2004a; Neethling *et al.*, 1985; Ramirez *et al.*, 2000). Jenkins *et al* (1993) reported several case studies of successful bulking control using chlorination. An AS plant in Georgia, USA, added chlorine (5 – 15 kg $Cl_2/10^3$ kg MLSS) into the RAS and primary effluent. They initially used chlorine at low levels and gradually increased dosage over a period of time. Sludge volume index were continuously monitored and increased to 230 mg/L. Chlorine overdose was observed by a high degree of SS in the secondary effluent. It was found that as the chlorine dropped from 4.7 to 2.9 kg $Cl_2/10^3$ kg MLSS, the SVI reduced between 100-130 mL/g. Another report on the Santa Clara water pollution control plant (California), dosed 2 - 4 kg $Cl_2/10^3$ kg MLSS; 1.5-3 mg Cl_2/L in the RAS stream. Results verified that nitrification was unaffected, with a decrease in SVI (Jenkins *et al.*, 1993).

Lakay *et al* (1988), have also reported chlorination in a laboratory scale system which was successfully used to control filamentous bulking (chlorination dose of 8 kg Cl_2/ 10^3 kg MLSS). The UCT BNR system used showed nitrification and phosphorus removal were unaffected (Lakay *et al.*, 1988). Alternatively, according to Daigger *et al* (1988), RAS chlorination at a chlorine dose of 2 kg $Cl_2/10^3$ kg MLSS for five days reduced sludge bulking from approximately 270 to 200 mg/L (Daigger *et al*, 1988). However, low Cl_2 doses showed poor phosphorus removal. It appeared that Cl_2 interfered with the PAOs ability to release and take up phosphorus in the anaerobic and aerobic zones respectively. A recent study reported doses of 4.7 mg Cl_2 $(gVSS)^{-1}$ at a 20 min contact time and previous work of Carevelli *et al* (2004) mentioned that floc forming bacteria were more sensitive than filamentous bacteria in pure culture studies. Hence, chlorination in the RAS stream should be applied with caution. Chlorine doses should be optimised to a degree that is high enough to eliminate the excessive filaments extending from floc to floc while at the same time not disrupting the floc (Caravelli *et al.*, 2004).

2.8.1.2. Advantages and Disadvantages of Chorine Dosing

Chlorine could be used as a useful technique, however, toxic compounds such as trihalomethanes (THMs) may be formed. Chlorine has been shown to be a common control agent and a very useful technique to control filamentous sludge bulking. (Caravelli *et al.*, 2006). However, some concerns have been noted and the major disadvantages of chlorine treatment in wastewater plants have been the formation of carcinogenic compounds and chlorine residuals which are toxic to aquatic life (Table 2.3).

Table 2.3: Advantages and disadvantages of chlorine (Gross and Farrell-Poe, 2005)

ADVANTAGES	DISADVANTAGES
Chlorination is a well-established technology.	The chlorine residual, even at low concentrations, is toxic to aquatic life and may require de-chlorination.
Presently, chlorine is more cost-effective than either UV or ozone disinfection (except when de-chlorination is required).	All forms of chlorine are highly corrosive and toxic.
The chlorine residual that remains in the wastewater effluent can prolong disinfection even after initial treatment and can be measured to evaluate the effectiveness.	Chlorine oxidizes certain types of organic matter in wastewater, creating more hazardous compounds (e.g. THMs).
Chlorine disinfection is reliable and effective against a wide spectrum of pathogenic organisms.	The level of total dissolved solids is increased in the treated effluent.
Chlorine is effective in oxidizing certain organic and inorganic compounds.	Chlorine residuals are unstable in the presence of high concentrations of chlorine-demanding materials, thus requiring higher doses to effect adequate disinfection.
Chlorination has flexible dosing control.	Chlorine resistant organisms.
Chlorine can eliminate certain noxious odours while disinfecting.	The long-term effects of discharging de-chlorinated compounds into the environment are unknown.

Recent findings point out that various WWTPs using chlorine as a disinfectant or form of treatment have been discharging significant levels of residual chlorine into the receiving wastewater effluent, causing major harm to the environment and human health. As a result, other treatment technologies have been investigated.

2.8.2. Ultraviolet Light Treatment

In the case of an Ultraviolet (UV) disinfection system, electromagnetic energy has been demonstrated to be transferred from a mercury arc lamp to the microorganisms, destroying the cells genetic material and its ability to reproduce. The effectiveness of a UV disinfection system depends on the characteristics of the wastewater, intensity of UV radiation, the time in which microorganisms are exposed to the radiation and the reactor configuration according to the Environmental Protection Agency (EPA, 1999b). Previous studies have shown a correlation between UV transmission at a wavelength of 254 nm and the degree of disinfection with different types of treated wastewater, however, no investigations have been

pursued to determine the effects of UV light on filamentous bacteria (Whitby and Palmateer, 1993).

2.8.2.1. Ultraviolet Light Dose

Evaluation of UV light treatment was undertaken to determine the percentage kill of filamentous bacteria at an effective UV dose. The amount of energy required to produce this effect in a given organism is referred to as the lethal dose. Ultraviolet dosage is the product of ultraviolet intensity (expressed as $\mu W/cm^2$ seconds). It was found that the logarithm of the survival fraction of filamentous bacteria was linear in respect to time (Severin, 1980).

2.8.2.2. Advantages and Disadvantages of Using Ultraviolet Light

The advantage of UV light treatment compared to chlorination has been postulated to be that it does not require the use of chemicals which could be toxic or detrimental to the environment. In addition, in contrast to chlorination and ozonation, UV light has not been shown to produce toxic by-products, and the effluent of a wastewater treatment would therefore be far less toxic to the environment (Martins *et al.*, 2004; Santos *et al.*, 2013). The use of this treatment in wastewater facilities also ensured the destruction of chlorine resistant bacteria (Macauley *et al.*, 2006).

The equipment used to generate UV light can be easily stored and maintenance is very low. The UV light has been shown to target surfaces of the flocs thus eliminating the majority of the filamentous bacteria extending out of the flocs, while stabilising the floc interior. Some of the major disadvantages of using UV light treatment in full-scale wastewater systems include power cost, replacement of UV-lamps, lamp fouling, the ability of the water to allow transmission of UV radiation and re-growth of microorganisms after treatment (Martin, 2004).

2.8.3. Ozone Treatment

Ozone is a highly unstable gas which easily degrades back to oxygen. During this conversion, a free oxygen atom is formed which is highly reactive and it has a short half-life, thus, ozone needs to be generated on site by the use of an ozone generator (Wijnbladh, 2007). Ozonation

have been demonstrated to successfully prevent bulking and could therefore be expected to improve AS processes (Collignon *et al.*, 1994; Leeuwen, 1989). Theoretically, O_3 is the strongest form of disinfection against microorganisms, due to its high redox potential. The O_3 has been shown to disintegrate the cell wall of the microorganism, thus, releasing cell constituents (Wijnbladh, 2007).

2.8.3.1. Case Histories of Ozone on Bulking Control

Application of ozone treatment on bulking control have been investigated in a 110 L/d pilot scale BNR system, the system operated as a three-stage Phoredox process. Ozone (10- 40 mg O_3/hr) was introduced into the 80 L aeration tank. Results firmly revealed that sludge settling was greatly improved (more compact flocs were formed) due to the decrease in filamentous bacteria abundance (Leeuwen, 1988). Studies revealed that ozone doses up to 70 mg O_3/gSS were found it to be significantly effective in improving sludge settleability (Järvik *et al.*, 2010; Nagare *et al.*, 2008). Saktaywin *et al*, (2005) found that ozone decreased microbial activity resulting in a 70% sludge inactivation with an ozone consumption of 30-40 mg O_3/gSS, emphasising that ozone at a much higher dose destroys most of the bacteria (Saktaywin *et al.*, 2005).

A pilot scale process by Van Leeuwen *et al*, (1988) discovered that 6-9 mg O_3/L was effective in improving sludge settling. There were no significant difference in settling at 9 mg O_3/L, hence, the former was taken as the optimum dose, concluding that filamentous bacteria can be controlled at a dose of 6 mg O_3/L. Ozone decreased filamentous abundance and results showed a significant relationship between sludge settling and filamentous bacteria. Microscopic analysis revealed that filamentous populations were much higher in the control than the ozonated sludge, thereby implying that filamentous growth could be controlled by low concentrations of ozone in AS systems.

2.8.3.2. Advantages and Disadvantages of Ozone Treatment

The major advantages of O_3 are that it is relatively pure in nature and it does not form harmful by-products which could possibly be detrimental to the environment (Wijnbladh, 2007). Recent studies have found that ozone significantly improved sludge settleability and

the treatment was more effective to inhibit filamentous bacteria than the floc formers in the ASP (Saktaywin *et al.*, 2005; Sankaran *et al.*, 2008). However, O_3 was also found to be an unstable gas and it had to be generated on-site for use. Ozone treatment can be costly in a full-scale system, since it is capital and power-intensive (EPA, 1999a).

2.9. Research Gaps Identified

Apart from filamentous bacteria being identified as problematic in bulking and foaming AS, in certain cases an excess of filaments could also be beneficial, in that certain types of filamentous bacteria could possibly be related to nutrient removal efficiency within WWTPs. Some filamentous bacteria could play a role in nitrification and phosphorous removal. From recent studies, Tian *et al* (2011) mentioned the use of limited filamentous bulking (SVI: 140-250 mL/g) under low DO as a stimulation of simultaneous nitrification and denitrification to enhance BNR and improve effluent quality. Experimental results showed that it was easy to maintain limited filamentous bulking by adjusting the DO. The dominant filamentous bacteria were *H. hydrossis.*, *S.natans.*, and Type 1701 (Tian *et al.*, 2011). Hence, limited filamentous bulking could be vital to WWTPs in order for the plant to function efficiently with respect to nutrient removal. In most cases it was found to be difficult to achieve this by controlling the plant operating parameters (Martins *et al.*, 2004; Tandoi *et al.*, 2006).

Due to the number of factors (e.g. chemical influent characteristics, industrial influent, climatic conditions and design modification of activated sludge systems), WWTPs in different locations with different configurations could harbour different types of microorganisms. There is a need to understand this in South African context, where the engineering aspects are well researched, but the microbiology within these systems is currently not as well elucidated.

Applying a suitable chemical/physical treatment method could also be an alternative method to eliminate undesired filamentous bacteria within the system. Under poor settling conditions, the RAS streams in full-scale wastewater treatment systems have been found to be contaminated with excess problematic filamentous bacteria, leading to a continuous bulking condition within the plant. Therefore, there is a need to understand the microbial contribution to this problem and evaluate selective methods (operational parameters) and non-selective

methods to determine their respective efficiencies in reducing the problem of bulking such that the growth of filamentous organisms is limited or suppressed.

2.10. Aim

To determine an efficient method to control or remediate filamentous bulking in wastewater treatments plants by evaluating specific and non-specific methods.

2.11. Objectives

- To identify the dominant filamentous bacteria in bulking samples in a full-scale BNR plant.
- To determine the relationships of the specific operational parameters to the predominance of filamentous bacteria in a full-scale biological nutrient removal plant using a cumulative logit model.
- To determine the effects of chlorine, ozone and UV light treatment on the viability filamentous bacteria.

3. PROFILING OF DOMINANT FILAMENTOUS BACTERIA IN A BIOLOGICAL NUTRIENT REMOVAL PLANT

3.1. Introduction

Currently, a vast number of filamentous bacteria are still very poorly understood due to the lack of cultivation methods, identification techniques and the absence of pure cultures. Hence, many of these organisms were not phenotypically characterised and taxonomically sanctioned (Martins *et al.*, 2004). The conventional identification of filamentous bacteria was based purely on morphological and staining characteristics such as trichome shape, Gram and Neisser staining reactions, attached growth, detection of intra-cellular storage products such as PHB, detection of sulphur deposits within the trichome, filament shape, filament length and the presence of a sheath covering the trichome. These identification methods (Table 2.2; Table 3.2) involved the use of standard staining and test reactions such as the evaluation of Gram staining, Neisser staining, PHB staining and the sulphur storage test (S-test) (Eikelboom, 2000; Jenkins *et al.*, 1993).

The above conventional techniques were found to be very useful in terms of identification of filamentous bacteria; however it could also have many drawbacks, since morphological characteristics and phenotype can infrequently match. The use of conventional methods were insufficient to further distinguish between filaments with the same morphology (Hug *et al.*, 2005). For instance, filamentous organisms with similar morphology can be different in their physiology and taxonomy. For example, filamentous bacteria *N. limicola I, II and III* were morphologically similar, but these types differed phylogenetically (Martins *et al.*, 2004). The detection of filamentous bacteria by FISH with specific rRNA targeted oligonucleotide probes was found to be a much more rapid and effective technique to identify filamentous bacteria, however, there were some known limitations (Pernthaler *et al.*, 2001).

Problems associated with FISH during image analysis included photobleaching and interferences by autofluorescence. Photobleaching was found to be caused by the destruction of the fluorophores at a high light intensity, whereas autofluorescence is a result of back ground fluorescence caused by certain natural biological structures emitting light. As a result,

these problems impose a considerable amount of biasness with regards to identification and quantification of the targeted microorganisms. Subsequent to the FISH procedure, quantification by image analysis can be very cumbersome and the results may not always be satisfactory. Due to the different levels of light signal, it can be difficult to discern the fluorescence of targeted microorganisms (Sanz and Kochling, 2007). Microorganisms with low ribosomal content reveal a low fluorescent signal, thus, indicating poor fluorescence and biasness with regards to quantification (Nielson *et al.*, 2009).

Despite the extensive research on filamentous bacteria, these organisms still pose an immense problem causing bulking and foaming, due to their excessive growth. The most common filamentous bacteria, dominating in bulking WWTPs in South Africa were found to be Type 0092, Type 0675, Type 0041, *M. parvicella*, Type 0914 and Type 1851 (Blackbeard *et al.*, 1988). Filamentous bacteria, Type 0092 and *M. parvicella* were frequently observed in low F/M conditions (long sludge age). The problem of sludge bulking has exacerbated when the system design was modified over the years, changing from plug flow systems, to completely mixed systems (Seviour *et al.*, 2010). To date, there are no solutions or profound strategies to control the problematic behaviour of these organisms (Martins *et al.*, 2004; Mielczarek *et al.*, 2012). In addition, the function and the physiological behaviour of these organisms are poorly understood. For the current study, a BNR plant in KwaZulu-Natal with frequent bulking and operational problems was selected to identify and understand the *in situ* behaviour of the dominant filamentous bacteria within the plant and to develop a control strategy for the excess filamentous growth.

In this study, Kingsburgh WWTP, a BNR plant treating domestic wastewater (95%) in the KwaZulu-Natal region was selected. The selected plant was designed for both biological nitrogen and phosphorus removal treating 3.4 ML/d average dry weather flow (ADWF) of domestic effluent. The plant was initially designed using the University of Cape Town (UCT) process configuration, however, the plant now operates by a simple restricted aeration mode without the internal recycle (a-cycle) from the aerobic to anoxic zone. Both nitrification and denitrification processes within the aeration tank were controlled using four sequentially operated aerators. The plant has been able to comply with the discharge standards but frequently experienced bulking and foaming problems due to excessive proliferation of filamentous bacteria.

3.2. Materials and Methods

3.2.1. Sampling and Plant Data

Mixed liquor sludge samples were collected bi-weekly from various sampling points which included the anaerobic, anoxic and aeration tanks, secondary clarifier and the return activated sludge (RAS) over a period of one year. Along with the sample, the most specific and time-related operational data were also collected (Table 3.3). The chemical analysis and wastewater characteristics were done using an Aquakem Gallery Photometric Auto-analyser (Thermo Scientific, Germany) according to protocols outlined by the environmental protective agency (EPA, 2010). Chemical oxygen demand (COD) and mixed liquor suspended solids (MLSS) were measured according to Standard Methods (APHA, 1998). The important parameters *viz*., Sludge Volume Index (SVI) and food to microorganism (F/M) ratio were calculated according to Jenkins, (2004). Dissolved oxygen and temperature were measured using a multi-parameter-YSI model, 556 MPS system (Yellow Spring Systems, USA). The mixed liquor sludge samples was immediately fixed in 96% ethanol and stored at -20°C for further microbiological analysis (Nielson *et al.*, 2009).

3.2.2. Identification of Filamentous Bacteria Using Conventional Staining and Microscopic Methods

Microscopic analyses to identify filamentous bacteria in the sludge samples were achieved by assessing the morphological characteristics using staining reactions based on key factors outlined by Jenkins *et al.* (1993) and Eikelboom (2000). Wet mount, Gram staining (Appendix 5) and Neisser staining (Appendix 6) was done to evaluate the AS floc shape, size, morphology, filament abundance and the effect of filaments on floc quality in connection to AS separability. The abundance of filamentous bacteria in each sample was attained using a subjective scoring method (Eikelboom, 2000). The assessment of dominant and secondary filaments was done on a seven point scale (0 - 6) termed the filament index (FI); Table2.1 (Jenkins et al., 1993). The organisms were classified as dominant and in high levels when ranked higher than level 4 on the scale (i.e. 5-20 filaments per floc). Additionally, PHB staining (Appendix 7) and the S-test (Appendix 8) was done for further presumptive identification of the filamentous bacteria (Eikelboom, 2000; Jenkins *et al.*, 2004a).

3.2.3. Molecular Identification of Filamentous Bacteria Using the Fluorescent *in situ* Hybridization Technique

3.2.3.1. Sample pre-treatment, fixation and dehydration

The mixed liquor samples were initially pre-treated in a fixed ratio containing 1X phosphate buffered saline (PBS) and 8% paraformaldehyde (1:3, v:v). Subsequently, samples were washed using phosphate buffered saline (PBS) and stored at -20°C in PBS/ethanol (1:1, v:v) until further use (Nielson *et al.*, 2009). A group of 16S rRNA oligonucleotide probes were selected to target different filamentous bacteria (Table 3.1). These probes were labelled with 5'(6)-carboxyfluorescein ester dye (MWG-Biotech, Germany; subsidiary company of Roche Products (PTY) LTD, South Africa).

The fixed samples were sonicated (2 W for 2 min) using an XL-2000 ultrasonic liquid processor (Trilab Support, USA) to break up the flocs. During the process of sonication, 2 mL samples were stirred by ultrasound waves at 2 W for 2 minutes, creating bubbles and this resulted in shearing the floc structure. After sonication, 10 μL of sample were applied onto the wells of teflon coated slides and dried at 48°C for 10 minutes. The slides were subjected to a dehydration step using ethanol in 50 mL polypropylene tubes (50%, 80% and 100% ethanol, 3 minutes in each), to remove the excess water from within the bacterial cells as to improve the resolution during microscopy.

Table 3.1: Oligonucleotide probes and target organisms (Nielson *et al.*, 2009)

Probe	Sequence (5'-3')	FA (%)	Target Filamentous Bacteria	Reference
G123T	CCT TCC GAT CTC TAT GCA	40	*Thiothrix* spp., *T. nivea, T. unzii, T. fructosivorans, T. defluvii,* Type 021N group I, II, III	(Kanagawa *et al.*, 2000)
G1B	TGT GTT CGA GTT CCT TGC	30	Type 021N group I	(Kanagawa *et al.*, 2000)
GNS B941	AAA CCA CAC GCT CCG CT	35	Type 0041, Type 0675	(Gich *et al.*, 2001)
CFX 1223	GGTGCTGGCTCC TCCCAG	35	Type 0092	(Speirs *et al.*, 2009)
SNA	CAT CCC CCT CTA CCG TAC	45	*Sphaerotilus natans*	(Wagner *et al.*, 1994a)
CHL 1851	AAT TCC ACG AAC CTC TGC CA	20	Type 1851	(Beer *et al.*, 2002)
MPA60	GGA TGG CCG CGT TCG ACT	20	*Microthrix parvicella*	(Erhart *et al.*, 1997)
NLIMI 91	CGC CAC TAT CTT CTC AGT	20	*Nostocoida limicola I*	(Liu *et al.*, 2001)
NLIMII 175	GGC TCC GTC TCG TAT CCG	40	*Nostocoida limicola II*	(Liu *et al.*, 2001)
NLIMIII 301	CCC AGT GTG CCG GGC CAC	20	*Nostocoida limicola III* strains	(Liu *et al.*, 2001)

*FA – Formamide 20 - 45%, for adequate hybridisation for probe binding, an optimum formamide concentration is used, thus, preventing the loss of fluorescence signal in the target cell (Pernthaler *et al.*, 2001).

3.2.3.2. *In situ* hybridization

Hybridization buffer (Milli-Q water, 5 M NaCl, 1 M Tris-HCl, 10% sodium dodecylsulfate, 20 - 45% formamide) was prepared for each percentage of formamide and the hybridization step was carried out with high stringency (Nielson *et al.*, 2009). The prepared hybridization buffer together with the specific gene probe (50 ng/μL, work solution) was added onto each well of the teflon coated slides and incubated overnight at 46°C. Following incubation, the slides were then rinsed with warm dH_2O and transferred onto a pre-warmed wash buffer (1M Tris/HCL, 10% SDS, 5 M NaCl, 0.5 M EDTA) in 50 mL polypropylene tubes. The tubes were incubated at 48°C for 45 minutes. After incubation, slides were counter-stained with the DNA stain 4′, 6′-diamidino-2-phenylindol (DAPI) (0.25 μg mL^{-1}). A volume of 10 μL DAPI

covered each of the wells on slides and they were left in the dark for 10 minutes; the slides were rinsed with dH_20 and left to air-dry. A drop of Vector-shield (mounting agent containing anti-bleaching) was added onto the slides and covered with a cover slip.

3.2.3.3. Image Analysis

Slides were examined using an Axiolab Apotome (Carl Zeiss, Germany) microscope containing the FLUOS fluorochrome filter set (Manz *et al*., 1992). Image analyses were examined with a Zeiss Axio Vision Release 4.6 imaging software.

3.3. Results

3.3.1. Identification of Dominant Filamentous Bacteria Using Conventional Microscopic Techniques

Bulk samples were analysed from various reaction tanks (*viz*., anoxic, anaerobic, aerobic, RAS and clarifiers). Filaments identified in the bulk samples are outlined in Table 3.2 with some of the common key characteristics observed, thus, supporting the presumptive identification of the organisms. Subsequently, filamentous bacteria were semi-quantified by the use of a subjective scoring scale (i.e. FI: 0 - 6).

Table 3.2: Filamentous bacteria identified in bulking samples of Kingsburgh WWTP, based on the morphological key characteristics observed

Filamentous bacteria	Key characteristics/ identification
Thiothrix spp.	Gram positive; filaments commonly extending out of the flocs; apical gonidia observed, no attached growth.
S. natans	Gram negative; Neisser negative; observed as large cells packed within a sheath.
Type 021N	Gram negative; Neisser negative; no attached growth; largest and longest filament commonly observed extending out of the flocs.
Type 1851	Weakly Gram positive; commonly observed in bundles with few or no attached growth.
Type 0092	Gram negative, Neisser positive; filamentous found dominating within the flocs.
Type 0041	Gram variable filaments, heavily attached growth observed.

Presumptive identification of filamentous bacteria were further confirmed with the FISH technique using species specific 16S rRNA targeted oligonucleotide probes (Nielson *et al.*, 2009); Table 3.1. Positive hybridization with species specific probes such as G1B (Type 021N), G123 (*Thiothrix* spp.), CFX 223 (Type 0092) and CFX mix (Type 0041) confirmed the identity of some of the filamentous bacteria (Figures 3.3. a, c, e and Figure 3.4 a respectively).

The composition of the filamentous bacterial population was stable over the study period with minor shifts in their population across the one year study period. The filamentous bacteria identified were found in all reaction tanks including anaerobic, anoxic, aerobic and RAS. Samples across all tanks showed only a slight variation in the filamentous population. Figure 3.1 illustrated the average filamentous bacteria abundance detected from the aeration tank and RAS (January- December 2011).

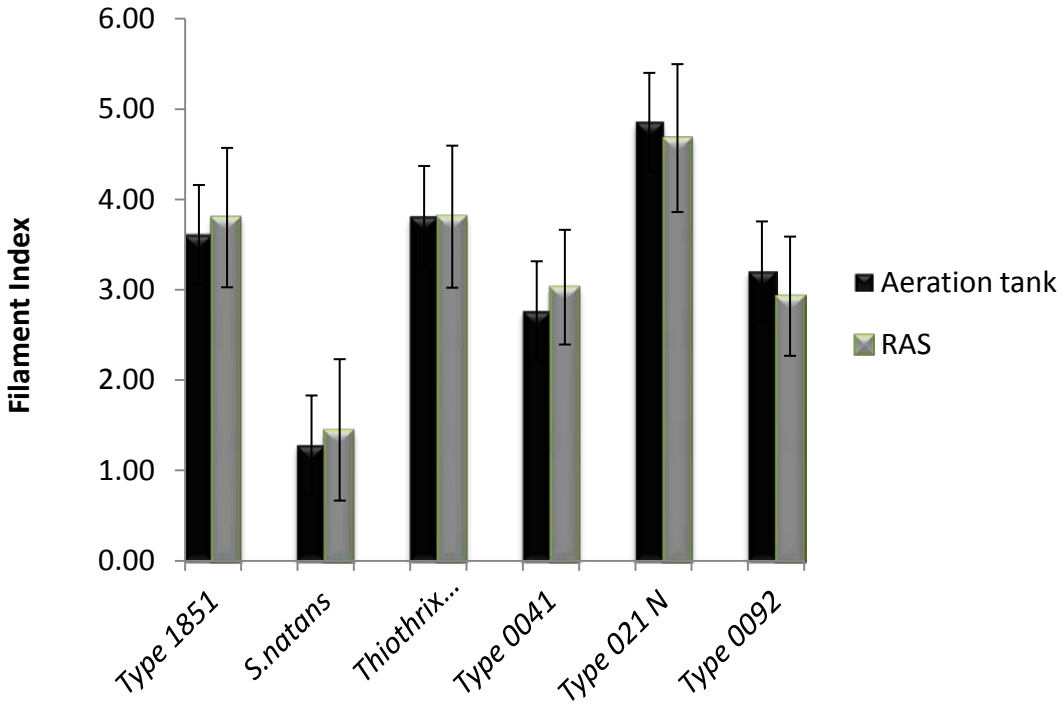

Figure 3.1: Average Filamentous bacteria detected from the aeration tank and RAS (January to December 2011), samples collected bi-weekly.

The population shift (FI: 1-6) of the various filamentous populations between the RAS and aeration tank showed only a small variation (Figure 3.1). Some of the filamentous bacteria which were morphologically identified by Gram and Neisser staining reactions which can be seen in Figure 3.2 (a - f). Type 021N has a distinguished ovoid to barrel shaped cells which are Gram negative and Neisser negative (Figures 3.2 a and b respectively), the filament distinctly revealed no attached growth. *Thiothrix* II clearly revealed defining features such as the formation of rosettes, this can be seen in Figure 3.2 d.

The FISH molecular technique with rRNA targeted oligonucleotide probes confirmed identification of the dominant filamentous bacteria and showed a strong green fluorescence (Figures 3.3, 3.4). The probe binding and fluorescence emitted by these organisms were not always the case due to some limitations (photobleaching, autofluorescence and insufficient permeabilization of the cell for the probe to penetrate). Hence, quantification using FISH was eliminated and filamentous bacteria were semi-quantified via the subjective scoring scale.

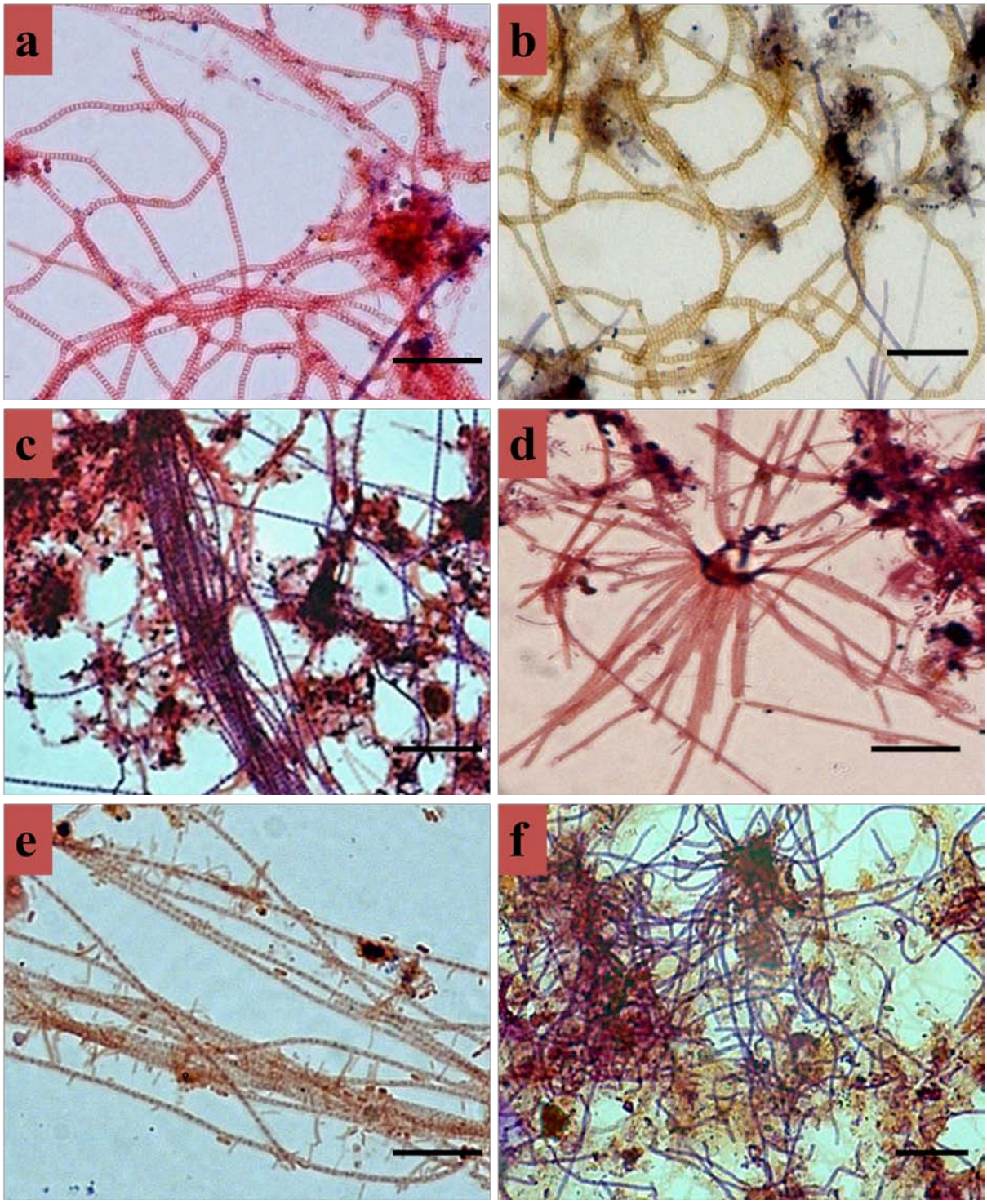

Figure 3.2: (a) Gram negative filamentous bacteria representing Type 021N, trichome is smoothly curved and most often found extending out of the floc (b) Neisser negative Type 021N, (c) *Thiothrix I* usually Gram negative however stains Gram positive when sulphur granules are present, (d) Gram negative *Thiothrix II* with rosette formation which were infrequently observed (e) weakly Gram Positive Type 1851 found in bundles with attached growth (f) Neisser positive Type 0092**,** which is found mostly within the floc, no attached growth. Scale bars = 10 μm.

3.3.2. Molecular identification of filamentous bacteria

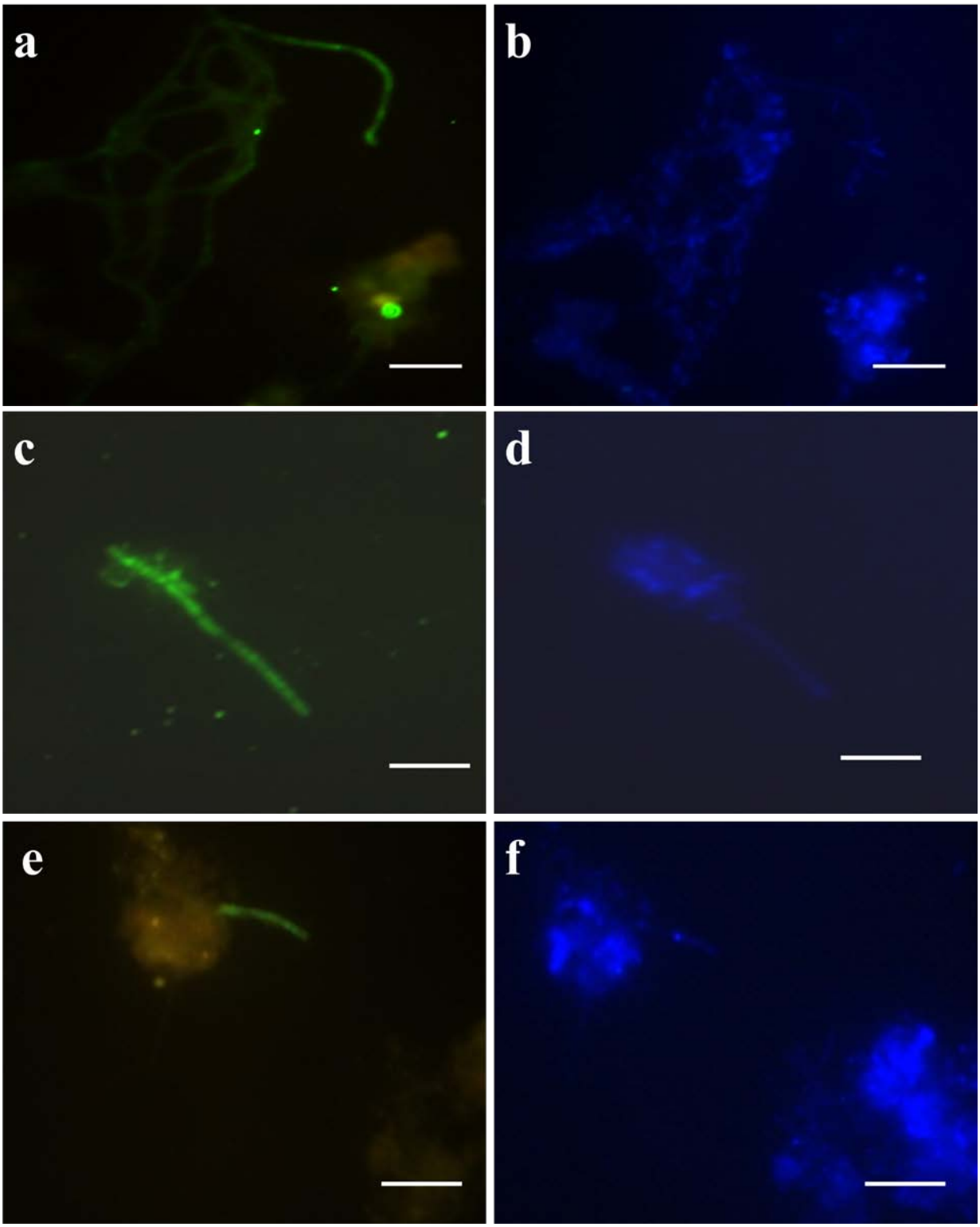

Figure 3.3: FISH on the dominant filamentous bacteria from the RAS sample (a) Typical Type 021N confirmed with G1B probe, (c) *Thiothrix* spp., confirmed with G123 probe (e) Type 0092 confirmed with CFX 223 probe, (FLOUS; green). Figures b, d & f represents DAPI images of the above mentioned filaments respectively. Scale bar = 10 μm.

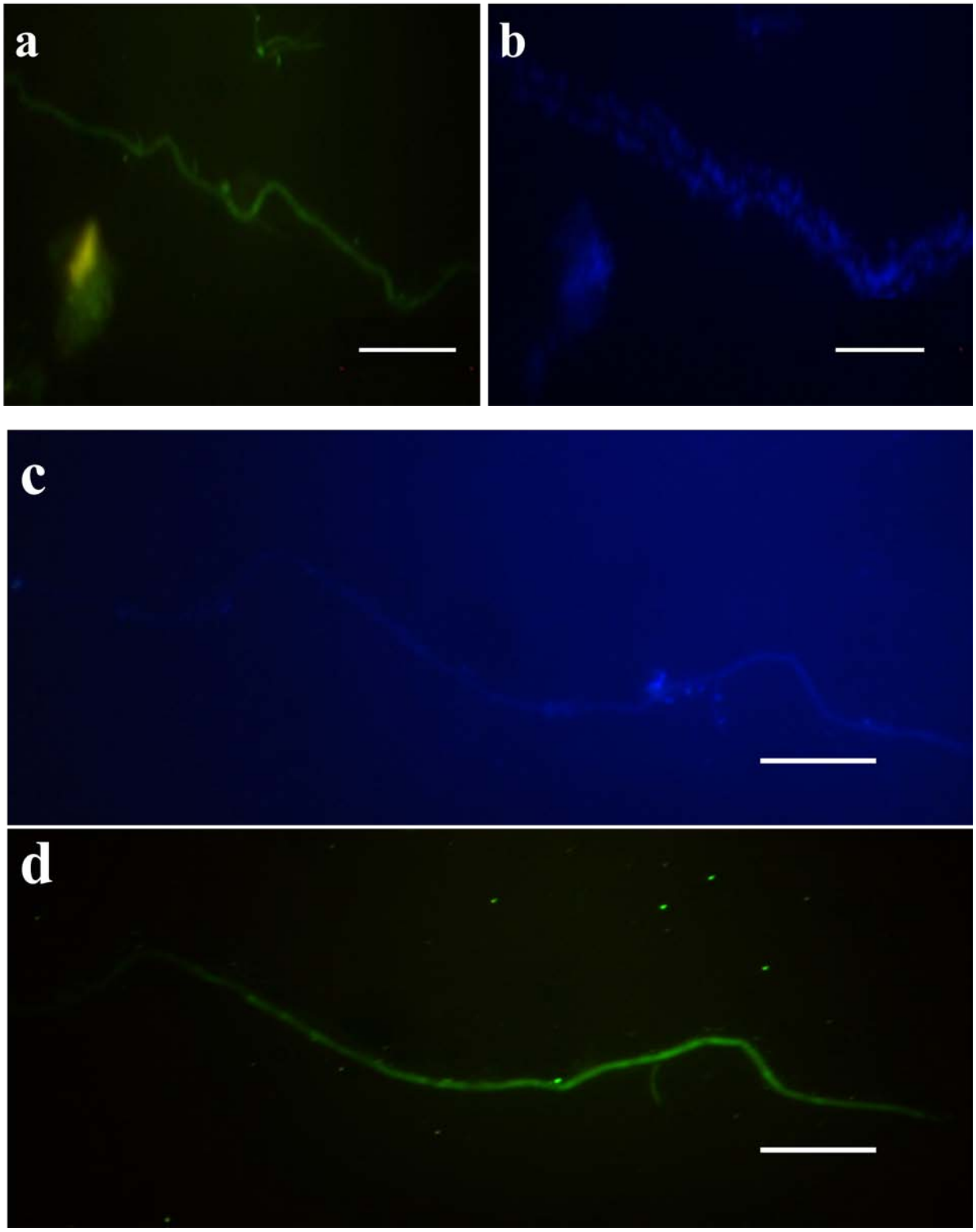

Figure 3.4: a and d indicating Typical Type 0041 detected with CFX mix, (FLUOS; green). Figures b and c - DAPI signal, blue. Scale bar = 20 μm.

3.3.3. The wastewater characteristics of the Selected Plant

The operational parameters, such as, COD, N-NH_4^+, MLSS, aerated phosphorus and SVI were recorded daily, whereas a few others (i.e. DO, pH and Temperature) were recorded bi-weekly, at the time of sampling (Table 3.3).

The selected WWTP showed optimum nutrient removal efficiencies in terms of COD, P and N removal, however, the plant frequently experienced bulking and foaming problems. A high variation in MLSS concentration (5014 ±1270 mg/L), which was greater than the original design MLSS concentration (4500 mg/L) was noted during the study period (Table 3.3). The increase in MLSS concentrations can be attributed to the change of operation control from the initial UCT Process design to a two stage Phoredox or anaerobic/oxic system to facilitate simultaneous nitrification and denitrification within the aeration reactor. At such instances, a higher MLSS concentration and a longer sludge age is required to maintain efficient nitrification and denitrification reactions. Apart from this, a low F/M ratio (ranging from 0.08 – 0.4) and a very low DO level (0.51 ±0.30 mg O_2/L) (Table 3.3) was also observed over the investigative period which ultimately could have been favouring conditions for the high abundance of the filamentous population.

Table 3.3: Plant operating conditions of the selected WWTP January- December (2011), indicating of operational parameters measured on a monthly or bi-weekly basis. Means and standard deviations are shown

	JAN	FEB	MAR	APR	MAY	JUN	JUL	AUG	SEP	OCT	NOV	DEC
influent COD (mg/L)	624.44 ±35.12	729.30 ±36.33	856.45 ±44.31	906.79 ±97.91	616.50 ±43.16	781.52 ±39.36	870.00 ±68.51	868.50 ±36.18	740.24 ±48.11	686.71 ±33.45	599.90 ±54.09	602.25 ±39.46
COD removal efficiency (%)	92.67 ±0.63	94.51 ±0.42	94.02 ±0.53	72.30 ±10.05	93.96 ±0.85	93.62 ±0.36	90.90 ±1.57	93.67 ±0.55	90.42 ±1.09	93.84 ±0.56	92.17 ±0.94	93.42 ±0.50
Influent N-NH_4^+ (mg/L)	23.35 ±2.21	39.05 ±2.14	43.37 ±1.59	40.59 ±4.43	34.18 ±4.36	34.29 ±2.82	34.19 ±3.30	42.77 ±2.86	38.05 ±2.03	40.38 ±2.42	29.37 ±2.89	31.17 ±2.68
NH_3 removal efficiency (%)	92.44 ±1.02	95.65 ±0.77	92.24 ±1.40	85.64 ±7.82	98.60 ±0.19	66.94 ±6.20	47.29 ±7.04	47.17 ±5.81	62.11 ±6.69	85.71 ±2.71	94.80 ±0.93	73.23 ±8.25
MLSS (mg/L)	5326 ±105	5936 ±108	5993 ±86	6078 ±190	5202 ±166	4838 ±171	4960 ±117	6369 ±111	7598 ±177	8575 ±194	6754 ±187	7031 ±159
P (mg P/L)	0.89 ±0.23	1.31 ±0.60	0.48 ±0.09	2.86 ±1.50	0.19 ±0.06	0.30 ±0.08	0.54 ±0.21	0.57 ±0.08	0.35 ±0.05	-	-	0.77 ±0.39
SVI (mL/g)	179.88 ±26.29	166.39 ±1.03	161.79 ±3.40	162.92 ±3.53	192.86 ±6.63	209.22 ±9.59	197.38 ±6.36	158.76 ±6.58	129.96 ±2.97	115.10 ±2.85	146.17 ±7.29	130.05 ±4.82
F/M ratio	0.11 ±0.01	0.12 ±0.01	0.14 ±0.01	0.12 ±0.01	0.11 ±0.01	0.17 ±0.01	0.18 ±0.01	0.14 ±0.01	0.11 ±0.01	0.08 ±0.01	0.08 ±0.01	0.08 ±0.01
DO (mg O_2/L)	0.90 ±0.10	0.50 ±0.20	0.80 ±0.15	1.20 ±0.25	1.40 ±0.25	0.25 ±0.15	0.21 ±0.10	0.35 ±0.14	0.24 ±0.13	0.58 ±0.05	0.37 ±0.04	0.25 ±0.04
pH	7.09 ±0.02	7.11 ±0.04	7.09 ±0.02	7.13 ±0.06	7.15 ±0.07	7.07 ±0.02	7.22 ±0.08	7.18 ±0.07	7.08 ±0.04	7.26 ±0.03	7.08 ±0.15	7.15 ±0.03
Temperature	24.01 ±0.04	24.10 ±0.01	24.80 ±0.03	23.30 ±0.01	20.70 ±0.02	19.30 ±0.30	17.50 ±0.01	19.80 ±0.03	20.30 ±0.04	22.10 ±0.02	22.90 ±0.10	24.50 ±0.01

3.3.4. The floc structure

The impact of filamentous bacteria on the AS floc structure was determined using light microscopy and almost all flocs were dominated by the presence of excessive filamentous bacteria. These filaments extended into the bulk solution leading to large diffuse flocs and bridging between flocs (Figure 3.5 a). The flocs were irregular and diffuse in the autumn and winter periods (Figure 3.6 a) and more compact during spring and summer (Figure 3.6 b). Under non-bulking conditions, the filamentous bacteria were still intact within the floc. However, during bulking conditions an open floc structure was visualized which also revealed bridging between the flocs (Figure 3.6 a), as a result, negatively affecting the sludge settling.

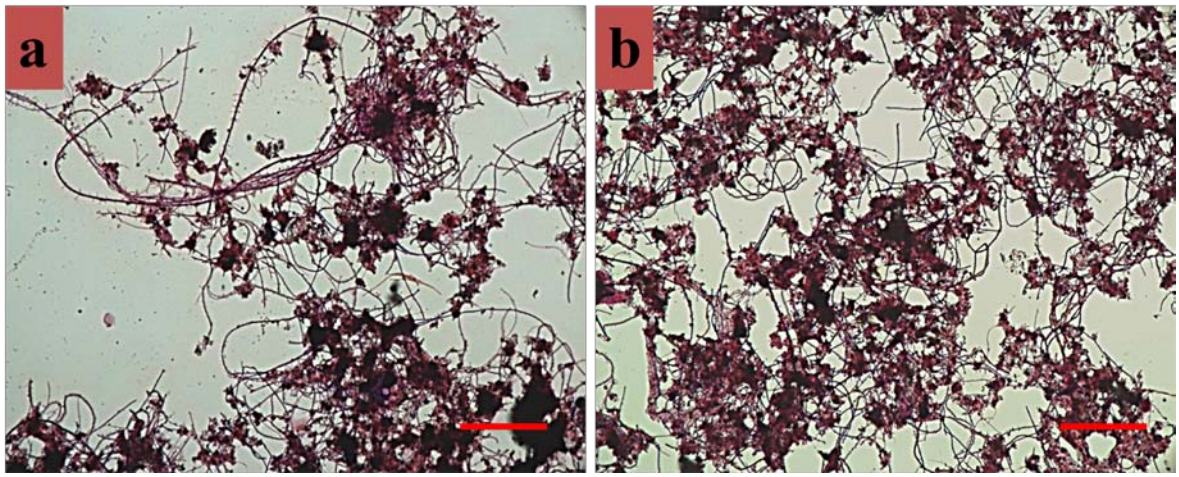

Figure 3.5: Floc structure during temporal changes, a) open floc structure showing irregular and diffuse flocs during winter; b) slightly compact flocs during spring. Scale bars = 5μm.

3.3.5. Temporal Variation

The temporal variation of the filamentous abundance was investigated on a weekly basis to monitor the filamentous bacteria population shift. Filamentous bacteria showed minor fluctuations in their population shifts during the four seasons (Figure 3.6).

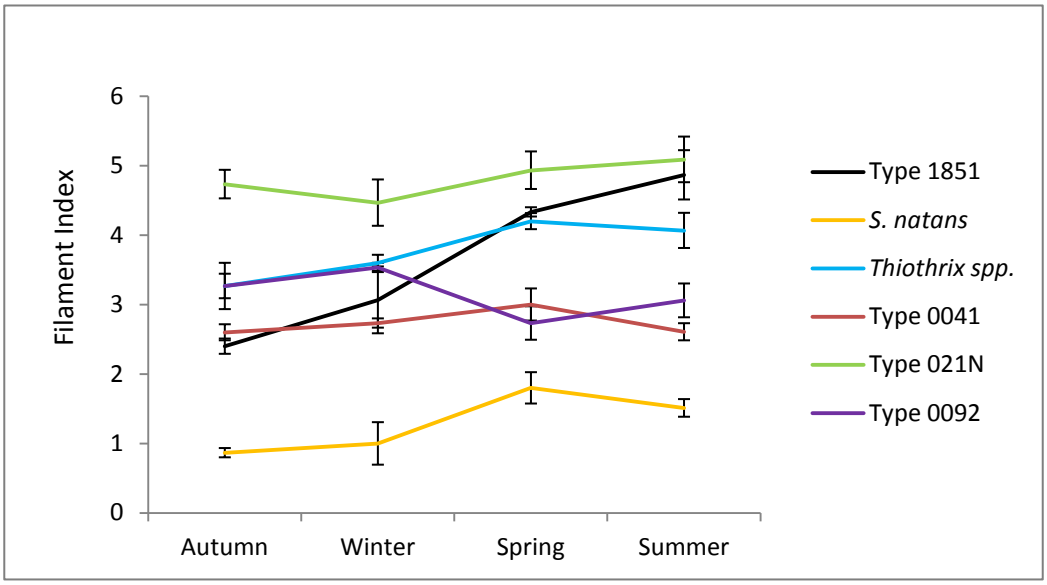

Figure 3.6: Abundance of filamentous bacterial populations (autumn, winter, spring and summer), averages for Kingsburgh WWTP are shown (2011).

The results showed that some of the filamentous bacteria *viz*., Type 1851 and *Thiothrix* spp. increased during the spring and summer periods (warmer temperature), while *S. natans* and Type 0041 showed a slight drop in their abundance during summer (Figure 3.7). Filamentous bacterium Type 1851, showed a gradual increase from autumn to summer.

3.4. Discussion

3.4.1. Dominant filamentous bacteria in bulking samples

Filamentous organisms perform several different roles in the nutrient removal process, some of which are beneficial and some are detrimental. When filamentous organisms were found in low concentrations they served to strengthen the floc particles thereby providing assistance to overcome the shearing forces as a result of mechanical action in the aeration tank. Conversely, if the filamentous organisms were in high concentration, they were found to cause bulking and foaming. An adequate balance between the core bacterial community was therefore essential to ensure an efficient process (Mesquita *et al.*, 2009). The settling phase was found to be a crucial stage of an activated sludge process, which allowed for the solid-liquid separation, hence, preventing biomass from being washed out in the final effluent. Filamentous bulking was found to be one of the most common problems in WWTPs plants

most frequently caused by the excessive growth of filamentous bacteria. Different types of filamentous bacteria were reported to be dominant in bulking samples depending on the wastewater characterisation and operational parameters (Kragelund *et al.*, 2008). Most of the filamentous bacterial requirements were very specific, thereby giving the plant a characteristic fingerprint (Mielczarek *et al.*, 2012).

Various factors have been identified that contribute to the high abundance of filamentous, some of them include DO deficiencies, nutrient deficiencies, reduced sulphur, long sludge ages and low F/M ratio (Aygun *et al.*, 2013; Jenkins *et al.*, 1993). The selected plant was operating at a high MLSS (5014 ±1270 mg/L), which was most often higher than the initial plant design (4500 mg/L). The plant also operated at a minimum DO concentration for facilitating nitrification and denitrification in the aeration tank. The process modification affected the overall plant performance (i.e. low F/M ratio, low DO level less than 1 mg O_2/L, and a longer sludge age greater than 25 days), and these conditions were favourable for most filamentous bacteria to proliferate.

Microscopic and FISH analysis of the scum and bulk samples revealed the presence of a diverse group of filamentous bacteria in the sludge samples (Jenkins *et al.*, 1993). Using the filament index (FI) scale (Jenkins *et al.*, 1993), *Thiothrix* spp., Type 1851, Type 021N and Type 0092 were the most dominant filamentous bacteria detected (FI: 3-6) (Table 3.2). Among the filamentous organisms identified, Type 021N and *Thiothrix* spp. (FI: 5-6) were reported to be abundant in septic WWTPs (Martins *et al.*, 2004; Richard *et al.*, 2003). Filamentous Type 1851 and *S. natans* were reported to dominate at low F/M (< 0.15) and low DO conditions (< 0.5 mg O_2/L) (Grady *et al.*, 1999; Jenkins *et al.*, 1993). Since all these conditions were prevalent in the selected WWTP, it was ideal for these organisms to proliferate.

The high abundance (FI: 4-6) of Type 021N and *Thiothrix spp*. in the WWTP could possibly be due to the high sulphide (50-60 mg/L) compounds which were found present in the influent wastewater, thus, leading to a septic sludge. It has been reported that, *Thiothrix I* (Figure 3.3 c) usually stains Gram positive as appose to Gram negative, due to the presence of high sulphur compounds within the filamentous trichome (Jenkins *et al.*, 1993), which was also evident in this study. Under nutrient deficient conditions these filaments exhibited rosettes (Figure 3.3 d) and contained PHB granules, this has been visualised microscopically.

In theory, *Thiothrix* spp. and Type 021N were found to proliferate under such conditions (Jenkins *et al.*, 1993) and were often reported from bulking samples worldwide (Aruga *et al.*, 2002). It has been reported that these two filaments organisms were physiologically and nutritionally versatile and they preferred similar readily biodegradable organic substrates and predominated in wastewater with low up to a moderate F/M ratios (Vaiopoulou *et al.*, 2007). Thus, the co-occurrence of these two species as the most dominant filamentous bacteria at the selected plant, could be explained based on their common characteristic requirements.

3.4.2. Sludge volume index and filamentous abundance

Filamentous bulking in practice is associated with a high SVI value. A close correlation between the filamentous bulking and the SVI values were well documented (Jenkins *et al.*, 1993; Liao *et al.*, 2004; Palm *et al.*, 1980; Sezgin *et al.*, 1978). An SVI, above 150 mL/g was found to be considered as bulking sludge (Jenkins *et al.*, 1993).

In this study, the distribution of SVI ranged between 181 mL/g – 218 mL/g, with an average of 182 mL/g from January to August (Table 3) and an average of 130 mL/g SVI for the remaining months (September to December) (warmer months). A considerable drop in SVI was recorded during the summer period even when the filament index was high (FI: > 4). Similar results were also reported previously by Mielczarek *et al.* (2012) on Danish WWTPs where, a high SVI value was recorded during winter and spring seasons compared to summer and autumn (Mielczarek *et al.*, 2012). A similar pattern has been observed in this study with an average of 176 mL/g during autumn and winter and an average of 130 mL/g during spring and summer. Additionally, a study conducted on the SVI-temperature prediction in Chongqing WWTP in China, also reported high SVI values during spring and low SVI values (non-bulking period) in summer (Lou and Zhao, 2012). This could be attributed to the fact that the competition between filamentous bacteria and floc forming bacteria were much higher during the warmer season, which may have resulted in compact flocs and hence better settling (Lou and Zhao, 2012).

3.4.3. Temperature Effects on Filament Abundance

Although, wastewater composition is one of the major operational parameters affecting filamentous growth, studies have also shown the impact of temperature on filamentous bacterial growth such as *M. parvicella* and *G. amarae* (Mamais *et al* 1998; Jenkins *et al*., 2004). Theoretically, with normal ambient temperatures (8 - 25°C) it can be stated that filamentous bacteria grow at a faster rate with an increase in temperature (Aygun *et al.*, 2013; Lee *et al.*, 2003). In this study, among the different dominant filamentous bacteria analyzed, Type 0092 showed more preference to colder temperatures as compared to the other filamentous organisms (Figure 3.6). Filamentous Type 0092 decreased subsequent to the winter period, reaching a filament index of less than 3 (Figure 3.6). The water temperature of the plant was always maintained within the optimum range and did not really deviate much. Therefore, since the WWTP was an open system, various other factors might have influenced their growth concurrently and hence many of these factors should be taken into consideration. Filamentous bacterium Type 1851 showed a rapid increase during spring and summer, where as only minor fluctuations were noted for the remaining filamentous types. Filamentous bacteria Type 1851 was found to be among the few filamentous organisms that can adapt to a wide temperature range (Jenkins *et al.*, 2004b). In this study, a gradual increase in temperature, in addition to low DO and low F/M, may have been the selective conditions jointly favouring the high abundance of this organism. It was found that the high temperatures could have increased the growth of floc forming bacteria as well as the filamentous bacteria, subsequently strengthening their interaction and competition (Aygun *et al.*, 2013; Lou and Zhao, 2012). Hence, firm and compact flocs were observed during the warmer seasons, thus, an increase in the settling velocity was also noted during this period. As a result, Type 1851 could possibly have won completion over floc formers and the remaining filamentous organisms over the nutrient substrate readily available. However, it was found that the specific mechanism of substrate competition was not fully understood (Ramothokang *et al.*, 2006a).

3.4.4. Operational parameters and filamentous abundance

In this study, low DO and F/M ratios (Table 3.2) seemed to be the main challenges of the plant. All the above filaments were reported to dominate with low F/M (0.01-0.15 kg COD/kg MLSS) and low DO levels (< 1 mg O_2/L); (Jenkins *et al.*, 1993; Scruggs and

Randall, 1998). Therefore, to predict the occurrence of the filamentous bacteria relative to the prevalent operational parameters, a statistical tool was developed. This was done to show how the operational factors (Temperature, pH, F/M, DO, $N\text{-}NH_4^+$ and COD) concomitantly affected the individual filamentous bacterial growth.

3.5. Conclusions

The investigation was carried out for a period of one year and the dominant filamentous bacteria identified from bulking sludge samples were; Type 021N, Thiothrix sp., Type 1851 and Type 0092. An open floc structure and severe bridging between the flocs were noted during bulking conditions. Filamentous bacteria showed minor shifts within their population during the four seasons. Among the different filamentous bacteria analyzed, Type 1851 showed more preference to the warmer temperature whereas Type 0092 dominated in colder temperatures. The WWTP plant showed exceptionally well COD removal efficiency throughout the study even during bulking episodes which strongly indicating the role of filamentous bacteria in COD removal from wastewater treatment plants. A major deviation from the initial design and a high variation in mixed liquor suspended solids (MLSS) concentration was observed which lead to an overall decrease in F/M ratios (< 0.5) and DO concentrations (< 1 mg O_2/L) which was lower than the optimal levels (±2 mg O_2/L). In contrast to the literature, it was also found that during summer when the SVI values were low (< 150 mL/g), the dominant filamentous bacterium was found to be very common to excessive (FI: > 4). A further study was conducted using a statistical logit model to understand the holistic effect of these operational parameters on the dominant filamentous bacteria (Chapter 4).

4. APPLICATION OF A CUMULATIVE LOGIT MODEL TO REDUCE PROBLEMATIC FILAMENTOUS BACTERIA

4.1. Introduction

Bulking is the most widespread AS settling problem caused by the enhanced propagation of filamentous bacteria worldwide (Martins *et al.*, 2004; Mielczarek *et al.*, 2012). Thus far, there are no universal strategies to overcome the increased abundance of filamentous bacteria (da Motta *et al.*, 2003; Mielczarek *et al.*, 2012) and the factors triggering filamentous growth still remain unclear (Lou and Zhao, 2012). Developing a successful tool to control bulking depends on identifying and monitoring the dominant filaments that grow under specific conditions and maintaining a balance between filamentous and floc-forming bacteria (Gulez and de Los Reyes, 2009). In addition, understanding the nutrient requirements and the conditions that enhance the activity of specific filamentous bacteria in full-scale wastewater treatment systems would be useful in controlling the excessive growth of these microorganisms (Gerardi, 2006b).

The distribution of filamentous bacteria varies from plant to plant and the presence of certain filamentous bacterial types is an indication of conditions prevalent within the system (Aonofriesei and Petrosanu, 2007; Jenkins *et al.*, 2004a; Richard *et al.*, 2003). Various surveys were performed to uncover the factors that contributed to the excessive growth of filamentous bacteria. These factors included wastewater composition, environmental conditions, process design (proper bio-selector design) and plant operating parameters (Govoreanu *et al.*, 2003; Guo *et al.*, 2010; Mielczarek *et al.*, 2012). Some of these organisms have been examined in the form of pure cultures (Kanagawa *et al.*, 2000; Kragelund *et al.*, 2008; Naidoo, 2005; Ramothokang *et al.*, 2003), however, to investigate such data in a full-scale treatment process can be very difficult and cumbersome. To date, pure culture studies have been ambiguous and limited, since isolating many of these filamentous bacteria have been reported unsuccessful (Mielczarek *et al.*, 2012). Many studies have been done directly towards filamentous bacteria in full-scale AS systems, however, knowledge about their *in situ* behaviour is still to be discovered.

Recent studies involve statistical modelling to investigate sludge settling properties in relation to operational parameters, in full-scale WWTPs (Lou and Zhao, 2012; Mielczarek *et al.*, 2012). For instance, SVI was correlated to operational parameters by the use of principal component regression analysis (PCR) and the artificial neural network (ANN) technique. Both techniques were structured to data-driven modelling, which was formed by computational programming, and the analysis of all data characterising a system under study. Lou and Zhao, (2012) concluded that the ANN technique performed better than the PCR technique when explaining sludge bulking problem in WWTPs, however, the approach still needed further validation.

Among the various statistical approaches, the cumulative logit model has been well explicated and used in the social and biomedical sciences where ordinal data is often collected. However, the model has not been implemented in activated sludge systems in relation to filamentous organisms. The work contained this chapter deals with the application of a cumulative logit model describing selective operational factors which significantly influenced the dominant filamentous bacteria using a statistical model. This study would enable researches to further expand in the surveys of filamentous bacteria and understand factors which may contribute to filamentous bacterial growth.

4.2. Materials and Methods

4.2.1. Sample collection and analysis

Mixed liquor sludge samples (1 L) were collected and analysed as described in Chapter 3, Section 3.2.1. Filamentous bacteria were identified using conventional microscopic techniques, and confirmed using the FISH technique (Chapter 3).

Wastewater characteristics were chemically analysed using an Aquakem Gallery Photometric Auto-analyser (Thermo Scientific, Germany) according to protocols outlined by the environmental protective agency (EPA, 2010). Chemical oxygen demand (COD) and mixed liquor suspended solids (MLSS) were measured according to Standard Methods (APHA, 1998). The important parameters *viz*., sludge volume index (SVI) and food to microorganism (F/M) ratio were calculated according to Jenkins, (2004). Dissolved oxygen and temperature were measured using a multi-parameter YSI model, 556 MPS system (Yellow Spring

Systems, USA). The sludge samples were immediately fixed using 96% ethanol and stored at -20°C for further microbiological analyses (Nielson *et al.*, 2009).

4.2.2. Statistical analysis

To determine the significant relationships of filamentous bacteria and the operational parameters, Logistic regression analysis was performed with statistical analysis software (SAS), version 9.2.

4.2.2.1. Ordinal Logistic Regression using SAS

Filamentous bacteria were quantified categorically (ranked from a scale of 1 to 6 i.e. very few to excessive, Table 2.2) hence, the logistic regression was selected as the method of choice for the analysis (Park, 2009). Since the categories were ordinal the "ordinal logistic regression analysis" was used (Kayri and Cokluk, 2010) to make significant predictions with the presence and abundance of an organisms and its growth requirements. This type of model has been successfully adopted by researchers especially when dealing with categorical data (Gardiner and Luo, 2011; Kayri and Cokluk, 2010).

The Akaike Information Criterion (AIC), is calculated as using the following equation (4.1.).

$$\text{AIC} = -2\,Log\,L + 2\,((k-1) + s) \qquad (4.1)$$

where *k* is the number of levels of the dependent variable and *s* is the number of predictors in the model. AIC is used for the comparison of non-nested models on the same sample. Ultimately, the model with the smallest AIC is considered the best, although the AIC value itself is not meaningful (SAS Annotated output 2012).

Schwarz Criterion (SC) is defined as:

$$SC = -2\,\text{Log}\,L + ((k-1) + s) * \log\,(\Sigma\,fi) \qquad (4.2)$$

where f_i's are the frequency values of the i^{th} observation, and *k* and *s* are defined above. Like AIC, SC penalizes for the number of predictors in the model and the smallest SC is most

desirable and the value itself is not meaningful. The Log-Likelihood (-2 Log L) is used in hypothesis tests for nested models and the value in itself is not meaningful (SAS Annotated output 2012).

The cumulative logits are defined as (Equation 4.3):

$$L_j = \text{logit}[F_j(x)] = \log\left(\frac{F_j(x)}{1-F_j(x)}\right) = \log\left(\frac{\pi_1(x)+\cdots+\pi_j(x)}{\pi_{j+1}(x)+\cdots+\pi_j(x)}\right),\ j = 1 \ldots, J-1. \qquad (4.3)$$

Each cumulative logit uses all J response categories. The cumulative logit L_j is an ordinary logit model for a binary response in which categories 1 to j form a single category, and categories j+1 to J form the second category. The cumulative logit model incorporates all J-1 cumulative logits for a J-category response into a single, parsimonious model. Let$\{L_j(x) = \text{logit}[F_j(x)]$, j=1,…, J - 1$\}$, where $F_j(x) = P(Y \leq j \mid x)$ is the cumulative probability for response category j, when the explanatory variables take value x (Yay and Akinci, 2009, Gardiner, 2011).

To include effects of the explanatory variables, proportional odds are expressed as follows (Equation 4.4):

$$L_j(x) = \alpha_j + \beta' x,\ j = 1, \ldots, J - 1. \qquad (4.4)$$

The $\alpha_j, \ldots\ldots, \alpha_{j-1}$ parameters are non-decreasing in j and are known as the intercepts or the "cut points" (Yay and Akinci,2009). The odds ratio of cumulative probabilities is called a cumulative odds ratio. The log of the cumulative odds ratio is proportional to the distance between the values of the explanatory variables, with the same proportionality constant applying to each cut point. The interpretation of the proportional odds model is that the odds of making response $\leq j$ are exp $[\beta'(x_1 - x_2)]$ times higher at x=x_1 than at x=x_2.

The model assumes a variable's effect on the odds of response below category j is the same for all j. Proportional odds cumulative logit model is found to be the most popular model for ordinal data. This describes the log-odds of two cumulative probabilities, one less-than and the other greater-than type. Proportional odds measures how likely the response is to be in category j or below versus in a category higher than j. The odds ratio considers the effects of the independent variable (SAS/STAT user-guide, 2008).

Table 4.1: Factors grouped accordingly for the ordinal logistic regression model (SAS), group 0 and group 1

Factors	group 0	group 1	References
Temperature	< 20°C	≥ 20°C	(Jenkins, 1993)
pH	< 7.15	≥ 7.15	
DO	< 1.1 mg O_2/L	≥ 1.1 mg O_2/L	(Martins *et al.*,2003)
F/M	< 0.1	≥ 0.2	(Jenkins *et al.*, 1993)
Influent COD	< 750 mg/L	≥ 750 mg/L	
Influent N-NH_4^+	< 32 mg/L	≥ 32 mg/L	

The operational parameters were grouped accordingly into low and high levels, groups 0 and 1 respectively (Table 4.1). The ranking order was made based on the data obtained from this study. This was done to achieve an overall perspective, thus, presenting clarity of how these conditions influence and show prevalence to the filamentous bacteria types. Based on the output data derived from the cumulative logit model, the operational parameters (high/ low levels) show simplicity in terms of how the factors in juxtaposition affect each of the filamentous bacteria. This has been clearly illustrated in the polybar plots (Figures 4.1- 4.7). Some of the operational parameters were categorised into high and low levels based on the median values (pH, COD, N-NH_4^+). Others (DO, F/M and temperature) were selected and categorised with the support of theoretical information and optimum conditions.

4.3. Results

4.3.1. Cumulative logit model analysis, to establish significant relationships between FI and operational parameters

An ordinal logistic model (PROC LOGISTIC, SAS V9.2) with a cumulative logit link function was used to determine the significant relationships of individual filamentous bacteria with the plant operating conditions (Table 4.3). The model convergence status was satisfied and was significant for each filamentous bacterium analyzed. The Akaike Information Criterion (AIC), Schwarz Criterion (SC) and the Log-Likelihood (-2 Log L) values of the

basic model decreased with the explanatory variable appearance of the model (Appendix 2). Results prove that the model fits the data adequately, lower AIC, SC and -2Log L values were shown with the intercepts and covariates compared to the intercepts only (Table 4.2). According to this type of analysis, a lower AIC value (outline figures in red) showed a better fit of the model and hence, the model seems to be highly significant for the current set of data.

Table 4.2: Model fit statistics for Type 1852 from the cumulative logit model with all factors grouped together

Model Fit Statistics		
Criterion	Intercept Only	Intercept and Covariates
AIC	186.155	160.236
SC	194.533	181.180
-2 Log L	178.155	140.236

The model fit statistics were carried out for each of the dominant organisms with individual factors and together with all factors combined (Appendix 2).

Individual factor analysis and a combination of all factors (COD, N-NH_4^+, DO, F/M, temperature and pH) were examined via the logit model. Significant relationships were obtained for a few operational parameters (F/M, influent COD, DO, N-NH_4^+, temperature) and the dominant filamentous bacterial populations (Table 4.3). Further, a maximum likelihood and a proportional odds ratio analysis were incorporated and analysed for each of filamentous bacterial populations against these operational parameters (Table 4.4 and 4.5).

Table 4.3: Cumulative logistic output, illustrating significant and non-significant relationships with the organism and operational parameters

Filamentous bacteria	Significant factors (Pr > ChiSq)						
	Influent COD	Influent $N\text{-}NH_4^+$	DO	F/M	Temp	pH	Phosphorus
Type 1851	*0.0059	*0.0077	*0.014	*0.0001	0.603	0.1939	*0.0163
T 021N	*0.0380	**0.0604	0.2277	*0.0064	0.2131	0.6833	0.3668
Type 0092	*0.0041	*0.0043	*0.008	*0.0160	*0.0028	0.3440	0.7628
Thiothrix spp.	*0.0172	0.3970	0.1844	*0.0033	0.7691	0.1860	0.1158
Type 0041	0.7663	*0.0123	0.7880	0.3850	0.5441	*0.0175	0.5086
S.natans	*0.0400	**0.0788	**0.0778	*0.0312	0.2280	0.5434	0.1732

*significant relationships (*p*-value < 0.05); **significant relationships (*p*-value < 0.1), indicating that the filamentous bacteria were highly significant to some of the operational parameters (i.e. COD, Temperature, F/M, DO, $N\text{-}NH_4^+$ and P).

Since a descending option on model selection was formatted into the maximum likelihood analysis. Thus, according to the output data generated using the above analysis, a positive coefficient (estimated by the maximum likelihood) indicates an increased chance that a subject (filamentous bacteria) with a higher score on the independent variable (COD, F/M, DO, $N\text{-}NH_4^+$, Temperature and pH) will be observed in a lower category (FI: 1-3); (Table 4.4 and 4.5). A negative coefficient indicates an increased chance that a subject (filamentous bacterium) with a higher score on the independent variable will be observed in a higher category (FI: 4-6) (Snedker *et al.*, 2010). For instance, COD showed a positive coefficient with the filament Type 1851 (maximum likelihood estimation =1.3884), hence the organism was most likely to be at a low index scale (FI: $\leq$ 3) when the COD is high. Only Filamentous bacteria Types 0092 and 021N showed negative coefficient values with a few operational parameters. The others showed positive coefficient values (this will be further explained in section 4.3.2 below).

Table 4.4: Maximum likelihood with std error and Odds Ratio (Type 1851, Type 021N, Type 0092)

Factors	Type 1851			Type 021N			Type 0092		
	Max likelihood	Std error	Odds Ratio	Max likelihood	Std error	Odds Ratio	Max likelihood	Std error	Odds Ratio
Inflluent COD	1.3884	0.5043	*4.009	1.0205	0.4919	2.775	-1.588	0.5532	0.204
Temp	-	-	-	-	-	-	1.8823	0.6286	6.569
F/M	3.4963	0.7333	32.992	1.6029	0.8672	4.968	-1.4602	0.6061	0.232
DO	1.7498	0.7125	5.754	-	-	-	-1.9217	0.7256	0.146
Influent $N\text{-}NH_4^+$	1.4931	0.5600	4.451	-1.0493	0.5588	0.350	1.7875	0.6286	0.167
P	1.8526	0.7710	6.377	-	-	-	-	-	-

*Odds Ratio indicates the relative differences among each of the operational parameters (i.e. COD, Temperature, F/M, DO, NH_3 and P) at high and low levels. For instance, The odds ratio analysis for influent COD "high" verses "low" (for Type 1851) showed that, with high COD levels the organism has *4.009 times the odds of being at a lower ranking, than low levels of COD.

Table 4.5: Maximum likelihood with std error and Odds Ratio (*Thiothrix* spp., Type 0041, *S. natans*)

Factors	*Thiothrix* spp.			Type 0041			S.natans		
	Max likelihood	Std error	Odds Ratio	Max likelihood	Std error	Odds Ratio	Max likelihood	Std error	Odds Ratio
Inflluent COD	1.2614	0.5043	3.530	-	-	-	1.0236	0.4984	2.783
Temp	-	-	-	-	-	-	-	-	-
F/M	1.9805	0.6742	7.246	-	-	-	1.3050	0.6058	3.688
DO	-	-	-	-	-	-	1.1529	0.6538	3.167
Influent $N\text{-}NH_4^+$	-	-	-	1.5334	0.6122	0.216	1.0324	0.4873	2.808
P	1.1415	0.7257	3.131	-		-	0.9017	0.6620	-

4.3.2. Relationships between chemical influent (COD, N-NH_4^+) levels and filamentous bacteria

Among the different operational parameters analyzed, influent COD and N-NH_4^+ illustrated significant relationships with the dominant filamentous bacteria (Table 4.3). The maximum likelihood estimation (Table 4.4 and 4.5) for influent COD versus filamentous bacteria were analysed. Results disclose, at a high COD level (≥ 750 mg/L), most of the filamentous organisms identified (Type 1851, Type 021, *Thiothrix* spp. and *S. natans* with estimated coefficients: 1.0205, 1.2614, 1.0236 respectively) were most likely to be at a lower ranking (FI: 1-3) except for Type 0092 (estimated coefficient: -1.588). Thus, indicating, at a high COD level, Type 0092 will most likely be ranked at high levels (FI: ≥ 4), provided that all the other operational parameters are held constant.

The odds ratio analysis for influent COD "high" versus "low" (for Type 1851) showed that, with a high COD level (≥ 750 mg/L) the organism has 4.009 times the odds of being at a lower ranking, than low levels of COD (< 750 mg/L) (Table 4.4). Meaning that, Type 1851 is most likely to be in a low ranking (FI: ≤ 3) when the COD was high (≥ 750 mg/L) as opposed to when the COD level was low (< 750 mg/L). The same relationships could be seen with Type 021N, *Thiothrix* spp. and *S. natans* with an odds ratio of 2.777, 3.530 and 2.783 respectively (Table 4.4 and 4.5). However, Type 0092 showed an odds ratio of less than 1 (OR: 0.204), indicating that the odds of Type 0092 ranked few to common (FI: 3-4) when the COD was high as opposed to a low COD, is within a ratio of 1:5, meaning that there was a greater chance of Type 0092 ranked few to common at a high COD level (Table 4.4).

A probability graph was obtained from the logit model analysis for each dominant filamentous bacterium against the significant operational parameters. Hence, the analysis explained above can be visualised more clearly in these polybar plots. In most cases filamentous bacteria showed a greater chance of being in lower rankings (FI: ≤ 3) when the operational parameters were high (Figures 4.1 – 4.3).

The plots (Figure 4.1 a - g) illustrate the observed rankings of each filamentous organism identified and is represented by the colours (blue, red, green, brown and purple, i.e. FI 2-6 respectively). Each bar colour represents a subjective filament index (FI). From the polybar plots in Figure 3 (a – c), it was observed that filamentous bacteria Type 021N, Type 1851 and

Thiothrix spp., showed an increased (> 50%) chance of being in a lower ranking when the influent COD was high (1). Whereas, Type 0092 showed a higher (> 80%) chance of being in a lower ranking when the influent COD was low (0) (Figure 4.1 d). Similarly, Figure 4.1 (e – g) represent filamentous abundance at low (0) and high (1) $N\text{-}NH_4^+$ levels. The influent $N\text{-}NH_4^+$ level ranged between 23 to 43 mgL^{-1} across the study period. From the maximum likelihood estimation and the odds ratio analysis, high $N\text{-}NH_4^+$ ($\geq$ 32 mgL^{-1}), reduced the likelihood of Type 021N at being in a lower ranking (MLE: -1.0493; OR: 0.350) (Table 5.1 and 5.2). It was therefore predicted that there is a higher chance of Type 021N being ranked at a higher filament index (FI: 4-6) when the $N\text{-}NH_4^+$ is high (1) ($\geq$ 32 mgL^{-1}). Conversely, filamentous Type 1851 and Type 0092 revealed a < 30% chance that these organisms could be at a higher ranking (FI: $\geq$ 4) when the $N\text{-}NH_4^+$ is high.

From the polybar plots indicated below 4.1 a - d, low COD (0) and high COD (1) levels are represented on the x-axis. Low COD represented by 0, specify < 750 mg/L and high COD levels represented by 1 specify $\geq$ 750 mg/L, the COD levels were grouped accordingly to BNR systems. The other factors were also grouped into high and low levels and are listed in Table 4.1. The plots (Figure 4.1) illustrate the measured rankings of each identified filamentous organism and this is represented by the colours (blue, red, green, brown and purple, i.e. FI 2-6 respectively). Each bar colour represents a subjective filament index, for instance, blue bar indicate FI = 2, red bar indicate FI = 3. Thus, indicating the probability of the organism being ranked at a possible level of abundance at a low COD (0) or high COD (1) level, whilst, holding all other operational parameters constant.

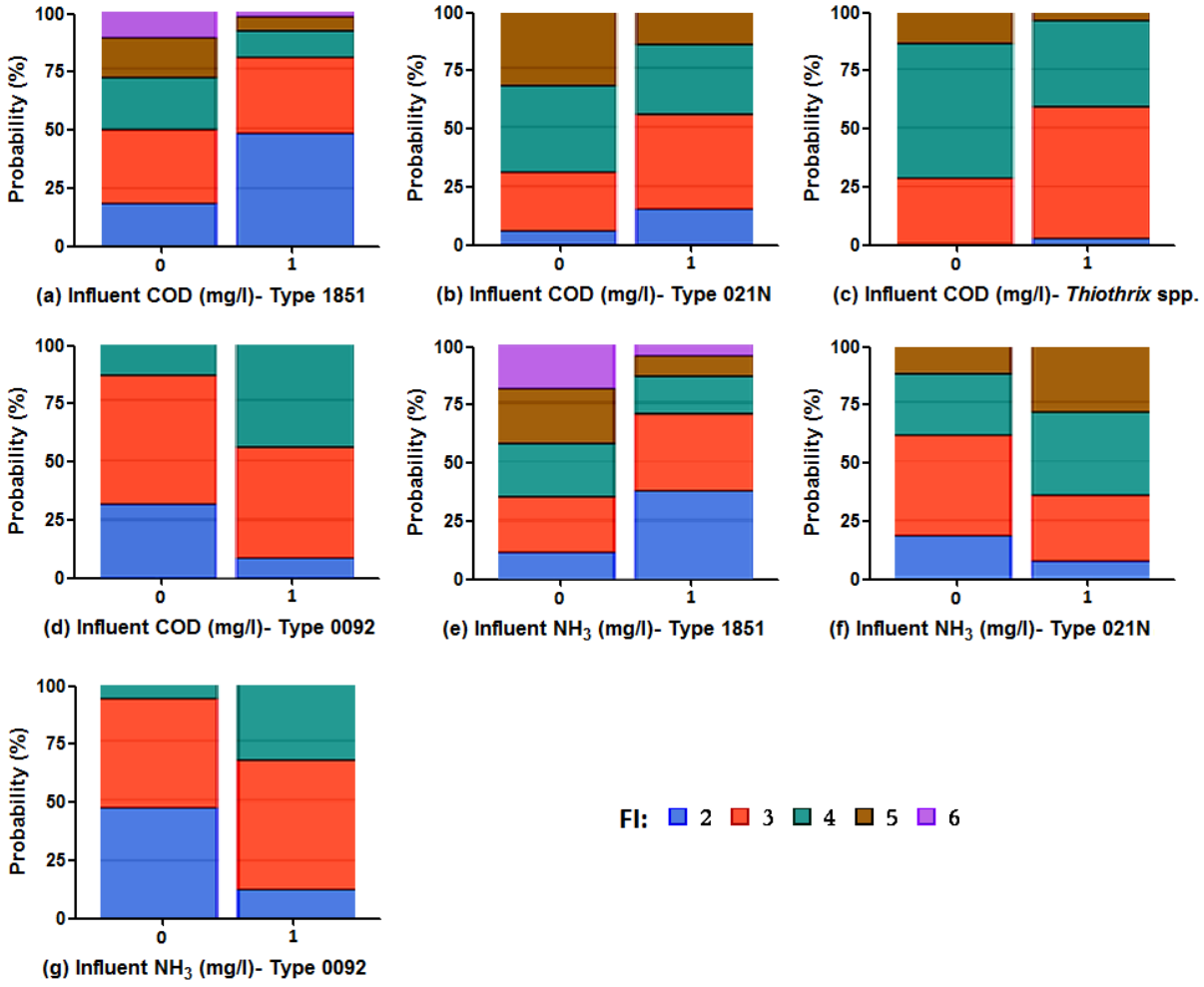

Figure 4.1: Polybar plots a - d illustrate the probability of each filamentous bacterial ranking *y* (FI: 2-6) at low (0) and high levels (1) of COD. The plots e - f indicate low N-NH_4^+ (0) and high N-NH_4^+ (1) levels. The *y*-axis indicate the probability percentage at different ranking levels.

From the polybar plots in Figure 4.1 a - c, it was observed that the filamentous bacteria Type 021N, Type 1851 and *Thiothrix* spp. respectively, showed a greater than 50% chance of being in a lower ranking when the influent COD was high (1). However, Type 0092 showed a higher (> 80%) chance of being in a lower ranking when the influent COD was low (0) (Figure 4.1 d).

Figures 4.1. e - g represent filamentous abundance at low N-NH_4^+ (0) and high N-NH_4^+ (1) levels, thus, indicating the influence of N-NH_4^+ on filamentous bacteria e) Type 1851, f) Type 021N and Type 0092 respectively. The influent NH_3 level with an average value of 36

mg/L fluctuated between 23 to 43 mg/L across the year. In this study, all filamentous bacteria identified (Type 1851, Type 021N, Type 0041, Type 0092 and *S.natans*), showed significant relationships with $N\text{-}NH_4^+$ except for the *Thiothrix* spp. (Table 4.3). From the maximum likelihood estimation, high $N\text{-}NH_4^+$ greater than 32 mg/L, increased the likelihood of Type 1851, Type 0092, Type 0041 and *S.natans* (estimated coefficients: 1.4931, 1.7875, 1.5334 and 1.032 respectively) at being in a lower ranking and reduced the likelihood of Type 021N at being in a lower ranking (MLE: -1.0493) (Table 4.4 and 4.5).

4.3.3. Operational parameters (DO, F/M) vs. filamentous bacterial index

Among the different filament bacteria analyzed, only a few filamentous types (*S.natans*, Type 0092 and Type 1851) showed significant relationships to the level of DO (Table 4.3). The dominant filamentous bacteria *Thiothrix* spp. and Type 021N showed no significant relationship with DO in the cumulative logit model.

The DO concentration at Kingsburgh WWTP was always kept at a low range (0.5 to 1.6 mg O_2/L) with the help of four sequentially-timed aerators to facilitate the denitrification process within the aeration tank. The estimated coefficients and odds ratio analysis (Table 4.4 and 4.5) showed that a higher DO (> 1.1 mg O_2/L), increased the probability of Type 1851 and *S.natans* ranked in a lower level (estimated coefficients: 1.7498, 1.1529 respectively), and decreased the log odds of Type 0092 (estimated coefficient: -1.9217) ranked in a lower level. This means that there was a greater chance of Type 0092 being in a higher ranking (FI: $\geq$ 4) with high levels of DO (Table 4.4). This can also be seen in Figure 4.2 b which clearly showed that Type 0092 revealed a greater than 60% chance at being in a higher ranking (FI: greater than 3) when the DO was high ($\geq$ 1.1 mg O_2/L), provided that all the other variables were held constant. In contrast, Type 0092 showed an increased 80% chance of the organisms being at low levels (FI: < 3) when the DO was low (< 1.1 mg O_2/L).

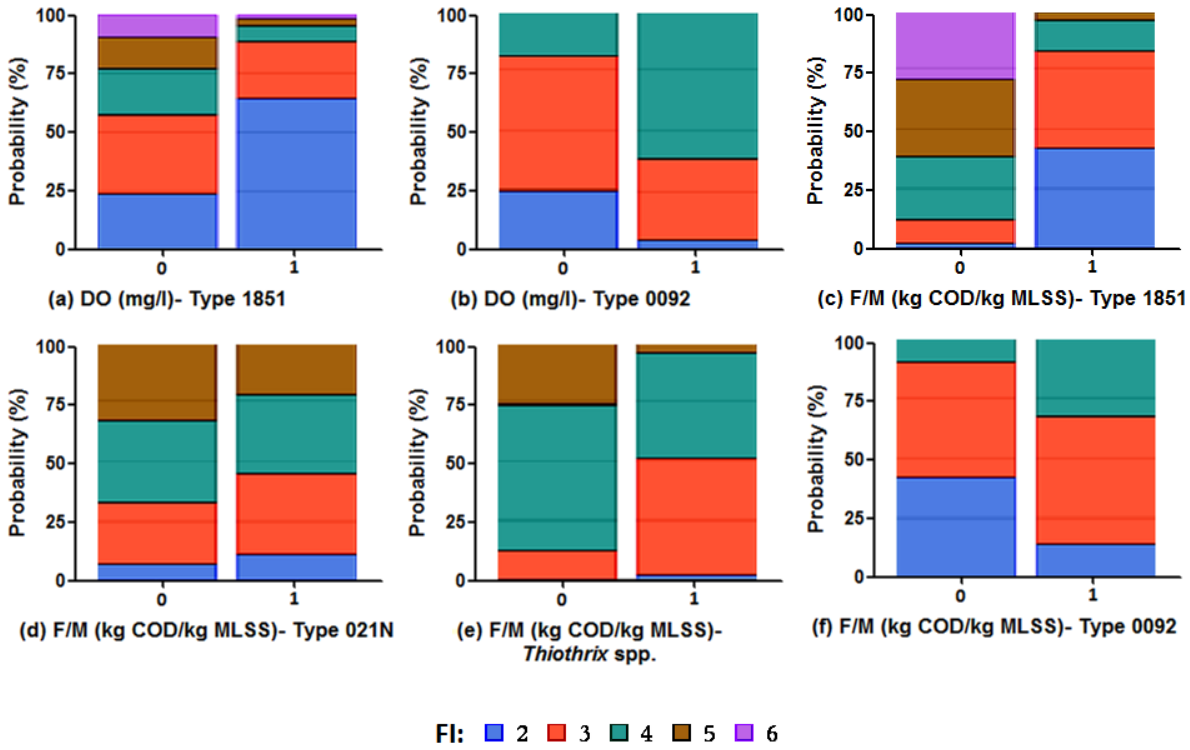

Figure 4.2: Polybar plots a & b illustrate the probability of each filamentous bacterial ranking *y* (FI: 2-6) at low DO (0) and high DO (1). The plots c - f indicate low F/M (0) and high F/M (1) levels. The *y*-axis indicate the probability percentage at different ranking levels.

The logit model showed a significant relationship with Type 1851, Type 0092, *Thiothrix* spp. and *S. natans* in relation to the F/M ratio. However, no significant relationships were obtained for Type 021N and Type 0047. Even though the F/M was considerably low (less than 0.2 kg COD/kg MLSS) throughout the study, the F/M was categorized as low (0) when F/M ratio was less than 0.1 kg COD/kg MLSS and high (1) when F/M was≥ 0.1 kg COD/kg MLSS (Table 3). The low F/M category increased the probability of filamentous Type 1851, Type 021N, *Thiothrix* spp. and *S.natans* (estimated coefficient: 3.496, 1.602, 1.980 and 1.305 respectively) at being in a lower ranking as opposed to Type 0092 (estimated coefficient: -1.460), provided that all other variables were held constant (Table 4.4, 4.5; Figures 4.2 c - f).

4.3.4. Environmental factors (Temperature and pH) vs. filamentous bacterial index

The influence of environmental factors such as temperature and pH on filamentous dominance was also investigated. The temperature and pH of the selected WWWTP ranged between 17°C to 25°C and 7.07 - 7.26 respectively, during the study period. Among the different filamentous bacteria analyzed, only Type 0092 showed a significant relationship with temperature shifts. The results indicated that temperatures≥ 20°C increased the probability of Type 0092 being in a lower ranking. Further, Type 0092 displayed an odds ratio of 6.60, also indicating the high odds of Type 0092 being ranked (FI: 1-3) at a lower scale when the temperature is ≥ 20°C (Figure 4.3).

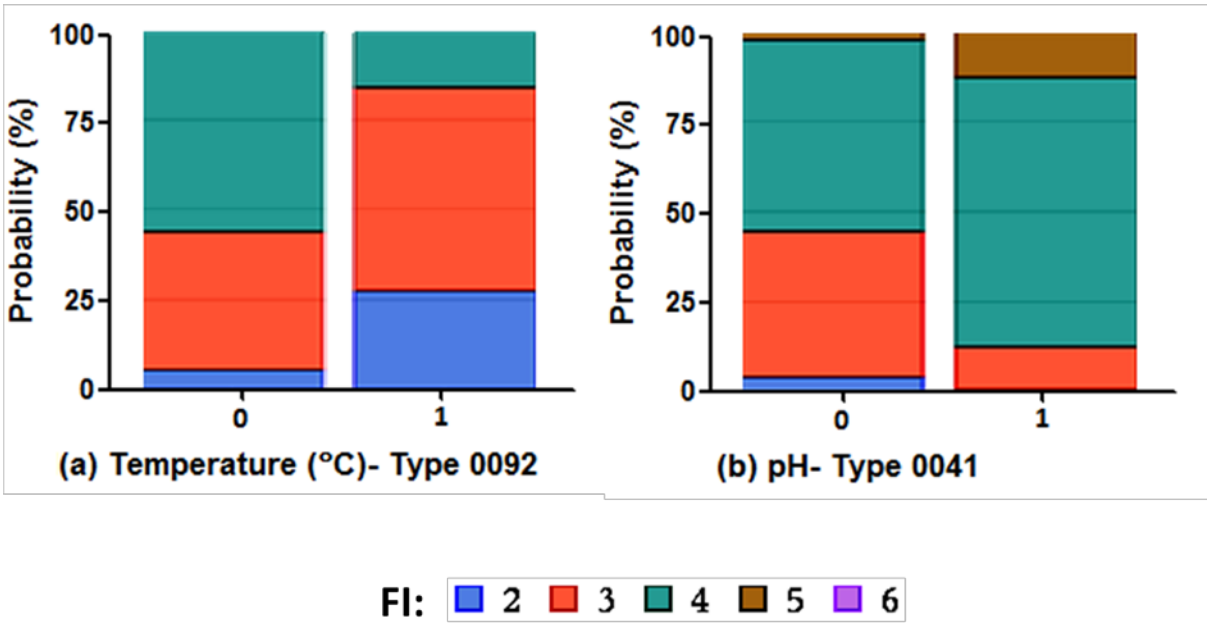

Figure 4.3: Polybar plot illustrates the probability of the filamentous bacterial ranking, *y* at low (0) and high (1) levels of temperature and pH respectively. The *y*-axis indicate the probability percentage at different ranking levels.

The proportional odds ratio model of Type 0092 showed a positive coefficient value (estimated coefficient: 1.8832); (Table 4.4). The high temperatures (≥ 20°C) increased the probability of Type 0092 being in a lower ranking, and lower temperatures (< 20°C) increased the probability (indicating a greater than 50% chance) of this filament being in a higher ranking (FI: 4). Type 0092 showed an odds ratio of 6.596, meaning that there is a

greater probability of Type 0092 ranked (FI: 1-3) in a lower scale when the temperature is high, provided that all other variables are held constant (Figure 4.3 a).

In Chapter 3, Figure 3.6, showed the effects of temporal variation on the filamentous bacteria identified. From the graph, was observed that Type 0092 were almost consistent during the autumn and winter period, however, there was a gradual decline in the filamentous population from winter to spring, followed by a steep increase into the summer period. Hence, predicting that colder temperatures was in favour of filamentous bacteria Type 0092.

4.3.5. Prediction of Filamentous Occurrence Based on Multiple Operating Parameters

Further analysis was done using cumulative polybar probability plots to obtain a more comprehensible prediction on the effect of these operational parameters on each of the dominant filamentous bacteria (Figures 4.4 - 4.7). The operational parameters (influent COD, F/M ratio, DO and $N\text{-}NH_4^+$) were chosen based on the significant relationships found with the dominant filamentous bacteria from the cumulative logit model.

4.3.5.1. Predicted Cumulative Probabilities of Type 1851

Type 1851 showed significant relationships with COD, DO, $N\text{-}NH_4^+$ and F/M ratios (Table 4.3). According to the predicted cumulative probability plot (Figure 4.4), when all the significant operational parameters were grouped at high levels (1), the plot (code- black elliptical outline in probability plot) clearly illustrated a greater than 90% chance of filamentous Type 1851 being at a lower filament index (FI: ≤ 3) scale.

In addition, the graph showed the maximum predicated cumulative probabilities (> 90% chance) of filamentous bacteria Type 1851 being at a low ranking (FI: ≤ 3), when DO and F/M are high (1) regardless of other operational parameters (COD and $N\text{-}NH_4^+$) being at low or high levels. From this, it was observed that a high F/M ratio together with a high DO level plays a significant role in maintaining Type 1851 in a lower ranking (FI: ≤ 3) (codes- black and grey elliptical outlines in probability plot, Figure 4.4). Alternatively, in all cases when DO was high (1) and F/M was low (0), regardless of all the other operational parameters, the graph showed only ±30% chance that the organism would be predicted at a lower scale. Thus,

the maximum probability of the organism being ranked low was obtained when F/M and DO were both at high levels.

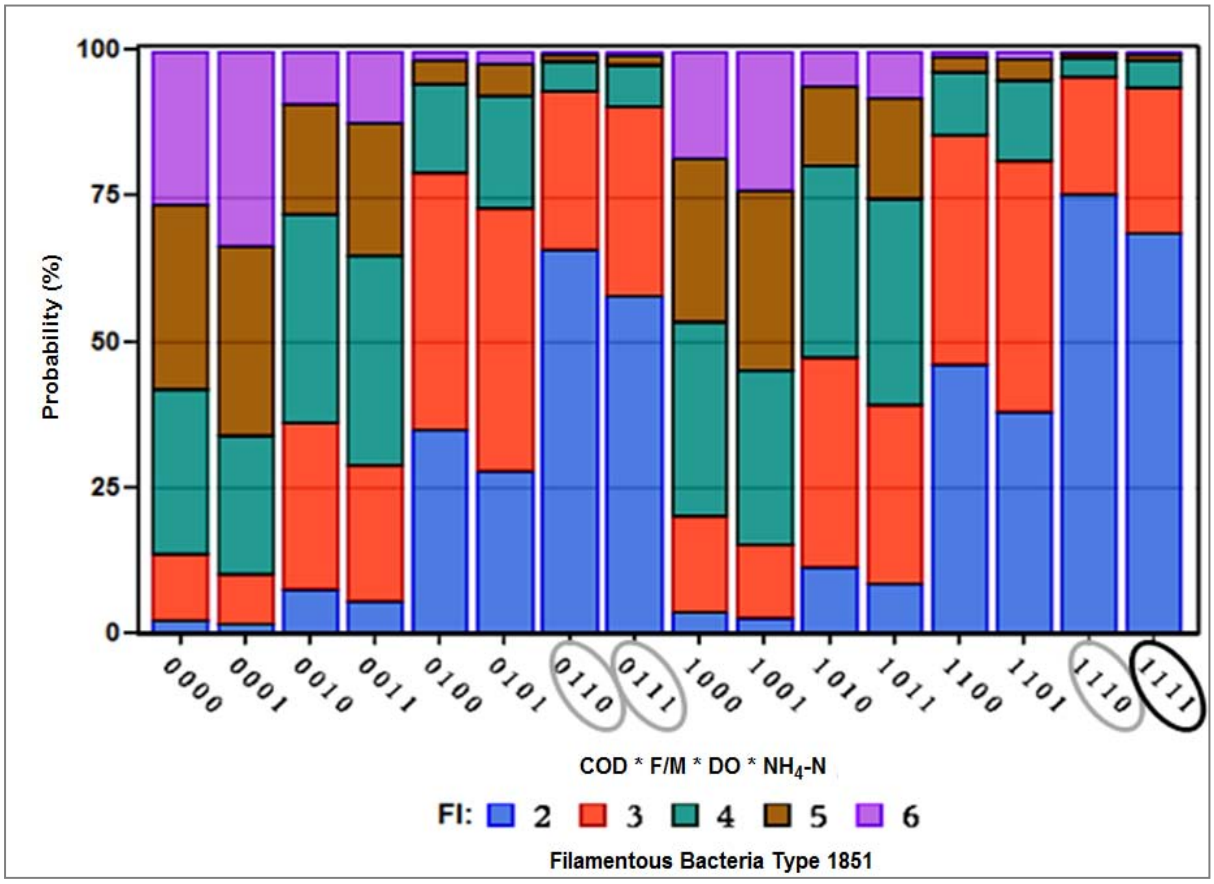

Figure 4.4: Plot indicating probability of occurrence of Type 1851 in Kingsburgh WWTP, a computational output with the cumulative low (0) and high (1) levels of four measured parameters. *N.B. 0 = low concentration, 1 = high concentration in the exact order of Influent COD: F/M ratio: DO: Influent N-NH_4^+. Each bar colour represents a subjective filament index, e.g. blue bar indicate FI=2, red bar indicate FI =3.

The plot also illustrated the occurrence and high abundance of Type 1851 when the parameters were reversed. In all cases, when both DO and F/M were low, the plot showed a greater than 80% probability of the organism being at a higher scale (FI: 4-6). As a result, the probabilities investigated, clearly indicated the role of DO and F/M over the other operational parameters in controlling filamentous Type 1851 once established in the system, thus, possibly controlling filamentous bulking if the organism is found to be highly excessive.

4.3.5.2. Predicted Cumulative Probabilities of Type 021N

According to the polybar plot for Type 021N (Figure 4.5), in all cases when influent COD was low (< 750 mg COD/L) and influent N-NH_4^+ was high (≥ 32 mg/L), the probability plot indicated a 95% chance that the organism would be at a higher scale (FI: 4-6). In retrospect, in all four cases, when influent COD was high and N-NH_4^+ was low, regardless of all other operational parameters, the plot illustrated the highest chances (> 25%) that the organism would be ranked low (FI: 1-3).

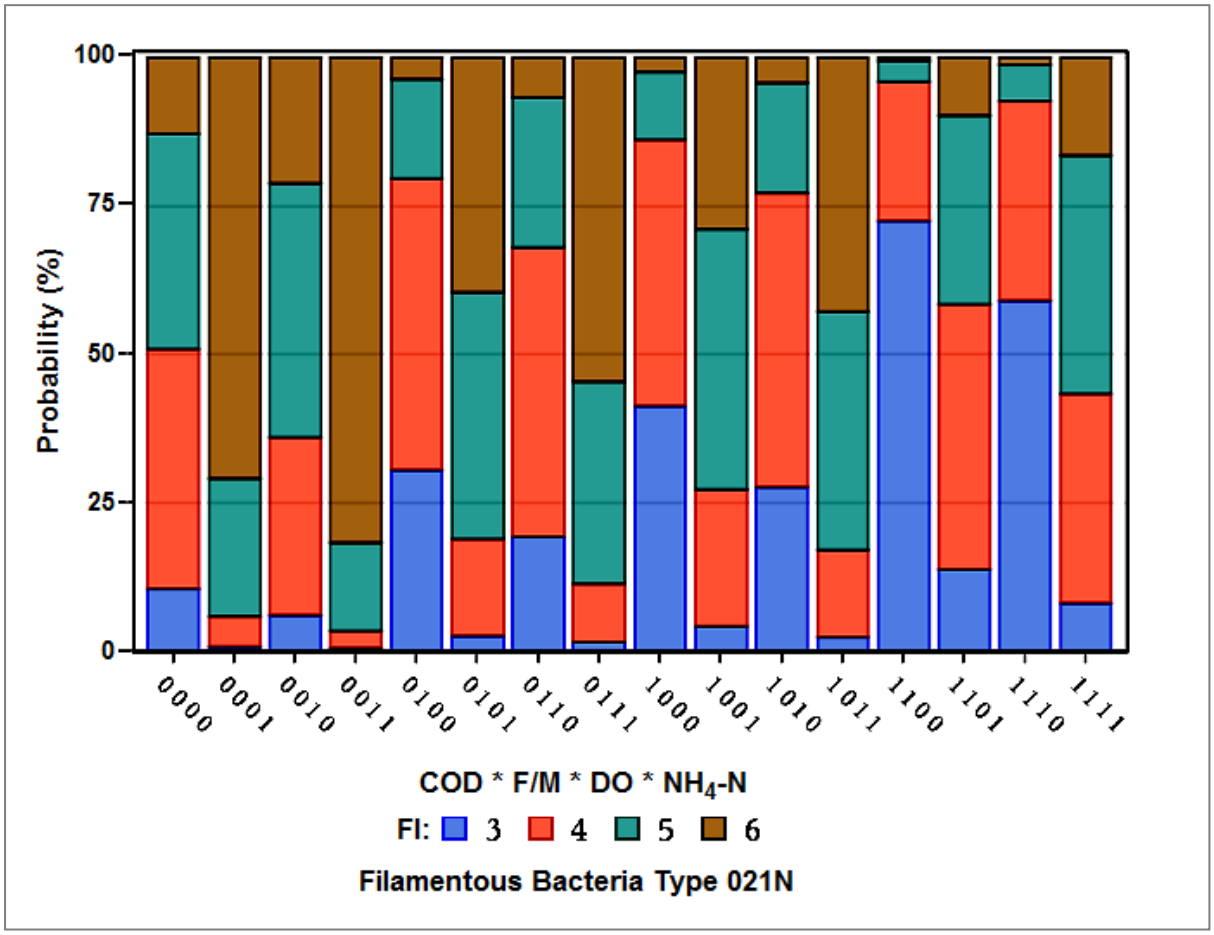

Figure 4.5: Plot indicating probability of occurrence of Type 021N in Kingsburgh WWTP, a computational output with the cumulative low (0) and high (1) levels of four measured parameters.

However, in all of the above cases (COD-high, N-NH_4^+-low), the two cases when F/M was high (regardless of DO) the plot showed a greater than 60% chance that the organism would be ranked low (FI: 1-3). With regards to the individual factor analysis, Type 021N showed

significant relationships with COD, F/M and N-NH_4^+. From the polybar plot, high COD, low N-NH_4^+ and a high F/M seems to be the most significant operational parameters in controlling filamentous Type 021N at a lower scale (FI: 1-3).

4.3.5.3. Predicated Cumulative Probabilities of *Thiothrix* spp.

According to the predicted probability plot, low F/M ratio and high N-NH_4^+ levels favoured *Thiothrix* spp. irrespective of all the other operational parameters being at low or high levels (Figure 4.6). The probability plot indicated > 70% chance of the organisms being on a higher scale (FI: ≥ 4) when the F/M ratio was low (< 0.1 kg COD/kg MLSS d^{-1}) and N-NH_4^+ level was high (≥ 32 mgL^{-1}).

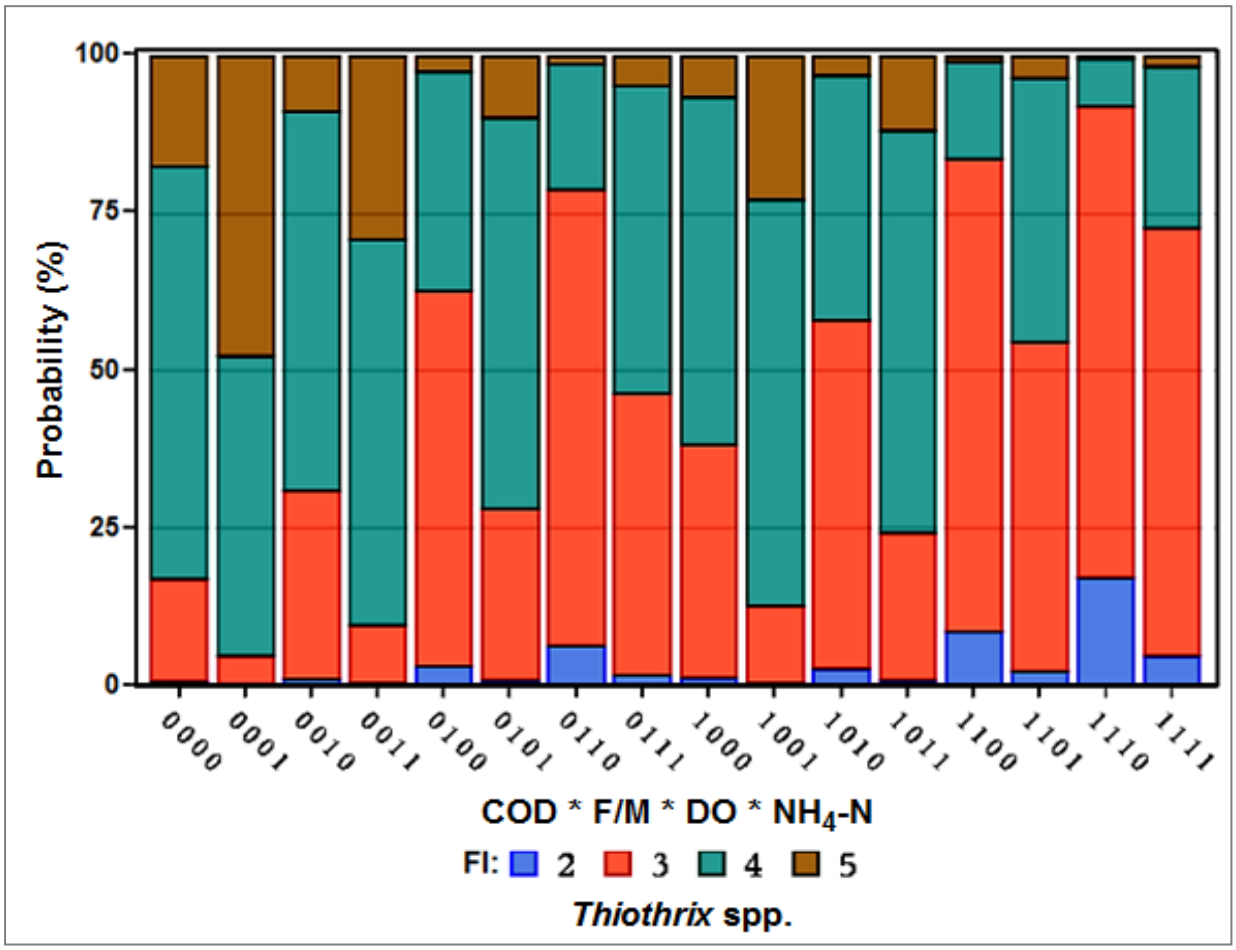

Figure 4.6: Plot indicating probability of occurrence of *Thiothrix* spp. in Kingsburgh WWTP, a computational output with the cumulative low (0) and high (1) levels of four measured parameters.

In contrast, the observations from the plot showed > 60% chance that the organism would be ranked low (FI: 1-3) in all cases when F/M was high and N-NH_4^+ low (regardless of COD and DO).However, it is important to note that the average sulphur content of the influent wastewater was 60 mg/L, which could possibly have triggered the over production of *Thiothrix* spp. apart from low F/M and ammonia conditions.

4.3.5.4. Predicted Cumulative Probabilities for Type 0092

Individual analysis of each parameter *viz*., COD, N-NH_4^+, DO, F/M and temperature showed their significant relationships with Type 0092 (Table 4.3). From the model, high temperature (< 20°C) seem to regulate the organism at a lower index scale (FI:≤ 3), whilst holding all the other variables constant. It was also noted that when the remaining operational parameters were low, the model predicted a greater than 80% chance of the organism being at lower levels (FI: ≤ 3). However, when considering all factors grouped together (Figure 4.7), it was noted that changes in the DO and temperature had the most significant impact on the occurrence of Type 0092. In all cases when the DO was low and temperature was high, irrespective of the operational parameters, the plot showed a greater than 90% chance that the organism would be at a lower scale (FI:≤ 3). In retrospect, when the conditions are reversed (i.e. when DO is high and temperature is low) the organisms has a greater chance of being at a filament index scale (FI: ≥ 4) (Figure 4.7).

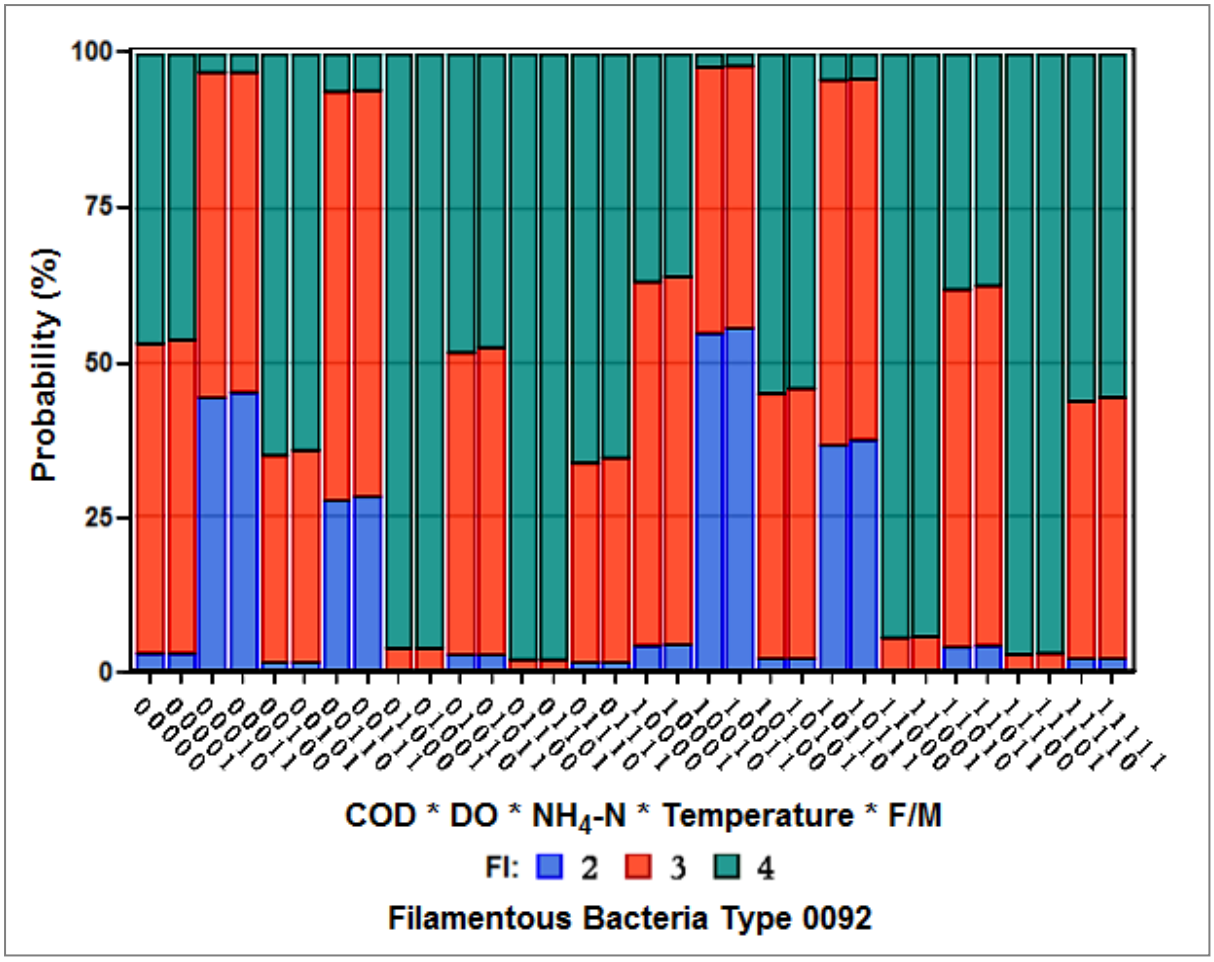

Figure 4.7: Plot indicating probability of occurrence of Type 0092 in Kingsburgh WWTP, a computational output with the cumulative low (0) and high (1) levels of four measured parameters.

4.4. Discussion

Despite much research carried over the last decade, filamentous bulking episodes are continuing to be a frequent problem in BNR plants worldwide. One of the main reasons for not finding an efficient yet general solution to bulking and foaming could be due to the lack of a set methodology to approach these events. According to the latest survey, each WWTP has its own characteristic “fingerprint” with regards to the filamentous population depending on the wastewater composition (Mielczarek *et al.*, 2012). A previous observation of a few domestic plants in KwaZulu-Natal have also shown similar findings. Although a few filamentous types were common in all BNR plants, a few other types were very specific to each BNR plant showing a characteristic fingerprint with regards to the filamentous bacterial

population at these plants (WRC report K5/2003). Based on these findings, the fundamental goal of this study was to identify the specific conditions which contribute to the proliferation of specific filamentous bacteria in a full-scale BNR plant using a statistical approach. A cumulative logit model was applied to find the significant relationships of filamentous bacteria to the specific operational parameters. Since the data obtained from the filamentous bacteria were categorical (ranked from a scale of 0 to 6), the ordinal logistic regression analyses (SAS) seemed to the best suitable method for this type of study (Alison, 1999; Kayri and Cokluk, 2010; Park, 2009).

4.4.1. Ordinal Logistic Regression Analysis of the Dominant Filamentous Bacteria (Type 1851, Type 021N, *Thiothrix* pp. and Type 0092)

4.4.1.1. Effect of Influent COD and N-NH_4^+ Level on the Dominance of Filamentous Bacteria

Theoretically, when COD levels are low, the filamentous bacteria proliferate due to the competition between the filamentous bacteria and the floc forming eubacteria for organic substrates. On the other hand, at higher COD levels, floc forming eubacteria out-compete filamentous bacteria due to their faster growth rate (Chudoba *et al.*, 1973; Martins *et al.*, 2004; Van Loosdrecht *et al.*, 2008). The estimated coefficient analysis (Table 4.4, 4.5) and the probability graph obtained for Type 1851, Type 021N and *Thiothrix* spp. (Figure 4.1 a, b and c respectively) showed a higher probability (greater than 50%) of these filaments being common to excessive (FI: 4-6) at a lower COD level (< 750 mg/L). It was found that, in completely mixed systems where substrate concentrations was low, filamentous bacteria had a higher specific growth rate and out compete floc forming bacteria for nutrients (Madoni *et al.*, 2000; Martins *et al.*, 2004; Van Loosdrecht *et al.*, 2008). However, the results for Type 0092 seemed contradicting to the above theory as there was a greater than 75% probability of Type 0092 being at higher scale at a high COD level as opposed to other filamentous bacteria analyzed in this study (Figure 4.1 d). This could have been due to the specific COD preferences for their growth. Type 0092 prefers slowly biodegradable substrates (SBCOD) as opposed to Type 021N, *Thiothrix* spp. and Type 1851 which favours readily biodegradable COD (RBCOD); (Jenkins *et al.*, 1993; Martins *et al.*, 2004). Thus, Type 0092 might have a competitive advantage over floc-forming and other filamentous bacteria at a higher

concentration of SBCOD. However, this needs further validation based on influent COD characterization.

Partial uptake of N-NH_4^+ and nitrite by filamentous bacteria has been previously reported (Jenkins *et al.*, 1993; Tian *et al.*, 2011). Among the different filamentous bacteria analyzed, Type 1851, Type 021N, Type 0092, Type 0041 and *S.natans* showed a significant relationships with N-NH_4^+ except for *Thiothrix* spp. (Table 4.3) in this study. A higher influent N-NH_4^+ increased the probability of Type 1851, Type 0092, Type 0041 and *S.natans* being on a lower ranking. However, a high N-NH_4^+ level (≥ 32 mg/L) reduced the probability of Type 021N being at a lower ranking (Figure 4.1 f) and there seemed to be a greater chance (±70%) of the organism being ranked at a higher level (FI ≥ 4). Thus, the model illustrated the proliferation of Type 021N, i.e. at a higher filament index scale, when the N-NH_4^+ levels were high.

As mentioned above, Type 021N also showed significant relationships with influent COD. The filamentous organism showed a higher probability of being common to excessive (FI: 4-6) at a lower COD level. Hence, type 021N seems to proliferate at a low COD (< 750 mg/L) and high N-NH_4^+ levels (≥ 32 mg/L). A study on filamentous bulking in South Africa on nutrient removal AS systems showed that with the effect of increased nitrates and nitrites at low COD levels, this increased the abundance of low F/M filaments (Musvoto *et al.*, 1999). Results confirmed the effect of the filamentous bacteria proliferation in a modified UCT process with the addition of N-NH_4^+ into the system, which resulted in an increased DSVI. A similar finding with filamentous Type 021N was derived from the logit model in this study. It was assumed that Type 021N have an advantage over floc formers in the rapid assimilation and storage of ammonium under stress conditions and thus compete successfully for energy and carbon sources under nutrient limited conditions (Jenkins *et al.*, 1993). In addition, a study was conducted by Jenkins *et al*., (1993) on the N-NH_4^+ uptake by Type 021N, results showed a much more rapid uptake of NH_3-N when the supply was intermittent rather than continuous. Since Kingsburgh WWTP is an open system, with N-NH_4^+ being irregular and fluctuating within the standard range, these conditions were favourable for the organism to proliferate.

4.4.1.2. Effect of DO Level and F/M Ratio on the Dominance of Filamentous Bacteria

Dissolved oxygen and F/M ratio are among some of the key factors known to be responsible for the most filamentous bacterial proliferation in the AS process (Martins *et al.*, 2003; Vaiopoulou *et al.*, 2007; Wilen and Balmer, 1999). Theoretically a low F/M ratio was potentially the most common cause to bulking and generally filamentous bacteria proliferate under low F/M ratios (Beer *et al.*, 2002; Jenkins *et al.*, 1993). The logit model showed a significant relationship with Type 1851, Type 0092, *Thiothrix* spp. and *S.natans* to the F/M ratio, however, no relationship was observed with the filamentous organisms Type 021N and Type 0041 to F/M ratios. Filamentous Type 021N and *Thiothrix* spp. predominate in low up to moderate F/M ratios and have been proven to take up nutrients rapidly under nutrient deficient conditions (Bitton, 2011). However, the F/M ratio of the plant investigated ranged between 0.08 to 0.18 kg COD/kg MLSS which were very low and well within the range of all these filamentous organisms to proliferate. In addition, these filamentous bacteria have a higher substrate removal rate, under nutrient stress conditions which could also explain the exceptional COD removal efficiency of this plant which was more often higher than 90%. The filamentous bacteria have been proven to take nutrients rapidly under nutrient deficient conditions and were anticipated to have a larger role in COD removal in BNR plants under limited bulking conditions (Bitton, 2011; Guo *et al.*, 2010).

S.natans, Type 0092 and Type 1851 showed a strong relationship with the DO levels (Table 4.3). Type 1851 and *S.natans* disclose a greater than 80% chance of being at a higher category when DO is low (less than 1.1 mg O_2/L). These filaments were also reported to grow at both low and mid-level DO (Vaiopoulou *et al.*, 2007), hence bulking can occur for a wide range of oxygen supply when there is one or more factors which are also in favour for these organisms to flourish. However, the most dominant filamentous bacteria in the currently investigated plant (*Thiothrix* spp. and Type 021N), showed no significant relationship with DO in the cumulative logit model (Table 4.3). These two filamentous bacteria are reported to proliferate when there is a sudden change or stress in DO levels (high or low) of the system (Gaval and Pernelle, 2003). Thus, the dominance of these two species in Kingsburgh plant could be due to this frequent stress in DO level due to the operation of sequentially-timed aerators to facilitate anoxic zones for denitrification. In addition, these filaments can take up both oxygen and nitrite as electron acceptors and can thrive well under

aerobic and anoxic conditions (Martins *et al.*, 2004). However, the probability model for Type 0092 differed from the rest, showing a greater than 60% chance of this filamentous bacteria ranked on a higher category scale (FI: 3-4) at a higher DO level. Type 0092 has already been characterized as an "all zone grower" and are able to grow in anoxic, anaerobic and aerobic conditions (Wanner and Grau, 1986). However, pure culture studies described them as being strict aerobes (Martins *et al.*, 2004; Speirs *et al.*, 2009). The cumulative model analysis showed Type 0092 as most likely to be ranked in a higher category at high a DO level, this could be attributed to its higher affinity towards oxygen as electron acceptors over nitrates, however this needs further validation. However, it has been reported that Type 0092 is able to synthesise and store poly *β*-hydroxyalkanoates which ultimately helps them to grow even in an oxygen deficient environment and compete with heterotrophic bacteria under high COD and low DO concentrations (Speirs *et al.*, 2009)

Bulking can therefore transpire from very low levels of oxygen in the aeration to a higher range with a greater stress level. However, a wider range is required to monitor the oxygen effect on filamentous bacteria which can only be done in a controlled system. Conversely, at limited bulking conditions, these filaments are also reported to be largely involved in nutrient removal process including COD and total nitrogen (Guo *et al.*, 2010).

4.4.2. A probability graph model to predict the growth of specific filamentous growth in a full-scale biological nutrient removal plant

Filamentous bacteria are crucially important to any WWTP due to its unique role in sludge settling (da Motta *et al.*, 2003; Eikelboom *et al.*, 1998; Madoni *et al.*, 2000). A model has been proposed in this study considering the plant operating parameters against each of the dominating filamentous bacteria. This was done to find relationships with the organisms and the combination of these significant variables that could have been playing a role in the selection of these filamentous bacteria. The proposed model can also be applied in general to all WWTPs to predict the filamentous bulking in full-scale systems. In achieving this, possible strategies to control the filamentous growth may very well be incorporated into these systems to specifically regulate the growth of bulking filaments.

According to the model with reference to Type 1851 established in the system, DO and F/M were the key factors that could possibly control the growth of the organism irrespective of all other factors that were considered in this study (Figure 4.4). The maximum probability of Type 1851 ranked on a lower scale (FI: 1-3) is obtained when the F/M and DO are both at higher levels. A combination of high DO (≥ 1.1 mg O_2/L) together with a high F/M (≥ 0.1 kg COD/kg MLSS) clearly showed a ±80% chance Type 1851 being in a lower ranking scale (FI: 1-3) irrespective of the other 2 factors. Type 1851 can use both oxygen and nitrate as electron acceptors and their growth rate is slow compared to the heterotrophs (Beer *et al.*, 2002; Jenkins *et al.*, 1993). Thus, at higher DO and higher F/M, the heterotrophic bacteria out compete this filamentous bacteria due to their faster growth rate.

Even though Type 021N showed significant relationships with COD, F/M and N-NH_4^+, when these factors were grouped together, COD and N-NH_4^+ seemed to be the most significant factors determining the dominance of this organism. It was noted that when the COD levels were low (< 750 mg/L) and N-NH_4^+ levels were high (≥ 32 mg/L) the chances of the organism ranked at a higher scale (FI: 4-6) was greater than 90%. It is assumed that Type 021N have an advantage over floc formers in rapid assimilation and storage of ammonium under stress conditions and thus compete successfully for energy and carbon sources under nutrient limited conditions (Jenkins *et al.*, 1993).

With reference to *Thiothrix* spp., according to the cumulative probability plot when all factors were grouped together, F/M ratio and the N-NH_4^+ levels seem to be the deciding factors to predict the dominance of this filamentous bacterium irrespective of the other factors considered. The model predicts that there is a greater than 90% chance that *Thiothrix* spp. was ranked on a lower scale (FI: 2-3) when both N-NH_4^+ and F/M were low. The probability graph showed > 75% chance that this organism would be ranked at high levels (FI: 4-5) when F/M is low together with a high N-NH_4^+ (≥ 32 mg/L). *Thiothrix* spp. uses readily biodegradable substrates and are capable of sulphide oxidizing, storing sulphur granules. The organism was reported to rapidly take up nutrients under nutrient deficiency (low F/M) (Aruga *et al.*, 2002; Jenkins *et al.*, 1993). However, the relationship of *Thiothrix* spp. abundance to N-NH_4^+ level needs further validation at a laboratory scale as no pure culture study was reported to support this statement. The mutual interaction of Type 021N and *Thiothrix* spp. in this system also needs further evaluation as their dominance followed a similar pattern.

Type 0092, are frequently being reported from BNR plants and are classified as “all-zone” growers (Wanner and Grau, 1986) as it can take up both oxygen and nitrates as the electron acceptors. They prefer slowly biodegradable substrates (SBCOD) and are usually found in environments with low F/M ratios (0.02-0.2) (Jenkins et al., 1993). In this study, the operational parameters including COD, $N\text{-}NH_4^+$, DO, F/M ratio and temperature showed significant relationships with Type 0092. However, as opposed to the other filamentous bacteria analyzed in this study, Type 0092 was ranked most common when all the above parameters (except temperature) were high. Aygun *et al.* (2013) found that Type 0092 increased in warmer temperatures during summer (> 15°C) and decreased in colder temperatures during winter. However, in the proportional odds ratio model, the high temperatures (> 20°C), increased the likelihood of Type 0092 being in a lower ranking, and lower temperatures increased the likelihood of having this filament at a higher ranking. When all the factors were grouped together, temperature together with DO specified preferable conditions for this bacteria to proliferate irrespective of all other factors being at high or low levels. From the probability graph (Figure 4.7), low temperatures (< 20°C) and high dissolved oxygen (> 1.1 mg O_2L^{-1}), predicted that Type 0092 had a greater than 90% chance of being at a higher level (FI: > 4). During low temperatures, the metabolic activity of microorganisms are slow, Type 0092 could assimilate a competitive advantage over these slow growing microorganisms since the organism also prefer SBCOD instead of RBCOD. As a result, during these conditions, Type 0092 could possibly out-compete heterotrophic bacteria.

4.5. Conclusions

The application of the CLM proved to be an efficient tool to predict excess filamentous bacteria under specific plant operational parameters. Methods and knowledge on filamentous bulking control is poorly understood, and thus a statistical data driven approach could be used as an effective tool to improve or remediate bulking problems in WWTPs. In this study, the CLM illustrated significant relationships and predictions between plant operational parameters such as F/M, influent COD, DO, influent $N\text{-}NH_4^+$, temperature and the dominant filamentous bacteria. The CLM predicted that filamentous Type 1851 dominated (FI: $\geq$ 4) during low DO (< 1 mg O_2L^{-1}) and low F/M (< 0.1 kg COD/kg MLSS d^{-1}) ratios. A low F/M ratio, high ammonia level together with septic sludge seemed to be the deciding factors which

favoured the dominance of *Thiothrix* spp., whereas high $N\text{-}NH_4^+$ levels (≥ 32 mgL^{-1}) favoured the growth of filamentous Type 021N. Type 0092 showed some unique characteristics, the organism seem to thrive during high DO (≥ 1.1 mg O_2L^{-1}) and low temperatures (< 20°C). This study has also shown the unique nature of the filamentous Type 0092 over other filamentous bacteria as they seemed to be more prevalent under optimum conditions as opposed to the other filamentous bacteria analyzed. Future studies will be focused on evaluating multiple WWTPs (domestic and industrial) using the CLM as to validate the current findings and to understand filamentous bacterial behaviour under different operational/environmental conditions. The observations will be compared to other models.

5. THE EFFECTS OF CHLORINE DOSE, UV LIGHT AND OZONE ON FILAMENTOUS BACTERIA

5.1. Introduction

The excessive growth of filamentous bacteria in the return AS streams is identified as one of the primary causes for continuous bulking and foaming problems in WWTPs. Various control strategies have been proposed to minimize bulking in full sale BNR plants (Jenkins *et al.*, 2004a; Leeuwen, 1992; Martins *et al.*, 2004) which included specific (managing operational parameters) and non-specific (implementing the use of chemical agents) control measures (Caravelli *et al.*, 2004; Saayman *et al.*, 1997; Saktaywin *et al.*, 2005).

When severe bulking occurs within a plant, dosing with chemicals such as chlorine, ozone, hydrogen peroxide (H_2O_2), and metal ions seems to be rapid treatment methods to reduce bulking (Jenkins *et al.*, 1993). Among these, chlorination, and ozonation were found to be the most commonly used non-specific methods to reduce bulking in AS wastewater treatment plants (Caravelli *et al.*, 2006; Leeuwen, 1988). Alternatively the use of UV light has most commonly been used in the final effluent of wastewater as a disinfectant (Martin, 2004). Chlorine was also frequently used as a disinfectant and vastly exploited in the treatment process to control bulking (Lakay *et al.*, 1988; Leeuwen, 1992; Madoni *et al.*, 2000). The mode of action of chlorine on the bacterial cell wall had been studied in great detail by researchers. Chlorine was found to diffuse within the cell wall of bacterial cells, damaging the cell membrane and inhibiting the enzyme activity, thus, inactivating the cells. Chlorine treatment proved to hamper protein biosynthesis and respiratory activity causing great impairment to the bacterial cells (Caravelli *et al.*, 2006; Ramirez *et al.*, 2000). Chlorine treatment in the RAS streams of WWTPs were highly sensitive, as it was found to affect both floc formers and filamentous bacteria. Heterotrophic bacteria involved in nitrogen and carbon removal are imperative to the wastewater process, a mere reduction in their population can affect the treatment process severely. Hence, the action of chlorine should be carefully monitored to predict an appropriate optimum dose to selectively target filamentous bacteria.

Filamentous bacteria has a much larger surface area compared to floc-forming bacterial cells, these microorganisms protrude out of the flocs and extend into the bulk solution causing inter-floc bridging. Thus, with the use of an optimum dose, filamentous bacteria were

considered more likely to make contact with these toxicants while floc formers could be protected within the extracellular matrix (Tian *et al.*, 2006).

Ultraviolet light has been an alternative advanced method for the disinfection of wastewater (Bilotta and Daniel, 2010; Oliver and Carey, 1976; Severin, 1980). The use of UV light has most commonly been used in the final effluent of wastewater to reduce pathogens and cysts which have not been eliminated during the treatment process. No research has been found to eliminate specifically filamentous bacteria using the UV treatment technology. The spectral range most effective for the destruction of bacteria and almost all known microorganisms are between range of 250nm and 265nm (EPA, 1999b).

When the UV radiation passes through the walls of the bacterial cell, the microorganisms are inactivated and the cells genetic material is disrupted, making it unable to reproduce (EPA., 1999). The primary effect of UV-light on bacteria is the formation of pyrimidine dimers (Gomez-Lopez *et al.*, 2007). A thymine dimer is most commonly formed thus distorting the structure and preventing transcription and translation enzyme attachment to the DNA (Giese and Darby, 2000; Severin, 1980). Cytosine-Thymine, and Cytosine dimers are also possible, however, they are less likely to be formed. Cells may also form an enzyme which enables the double bond in the thymine dimers to be repaired.

Theoretically, ozone has greater disinfection efficiency on microorganisms since it can act as a strong oxidising agent and can work over a wide pH range. The functionality of this treatment is that it attacks the dehydrogenase enzyme responsible for the respiratory system. The mode of action of ozone is similar to chlorine. When Ozone reacts with water it forms OH^- radicals and HO_2^- radicals thus playing a significant role in bacterial destruction. Studies reveal that ozone targets the cell membrane altering the cells permeability and thus resulting in the leakage of the cells constituents, while some researches make note of ozone directly diffusing through the cell membrane and reacting with the bio-molecules, damaging the chromosomal DNA (Caravelli *et al.*, 2006).

Oliver and co-workers (1976) conducted an experimental set up to test the effectiveness of ozone (0-70 mg O_3/L) on sludge reduction, their findings concluded that the introduction of ozone into the aeration tank effectively improves effluent quality and sludge production (Oliver and Carey, 1976). In a recent study, Caravelli *et al.* (2006) reported that 87% of

filamentous bacteria were reduced at an ozone dose of 18 mg O_3/g volatile suspended solids (VSS) sufficient to control filamentous bulking. Ozone was found to be widely used for disinfection purposes, however, clearly not much information was known on the effect of ozone to control filamentous bacteria.

The scope of this study was to evaluate the effects of chlorine, UV light and ozone gas on the dominant filamentous bacteria and to find the minimum dosage required to control excess filamentous growth.

5.2. Materials and Methods

5.2.1. Sampling

Mixed liquor samples (1 L) were collected from the Return Activated Sludge (RAS) stream of Kingsburgh KWWTP. The conventional AS plant is situated in the southern region of KwaZulu-Natal, treating predominantly domestic wastewater. Filamentous bacteria were found in excessive amounts, thus, causing frequent bulking problems. The filamentous bacteria were identified according to keys proposed by Jenkins *et al* (2004) and Eikelboom (2000). To confirm identification, the FISH technique (Appendix 3) was implemented using a range of 16S rRNA targeted oligonucleotide probes (Chapter 3: Table 3.1), these probes were selected to specifically target different filamentous bacteria.

5.2.2. Concentration of filamentous bacteria using filtration

Immediately after sampling, a diluted wastewater sample (1:1) was initially filtered through a filtration unit to concentrate filamentous bacteria (Figure 5.1 a). The filtration unit was arranged with a vacuum pump (BOECO, Germany) connected to a 1 L flask and sealed with parafilm to prevent air from escaping. A range of micron filter sieves (Labotec suppliers, South Africa) composed of wire netting was used with various pore sizes i.e. 425 μm, 355 μm, 300 μm, 150 μm, and 75 μm and 53 μm (Figure 5.1 b). The sample was passed through these filters respectively. The filters were spray washed (500 mL distilled water) to get maximum concentration of the filamentous bacteria. The most suitable filter sieve to retain predominantly filamentous bacteria was selected based on microscopic analysis. The filtered samples where then subjected to the different treatments i.e. Chlorination, UV light and ozonation.

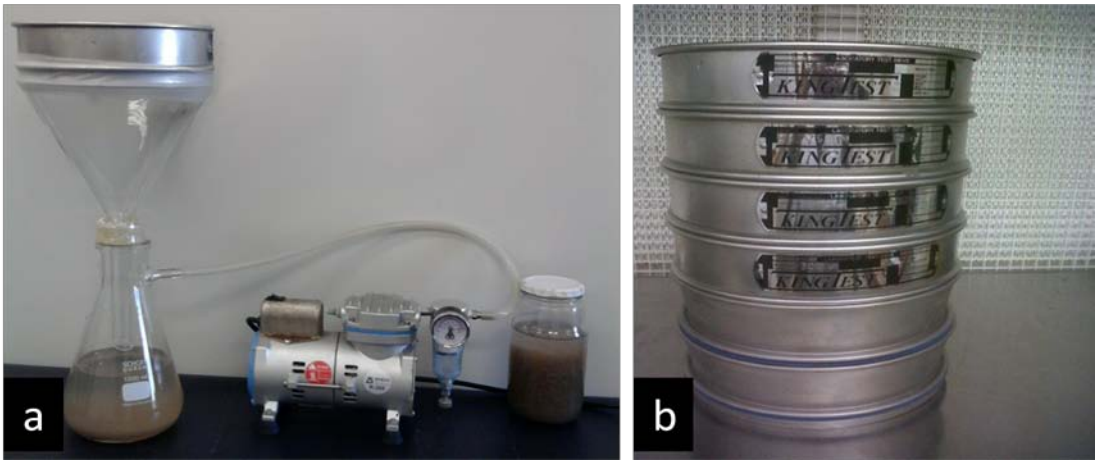

Figure 5.1: (a) Filtration unit used to separate filamentous bacteria from flocs. (b) stainless steel sieves (425 μm, 355 μm, 300 μm, 150 μm, and 75 μm and 53 μm).

5.2.3. Optimization of the Live/ Dead staining of filamentous bacteria

The concentration of each dye was optimised following the procedure by means of the commercial Live/Dead cell double staining kit (Appendix 4). A working solution of Calcein-AM (1 μM) and PI (1 μM) was prepared using PBS buffer (100 μL). To determine the viability of the filamentous bacteria, each of the solutions (2 μL) were added to 100 μl cell suspension and incubated in the dark at 37°C for 15 min. The samples were then analyzed

with an Axiolab Zeiss fluorescent microscope using the FLOUS and Rhodamine filter sets (Carl Zeiss, Germany), in doing so, viable and dead cells were visualised respectively.

5.2.4. Assessment of Chlorine effects on Filamentous Bacteria

The effects of chlorine dosing on the growth of filamentous bacteria were established via batch tests, a 15% Sodium hypochlorite (NaClO) solution was used as a source of free chlorine, in the wastewater samples. A stock solution of 277 mg Cl_2/L was prepared by dilution with distilled water. Sodium hypochlorite, dH_2O and 5mL sample were spiked into 50 mL polyethylene tubes to achieve doses of (0) control, 2, 5, 10, 15, 20, 25, 30 and 40 mg Cl_2/L. The sample contained a high concentration of filamentous bacteria and were introduced into each of the polyethylene tubes. Total chlorine and free chlorine was analysed at 20 min, 1 hr and 2 hrs intervals using a chorine pocket colorimeter meter (Hach, USA). Samples were vortexed and allowed to react for 20 min at room temperature; subsequently 1 mL together with an additional 5 mL of homogenised mixed liquor was withdrawn from each of the vials. These were measured for viability staining and chlorine testing respectively. The colorimeter meter was used to monitor the chlorine dose within the sample.

5.2.5. Germicidal effect of Ultraviolet Light on Filamentous Bacteria

A Thermorex Ultra Violet steriliser unit (Figure 5.2 a & b) composed of a T-UV-15W low pressure mercury vapour germicidal lamp (Laboratory Equipment & Suppliers) was used to inactivate the filamentous bacteria. The UV lamp was situated in the chamber 10 cm above the samples and radiated energy in the 2537 A° wavelength (Figure 5.2 b).

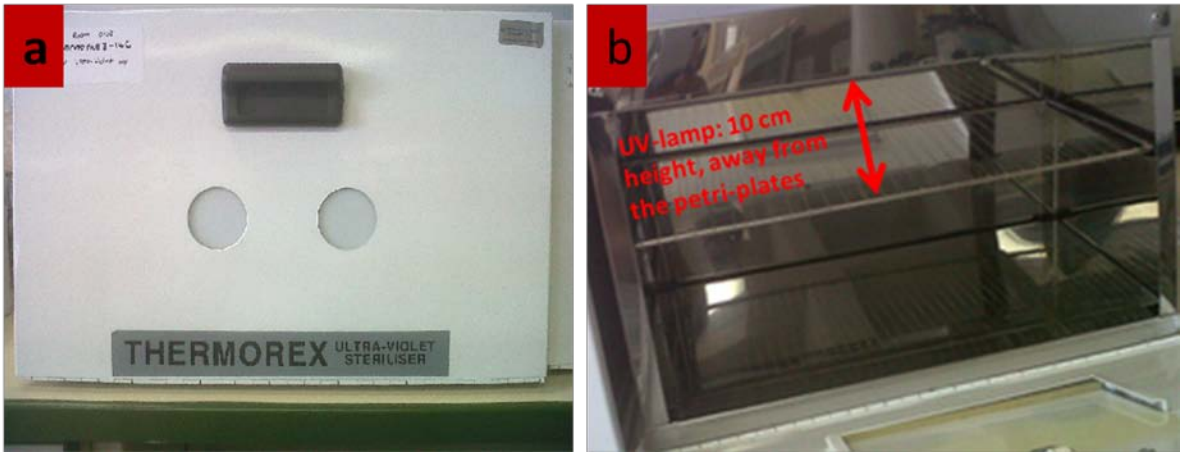

Figure 5.2: Low pressure germicidal UV lamps used to inactivate filamentous bacteria; a) UV steriliser unit, b) Filamentous bacterial samples in 20 mL portions were placed open in disposable Petri-plates10 cm below the UV light.

The unit provided a highly reflective inner surface to increase the intensity of the lamp, therefore ensuring optimum exposure of UV to the filamentous bacteria. The light intensity of the lamp was 5.4 microwatt seconds per square centimeter (mW/ cm^2). Kill rates were analysed at different time intervals. Sample within the petri plates were homogenized regularly. To evaluate the effect of UV dose on filamentous bacteria, UV irradiation was carried out at a distance of 10 cm at various exposure times.

An aliquot of 100 ul were periodically drawn from the petri-plates at 5, 10, 20, 30, 45, 60 and 80 min to perform live/dead staining. This method gave a relative measure of the filamentous bacteria present after various UV exposure times. UV dose rate was calculated as the product of light intensity together with reaction time (Braunstein *et al.*, 1996).

5.2.6. Ozone Test

Sludge samples were treated by ozone treatment in 50 mL Erlenmeyer flasks. An ozone gas stream was bubbled through into an ozone receiving solution (Millipore water with 10mM KH_2PO_4, adjusted to pH 7.7 + sample); Macauley *et al.* (2006). The ozone gas was saturated into the aqueous phase for 5 min with different concentrations of ozone at 2,5, 5.8, 9.5, 14.8, 21.7, 31.4, 38, 52 and 60 mg/L. The ozone generator controls gas production via the outlet by controlling the L/min and the percentage output. Ozone residual were not measured,

theoretically, ozone decay is within 3 minutes, and wastewater samples produce significant amounts of organics which therefore allows ozone to be depleted (Macauley *et al.*, 2006).

5.2.7. Viability (Live/Dead) Staining Technique

The effectiveness of chlorine, UV light and ozonation were evaluated by a viability staining technique using a commercial Live/Dead cell double staining kit (Sigma-Aldrich Chem GmbH, CH-9471 Bucshs/ Switzerland). The kit contains Calcein-AM and Propidium Iodide (PI) solutions, which stained viable and dead cells respectively. The stains differentiate between organisms with intact cell membranes (stained green and scored alive) and organisms with damaged cell membranes (stained red and scored dead), viewed by fluorescence microscopy.

Calcein-AM is a dye used to determine viable cells, by emitting a strong green fluorescence (emission maximum at 515 nm). Propidium Iodide (PI) stains the nuclei of a cell and is unable to pass through an intact cell membrane, it enters the nucleus by passing through the disordered membrane and binds to the cell DNA. Propidium Iodide emits a bright red fluorescence (emission maximum at 617 nm). Both dyes can be excited with a 490 nm light, hence viable and dead cells can be viewed simultaneously with a fluorescence microscope. The concentration of each reagent were optimized for filamentous bacterial staining to be, 1 μM Calcein-AM and 1 μM PI into 100 μL of homogenized mixed liquor sample. The solution was incubated at room temperature in the dark for 15 min, a wet mount, contained 20 μL sample was prepared onto microscopic slides and subsequently examined under a fluorescent microscope. A total of ten images per sample for each of the three treatment test (Chlorine, UV light and Ozone) were captured using a MRC Axiocam (Carl Zeiss, Germany) camera, the viable and dead filaments were quantified by image analysis using an Axiovision 4.8 imaging system (Carl Zeiss, Germany).

5.2.8. Statistical analysis

Statistical analysis using Graphpad prism version 5 was used to determine the effectiveness of each treatment against filamentous bacteria.

5.3. Results

5.3.1. A Rapid Technique to Concentrate Filamentous Bacteria from Mixed Liquor Samples

The dominant filamentous bacteria ascertained from the RAS streams were Type 021N, Type 1851, *Thiothrix* spp. and Type 0092. The dominant filaments were successfully separated from the flocs through the filtration unit (Figure 5.1). Mixed liquor sample (1 L) was passed through a range of filters (425 - 53 μm), using vacuum pressure (R-300 pressure pump, BOECO, Germany). Most of the debris and larger particles were retained on the stainless steel filters with the larger pore sizes (425-300 μm). Both floc formers and filamentous bacteria were exceedingly dense on the 53 μm pore size filter (Figure 5.3 c), hence the 75 μm pore size filter was utilized to achieve an optimum concentration of the filamentous bacteria population. The filamentous bacteria retained on the stainless steel filter sieve were washed with distilled water and subsequently used as the test cultures for further treatment.

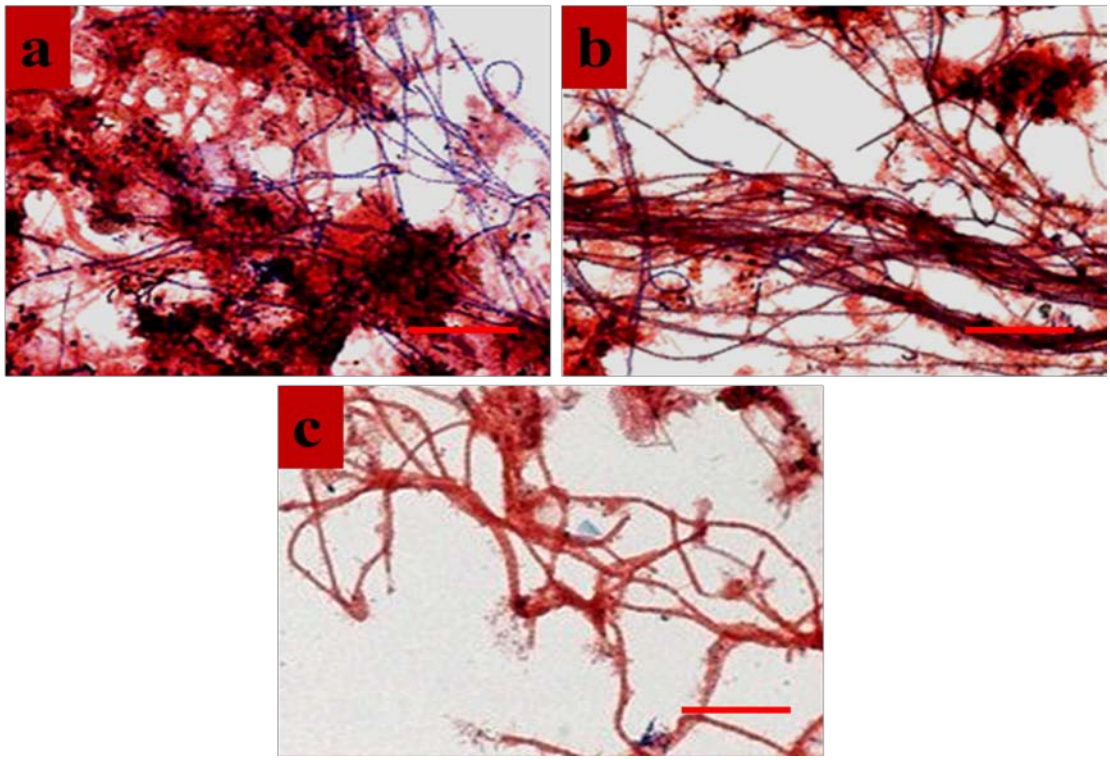

Figure 5.3: Light microscopic images of (a) non-filtered sample; (b) and (c) indicating filtrated samples from the 53 μm and 75 μm stainless steel filters respectively (1000 X maxnification). Scale bars = 5 μm.

5.3.2. Assessment of Chlorine Inactivation of Filamentous Bacteria

The effects of chlorine dosing on the filamentous bacteria were established via batch tests. The Live/Dead staining procedure was performed at 0 min, 20 min, 1 hour and 2 hours for each dose. The results obtained showed the reduction of the dominant filamentous bacteria as chlorine dose increased from 2 to 25 mg Cl_2/L, in addition an appropriate dose to minimise filamentous bacteria can be predicted. At a chlorine dose of 5 mg Cl_2/L, filamentous bacteria showed a significant reduction, damaged cells were observed within the trichome (red cells) of the filamentous bacteria (Figure 5.4 c).

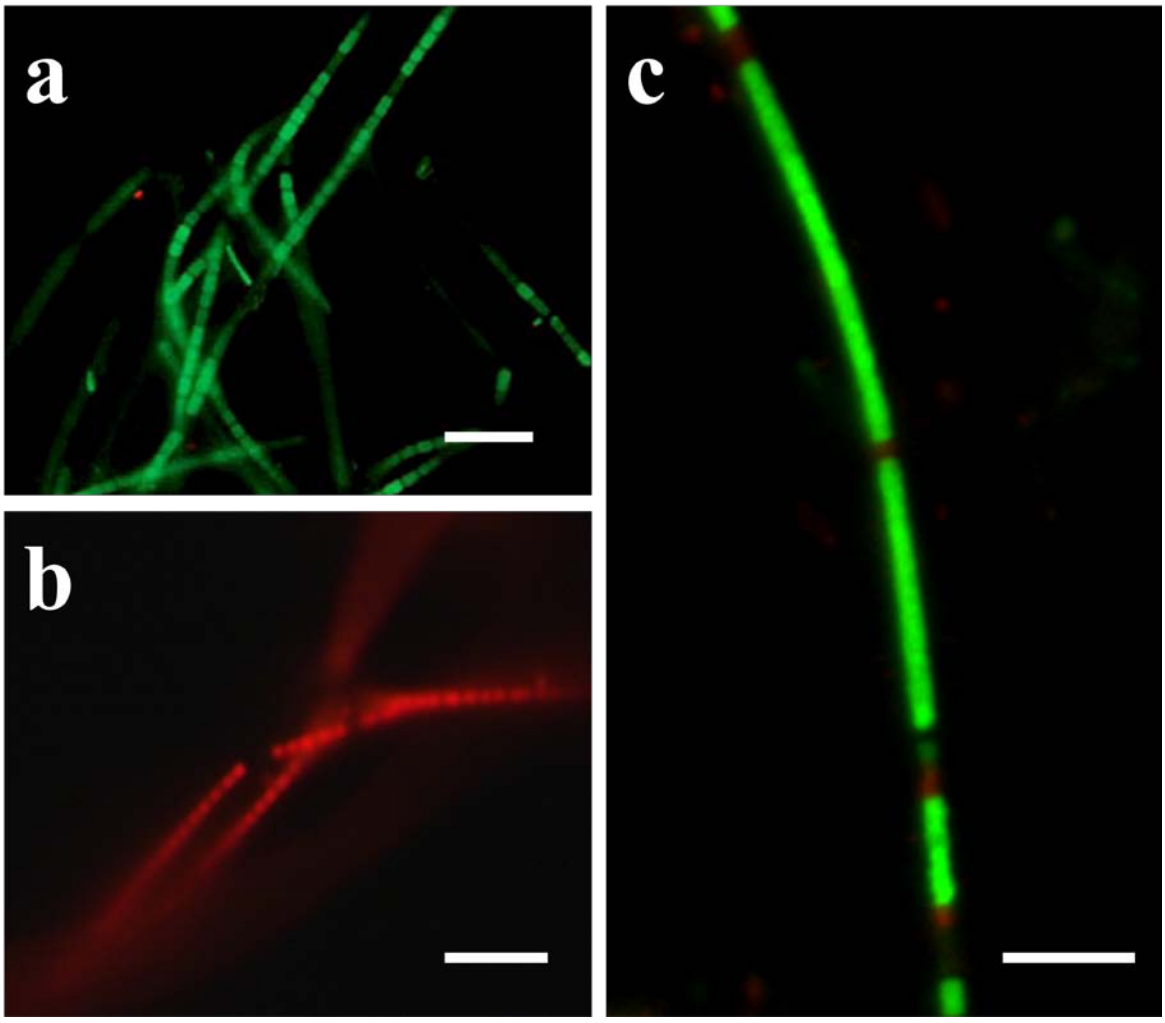

Figure 5.4: Micrographs a, b and c depicting the effects of chlorine on filamentous bacteria via Live/dead staining. (A) Control (live) green cells of filamentous bacteria, (B) treatment at 20 mg CL_2/L (dead) red cells. (C) treatment with 5 mg Cl_2/L, injured filament recognised by the red cells between the filament. Scale bars = 5 μm.

At the chlorine dose range between 10-20 mg Cl_2/L, most flocs were significantly affected and after 2 hours all filaments together with the floc formers were destructed at 20 mg Cl_2/L (Figure 5.4 b; Table 5.1).

Table 5.1: Chlorine effect on filamentous bacteria survival rate at 20min, 1 hr and 2 hrs

Chlorine dose (mg Cl_2/L)	20 min (mg Cl_2/L)	1 hr (mg Cl_2/L)	2 hr (mg Cl_2/L)
0	100.00	98.05	97.10
2	91.80	95.23	83.33
5	85.19	60.00	63.37
10	35.52	33.33	39.05
15	38.89	8.88	14.29
20	9.10	6.67	0.00
25	0.00	0.00	0.00

The graph in Figure 5.5 reveal the variance of chlorine dose rate at three different time intervals (20 min., 1 hour and 2 hours), illustrating the effect of chlorine dose on filamentous bacteria population (%) at different time intervals.

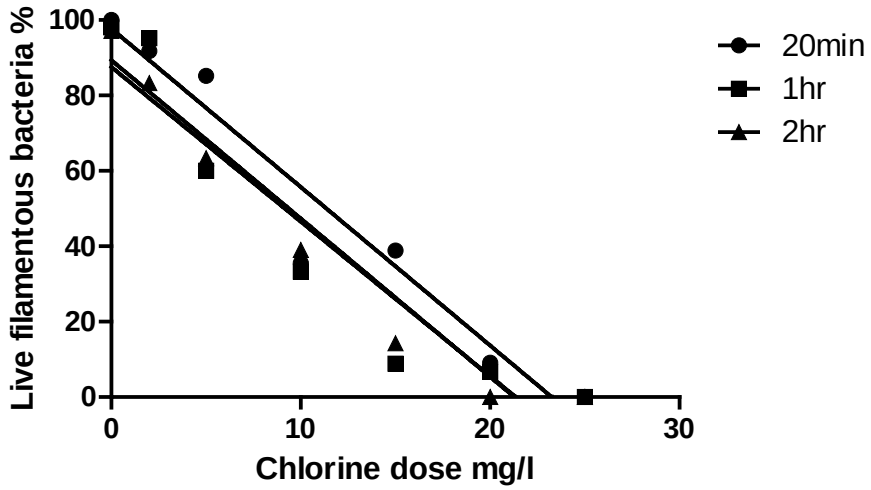

Figure 5.5: The relationship between filamentous bacterial survival rate and varying chlorine dose (0-25 mg Cl_2/L) at different time 20 min, 1hr and 2 hrs.

A test for deviation from linearity for each of the curves (20 min., 1 hr, 2 hrs) was performed and the *p*-values for each of the curves were found to be 1, 0.2 and 0.2 respectively. Since all of these *p*-values are > 0.05, it could be concluded that there was insufficient evidence to indicate that any of the curves deviate from linearity. Hence, linear regression was not inappropriate to use. The coefficient of determination (R^2) values for each of the curves (20 min, 1 hr, 2 hrs) were 0.9416, 0.8952 and 0.9401 respectively. The R^2 is an estimate of the proportion of variation in the dependent variable (filamentous bacteria alive) that is being explained by the independent variable (chlorine dose). This means that 94.16% of the variation in the percentage of viability of filamentous bacteria can be explained by the chlorine dose at 20 min of exposure. Likewise 89.52% of the variation in the percentage of filamentous bacteria alive can be explained by the chlorine dose at 1 hour of exposure and 94.01% of the variation in the percentage of filamentous bacteria alive can be explained by the chlorine dose at 2 hours of exposure.

For each of the curves (20 min, 1 hour and 2 hours), when testing whether the effect of chlorine dose on the percentage of filamentous bacteria alive is significant, the *p*-values observed are 0.0003, 0.0013 and 0.0003 respectively. Since the *p*-values are all less than 0.05, it can be concluded that for all three lengths of exposure that there is a significant effect of chlorine on the percentage of filamentous bacteria alive.

Lastly it is of interest to determine whether there is a significant difference between the three curves. When testing whether the slopes are different, a p-value of 0.9878 is observed. Since this *p*-value is greater than 0.05 (noticeably large), it can be concluded that there is no significant difference between the slopes of the three curves. When testing whether the intercepts are different, a *p*-value of 0.2889 is observed. Since this *p*-value is greater than 0.05, it can be concluded that there is insufficient evidence to indicate that there is a difference between the intercepts. Since it has been shown that the slopes are not different and neither are the intercepts, a pooled slope and a pooled intercept can be used for all the data, i.e. one curve can be used to represent all the data, regardless of the time of exposure.

This curve is modelled by the equation 5.1:

$$y = 91.5311 - 4.16603\,x \qquad (5.1)$$

From the equation 5.1, we can see that the estimated percentage of bacteria alive reaches 0 at a chlorine dose of 22 mg Cl_2/L. The above model was cross validated for all the different chlorine levels, this can be clearly seen in Table 5.1.

Table 5.1: Model Estimated Percentage of Filamentous Bacteria Alive at the Different Chlorine Doses

Chlorine dose (mg Cl_2/L)	Filamentous bacteria Alive (%)
0	91.53
2	83.20
5	70.70
10	49.87
15	29.04
20	8.21
22	0.00

The chlorine residual were monitored during the reaction, Table 5.2 illustrates a residual total chlorine of 0.5 mg Cl_2/L after the 2 hour treatment period at the highest dose of 40 mg Cl_2/L. At the optimum dose of 25 mg Cl_2/L to achieve full inactivation of filamentous bacteria, a mere 0.2 mg Cl_2/L total chlorine is left over after 2 hours.

Table 5.2: Total chlorine residual after treatment

Chlorine (mg Cl_2/L)	15 min (mg Cl_2/L)	30 min (mg Cl_2/L)	1hr (mg Cl_2 /L)	2hr (mg Cl_2/L)
0	0	0	0	0
2	0	0.1	0	0
5	0.4	0.2	0.2	0.1
10	0.5	0.3	0.2	0.2
15	0.4	0.6	0.2	0.3
20	0.5	0.6	0.2	0.2
25	0.7	0.9	0.2	0.2
30	1	0.9	0.3	0.3
40	1.4	1.2	0.4	0.5

5.3.3. Germicidal Effect of Ultraviolet Light on Filamentous Bacteria

The germicidal effectiveness of UV-light on filamentous bacteria was optimized for different time intervals (5-80 minutes). The UV-light Tests were done in replicates (T1, T2 and T3); (Table 5.3). The results illustrated after 80min in T1 and T2 all microorganisms were killed (Figure 5.6 c), however, T3 revealed a 16% survival rate of the filamentous bacteria.

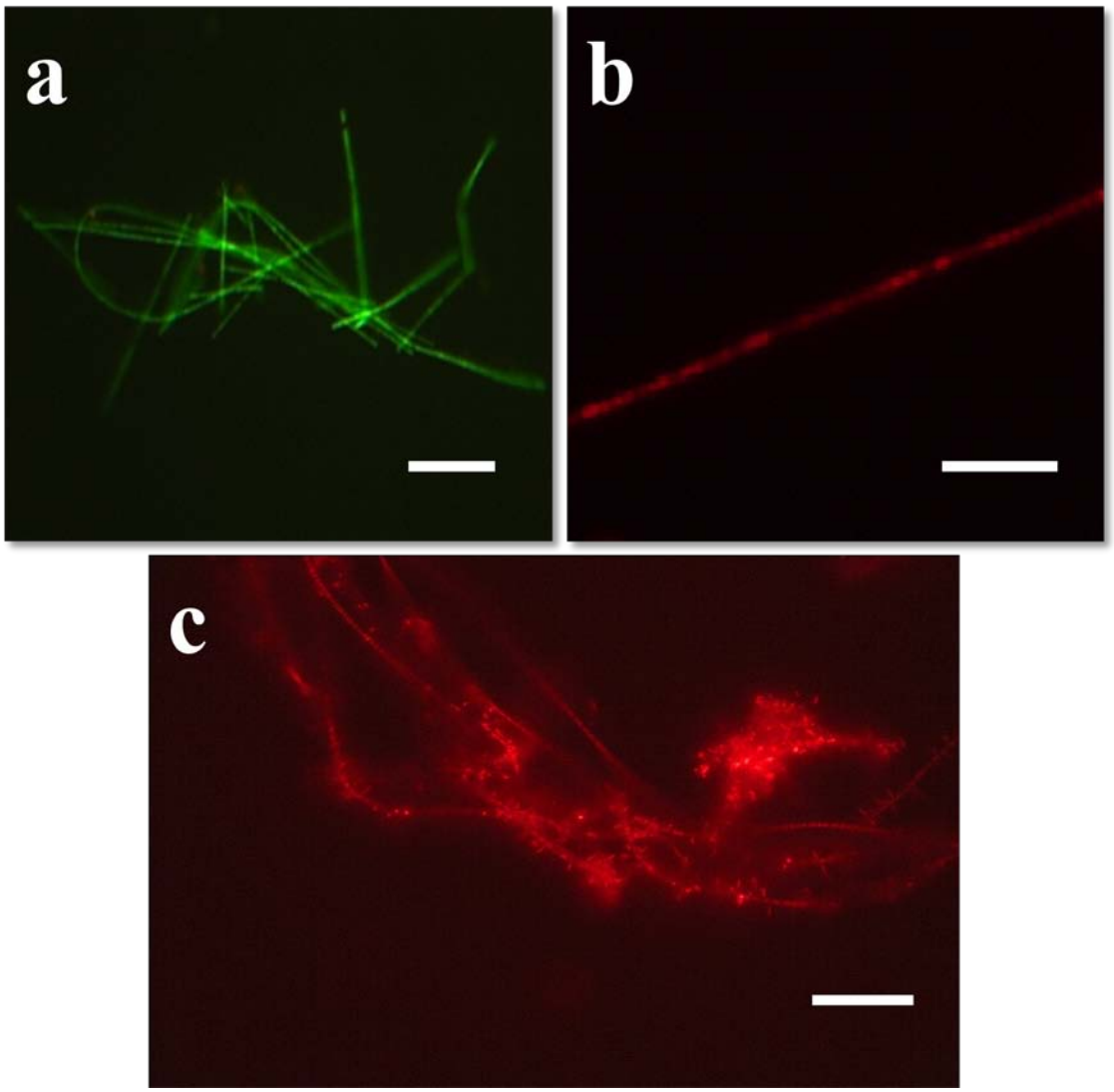

Figure 5.6: (a) Control live (green) filamentous bacteria; (b) dead (red) filament after 20 min exposure (c) after 80 min exposure dead floc and filamentous bacteria. Scale bars = 5 μm.

The slight variation in the results of replicate (T3) can be attributed to insufficient mixing of the samples, since UV light is most effective on the surface. Hence, a depth wise procedure with a larger volume should have been exercised to achieve a greater understanding on the effect of UV on the filamentous organisms. It is important to acknowledge the effect of UV transmission, as this plays an integral role in understanding the overall effect of UV-light on the filamentous bacteria. A steep decline in the filamentous bacteria survival rate was also observed from 20-60 minutes (Figure 5.7).

Table 5.3: Ultraviolet light (554nm) effect on filamentous bacterial survival rate (%)

UV light Exposure (min)	Survival (%) T1	Survival (%) T2	Survival (%) T3
0	100	100	98
5	93	93	70
10	87	90	57
20	79	77	58
30	60	64	57
45	22	30	46
60	20	14	29
80	0	0	16

The survival rate of filamentous bacteria with UV exposure at different lengths of time was plotted on a scatter plot, Figure 5.7 graphically represent the filamentous bacteria survival rate affected by the different dose of UV light (product of UV light intensity and exposure time).

A test for deviation from linearity for the curve was performed and the p-value was found to be 0.6286. Since the p-value is greater than 0.05, we can conclude that there is insufficient evidence to indicate that the curve deviates from linearity. Hence, linear regression is not inappropriate to use. The coefficient of determination (R^2) value was estimated to be 0.9060. The R^2 is an estimate of the proportion of variation in the dependent variable (filamentous bacteria alive) that is being explained by the independent variable (UV exposure). This means that 90.60% of the variation in the percentage of filamentous bacteria alive can be explained by the length of UV exposure.

When testing whether the effect of UV exposure on the percentage of filamentous bacteria alive is significant, the p-values observed is less than 0.0001. It can thus be concluded that the length of UV exposure has a significant effect on the percentage of filamentous bacteria alive.

This curve is modelled by the equation 5.2:

$$y = 93.42 - 1.165\,x \qquad (5.2)$$

From the equation 5.2, the estimated percentage of bacteria alive reaches 0 at a UV exposure of 80 minutes.

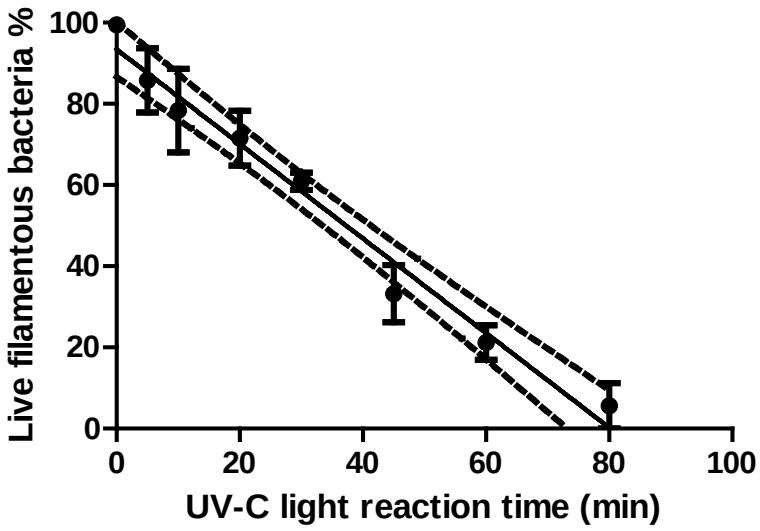

Figure 5.7: Graph representing changes in filamentous bacterial survival – UV dose.

It was observed that with an increase in time, UV dose increases (i.e. UV dose directly proportional to time) and filamentous bacteria are destroyed (Figure 5.7). The energy required (UV dose) is measured in micro-watt seconds per square meter (mW Seconds/cm^2).

UV dose = Light Intensity X Time

Light intensity is given by the wattage of the bulb divided by $4\pi r^2$. UV light intensity was thus calculated as follows: The point at which the light intensity is measured was 10 cm away

from the light source, hence distance between the UV light and the sample was 0.1 meters. The value is characterized as the radius of a sphere surrounding the bulb. To calculate the light intensity, the surface area of a sphere is equal to $4\pi r^2$ (0.1256) divided by the wattage of the UV-C germicidal lamp (15 W). UV light intensity was thus 119.43 watts/cm^2. As a result, UV dose required to kill 99.9% filamentous bacteria is the product of 119.3 watts/cm^{2}at 80 min which is 9554.4 μW seconds/cm^2. However, the live dead stain analysis show, at a dose of 9554.4 mW Seconds/cm^2 (UV-light at 80 min) all flocs were completely dead.

With the equation $5.2, (\ y = 93.42 - 1.165x)$ the average UV dose applied can be approximated to kill of a certain percentage of filamentous bacteria. It was clear, that the maximum filamentous reduction (99%) was observed after 80 minutes of exposure, however a 50% reduction was after 37 minutes exposure time. It was thus evident from the equation obtained in the regression analysis (Equation 5.2), a UV dose of 4418.91 μw/cm^2 was required to effectively kill 50% of the filamentous bacteria, at an exposure time of 37 min. Disadvantages encountered thus far, UV dose was subjected to a thin film of sample (1 cm). For a further improvement, a depth wise procedure should have been configured to determine the survival of filamentous bacteria at different depths.

5.3.4. Assessment of Ozone Inactivation of Filamentous Bacteria

The ozone test was done in replicates (T1, T2 and T3) and in all the tests, filamentous bacteria showed a rapid decrease from 2.5 - 34 mg O_3/L (Table 5.4). Thereafter, the death rate of the filamentous organisms was more or less at a steady state. The control test experiment contained a large number of viable filamentous bacteria (Figure 5.8 a). At the highest ozone dose (60 mg O_3/L), the Live/Dead stain still revealed a few viable filaments, however all floc forming bacteria were dead (Figure 5.8 c). The microbial flocs were significantly damaged at an increased ozone dose.

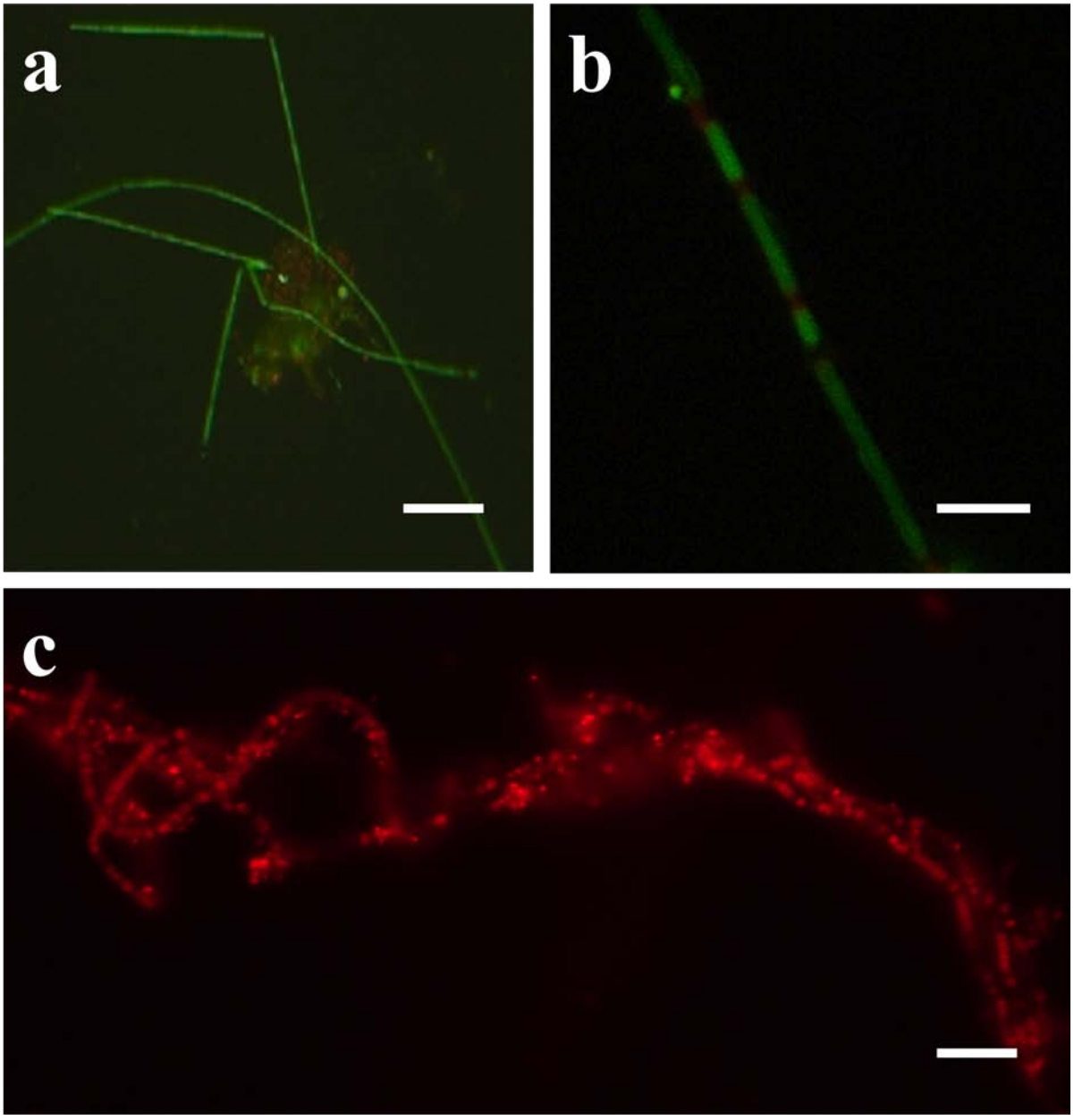

Figure 5.8: Epifluorescent micrographs depicting (a) control- live (green) filaments; (b) 5.8 mg O_3/L damaged filaments and (c) 60 mg O_3/L dead (red) filamentous bacteria. Scale bars = 5 μm.

Table 5.4: Ozone effect on filamentous bacteria

Ozone contact time mg O_3/L	Survival (%) T1	Survival (%) T2	Survival (%) T3
0.0	100	100	100
2.5	87	95	70
5.8	65	70	43
9.5	51	62	34
14.8	72	76	53
21.7	50	66	48
31.4	10	26	10
38.0	14	38	11
52.0	14	22	24
60.0	4	16	8

The coefficient of determination (R^2) value was estimated to be 0.88. This means that 88% of the variation in the percentage of filamentous bacteria alive can be explained by the ozone dose. Results reveal a quick decline in the filamentous population at low levels of ozone and at higher levels, the ozone effect on filamentous bacteria is more or less sluggish, between 20- 60 mg O_3/L. At 9.5 mg O_3/L, more than half the population survived, however once the dose increases 2-fold (21.7 mg O_3/L), results show a very small decline in filamentous population (Table 5.4, Figure 5.9). The results indicate that a low ozone dose seems to be most effective in the destruction of filamentous organisms.

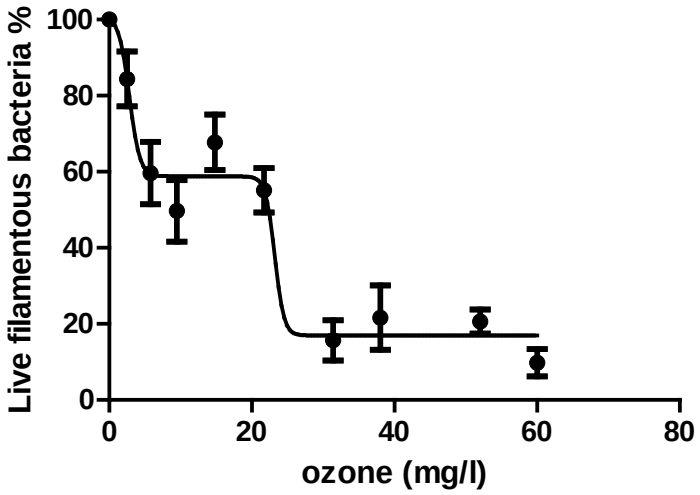

Figure 5.9: Ozone consumption (mg O_3/L), bell-shaped curve (R^2= 0.88).

5.4. Discussion

Researchers have been trying to solve the filamentous bulking problem for over a century by looking at various control measures. To date, these methods are intractable issues and an easy and cost effective solution for their control has not yet been identified. Both, specific and non-specific control methods are in practise to control the growth of these organisms. Specific methods to control filamentous bulking include; DO, F/M ratio, nutrient deficiency and septic influent (Jenkins *et al.*, 2004a; Richard *et al.*, 2003). Specific methods have been covered extensively in chapters 3 and 4. Chlorination, ozonation and hydrogen peroxide are some of the common toxicants exercised as non-specific methods to control filamentous bulking (Leeuwen, 1989; Martins *et al.*, 2004). UV light has been applied as a disinfectant in the final effluent of wastewater treatment, however the method has not been investigated in filamentous bulking. Thus, the scope of this chapter focuses on evaluating three existing technologies (i.e. Chlorine, UV-light and Ozone) which can be used to minimize the filamentous population in RAS samples.

Due to the difficulty in achieving pure cultures on the filamentous bacteria, a concentrated filamentous culture was obtained using the filtration technique, however, some flocs with a high density of eubacteria were also retained. This could be possible due to the sticky EPS layer binding these organisms together, hence, some of the larger flocs were also trapped onto the filter. The samples were passed through a range of stainless steel filter sieves of various

pore sizes as to gain a maximum concentration of the filamentous population, the 75 μm pore size filter showed the best results (Figure 5.3 c). This is the first report on the successful use of a filtration technique to concentrate filamentous bacteria. The filtered samples were subsequently used as the test cultures for this study.

Of all the treatment methods on AS bulking in full-scale wastewater systems, chlorine has been meticulously investigated. Theoretically, chlorine was one of the first technologies proposed to control filamentous bulking and it is also commonly used as a disinfectant in the effluent wastewater in the form of chlorine gas. In this study, the effect of chlorine dose on the inactivation of filamentous bacteria at various time intervals was examined and results are shown in Figure 5.4, 5.5. It was evident that the estimated percentage of bacteria alive reaches 0 at a chlorine dose of 22 mg Cl_2/L, given by the equation 5.1, $y = 91.5311 - 4.16603\ x$ and to achieve at least 50% reduction of filamentous bacteria, an effective dose of approximately ±10 mg Cl_2 /L was required (Figure 5.5).

Theoretically, low F/M filamentous bacteria like Type 021N showing resistance to chlorine and maintaining cell integrity up to doses of 80 mg Cl_2/g SS (Guo and Zhang, 2012; Neethling *et al.*, 1985; Seka *et al.*, 2001). The dominant filamentous bacteria used for the current study *viz*., Type 021N, Type 0092, *Thiothrix* spp. and Type 1851 are classified as low F/M filaments. However, the results obtained during the current study showed that at a chlorine dose of 22 mg Cl_2/L, all bacteria including filaments were killed. Within a 30min reaction time, a total chlorine dose of 0.9 mg Cl_2/L total was left over. Subsequently the chlorine residual dropped to 0.2 mg Cl_2/L after 2 hours which is well below the acceptable discharge limit. At a lower dose of 10 mg Cl_2/L chlorine, there was a reduction of up to 50% of the filamentous bacteria which seems to be an effective dose to target filamentous bacteria.

Several reports have been investigated to monitor chlorine effects on filamentous bulking, Neethling (1985), illustrated an efficient bulking control with a chlorine dose between 0.7 – 20 mg Cl_2/L. Lakay et al (1988) and Van Leeuwen has reported a laboratory scale system showing a chlorination dose of 6-8 kg Cl_2/ 10^3 kg MLSS which was successful to control filamentous bulking. More recently Seka (2003) predicted the effects of chlorine dosing on filamentous bacteria coupling the Live/Dead stain with the NA and/or OUR test, a sharp decline in filamentous organisms were observed at a chlorine dose between 2-10 mg Cl_2/L and almost the entire filamentous population and flocs were killed and highly damaged at a

chlorine dose between 15-40 mg Cl_2/L. Furthermore, results showed no effect on nitrification and phosphorus removal at the low chlorine dose, i.e. less than 10 mg Cl_2/L (Lakay *et al.*, 1988; Neethling *et al.*, 1985; Seka *et al.*, 2003).

Chlorine treatment proved to be promising for eliminating filamentous bacteria, however, this method has limitations when applied to full scale systems. The major challenge is that chlorine reacts with N-NH_4^+ to form monochloroamine (Jenkins *et al.*, 1993), which is much less potent than Cl_2. When high N-NH_4^+ concentrations are present, the chlorine oxidises N-NH_4^+ nitrogen to free nitrogen gas thus preventing it from killing filamentous bacteria. In addition, it has also been reported that chlorine doses higher than 2.5 mg Cl_2/g SS caused limitation in the substrate uptake rate and phosphorus removal (Dionisi *et al.*, 2006). Due to these problems other treatment methods have been exploited.

The UV-light germicidal lamps have been widely used as a sterilization and disinfection techniques to reduce pathogens and cysts which cannot be destroyed by the use of chemical treatment. The application has significantly evolved in many WWTPs, and has proven to be more feasible and cost effective than chlorine (Braunstein *et al.*, 1996; Scheible, 1987). However, the UV-treatment method has not been applied to specifically reduce filamentous bacteria.In this study, an alternative to chlorine treatment, a germicidal UV-lamp of 254nm wavelength were used to reveal its effectiveness on filamentous bacteria at different times (0-80 min). A plot of the survival percentage of filamentous bacteria against the different UV-dosage is showed a negative slope (p value < 0.05), with filamentous bacteria decreasing as the time of exposure increases (Figure 5.7). UV dose expressed as $\mu w/cm^2$ is the product of ultraviolet light intensity and time of exposure (UV-dose = Intensity × Time). Results revealed that 99.99% filamentous bacteria were killed at an effective dose of 9554.4 μw seconds/cm^2. The equation (5.1), obtained from the regression analysis disclose that to effectively kill 50% of the filamentous bacteria, a UV exposure 4418.91 $\mu w/cm^2$ for 37 minutes was required.

This treatment process should be scaled-up via the installation of UV-light on a pilot scale sequencing batch reactor system, evaluating samples from the return AS stream with an enriched filamentous bacterial culture. This would clearly present a holistic approach of the UV-light treatment on filamentous bacteria, at a minimum UV-dose so as to target the surface extending filaments while still rendering the internal structure of the floc active and at the

same time monitoring the effect of UV-dose at different depths. Some of the limitations to apply UV-treatment onto full-scale systems include; the limited penetration of UV into the sludge, depending on the thickness of the biomass. Hence, this treatment method should be applied to a well-mixed system, also taking into consideration the hydraulic retention time. A major advantage of UV-light in contrast to chlorination, is that the final effluent wastewater is free of any toxicants (Oliver and Cary., 1976). Results reveal that a further investigation on UV-light treatment should be taken as this could be a very useful technique to reduce filamentous bacteria.

The ozone treatment to reduce filamentous bulking has proven to be partially successful in full-scale WWTPs (Leeuwen, 1988; Wijnbladh, 2007). The treatment does not interfere with normal wastewater treatment functions *viz*., nitrification and phosphorous removal. A study conducted by Wijnbladh (2007) showed that an optimized ozone dose can be successfully applied to the aeration tank to improve sludge quality without interfering with the normal functioning of the plant. The scatter plot in Figure 5.9 illustrated, an increase in the ozone supply, resulted in a decrease in the filamentous bacteria population. Results are indicative of a non-linear relationship which is strongly significant (p value < 0.05; R^2= 0.88). The dose response fit a bell shaped curve and the results revealed that at a dose of ±20 mg O_3/L, approximately ±50% of filamentous bacteria were killed. According to the earlier reports, the most effective dose required to control filamentous bulking was 66 mg O_3.$gVSS^{-1}$.min^{-1} (Caravelli *et al.*, 2006).

From the study, it can be seen that ozone had almost an immediate effect on the death rate of filamentous bacteria with a close to 40% drop after 5 mg O_3/L was applied. This could be due to the fact that O_3 is highly oxidisable. The results obtained from the analysis showed that 30 mg O_3/L ozone gas, at a constant rate effectively destroys 80% of the filamentous population. Further increasing ozone above 30 mg O_3/L showed little or almost no effect. Batch ozone experiments have been previously conducted to evaluate the effectiveness on AS, 15-70 mg O_3/L were applied to the batch system (Järvik *et al.*, 2010). Sludge treatment also evaluated by Nagare *et al*, (2008) and showed an improvement in settling properties at an ozone range between 20-40 mg O_3/g SS.

Recently there have been many attempts to apply ozonation in wastewater plants to improve process efficiency. Ozone doses up to 30 mg O_3/L were subjected to the process to improve

effluent quality (Oliver *et al,* 2010). Ozone has to be generated on site and it is a very expensive process. Van Leeuwen mentions on a review of non-specific control measures to reduce bulking, an ozone dose of 2 mg O_3/L was effective enough to improve settling. Apart from ozone being effective in eliminating filamentous bacteria, it is also been reported that doses up to 30 mg O_3/L does not affect the nutrient removal process (Leeuwen, 1988).

The next testing stage of these three treatments will be applied to a pilot scale SBR system. Analyses will include the effective dose rates of each treatment on both filamentous bacteria as well as floc forming bacteria. Total cost of each treatment, as well as the best efficient treatment to a level of a full-scale WWTP would be considered.

5.5. Conclusions

Non-specific methods were evaluated to eliminate or control filamentous bacteria. These methods include Chlorine, UV light and Ozone. Live/dead staining appeared to be an appropriate rapid method which determined the viability of filamentous bacteria. All three methods evaluated were found to be effective in inactivating filamentous bacteria. It was observed that a chlorine dose of 10 mg Cl_2/L were effective in killing 50% of the filamentous bacteria. Results revealed that 99.99% filamentous bacteria were killed at an effective UV-dose of 9554.4 μW seconds/cm^2. The regression analysis disclosed that to effectively kill 50% of the filamentous bacteria, UV exposure 4418.91 μW/cm^2 for 37 minutes was required. An Ozone dose of ±20 mg O_3/L was required to reduce approximately ±50% of filamentous bacteria. Low concentration of ozone seemed to be most suitable and effective in eliminating filamentous bacteria.

6. CONCLUSIONS AND FUTURE RECOMMENDATIONS

6.1. Conclusions

- The dominating filamentous bacteria identified via microscopic and molecular techniques at Kingsburgh WWTP were found to be; Type 021N, *Thiothrix* sp., Type 1851 and Type 0092.
- The ambient temperature changes during full-scale observations (17 - 25°C) showed that the warmer seasons significantly increased the filamentous bacteria Type 1851. However, Type 0092 was favoured during colder temperatures (during the autumn and winter season). The remaining dominant filamentous bacteria (i.e. Type 021N and *Thiothrix* spp.) showed minimal fluctuations to the temporal changes.
- High SVI values with an average of 176 mL/g were noted during autumn and winter, (indicating bulking) and an average of 130 mL/g during spring and summer (non-bulking). However, the filamentous bacteria were found to be highly excessive reaching a score of 6 on the filamentous abundance scale, hence possibly a DSVI should have been attempted to reflect a more authentic assessment of sludge settling.
- The cumulative logit model proved to be an efficient tool to understand the growth pattern of the dominant filamentous bacteria based on operational parameters experienced in full-scale conditions. Thus, the growth of filamentous bacteria can possibly be predicted and controlled in this system.
- Using the cumulative logit model, significant relationships were obtained for a few operational parameters (F/M, influent COD, DO, influent N-NH_4^+, temperature) against the filamentous bacterial population.
- *Thiothrix* spp. showed significant relationships with many operational parameters, however, when all these parameters were grouped together, F/M and N-NH_4^+ at high levels seems to be the deciding factors to predict the dominance of this organism regardless of the other factors being at high or low levels.
- The model clearly explicates that N-NH_4^+ significantly influenced *Thiothrix* spp. and Type 021N, the model predicted a high probability of these organisms being very common to excessive when the N-NH_4^+ level was high. Low COD levels with an increase in ammonia were the suitable conditions which predicted filamentous Type 021N to be excessive.

- Findings from the model were consistent with literature.
- Live/dead staining and image analysis system allowed a rapid assessment of the effect of the non-specific treatment methods on the viability of the filamentous bacteria.
- Findings confirmed a chlorine dose of 10 mg Cl_2/L is required to achieve at least a 50% reduction of the filamentous bacteria.
- An effective UV and ozone dose of 4418.91 μW seconds/cm^2 and ±20 mg O_3/L respectively, was required to kill 50% filamentous bacteria.

6.2. Recommendations

- The development/design of a CLM model to control filamentous bacteria would be beneficial, as it is very difficult to cultivate these organisms in pure culture. Applying the model to full-scale biological systems would illustrate their functionality and improve their accuracy when controlling filamentous bulking. Therefore, further development of the CLM using full-scale specific operational parameters should be further investigated in order to improve the applicability of the model.
- In this study, chemical and physical treatment methods used to reduce filamentous bacteria via batch tests. Applying these methods to a full-scale system would determine the reproducibility of the current findings at a larger scale with respect to non-specific control methods.
- A comparison of these methods with reference to filamentous bacteria and floc forming bacteria in pilot scale systems, coupling the live/dead staining together with the microbial metabolic activity (oxygen uptake rate) has not been reported in literature and in this study. Therefore, further studies showing the effect of these treatment methods on both the floc formers and the filamentous bacteria would greatly contribute to determine the correlation degree between these techniques and to discern the possible mechanism of action for the destruction of filamentous bacteria in full-scale systems.

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APPENDIX 1: FILAMENTOUS BACTERIA IDENTIFICATION KEYS

-based on morphological and staining techniques (Jenkins *et al.*, 1993)

Filamentous type	Gram stain	Neisser Stain		Trichome Shape	Key characteristic
		Trichome	Granules		
S.natans	-	-	-	Straight or smoothly curved	Attached growth when the organism is slow-growing, cells are large and tightly packed within a sheath, False branching.
Type 1701	-	-	-	Straight, bent	Occasionally occurring out of the floc without attached growth, however when the filament is stagnant a dense epiphytic bacteria attach to the trichome.
Type 0041	+,-	-	-,+	Straight, smoothly curved	Excessive attached growth.
Type 021N	-	-	-,+	Straight, smoothly curved sometimes coiled	No attached growth, one of the largest and longest filament. Rosettes and gonidia may occur.
Thiothrix I	-,+	-	-,+	Straight, smoothly curved	One of the thickest filaments, sulphur granules usually present, can be detected by the S-test. Apical gonidia and rosettes commonly observed.
Thiothrix II	-	-	-,+	Straight, smoothly curved	Small amounts of attached growth, apical gonidia and rosettes are commonly observed.
M.parvicella	+	-	+	Coiled	No attached growth
H.Hydrosis	-	-	-	Rigidly straight or bent	Very thin small filaments occasionally growing out of the floc, no attached growth.
Type 1851	+ weak	-	-	Straight, smoothly curved	Commonly occurs in bundles.
Type 0092	-	+	-	Straight or bent	Always inside the floc, no attached growth.

NB: Type- refers to filamentous morphotypes that have been identified by conventional methods, however, these filamentous bacteria are not fully characterised and classified.

APPENDIX 2: MODEL CONVERGENCE STATUS

Cumulative logit mode: Model fit Statistics- determination of the model convergence describing , data output from the SAS program in this study. AIC values lower than the SC and -2Log L values indicated model best fit.

I**ntercept and co-variates** corresponds to the respective criterion statistics for the fitted model. A fitted model includes all independent variables and the intercept (SAS Annotated output: 2012).

Table 1: Model fit statistics with AIC, SC and -2Log L, Type 021N

Operational conditions	**Intercept and Covariates**		
	AIC	SC	-2logL
All Factors combined	153.890	172.739	135.890
COD	160.382	168.760	152.382
DO	163.232	171.610	155.232
$N\text{-}NH_4^+$	161.283	169.661	153.283
pH	164.695	173.072	156.695
Temperature	163.252	171.629	155.252
F/M	163.957	172.335	155.952
Phosphorus	137.382	145.030	129.382

Table 2: Model fit statistics with AIC, SC and -2Log L, Type 0092.

Operational conditions	**Intercept and Covariates**		
	AIC	SC	-2logL
All Factors combined	108.880	125.635	92.880
COD	119.933	126.216	113.933
DO	121.067	127.350	115.067
$N\text{-}NH_4^+$	120.101	126.384	114.101
pH	128.086	134.369	122.086
Temperature	118.884	125.167	112.884
F/M	122.733	129.016	116.733
Phosphorus	105.698	111.434	99.698

Table 3: Model fit statistics with AIC, SC and -2Log L, Type 1851

Operational conditions	**Intercept and Covariates**		
	AIC	SC	-2logL
All Factors combined	160.232	181.180	140.236
COD	179.918	190.450	169.978
DO	180.820	191.292	170.820
$N\text{-}NH_4^+$	181.500	191.972	171.500
pH	186.366	196.838	176.366
Temperature	187.857	198.329	177.857
F/M	157.480	167.952	147.480
Phosphorus	139.422	148.982	129.422

Table 4: Model fit statistics with AIC, SC and -2Log L, Thiothrix spp.

Operational conditions	**Intercept and Covariates**		
	AIC	SC	-2logL
All Factors combined	121.029	139.878	103.029
COD	123.939	132.316	115.939
DO	128.048	136.425	120.048
$N\text{-}NH_4^+$	129.242	137.619	121.242
pH	128.144	136.521	120.144
Temperature	129.879	138.256	121.879
F/M	119.934	128.312	111.934
Phosphorus	101.320	108.969	93.320

APPENDIX 3: FISH PROTOCOL

(Amann *et al*., 1995)

a) PRE-TREATMENT OF SLIDES

- Acid wash, Teflon coated slides(Merck, Germany).
- The slide surface was cleaned by soaking them into warm detergent solution for an hour.
- Washed slides were placed in a 1: 10 diluted solution Poly-L-Lysinè and allowed to soak for 5 min @ room temperature (Sigma Diagnostics, USA).
- Treated slides were removed and air dried.

b) CELL FIXATION

Solutions:

- 1 x Phosphate Buffered Saline (PBS) pH 7.2
  - 10 mL 10 x PBS
  - 90 mL sterile deionized water

- 3 × Phosphate Buffered Saline (PBS) pH7.2
  - 30 mL 10 x PBS
  - 70 mL sterile deionized water

- 99% Ethanol

- 4% Paraformaldehyde
  - Preparation of paraformaldehyde Fixative:
    - heat 33 mL of deionized water to 60-65°C
    - add 2 g paraformaldehyde while stirring
    - add 2 M NaOH drop-wise until paraformaldehyde is dissolved completely
    - add 16.5 mL 3 x PBS buffer
    - adjust the pH to 7.2 - 7.4 at 20°C

- filter through 0.45 μm filter
- use within 24hrs, store in ice until use

Fixation:

- Sample centrifugation (3500 rpm, 5 minutes) and discard the supernatant.
- wash the pellet with 1 x PBS, centrifuge and re-suspend pellet in an appropriate volume of fresh 1 x PBS
- add 3 volumes of fresh paraformaldehyde
- incubate for 3 hours or overnight at 4°C
- spin down the cells (5 minutes)
- dislodge supernatant
- wash pellet with 1 x PBS, centrifuge again
- add 1 volume of ice-cold EtOH$_{abs}$
- store at -20°C

- Ethanol Fixative for Gram positive bacteria: Paraformaldehyde fixative is most suitable for Gram negative cells, Gram positive cells are fixed ethanol only.
  - Harvest cells by centrifugation
  - Wash the pellet in 1 x PBS, and centrifuge
  - Re-suspend the pellet in an appropriate volume of fresh 1 x PBS.
  - Add one volume of ice-cold EtOHabs
  - Store at -20°C

c) IMMOBILISATION OF CELLS

- Add 10 μL diluted, fixed sample onto each of the wells
- Allow to dry for about 10 minutes @ 46°C
- Dehydrate the cells by successive passages through increasing alcohol series 50, 80 and 99.99% ethanol washes for 3 minutes each.
- Allow to air dry. The slides can be stored at room temperature.

d) WHOLE CELL HYBRIDISATION

- Materials
  - 50 mL Polypropylene screw top tube
  - Whatman 3MM paper
  - Hybridisation buffer pH 7.2
    - 5 M NaCl 360 μL
    - 1 M Tris HCl 40 μL
    - Add formamide and ultra pure water depending on applied stringency

- Preparation of hybridisation buffer for *in situ* hybridisation at 46°C:
- Prepare mixture into a 2 mL Eppendorf tubes:
  - 5 M NaCl (360 μL)
  - 1 M Tris HCl pH 8.0 (40 μL)
  - Add formamide and MQ (ultra pure water), depending on the applied stringency

% formamide(v/v)	Formamide (μL)	MQ (μL)
0	0	1.600
5	100	1.500
10	200	1.400
15	300	1.300
20	400	1.200
25	500	1.100
30	600	1.000
35	700	0.900
40	800	0.800
45	900	0.700
50	1000	0.600
60	1100	0.500
65	1200	0.400
70	1300	0.300

- 10% (w/v) SDS 4 μL (Add last; add onto lid of the tube).
- Defrost the oligonucleotide probes
- Add 9 μL hybridization buffer to the wells
- Add 1 μL probe (working solution, concentration 50 ng/μL for FLOUS labelled probes) without scratching the Teflon-coated onto the surface (Mix the 1 μL probe

with 9 μL of the hybridization buffer. Foe EUB, add 1 μL of each probe to 7 μL of the hybridization buffer

- Prepare a hybridization tube (50 mL Polypropylene tube) by folding a piece of tissue or Whatmann 3 MM paper, put it into the tube and pour the rest of the hybridization buffer onto the tissue
- Immediately transfer the slide into the hybridization tube (chamber) and incubate in the hybridization oven (46°C) for 3 hours
- Protect the slide in chamber with foil from hybridization oven and transfer into washing buffer very quickly

d) Prepare the washing buffer and preheat this buffer at 48°C in a water bath.

- Preparation of washing buffer for *in situ* hybridisation at 48°C
  - Mix in a 50 mL Polypropylene tube:
  - 1 mL of 1M Tris/HCl pH 8.0
  - 5 M NaCl and 0.5 M EDTA pH 8.0 according to the following table:

% Formamide in hybridization buffer	NaCl (mol/L)	NaCl (µL)
0	0.900	9.000
5	0.636	6.300
10	0.450	4.500
15	0.318	3.180
20	0.225	2.150
25	0.159	1.490
30	0.112	1.020
35	0.080	0.700
40	0.056	0.460
45	0.040	0.300
50	0.028	0.180
55	0.020	0.100
60	0.008	0.400
70	0.000	350 μL EDTA

  - for formamide concentrations of 20% and higher in the hybridization buffer add 500 μL 0.5M EDTA (for stabilization of probe).
  - 50 μL of 10% (w/v) SDS
  - preheat the washing buffer at 48°C prior to use

- Rinse the hybridization buffer with the washing buffer from the slide and incubate the slide in the washing buffer for 10mins in a 48°C preheated water bath
- Remove the washing buffer with distilled water without detaching the cells and dry the slide quickly with compressed air
- Embed the slide with embedding liquid and put a cover slip onto the slide.

DAPI STAINING (Hicks *et al*., 1992; modified)

- Spread 10 μL of DAPI (0.25 μg/mL) on each well and allow to stain for 5 minutes.
- Wash the slides with 1 x PBS and allow to air dry.
- Mount the slide with Vectashield Mounting Media (Vector Laboratories, Burlingame) and cover with a cover slide.
- View immediately.

APPENDIX 4: LIVE/DEAD STAINING

(Sigma-aldrich.com 2012)

Content

Solution A: 4 vials
Solution B: 1 vial

Methods

- Add 10 μL Solution A and 5 µl Solution B to 5 mL PBS to prepare assay solution (a)
- Prepare a cell suspension with a trypsin-EDTA treatment if cells are adhered to a culture plate (b)
- Centrifuge the cell suspension at 1,000 rpm for 3 min.
- Wash the cell pellet with PBS several times to remove residual esterase activity.
- Prepare a cell suspension with PBS in which the cell density is 1 x 105 to 1 x 106 cells/mL.
- Mix 200 μL of cell suspension and 100 µL of assay solution and incubate the mixture at 37 ºC for 15 min.
- Detect fluorescence using a fluorescence microscope with 490 nm excitation for simultaneous monitoring of viable and dead cells. With 545 nm excitation, only dead cells can be observed.

a) The concentration of each reagent should be optimized. Following steps may be necessary to determine the suitable concentration of each reagent:
1. Prepare dead cells by 10 min incubation in 0.1% saponin or 0.1-0.5% digitonin or by 30 min incubation in 70% ethanol.
2. Stain dead cells with 0.1-10 µM PI solution to find a PI concentration that stain nucleus only, does not stain cytosol.
3. Stain dead cells with 0.1-10 µM Calcein-AM solution to find a Calcein-AM concentration that does not stain cytosol. Then stain viable cells with that Calcein-AM solution to check whether the viable cell can be stained.

b) Or you may remove culture medium and wash cells with PBS several times. Add assay solution and incubate at 37 ºC for 15 min.

Storage

- Reagent solution is stable for 12 months at -20°C with protection from light and moisture.
- Since the buffer solution of Calcein-AM is gradually hydrolyzed to generate fluorescent Calcein, the working solution is not storable.

Close the bottle cap tightly after using a portion of Calcein AM solution to avoid moisture.

APPENDIX 5: GRAM STAINING

(Jenkins *et al*., 1993)

Reagents

Solution (1)

a) Crystal violet 2g
Distilled water 80 mL

b) Ammonium oxalate 0.8 g
Ethanol, 95% 20 mL

Solution (2)

Iodine 1g
Potassium Iodide 2g
Distilled water 300mL

Solution (3)

Safranin O (2.5% w/v dissolved in 95% ethanol) 10mL
Distilled water 100mL

Method

- Add diluted sample onto the microscope slides, leave to air dry.
- Add 1 mL solution (1) for 60 seconds; rinse with H_2O.
- Add 1 mL solution (2) for 60 seconds: rinse well with H_2O.
- Decolorize with 95% ethanol onto the smeared sample for 30 seconds, hold slide at an angle and adding the ethanol in a drop-wise procedure.
- Add solution (3) for 60 seconds, rinse well and blot dry.

APPENDIX 6: NEISSER STAINING

(Jenkins *et al*., 1993)

Reagents

Solution (1):

a)	Methylene Blue	0.1g
	Ethanol, 95%	5 mL
	Glacial acetic acid	5mL
	Distilled water	100 mL

B)	Crystal violet (10%w/v in 95% ethanol)	3.3 mL
	Ethanol 95%	6.7 mL
	Distilled water	100 mL

Two parts by volume of (a) are mixed with 1 part by volume of (b). fresh stock to be prepared monthly.

Solution (2):

Bismark Brown (1% w/v aqueous)	33.3 mL
Distilled water	66.7 mL

Method:

- Prepare thin sample smears on microscope slides and allow to air dry.
- Then stain slides for 30 seconds with solution 1 and then rinse with water for 1 second.
- Stain this preparation with solution 2 for 1 minute, rinse well with water and then blot dry.

APPENDIX 7: PHB STAINING

(Jenkins *et al*., 1993)

Reagents

Solution (1): Sudan Black B (IV) 0.3% w/v in 60% ethanol

Solution (2): Safranin 0 0.5% w/v aqueous

Method:

- Add diluted sample on microscopic slides and allow to air dry.
- Subsequently, add solution (1) onto slide for 10 min, rinse well with water for 1 second.
- Stain this preparation with solution 2 for 10 seconds, rinse well with water and blot dry.

APPENDIX 8: SULPHUR TEST

(Jenkins *et al*., 1993)

Solution 1: Na_2S solution

200 mg of Na2S. 7 H2O per 100 mL

Method:

Mix equal volume of activated sludge together with Solution 1.

Mix well and keep the sludge in suspension. Examine slides microscopically.